The gut microbiome and its relationship to migratory behaviour and fate in wild fishes

by

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Abstract

Gut microbiota play an essential role in modulating host physiological processes that contribute to host health and fitness. Wild, migratory species offer a unique opportunity to examine the gut microbiome under an additional layer of complexity. Changing external environments, compounded by migration-associated physiological changes in the host, may be associated with variations in the microbial community and differentially impact fish health and fitness. The objective of this thesis was to investigate the hypothesis that the hindgut microbial communities vary relative to migratory behaviour and fate in wild fishes. Specifically, I assessed hindgut microbial communities in three fish species that exhibit different migratory behaviours using 16S rRNA gene amplicon sequencing: white sucker (*Catostomus commersonii*), sockeye salmon (*Oncorhynchus nerka*), and brown trout (*Salmo trutta*). Further, I highlighted the importance of transitioning to non-lethal sampling methods when studying wild fish microbiomes, especially in relation to studying behaviours. Gut microbial analysis revealed that potamodromous white suckers were dominated by the genus Aeromonas. Further, late migrants were found to be less diverse than individuals arriving during peak migration and contained a significantly different community composition, driven by the genus *Mycoplasma.* For anadromous migrations, there was weak evidence that the hindgut microbial composition of adult sockeye salmon varied between spawning populations with a relatively short migration (Weaver Creek) versus the most challenging migration (Chilko River). Differential abundances of marine-associated microbes characterized the Weaver population, whereas the potential pathogenic genus

Flavobacterium was associated with the Chilko group. Comparatively, *Mycoplasma* appeared as a dominant taxon across this species. Examining whether the fecal microbiota was associated with migratory status in a partially anadromous juvenile brown trout population revealed no association between the gut microbial diversity or composition and migratory status, though finer-scale site-specific differences were observed. The results presented in this thesis are novel in that they are the first to attempt to characterize the gut microbiome in relation to migratory behaviours and fate in wild fishes. Moreover, this thesis highlights the importance of incorporating a microbial perspective in fish physiology and behaviour research and demonstrates the value of incorporating a holobiont approach.

Acknowledgements

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Preface

All research conducted as part of this thesis was in accordance with the animal welfare guidelines and policies of Carleton University's Animal Care Committee and followed protocols approved by Carleton's Animal Care Committee, the University of British Columbia, the Danish Technical University, and Fisheries and Oceans Canada.

In 2021 the International Committee on Systematics of Prokaryotes (ICSP) updated the classification of 42 phyla of bacteria and archaea. I have kept to the old classification system to maintain clarity with the previously published literature.

Thesis Format

This thesis contains six chapters, four of which are written in manuscript-format (Chapters 2-5). Chapter 1 outlines a general introduction and includes thesis objectives and hypotheses. Chapter 2 examines the hindgut microbial composition and diversity of white suckers in relation to migration timing, sex, and tumor presence. Chapter 3 examines the hindgut microbial composition and diversity in adult sockeye salmon from three spawning populations in the Fraser River Watershed. Chapter 4 examines the fecal microbiota of partially anadromous brown trout in relation to life-history strategy. Chapter 5 offers a field ecologist perspective in transitioning to non-lethal microbiome sampling methods, with a particular focus on fish. Chapter 6 summarizes my general conclusions and project limitations and proposes future research based on the results generated in the present thesis. This thesis contains all my own research and writing but was conducted in collaboration with various other researchers, to which I have provided a summary list of their contributions below. In addition, acknowledgements from each data chapter have been included in the thesis acknowledgement section.

Chapter 1: General Introduction

Chapter 2: The hindgut microbial composition and diversity of a potamodromous freshwater fish varies by migration timing, but not sex or tumor presence.

Kelly, L.A., Russell, J.N., Loos, K.D., DeBruyne, J.E., Yost, C.K., and Cooke, S.J.

L.A.K, C.K.Y, and S.J.C conceived the project. L.A.K performed the sample collection. L.A.K, J.N.R, and J.E.D performed DNA extraction. K.D.L and J.N.R performed 16S rRNA sequencing. L.A.K performed data analysis, with help from J.N.R. L.A.K drafted the manuscript, figures, and tables. C.K.Y, J.N.R, and S.J.C reviewed the manuscript. This manuscript is in prep for journal re-submission.

Chapter 3: The hindgut microbial composition, but not diversity, in adult sockeye salmon (Oncorhynchus nerka) varies among three spawning populations in the Fraser River watershed

Kelly, L.A., Russell, J.N., Loos, K.D., DeBruyne, J.E., Healy, S., Patterson, D., Yost, C.K., and Cooke, S.J.

L.A.K, D.P, and S.J.C conceived the project. D.P and S.H performed sample collection and blood plasma analysis. L.A.K, J.N.R, and J.E.D performed DNA extraction. K.D.L and J.N.R performed 16S rRNA sequencing. L.A.K performed data analysis, with help from J.N.R. L.A.K drafted the manuscript, figures, and tables. C.K.Y, J.N.R, and S.J.C reviewed the manuscript. This manuscript is in prep for journal submission.

Chapter 4: Does the fecal microbiome predict migratory status in a population of brown trout (Salma trutta) displaying partial migration?

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Chapter 5: Challenges and opportunities with transitioning to non-lethal sampling of wild fish for microbiome research

Kelly, L.A., Yost, C.K., and Cooke, S.J.

L.A.K and S.J.C conceived the paper. L.A.K drafted the manuscript and figures. C.K.Y and S.J.C reviewed the manuscript. All authors read and approved the final manuscript. This manuscript is in prep for journal submission.

Chapter 6: General Conclusions and Future Directions

Table of Contents

Abstract .... **ii**

Acknowledgements .... **iv**

Preface .... **vi**

Thesis Format and Co-authorship .... **vii**

Table of Contents .... **x**

List of Tables .... **xiv**

List of Figures .... **xvii**

List of Appendices .... **xxii**

Chapter 1: General Introduction **1**

1.1 Gut microbial communities of fish 1

1.2 Historical overview of fish gut microbiome research and methodological approaches . 7

1.3 Fish migratory behaviours 10

1.4 Objectives and hypotheses 14

1.5 Challenges 15

1.6 Scientific contribution 17

Chapter 2: The hindgut microbial composition and diversity of a potamodromous freshwater fish varies by migration timing, but not sex or tumor presence. **19**

2.1 Abstract 19

2.2 Introduction 20

2.3 Methods 23

2.3.1 Fish and microbiome sample collection 23

2.3.2 DNA extraction 24

2.3.3 16S rRNA gene sequencing 25

2.3.4 Sequencing data processing and statistical analysis 26
2.4 Results 28
2.4.1 Sequence quality 28
2.4.2 Hindgut microbial community composition and diversity 28
2.5 Discussion 30
2.5.1 Limitations of study 35
2.5.2 Conclusions 35
Chapter 3: The hindgut microbial composition, but not diversity, in adult sockeye salmon (*Oncorhynchus nerka*) varies among three spawning populations in the Fraser River Watershed 46
3.1 Abstract 46
3.2 Introduction 47
3.3 Methods 51
3.3.1 Study system and sample collection 51
3.3.2 Blood plasma processing 52
3.3.3 DNA extraction and 16S rRNA amplicon sequencing 52
3.3.4 Sequencing data processing and statistical analysis 54
3.4 Results 55
3.4.1 Microbiome amplicon sequence quality 55
3.4.2 Characterizing community composition and diversity patterns among different spawning populations 56
3.4.3 Physiological status of different spawning populations 58
3.4.4 Integrating physiological metrics and composition of bacterial communities 59
3.5 Discussion 59
3.5.1 Conclusion 65

Chapter 4: Does the fecal microbiome predict migratory status in a population of brown trout (Salma trutta) displaying partial migration? 77

4.1 Abstract 77

4.2 Introduction 78

4.3 Methods 82

4.3.1 Study location 82

4.3.2 Fish sampling and tagging 83

4.3.3 Evaluation of migration 84

4.3.4 DNA extraction and 16S rRNA amplicon sequencing 84

4.3.5 Sequencing data processing and statistical analysis 86

4.4 Results 87

4.4.1 Life-history strategy and sample sequence quality 87

4.4.2 Hindgut microbial community composition and diversity by migratory status and body condition 88

4.4.3 Hindgut microbial community composition and diversity by sample site 90

4.5 Discussion 91

4.5.1 Conclusions 97

Chapter 5: Challenges and opportunities with transitioning to non-lethal sampling of wild fish for microbiome research 115

5.1 Abstract 115

5.2 Introduction 116

5.3 Non-lethal sampling methods 118

5.4 Opportunities of non-lethal microbiome sampling 121

5.5 Challenges of non-lethal microbiome sampling 124

5.6 Conclusions 127

Chapter 6: General conclusions and future directions 130

6.1 General conclusions 130

6.2 Limitations 137

6.3 Future Directions 139

Appendices 142

Appendix A Supplementary Tables 142

Appendix B Citations of non-thesis publications during doctoral studies 144

References 146

List of Tables

Table 2-1. Description of migrating adult white sucker fish sampled for this study........ 37

Table 2-2. Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance of hindgut bacterial communities of white suckers for groupings: Migration time, sex, and tumors. Df = degrees of freedom, SS = sum of squares, MS = mean squares, F. Model = F-value by permutation, R2 = percentage of variance explained by the groups, Pr(>F) = p-value for F-statistic. P-values based on 999 permutations. *Difference is significant at 0.05 level. 38

Table 2-3. Analysis of multivariate homogeneity of group dispersions (betadisper) based on Bray-Curtis distance of hindgut bacterial communities of late and peak migrating white suckers. Df = degrees of freedom, SS = sum of squares, MS = mean squares, Pr(>F) = p-value for F-statistic. 38

Table 3-1. Description of adult sockeye salmon fish sampled for this study with a minimum of 800 reads. 67

Table 3-2. Prevalence of phylum-level taxa across the sampled adult sockeye salmon population with a relative abundance greater than 1% of total OTU sequences. 68

Table 3-3. Prevalence of genus-level taxa across the sampled adult sockeye salmon population with a relative abundance greater than 1% of total OTU sequences. 68

Table 3-4. Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance of hindgut bacterial communities of adult sockeye salmon for groupings: location, sex, glucose, lactate, hematocrit, and condition factor. Df = degrees of freedom, SS = sum of squares, MS = mean squares, F. Model = F-value by

permutation, R2 = percentage of variance explained by the groups, Pr(>F) = p-value for F-statistic. P-values based on 999 permutations. *Difference is significant at 0.05 level.69

Table 3-5. Analysis of multivariate homogeneity of group dispersions (betadisper) based on Bray-Curtis distance of hindgut bacterial communities of adult sockeye salmon from three spawning locations. Df = degrees of freedom, SS = sum of squares, MS = mean squares, Pr(>F) = p-value for F-statistic. 69

Table 3-6. Pairwise comparisons based on the location grouping from the PERMANOVA analysis. Df = degrees of freedom, SS = sum of squares, MS = mean squares, F. Model = F-value by permutation, R2 = percentage of variance explained by the groups, Pr(>F) = p-value for F-statistic. P-values based on 999 permutations. *Difference is significant at 0.05 level. 69

Table 4-1. Description of juvenile brown trout fish retained for this study. 99

Table 4-2. Prevalence of family-level taxa across sampled population with a relative abundance greater than 1% of total OTU sequences. 100

Table 4-3. Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance of fecal bacterial communities of brown trout for groupings: migratory status, study site, and body condition. Df = degrees of freedom, SS = sum of squares, MS = mean squares, F. Model = F-value by permutation, R2 = percentage of variance explained by the groups, Pr(>F) = p-value for F-statistic. P-values based on 999 permutations. *Difference is significant at 0.05 level. 100

Table 4-4. Analysis of multivariate homogeneity of group dispersions (betadisper) based on Bray-Curtis distance of fecal bacterial communities of juvenile brown trout from

different sampling sites. Df = degrees of freedom, SS = sum of squares, MS = mean squares, Pr(>F) = p-value for F-statistic. 101

Table 4-5. Pairwise comparisons: Observed p-value below diagonal, permuted p-value above diagonal. 101

List of Figures

Figure 1-1. Number of publications between 2002 and 2022 gathered from the Web of Science database. Data was collected using the following search string for aquaculture: TS=((captive OR domestic OR aquaculture OR culture$ OR farm*) AND (fish$ OR teleost) AND ("*gut microb*" OR "*intestinal microb*" OR "fecal microb*" OR "faecal microb*") NOT (shrimp) NOT (human)), which totaled 1660 publication records; and wild: TS=((wild) AND (fish$ OR teleost) AND ("*gut microb*" OR "*intestinal microb*" OR "fecal microb*" OR "faecal microb*") NOT (shrimp) NOT (human)), which totaled 271 publication records. Plot was created using R (v4.0.5) and ggplot (v3.3.5). 18

Figure 2-1. Study site map showing A) location of Cobourg within the Great Lakes system, and B) location of the weir where sampling occurred, approximately 800m from the entrance to Lake Ontario. Figure was created using Adobe Illustrator version 25.0; base-map data from Google maps ©2021 39

Figure 2-2. Rarefaction curve showing the number of OTUs on the vertical axis against the number of sequences (sample size) on the horizontal axis for the 14 white sucker fish samples. 40

Figure 2-3. Relative abundance of the major phyla present across all samples grouped into peak and late migration timing. 41

Figure 2-4. Relative abundance of the major families present across all samples grouped into peak and late migration timing. 42

Figure 2-5. Shannon alpha diversity measure on operational taxonomic units (OTUs) of hindgut bacterial community of peak and late migrating white sucker fish. 43

Figure 2-6. Two-dimensional non-metric multidimensional scaling (NMDS) plots with 95% confidence ellipses around each group centroid of white sucker hindgut bacterial communities using a Bray Curtis distance matrix to show dissimilarity between individual fish samples grouped by peak and late migration times. 44

Figure 2-7. Linear discriminant analysis effect size (LEfSe) analysis showing OTUs likely driving the dissimilarity observed in the hindgut bacterial community structure between Peak and Late migrating white suckers. A negative LDA score shows OTUs with significantly higher abundances in Peak migrants, while positive LDA scores show OTUs with significantly higher abundances in Late migrants... 45

Figure 3-1. Study site locations where adult sockeye salmon were sampled at the terminal end of their migration run representing three separate stocks. Blue triangle is situated at Weaver Creek Spawning Channel, yellow square at the lower Adams River, and red circle at the Chilko River StAD Camp. 70

Figure 3-2. Rarefaction curve showing the number of OTUs on the vertical axis against the number of sequences (sample size) on the horizontal axis for all sockeye salmon hindgut swab samples. 71

Figure 3-3. Relative abundance of the major genera present across adult sockeye salmon fish from three separate spawning stocks (Weaver Creek, lower Adams River, and Chilko River; representing more than 1.0 % relative abundance). 72

Figure 3-4. Alpha diversity indices showing A) Observed Species Richness and B) Inverse Simpson index on the operational taxonomic units (OTUs) of the hindgut bacterial community of adult sockeye salmon at three spawning sites (Weaver Creek, lower Adams River, and Chilko River). 73

Figure 3-5. Two-dimensional non-metric multidimensional scaling (NMDS) plots with 95% confidence ellipses around each group centroid of adult sockeye salmon hindgut bacterial communities using a Bray Curtis distance matrix to display dissimilarity between individual fish samples grouped by their respective spawning locations........... 74

Figure 3-6. Linear discriminant analysis effect size (LEfSe) analysis showing OTUs likely driving the dissimilarity observed in the hindgut bacterial community structure between adult sockeye salmon sampled on the Weaver Creek spawning ground vs. Chilko spawning ground (LDA scores greater than 3; A), and between sockeye salmon with below average and above average condition factors (LDA scores greater than 2.5; B). A negative LDA score shows OTUs with significantly higher abundances in fish from Weaver Creek and fish with below average condition factors, while positive LDA scores show OTUs with significantly higher abundances in fish from Chilko River................. 75

Figure 3-7. Blood and body composition physiological variables taken from adult sockeye salmon on three spawning grounds at the terminal end of their migration run. A) plasma glucose (mmol/L) with overall and pairwise comparison significance values, B) plasma lactate (mmol/L) with overall and pairwise comparison significance values, C) plasma hematocrit (% estimate) with overall and pairwise comparison significance values, and D) condition factor with overall and pairwise comparison significance values. 76

Figure 4-1. Study site location, Gudsø stream, southeastern Jutland, Denmark. Two PIT stations (S1 and S2) are located just upstream of the mouth into Kolding Fjord. The shaded box in the top box represents the area of stream sampled, which is blown up in the

bottom box to show the location of the sampling sites (A-D), as well as the containment pond located just upstream from Site D. 102

Figure 4-2. Rarefaction curve showing the number of OTUs on the vertical axis against the number of sequences (sample size) on the horizontal axis for all juvenile brown trout fecal samples. 103

Figure 4-3. Relative abundance of the major phyla present in migrant and resident juvenile brown trout fecal samples (representing more than 1.0 % relative abundance). 104

Figure 4-4. Relative abundance of the major phyla present across fish with low body condition (k<1), normal body condition (k=1), and high body condition (k>1; representing more than 1.0 % relative abundance). 105

Figure 4-5. Relative abundance of the major families present across migrant and resident fish (representing more than 1.0 % relative abundance) 106

Figure 4-6. Relative abundance of the major families present across fish with low body condition (k<1), normal body condition (k=1), and high body condition (k>1; representing more than 1.0 % relative abundance). 107

Figure 4-7. Shannon alpha diversity measure on operational taxonomic units (OTUs) of fecal bacterial community of migrant and resident juvenile brown trout. 108

Figure 4-8. Shannon alpha diversity measure on operational taxonomic units (OTUs) of fecal bacterial community of juvenile brown trout with body condition scores of k<1 (low), k=1 (normal), and k>1 (high). 109

Figure 4-9. Two-dimensional non-metric multidimensional scaling (NMDS) plots with 95% confidence ellipses around each group centroid of juvenile brown trout fecal

bacterial communities using a Bray Curtis distance matrix to show dissimilarity between individual fish samples grouped by A) Migratory Status, B) body condition categories, and C) sampling site.. 110

Figure 4-10. Relative abundance of the major phyla present across fish captured at four different sampling sites (representing more than 1.0 % relative abundance). 111

Figure 4-11. Relative abundance of the major families present across fish captured at different sampling sites (representing more than 1.0 % relative abundance). 112

Figure 4-12. Shannon alpha diversity measure on operational taxonomic units (OTUs) of fecal bacterial community of juvenile brown captured at different sampling sites. 113

Figure 4-13. Linear discriminant analysis effect size (LEfSe) analysis showing OTUs likely driving the dissimilarity observed in the fecal bacterial community structure between fish captured at sampling sites B and D (top) and C and D (bottom). A negative LDA score shows OTUs with significantly higher abundances in fish from sample sites B (top) and C (bottom), while positive LDA scores show OTUs with significantly higher abundances in fish from site D... 114

Figure 5-1. The four main non-lethal fish microbiome sample types............................ 129

Figure 5-2. Example of hindgut swabbing on a white sucker fish (*Catostomus commersonii*).. 129

List of Appendices

Appendix A. Supplementary Tables……..…………………………………………..…… 142

Appendix B. Citations of non-thesis publications during doctoral studies………..…. 144

Chapter 1: General Introduction

This thesis examines the hindgut microbial communities of wild fishes exhibiting migratory behaviours and attempts to determine if gut microbial communities are related to their behaviour and fate. In the context of this thesis, "fate" includes outcomes related to migration failure (e.g., mortality) and life-history status (e.g., assuming a resident or migratory status). More specially, I investigated the hindgut microbial composition and diversity in relation to adult anadromous and potamodromous spawning migrations and juvenile anadromous feeding migrations in salmonid and catostomid fishes. In this general introduction, I provide the necessary background information for understanding the concepts, objectives, and hypotheses developed and explored in this thesis. The first section provides a comprehensive overview of the roles and functions associated with gut microbial communities of fish, as well as describes in detail the exogenous and endogenous factors that structure these communities (Section 1.1). I then give a historical overview of gut microbial research and approaches used to study them (Section 1.2). Next, I discuss fish migratory behaviours relevant to this thesis in the context of my study species (Section 1.3). Lastly, I will outline my hypotheses and objectives (Section 1.4), as well as challenges (Section 1.5), and highlight potential scientific contributions arising from this work (Section 1.6).

1.1 Gut microbial communities of fish

Vertebrates harbour a diverse microbial community within their intestinal tract, comprised mainly of bacteria, but also include species of archaea, viruses, and fungi

(Walter et al. 2011; Sommer and Bäckhed 2013). Humans and other mammals have been the primary focus of gut microbiome research to date, and results from these studies have revealed that the gut microbiota play a role in supporting the health and fitness of their host by contributing to host physiology, energy metabolism, vitamin synthesis, development, behaviour, and immune function (De Filippo et al. 2010; Lozupone et al. 2012; Ridaura et al. 2013; Johnson and Foster 2018). The Human Microbiome Project has been instrumental in advancing our understanding of the commensal microbiota and laid the foundation for investigating associations between our microbes and health and disease (Human Microbiome Project, 2012). One of the goals of that project was to determine whether a healthy core microbiome was present across the participants. Since then, the core microbiome has been examined in relation to health and disease states in mammalian hosts (Kuczynski et al. 2010). The core microbiome describes a common "core" of bacterial taxa present in the gut microbiome of individuals across a species, regardless of whether individuals were captive or wild, from different locations, or had different diets – suggesting host-specific selection (Roeselers et al. 2011; McDonald et al. 2012; Star et al. 2013; Wong et al. 2013). It is likely that this "core" provides a minimum functionality as it is conserved across many exogenous factors (Star et al. 2013). It is now believed that this core may be represented by a common microbial gene pool rather than a core centered on conserved phylogeny (Barko et al. 2018). Conversely, inter-individual variation in microbial composition can potentially act as a selective pressure for host adaptation, fitness, and evolution (Suzuki 2017). Changes in the composition of the gut microbial community could have downstream positive or negative repercussions on host phenotypic plasticity, leading to differential survival of a population during periods of

adverse environmental change (Alberdi et al. 2016). The idea that an individual's microbial community is an agent of adaptation is a recent development in organismal physiology and evolution and changes the perspective that an individual is not an autonomous organism but a complex integrated assemblage of host and symbionts – termed the holobiont (Bordenstein and Theis 2015).

While human-based studies dominate the vertebrate microbiome literature, mammals comprise only 10% of vertebrate species. Conversely, fish represent almost 50% of vertebrates, yet research lags far behind their mammalian counterparts (Sullam et al. 2012). There is some overlap at the phylum level between mammals and fish gut microbiota, and as fish represent a more ancestral line of the vertebrate subphylum, it is vital to understand these host-microbe relationships in terms of co-evolution at a more basal level (Ley et al. 2008a; Sullam et al. 2012). Further, the gut microbial communities of fish tend to be less diverse than mammals (Holben et al. 2002), which may make them a simpler model for understanding evolutionary relationships and host-microbiota interactions (Rasmussen et al. 2023). Like mammals, fish gut microbiota play an essential role in host digestive processes and metabolism, vitamin synthesis, epithelial renewal, and immune function (Hansen and Olafsen 1999; Gómez and Balcázar 2008; Nayak 2010; Mouchet et al. 2012; Wu et al. 2012; Llewellyn et al. 2014; Dehler et al. 2017a). In fact, the gut microbiota has been shown to regulate the expression of at least 212 genes in fish (Rawls et al. 2004), and gnotobiotic fish studies have demonstrated that the absence of gut microbiota led to reduced physiological functions, such as issues with intestinal epithelial cell renewal (Rawls et al. 2004, 2006; Cheesman et al. 2011) and nutrient absorption (Bates et al. 2006). Two distinct groups of bacteria are present within the

intestinal tract: autochthonous, which attach to the intestinal mucosa and are a more stable community (Nayak 2010), and allochthonous, which are more transient and typically found in feces (Ringø and Birkbeck 1999). The autochthonous bacteria are more directly involved in interactions with the host because they are more closely associated with the epithelial cells lining the intestinal mucosal wall (Ringo and Birkbeck 1999). These bacteria provide beneficial services, such as inhibiting colonization of pathogenic bacteria through competitive exclusion or the production of toxic secondary metabolites, as well as interactions with the immune system (Gatesoupe 1999; Balcázar et al. 2006; Ringø et al. 2006a, 2006b; Caipang et al. 2010; Llewellyn et al. 2014).

Microbial colonization begins during the egg stage, where glycoproteins present on the egg surface may allow species-specific bacteria to adhere to the egg surface (Hansen and Olafsen 1989; Romero and Navarrete 2006). Once fish larvae begin active feeding, the number of bacteria in the gut increases substantially, and there is a shift in composition, suggesting that diet is an important source for colonization and establishment of the gut microbiota of young fish (Munro et al. 1994; Romero and Navarrete 2006; Nayak 2010; Ingerslev et al. 2014). Microbes from the local environment are also important early colonizers of the fish gastrointestinal tract, where fish larvae receive microbial inputs through the ingestion of water (Liston 1957; Nieto et al. 1984; Hansen and Olafsen 1999; Ringø et al. 2006b; Fjellheim et al. 2007; Sullam et al. 2012). The retention of microbes within the gastrointestinal tract is also partially constrained by host gut physiological conditions (Hansen and Olafsen 1999). Further, the gut microbiota are not a static community once microbial colonization and establishment have occurred but shift in composition under a range of different factors, including host-

specific factors (e.g., development stage, digestive physiology, weight, and immunity; Cahill 1990; Verner-Jeffreys et al. 2003; Romero and Navarrete 2006; Li et al. 2012, 2014; Navarrete et al. 2012; Bolnick et al. 2014a; Stephens et al. 2016), environmental factors (e.g., water salinity, geographic location, season, antibiotics; Hagi et al. 2004; Hovda et al. 2011; Sullam et al. 2012; Zarkasi et al. 2014; Ringø et al. 2016; Dehler et al. 2017b), and dietary factors (e.g., diet, trophic level, starvation; Brunvold et al. 2007; Reid et al. 2009; Xia et al. 2014; Ye et al. 2014). The gut microbiota also differs within different gastrointestinal tract regions based on the physio-chemical conditions present (Zhou et al. 2007; Wang et al. 2018). Further, there is a general trend of increasing bacterial abundance as you move from foregut to hindgut (Cahill 1990; Molinari et al. 2003; Das et al. 2014).

So far, I have discussed a wide variety of factors that impact the structure of the gut microbiota of fish. Because of the diverse nature of the gut microbiota across fish species, I did not go into specific details regarding taxonomic composition differences for each of these factors between or among fish. Instead, I outlined the general forces that exert change on the gut microbiota. Here, I will broadly discuss the taxonomic patterns observed across teleosts as an introduction to the general bacterial biodiversity observed in the fish gastrointestinal tract. Overall, fish gut microbiomes are dominated by the phyla Proteobacteria, Fusobacteria, Bacteroidetes, Firmicutes, and Actinobacteria (Llewellyn et al. 2014; Ghanbari et al. 2015; Egerton et al. 2018; Legrand et al. 2020a). This suggests that members of these phyla are particularly well-suited to the fish gastrointestinal tract and likely provide beneficial services to the host. Differences in bacterial composition have been observed between the gastrointestinal microbiota of

freshwater and marine fishes. Freshwater fish species are more generally associated with genera such as *Aeromonas*, *Pseudomonas*, *Bacteroides* type A, *Plesiomonas, Enterobacteriaceae, Micrococcus, Acinetobacter,* and *Clostridium* (Sugita et al. 1985; Nayak 2010). Conversely, marine fishes typically have representatives from *Vibrio, Pseudomonas, Achromobacter, Corynebacterium, Alteromonas,* and *Flavobacterium* (Cahill 1990; Verner-Jeffreys et al. 2003; Nayak 2010). The trophic level of fish also plays a role in structuring the microbiota of the gastrointestinal tract, where diversity has been reported to increase from carnivorous fish to omnivorous fish, and is highest in herbivorous fish (Ward et al. 2009; Larsen et al. 2014; Li et al. 2014; Miyake et al. 2015; Wang et al. 2018). Higher diversity in herbivorous fish is likely due to the requirement of cellulose-degrading bacteria such as *Clostridium* and others, which assist with digestion of plant material (Liu et al. 2016). In contrast, carnivorous fish can readily digest and assimilate amino acids and typically have low gastrointestinal bacterial diversity (Wang et al. 2018; Huang et al. 2020). In salmonids, the genus *Mycoplasma* often dominates the intestinal microbiota, and recent metagenomic studies have discovered that this genus likely plays a role in synthesizing B_{12} vitamins (Rasmussen et al. 2023).

Most fish microbiome research to date has been conducted on farmed species, including Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), and others (Ringo and Olsen 1999; Heikkinen et al. 2006; Ringø et al. 2006b; Han et al. 2010; Desai et al. 2012; Neuman et al. 2016; Schmidt et al. 2016). The importance of microbiome research in aquaculture is immediately apparent due to the continual effort required to maintain fish health and reduce disease under high rearing densities. Pre/probiotic treatments to

modulate the gut microbiome in an effort to reduce pathogenic bacteria and maintain fish health as alternatives to antibiotics have been investigated in several aquaculture studies (Kumar et al. 2008; Barbosa et al. 2011; Dimitroglou et al. 2011; Boutin et al. 2013; Geraylou et al. 2013; Dawood et al. 2016). However, while the study of farmed fish species is important, and there are many economic benefits to improving the gut microbiome and overall fish health, there are differences in the gut microbiota between farmed and wild species (Dhanasiri et al. 2011; Kormas et al. 2014; Eichmiller et al. 2016; Ramírez and Romero 2017). Therefore, farmed species cannot be used as a proxy for the gut microbiome composition of wild species. Research into the gut microbiome of wild species lags far behind that of their cultured counterparts (Figure 1-1). However, studies conducted thus far offer important insights into host-microbe co-evolution, diet significance, habitat, and other environmental and social factors that have been shown to help shape the composition of the gut microbiome (Nelson et al. 1999; Smriga et al. 2010; Mouchet et al. 2012; Amato 2013; Star et al. 2013; Ye et al. 2014; Bolnick et al. 2014b; Miyake et al. 2015; Smith et al. 2015; Llewellyn et al. 2016).

1.2 Historical overview of fish gut microbiome research and methodological approaches

The first fish gut microbiota studies were based on conventional culture-dependent methods (Cahill 1990), whereby gut samples were cultured on nutrient agar and incubated for varying lengths of time based on the environmental conditions of the host (Sugita et al. 2005; Ringø et al. 2006a). After the bacteria had grown for the appropriate length of time, colonies were counted, and a representative sample was re-

streaked on nutrient agar for isolation. After which, biochemical assays and/or phenotypic studies would be used to identify the bacterial species present (Zhou et al. 2014). While culture-based approaches defined the earlier generation of fish microbiome research and established our baseline information in this field, these studies presented a skewed and limited view of gut microbial communities due to the low cultivability of fish microbes, estimated at less than 0.1% of the total microbial communities present in the gastrointestinal tract of some fish species (Shiina et al. 2006).

Culture-independent molecular-based techniques, such as fluorescence *in situ* hybridization (FISH), DNA fingerprinting, and DNA sequencing of the 16 small subunit ribosomal RNA (rRNA) gene, have advanced the field of fish gut microbiome research over the past 20 years and led to exponential increases in the understanding of the composition and function of these microbial communities (Zhou et al. 2014; Ghanbari et al. 2015). Most studies use the 16S rRNA gene as it is present in all bacteria and contains multiple conserved and hypervariable regions that allow for phylogenetic differentiation (Woese and Fox 1977). I have chosen these three techniques to briefly describe as they appear most commonly in the literature, though it should be noted that this is not an exhaustive list of molecular techniques used in fish gut microbiome research.

FISH uses fluorescent-labelled probes to target regions of the 16S rRNA genes of bacteria for observation using fluorescence or confocal microscopy (Amann et al. 1995). While it is a low throughput method, it has high specificity and can achieve single-cell resolution (Shi et al. 2021). This has utility in tracking specific strains, such as different probiotic species in aquaculture studies (Del'Duca et al. 2013) or infectious agents (Levsky and Singer 2003).

Next, DNA fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE; Zhou et al. 2009), were commonly used in early fish bacterial community analysis research to ascertain the complexity of microbial communities (Tarnecki et al. 2017). However, results are typically more qualitative and not informative at the individual Operational Taxonomic Unit (OTU) level. DNA fingerprinting is considered a medium to high-throughput analysis (Tarnecki et al. 2017).

Finally, and most relevant to this thesis, are the DNA sequencing approaches, specifically technologies that sequence 16S rRNA gene amplicons, providing a means to identify whole communities of bacteria in a sample (Fraher et al. 2012). Sanger sequencing was the first generation using this approach, and involved sequencing individual 16S clone libraries, which was both labour intensive and expensive (Tarnecki et al. 2017). Next generation sequencing (NGS) quickly became the preferred method for fish microbiome studies as it could generate large volumes of data in parallel, making it very cost-efficient (Ghanbari et al. 2015). The predominant technology for the application of NGS is Illumina short-read sequencing, using either the HiSeq or MiSeq platforms (Ghanbari et al. 2015). Illumina uses reversible terminator sequencing-by-synthesis chemistry to produce short-length DNA sequences (Ghanbari et al. 2015). Some limitations to note with NGS approaches are that the interpretation of the bacterial communities can be affected by the DNA extraction method and primer selection used, as well as PCR amplification bias (Kuczynski et al. 2016).

Overall, these molecular techniques provide high-resolution methods of characterizing the entire microbial community found within a sample from the intestinal tract and allow for identifying rare and previously unknown bacteria (Navarrete et al.

2010; Nayak 2010; Qin et al. 2010). It was imperative to quickly summarize the wide array of methods used in fish microbiome studies to give context to the issues arising from different methodologies used in the literature, which can make it difficult for direct comparisons. Even between studies using 16S rRNA gene sequencing, there is a wide range of variation that exists in methodology, including which hypervariable region is sequenced (V1 through V9; Kim et al. 2011), which region of the gastrointestinal tract and sample type (digesta vs. mucosa), preservation and storage method, DNA extraction protocol, sequence platform, and processing, all of which introduce bias and affect downstream analysis of microbial communities (Tarnecki et al. 2017).

1.3 Fish migratory behaviours

Migration is a widespread phenomenon involving a diverse array of species across the globe (Dingle 1980). There are many reasons for an individual to exhibit migratory behaviour, including reproduction, growth, and survival (Dingle and Drake 2007). Migration often involves movement across many different landscapes and diverse habitats, potentially exposing individuals to a wide range of environmental pollutants, water qualities, parasites, and disease pathogens (Altizer et al. 2011; Chapman et al. 2011). Two general types of migration patterns involve a freshwater component. The first is potamodromy, migrations that occur solely in freshwater (Lucas and Baras 2001). The main drivers of these freshwater migrations are feeding opportunities, refuge-seeking behaviours (such as overwintering), and spawning (Lucas and Baras 2001). Many potamodromous fish species are also iteroparous, meaning they carry out multiple reproduction events and spawning migrations during their lifetime (Thalinger et al.

2019). White sucker (*Catostomus commersonii*) is an example of a potamodromous, iteroparous species (Corbett and Powles 1983). These fish carry out spawning migrations in the spring once water temperatures reach 10°C (Corbett and Powles 1983). White sucker travel from lakes and rivers into spawning tributaries, the length of which can vary from just a few hundred meters up to 40 km (Doherty et al. 2010).

The second type of migration with a freshwater component is diadromy, which occurs between freshwater and marine habitats. Diadromous migrations are further split between anadromy and catadromy. Anadromous fish feed and grow at sea, followed by an adult spawning migration into freshwater. Anadromous species include sockeye salmon (*Oncorhynchus nerka*) and brown trout (*Salmo trutta*). Conversely, in catadromy, feeding and growth occur in freshwater prior to the initiation of an adult spawning migration to sea. Catadromous migration is practiced by species such as the North American eel (*Anguilla rostrata*) and European eel (*A. anguilla*; Lucas and Baras 2001). As two of my study species are anadromous, this shall be the focus of the diadromous aspects of migration going forward. Anadromous species, such as salmonids, can exploit different habitats for growth and reproduction. The ocean provides rich feeding grounds that allow fish to maximize growth opportunities to optimize gonadal development and fitness potential (Jensen et al. 2014). However, migrations are often very challenging from a physiological standpoint and are energetically costly for individuals, resulting in differential migration success due to variations in individual fitness (Hinch et al. 2006) and increased predation risks en-route (Alerstam et al. 2003). In some species of salmonids, such as brown trout, anadromous migration has evolved to be facultative, where populations display both migratory and resident phenotypes (Archer et al. 2019).

The decision to migrate occurs in the juvenile stage, though the proximate mechanisms underlying this decision are still being investigated. It is thought to be related to food limitation (Olsson et al. 2006; O'Neal and Stanford 2011) and low body condition/high metabolic rates (Cucherousset et al. 2005; Boel et al. 2014; Peiman et al. 2017; Birnie-Gauvin et al. 2021). While smoltification is a stressful and energetically demanding process, benefits include increased growth from superior ocean feedings grounds and the avoidance of harsh freshwater winter environments with high levels of overwinter mortality (Klemetsen et al. 2003; Shuter et al. 2012). As adults returning to spawn, anadromous salmonids typically cease feeding upon entering freshwater (Lucas and Baras 2001). Capital breeders, such as sockeye salmon, rely solely on endogenous energy supplies to fuel their migration run and spawning activities (Brett 1995; Hinch et al. 2006). Sockeye salmon also demonstrate semelparity, meaning they return to their natal headwaters for a single reproductive event before dying (Dingle 1980); therefore, if premature mortality occurs en-route or before spawning, their lifetime fitness is zero.

Migration acts as an additional layer of complexity to studying the gut microbiomes as exogenous (e.g., heterogenous landscapes) and endogenous (e.g., physiological changes associated with migration) forces likely exert multi-factorial impacts on the structure and composition of the gut microbiome. Recent studies have assessed gut microbiomes in relation to migrating fish (Llewellyn et al. 2016; Le Doujet et al. 2019; Element et al. 2020a, 2020b; Le and Wang 2020; Ying et al. 2020; Liu et al. 2021). However, there is still a general lack of knowledge on downstream impacts of microbiomes on host health and fitness as studies often employ lethal sampling methods in a cross-sectional design, not allowing for subsequent observations on migration and

spawning outcomes. Transitioning to non-lethal sampling in fish microbiome research will be a valuable technique to explore the impacts of variations in migratory and spawning success in relation to microbiome compositions.

As mentioned earlier in this introduction, organismal physiology would benefit from looking at individuals through a holobiont perspective, that is, the host and its resident microbiota. This is particularly relevant when examining variation in organismal level fitness and associated fitness proxies such as migration success or failure. En-route mortality during spawning migrations is common among a variety of taxa. However, the effects in terms of lifetime fitness consequences are particularly apparent for semelparous species, such as Pacific salmonids (Cooke et al. 2006), where failure to reach the spawning grounds results in a negligible lifetime fitness (Dingle 1980). En-route mortality can occur due to several physiological factors, such as low osmoregulatory readiness, early depletion of endogenous energy reserves, or early senescence (Brett 1995; Carruth et al. 2002; Cooke et al. 2006; Hinch et al. 2006). Differences in migration difficulty, such as areas of increased river flow or temperature conditions, can further deplete limited energy reserves of migrating fish and cause premature mortality (Rand and Hinch 1998; Lee et al. 2003; Crossin et al. 2004).

Cooke et al. (2006) examined the mechanistic basis of individual mortality in sockeye salmon along their migration route by using telemetry and physiological assessments (of fish intercepted in the ocean) to compare successful migrants against those that died en-route. They found that early mortality was related to a high-stress state (e.g., elevated plasma lactate, glucose, and cortisol) and high somatic energy levels in fish that did not enter the river system. Further, high plasma osmolality and lower levels

of reproductive hormones were associated with fish that did not leave the lower and upper reaches of the Fraser River, respectively. Typically, these comparative studies have examined salmonid migration from a purely physiological viewpoint. Now, we understand that the gut microbiome plays an important role in modulating host physiology, energy metabolism, and immune function (Hansen and Olafsen 1999; Gómez and Balcázar 2008; Nayak 2010; Mouchet et al. 2012; Wu et al. 2012; Llewellyn et al. 2014; Dehler et al. 2017a). While mechanistic studies investigating the relationship of the gut microbiome with physiological factors that contribute to differential mortality for salmon undergoing spawning migrations are still lacking, this thesis examines more broadly the extent to which the gut microbiome may be associated with (or explain) variations in fitness. We see this as the first step in linking physiological and behavioural aspects of migration using a holobiont perspective lens.

1.4 Objectives and hypotheses

Characterizing the microbiome of transient species, such as those that initiate migration runs, is important because there are many dynamic factors at play that exert differential forces in shaping the gut microbiome. What is the effect of changing habitats, and very likely, changing microbial loads in the water, compounded with the effects of physiological changes and stress exerted on individuals on their migration run? The main objective of this Ph.D. is to investigate the hypothesis that the composition of the gut microbiome is related to the migration behaviour and fate of wild fishes. Specifically, I will assess hindgut microbial communities in three migratory fish species: white sucker (*Catostomus commersonii*), sockeye salmon (*Oncorhynchus nerka*), and brown trout

(*Salmo trutta*). Chapters in this thesis will be arranged logically in a framework that first examines spawning adult migrations, both in the context of potamodromous and anadromous migrations, where I will: (Chapter 2) characterize differences in the gut microbial community between individuals with different migratory timings, and (Chapter 3) characterize differences in the gut microbial community between individuals from different spawning populations. Examining anadromous migration from a juvenile standpoint, I will next (Chapter 4) characterize differences in the gut microbial community between individuals that migrated and those that remained resident. I will also provide a scientific perspective (Chapter 5) for transitioning towards non-lethal sampling methods for studying the gut microbiota of fish, particularly in the context of fish behaviour. Finally, in the general conclusion (Chapter 6), I will synthesize my overall findings, place them into context with the current literature base, and discuss relevant future directions.

1.5 Challenges

There were some logistical challenges in completing four chapters that resulted in them being excluded from this thesis, which also led to changes in the original research objectives and questions I addressed. Initially, spawning success was also a fitness proxy that was included in assessing the relationship of the gut microbial communities on host fitness. I collected data for this on female sockeye salmon in the Gates Creek spawning channel in both 2017 and 2018 by sampling the hindgut microbiota non-lethally from fish being held at the channel gate prior to entering, as well as externally tagging fish with anchor tags for identification. I was able to follow their behaviour over the subsequent

days due to the contained nature of the channel, and once the tagged fish died, I cut open the abdomen and checked if the fish had successfully spawned (no eggs retained) or were unsuccessful (full or most egg retained). I successfully collected two years' worth of data for this chapter. However, technical challenges during sample processing and sequencing of these samples (which I will discuss further in my general conclusions) resulted in unviable data for analysis. The same technical challenges with samples in the laboratory resulted in the exclusion of my non-lethal sampling methodology chapter, where I was comparing hindgut microbial communities using lethal vs. non-lethal sampling methods in white sucker. In place of this, I used a subset of the data with adequate sample reads to characterize the hindgut bacterial composition and diversity of migrating white sucker as an alternate chapter. Further, I attempted to do a temporal study using these non-lethal methods, where I externally tagged white suckers en-route to their spawning grounds and took hindgut bacterial samples, with the aim of repeat sampling the following year on a subset of the tagged individuals as they exhibit some sight fidelity. However, the following year only two returning adults were caught (and in general, it was the worst year for adult white sucker numbers since monitoring started at this location in 2010). Therefore, there were inadequate sample numbers for any temporal comparison. Next, I attempted a pilot experimental study, where I treated juvenile brown trout with either antibiotics and/or cortisol in a pen study to examine how the fecal bacterial communities changed under these experimental treatments. I experienced the same laboratory challenges on a large portion of the samples, and since it was a small-scale experiment, there were too few samples with adequate reads to perform statistical analysis. During the same timeframe, we completed the larger-scale experimental work on juvenile brown

trout in the Danish stream Kastbjerg, where we wished to examine the effects of antibiotics and/or cortisol on disrupting the fecal bacterial community and the resulting migratory decisions to outmigrate to sea or remain resident, against a control group of fish. Unfortunately, upon re-sampling the stream after the migration period, it was found that the stream had been greatly disturbed by the likely introduction of fertilizer, as it was completely overgrown with thick algae, and no fish were found instream (neither tagged fish from our experiment nor any brown trout in general). As a result of these technical and logistical challenges, these chapters were also excluded from the final thesis. In lieu, I completed a perspective chapter (Chapter 5), which discusses transitioning to non-lethal sampling methods, especially in the context of studying behaviour when studying wild fish.

1.6 Scientific contribution

The gut bacterial community under migratory influences has only started to be touched on in fish (Llewellyn et al. 2016; Le Doujet et al. 2019; Element et al. 2020a, 2020b; Le and Wang 2020; Ying et al. 2020; Liu et al. 2021). It is an interesting avenue for microbiome research as both abiotic and biotic processes under migration are highly variable and dynamic. This has significant consequences for the fitness of animals if the microbiome composition shifts unfavourably in response to migration conditions, which can have downstream unknown evolutionary effects. Further, the idea that variation in microbiome composition could be associated with (or explain) variation in fitness using a migratory model has yet to be addressed in the literature. This thesis attempts to characterize the hindgut bacterial communities of three migratory species from their wild

habitats. In addition, there does not appear to be any publications of the white sucker gut bacterial community; therefore, this represents a novel species account to add to the literature base.

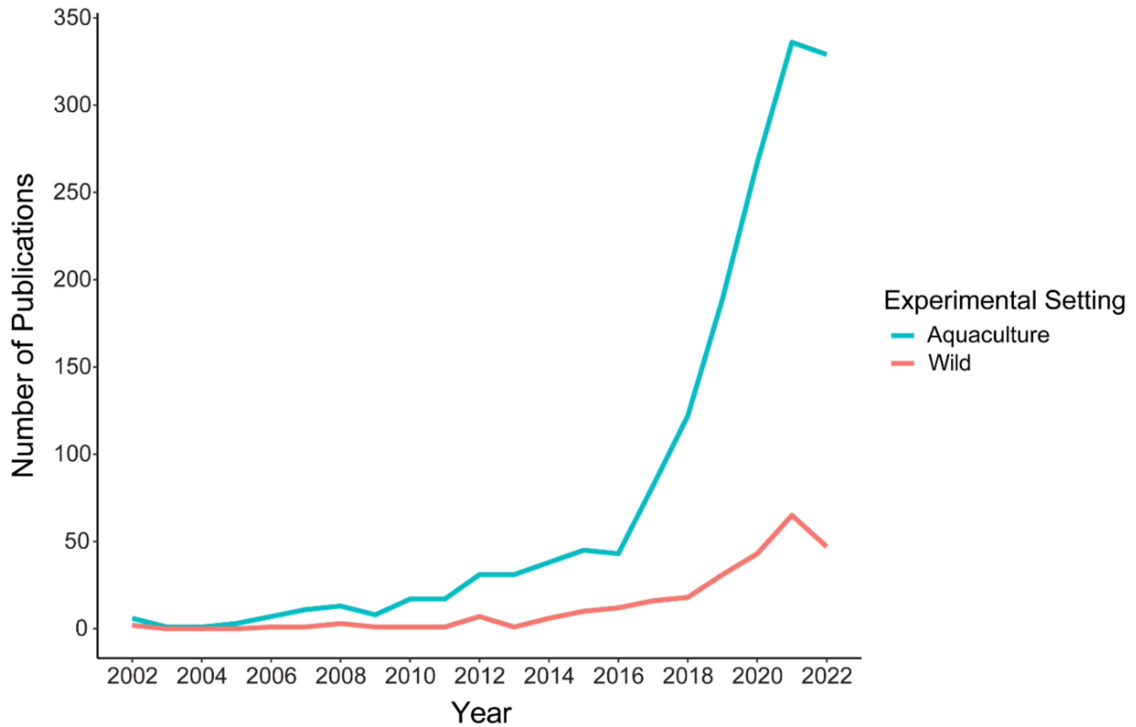

Figure 1-1. Number of publications between 2002 and 2022 gathered from the Web of Science database. Data was collected using the following search string for aquaculture: TS=((captive OR domestic OR aquaculture OR culture$ OR farm*) AND (fish$ OR teleost) AND ("*gut microb*" OR "*intestinal microb*" OR "fecal microb*" OR "faecal microb*") NOT (shrimp) NOT (human)), which totaled 1660 publication records; and wild: TS=((wild) AND (fish$ OR teleost) AND ("*gut microb*" OR "*intestinal microb*" OR "fecal microb*" OR "faecal microb*") NOT (shrimp) NOT (human)), which totaled 271 publication records. Plot was created using R (v4.0.5) and ggplot (v3.3.5).

Chapter 2: The hindgut microbial composition and diversity of a potamodromous freshwater fish varies by migration timing, but not sex or tumor presence.

2.1 Abstract

Gut microbiota play an essential role in maintaining host health by providing many beneficial services, such as nutrient absorption, energy metabolism, and modulating innate immune responses. These functions may play a role in supporting the fitness of migrating and spawning fish such as the white sucker (*Catostomus commersonii*), an abundant but understudied freshwater teleost species. We analyzed the bacterial communities of migrating white suckers using 16S rRNA amplicon sequencing to uncover associations between bacterial community composition and migration timing, sex, and tumor prevalence in white sucker fish. Proteobacteria, specifically operational taxonomic units delineated as *Aeromonas,* predominated across the sampled population. No differences in bacterial diversity or community composition were observed between sex or tumor prevalence. However, the late migrant group displayed lower diversity and a significantly different community composition, driven by the presence of the OTU delineated as *Mycoplasma*. Higher abundances of 13 OTUs associated with the peak migrant group further contributed to differences in community composition. This is an important first step for integrating microbiota characterization into physiological and behavioural studies and provides a more holistic view of fish health and fitness, which warrants further study.

2.2 Introduction

Vertebrates harbour a diverse microbial community within their intestinal tract, termed the gut microbiome, that is known to play a role in supporting the health and fitness of their host (Walter and Ley 2011; Sommer and Bäckhed 2013). In general, the community structure of the gut microbiome is influenced by both exogenous (i.e., environment, host diet) and endogenous (i.e., host genetics) factors (Spor et al. 2011). Some species have been shown to share a core gut microbiome, defined as the common microbial members, genes, or functions shared across different habitats (Roeselers et al. 2011; Shade and Handelsman 2012). This core microbiome may provide an adaptive advantage to the host (McDonald et al. 2012; Wong et al. 2013; Romero et al. 2014; Risely 2020), with inter-individual variation potentially acting as selective pressure for host adaptation, fitness, and evolution (Suzuki 2017). Because of the potential role that gut microbes play in host fitness and behaviour, plasticity of the gut microbiota may have downstream effects on host phenotypic plasticity and differential survival during times of rapid environmental change (Alberdi et al. 2016). As such, there has been a recent interest in looking more holistically at organismal physiological and evolutionary research through the lens of the host-microbiome symbiotic relationship (Bordenstein and Theis 2015). Much of this research has been generated using mammalian models, particularly humans, however fish comprise a significant portion of vertebrate species, and microbiome research in this area is still under-appreciated (Llewellyn et al. 2014; Luna et al. 2021).

The bulk of fish gut microbiome research has been anchored to fish species relevant to the aquaculture industry (Burtseva et al. 2021), where case studies have

shown that manipulating fish diets can improve fish health and disease resistance (Kumar et al. 2008; Barbosa et al. 2011; Dimitroglou et al. 2011; Boutin et al. 2013; Geraylou et al. 2013). However, aquaculture environments are dissimilar to wild environments; fish densities are artificially high, and fish physiology and behaviour are altered (Llewellyn et al. 2014). Further, therapeutics and antimicrobial drugs are often used to prevent and treat disease outbreaks (Yukgehnaish et al. 2020). As such, there are differences in the gut microbiota of aquaculture vs. wild fish species (Dhanasiri et al. 2011; Kormas et al. 2014; Eichmiller et al. 2016; Ramírez and Romero 2017), creating a need to diversify fish microbiome research to other species with different life cycles.

Of particular relevance are migratory fish species, as migration acts as an additional layer of complexity to the study of gut microbiomes because these animals are exposed to a range of different microbes as they move across heterogeneous environments and encounter different food sources (Risely et al. 2017). During this time, animals undergo physiological changes to accommodate their changing environments (Evans et al. 2011; Lennox et al. 2016), and in the case of spawning migration, sex-specific variations in endocrinology, morphology, and behaviour are often observed (Scott et al. 1984; Fitzpatrick et al. 1986; Hanson et al. 2008). In addition, migration is an energetically-taxing event that impairs immune function of the host, leading to differential disease expression across populations, with potential downstream fitness consequences if individuals die en-route (Altizer et al. 2011). Independently, these exogenous and endogenous factors are all key contributors to shaping the gut microbiome of an animal host (Ley et al. 2008b; Sullam et al. 2012; Sommer and Bäckhed 2013; Miyake et al. 2015; Eichmiller et al. 2016; Dehler et al. 2017a; Kelly and Salinas 2017),

and so, migration likely exerts a multi-factorial impact on the structure and composition of the gut microbiome. Recent research has begun to investigate the role of migration on the gut microbiome in fish (Llewellyn et al. 2016; Rudi et al. 2018; Le Doujet et al. 2019; Element et al. 2020a, 2020b; Le and Wang 2020; Le et al. 2020; Ying et al. 2020; Liu et al. 2021), however, there is still a general lack of knowledge on downstream impacts of altered microbiomes on host health and fitness.

One example of a migratory species is the white sucker (*Catostomus commersonii*), a common teleost iteroparous species found in lakes and rivers of North America (Corbett and Powles 1983). Migration begins in early spring, when fish undertake a potamodromous migration from lakes and rivers into spawning tributaries, travelling up to 40 km (Doherty et al. 2010), and peak numbers occur once the daily maximum water temperatures reach 10°C (Corbett and Powles 1983). White suckers have been investigated for their potential to be used as aquatic ecosystem indicator species, as they are prone to epidermal papillomas and lip tumors in polluted waters (Smith and Zajdlik 1987; Baumann 1992). The pathology of these papillomas and tumors appears to be an unknown viral etiology (Premdas and Metcalfe 1996) and possibly linked to immunosuppression due to environmental stressors (Reizenstein 1983; Anderson 1990) or chemical contaminants (Anderson 1990). The gut microbiome is involved in the modulation of the host's immune system (Gómez and Balcázar 2008) and water pollution, such as sewage (Giang et al. 2018; Sakalli et al. 2018) and heavy metals (Dahan et al. 2018; Cheaib et al. 2020) have been shown to cause gut microbiome dysbiosis in fish. However, the connection between water pollution, gut microbiota composition, and tumor prevalence is currently unknown.

As a potamodromous migrating species, white suckers provide a good model to understand the links between migration and the gut microbiota in freshwater environments. Conversely, the transition from saltwater to freshwater (or vice versa) that occurs in anadromous migrations introduces additional confounding variables as the microbial communities of these environments are so vastly different (Fortunato et al. 2012; Dehler et al. 2017b; Rudi et al. 2018). The present study investigates associations between migration timing, sex, and tumor prevalence with the hindgut microbial diversity and composition of a migrating population of white sucker fish in their natural environment.

2.3 Methods

2.3.1 Fish and microbiome sample collection

Migrating white sucker fish were sampled between April and June 2017 from a weir trap on Cobourg Creek (43° 57’33.696” N, 78° 10’55.884” W), a tributary of Lake Ontario (Figure 2-1). Fish caught in the weir trap overnight were promptly removed each morning with a dip net, placed in a 150 L cooler of water with an oxygenator, and processed individually. Fish were euthanized via cerebral percussion and then weighed, measured, and the general health status of the fish (female/male, mature/immature, healthy/injured/diseased) was noted. Following this, the exterior ventral surface of the fish was cleaned with ethanol before full intestines were removed aseptically and placed on a sterile surgical pad. A sterile scalpel was then used to make an incision in the hindgut, approximately 8 cm from the anus, and a sterile cotton swab (Puritan, Guilford, ME) was inserted, and a 4 cm section was swabbed thoroughly, ensuring to scrape the

mucosal layer of the tract so that both the digesta (if present) and mucosal bacterial communities could be sampled. The swab tip was broken off within a sterile 2 ml cryovial (Thermo Fisher Scientific*,* San Diego, CA*)* containing 1.5 ml of RNAlater stabilization solution (Invitrogen, Carlsbad, CA). The vials were then capped and placed on ice before transfer to -20 °C within 6 hours of sampling. Care was taken to avoid any environmental contamination of the swab, and any potential contamination was noted. Post-field season, samples were transported to the laboratory for long-term storage at -20 °C until processing.

2.3.2 DNA extraction

Swab samples were thawed at room temperature and centrifuged for 7 minutes at 12000 RPM. 1 ml of RNAlater was removed and replaced with PBS buffer, and samples were vortexed for 10 minutes. Swabs were then removed from their original vials and put into a powerbead tube from the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). The original vials were centrifuged for 7 minutes to produce a small pellet. PBS buffer was removed and discarded, and the pellet was resuspended in 100 µl PBS buffer and transferred to the powerbead tube containing the swab. DNA extractions were performed using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the following modifications; Step 2: 60 µl of C1 was added to the powerbead tube, vortexed briefly, and then incubated at 65 °C for 15 minutes. Steps 3 and 4: In lieu of vortexing for 10 minutes, a Retsch MM 400 Mixer Mill (Thermo Fisher Scientific, MA, USA) was used for one minute of 30 cycles/second to mix and homogenize the samples. Step 19: 50 µl of elution buffer was added to the white

filter membrane and incubated at room temperature for 5 minutes before centrifuging at 12000 RPM for 1 minute. DNA was quantified using the Qubit™ dsDNA HR Assay Kit (Thermo Fisher Scientific, MA, USA), and due to the relatively low DNA yields following extraction, samples included for sequencing were concentrated using a Savant DNA 120 SpeedVac Concentrator (Thermo Fisher Scientific, MA, USA) for 30 minutes.

2.3.3 16S rRNA gene sequencing

Amplification of the variable region V4 of the 16S rRNA gene was performed to characterize the microbial communities sampled from individual fish using a sequencing protocol previously described by Kozich et al. (2013). Briefly, PCR was conducted using 12.5 µL of NEB Q5® Hot Start High Fidelity 2X Master Mix polymerase (New England Biolabs, Ipswich, USA), 2.5 µL of 5 µM Forward and 5 µM Reverse index primer pool, 8 µL of nuclease-free water, and 2 µL of template DNA. Thermocycling conditions for the amplicon PCR were as follows: initial denaturation at 98°C for 30 seconds, then 98°C for 10 seconds, 30 annealing cycles of 55°C for 30 seconds and 72°C for 20 seconds, and a final extension of 72°C for 5 minutes, followed by an 8°C hold.

PCR reaction clean-up was performed on the libraries using AMPure XP PCR clean-up beads (Beckman Coulter, Indianapolis, USA) according to the manufacturer's specifications. Five µL from all PCR amplicons were pooled together to create the library and concentrated in the Savant DNA 120 SpeedVac Concentrator (Thermo Fisher Scientific, MA, USA) for approximately 3 hours to reach a volume of 30 µL. The pooled libraries (approximately 390 bp in length) were further purified using e-gel size select 2% gel on an E-Gel® iBase™ (Invitrogen, CA, USA), and the library size distribution was

assessed using the Agilent High Sensitivity DNA kit (Agilent, Santa Clara, USA) on a 2100 Agilent Bioanalyzer (Agilent, Santa Clara, USA). The final library molarity was assessed using the NEBNext® Library Quant Kit (New England Biolabs, Ipswich, USA) for Illumina® using ROX for normalization on an Applied Biosystems StepOnePlus™ System (Applied Biosystems, MA, USA) and the resulting library concentration was 2 nM.

Samples were sequenced on an Illumina MiSeq system using a MiSeq® Reagent Kit v3 with 500 cycles (2x250; Illumina, San Diego, USA), following the manufacturer's protocol and using a 15 % spike of PhiX Control v3 (Illumina, San Diego, USA).

2.3.4 Sequencing data processing and statistical analysis

Sequences were processed in mothur (version 1.35.1), as referenced by Schloss et al. (2009). Operational taxonomic units (OTUs) were assigned based on 97 % similarity, and taxonomy was assigned using the SILVA reference database (version 138; Quast et al. 2013). Samples containing less than 569 reads were discarded, and all remaining samples were rarified to 569 reads. This number was chosen to balance the inclusion of the maximum number of samples and sufficient read depth. Rarefied OTU tables were used for all downstream analyses in RStudio (v1.4.1106) for R (R Core Team 2021; v4.0.5), barring the LEfSe analysis that used the unrarefied OTU table (Segata et al. 2011).

Relative abundance of the top bacterial phyla and families among sampled white sucker fish was visualized using phyloseq (McMurdie and Holmes 2013; v1.34.0) and ggplot (Wickham 2016; v3.3.5). Alpha (Shannon-Weiner index) and beta diversity were

calculated in phyloseq (McMurdie and Holmes 2013; v1.34.0). A non-parametric Wilcoxon rank-sum test was used to test for significant differences ($p < 0.05$) in alpha diversity between groups (migration time, sex, presence of tumors) of white suckers. Beta diversity was assessed through non-metric multi-dimensional scaling (NMDS) ordination performed on a Bray-Curtis distance matrix to visualize the dissimilarity within and between the white sucker groups (migration time, sex, presence of tumors), using two dimensions (Bray and Curtis 1957). To assess if there was a significant difference in beta diversity between the white sucker groups (migration time, sex, presence of tumors), a permutational multivariate analysis of variance (PERMANOVA; $p < 0.05$) was performed with 999 permutations on the Bray-Curtis distance matrix in the R vegan package (Oksanen et al. 2020; v2.5-7). The variability of microbial community composition among samples of white sucker fish between migration times was assessed using an analysis of multivariate homogeneity of group dispersions (betadisper; $p < 0.05$) on the Bray-Curtis distance matrix (Anderson 2006) in the R vegan package (Oksanen et al. 2020; v2.5-7).

Finally, differentially abundant OTUs were determined using a linear discriminant analysis (LDA) in LEfSe with an alpha value of 0.05 and threshold logarithmic LDA score of 3 (Segata et al. 2011). LEfSe is a non-parametric statistical test that first determines which OTUs have significantly different abundance between groups and then ranks them based on their logarithmic LDA score.

2.4 Results

2.4.1 Sequence quality

The sequencing run produced a total of 42,185 raw reads across 23 input libraries. Due to low read numbers, nine samples were excluded after normalization to 569 reads, which was the read number of sample LK188, so that it could be retained in the dataset. After rarefaction, the final dataset contained 7,966 reads from 14 samples for downstream analysis, corresponding to 551 unique OTUs. An overview of the corresponding sample metadata is provided in Table 2-1, and the rarefaction curve in Figure 2-2.

2.4.2 Hindgut microbial community composition and diversity

The bacterial composition of the hindgut of white suckers was characterized using the relative abundance of OTUs from all rarefied samples. At the phylum level, this constituted a total of 15 phyla, of which seven were dominant and accounted for 97.67 % of the OTU sequences (Figure 2-3). The most abundant phylum of bacteria across samples was Proteobacteria (77.62%), followed by Fusobacteria (7.62%), an unclassified bacterial phylum (4.43%), Firmicutes (2.26%), and Tenericutes (2.22%). Even at the phylum level, some inter-specific variation was observed among individuals and between late and peak migrants (Figure 2-3). Fish that migrated during the peak migration time had gut microbiota compositions that were more diverse than fish that migrated at the end of the migration run; this is indicated by the higher proportion of "Other" phyla in the peak migrants' relative abundance compared to the late migrants (Figure 2-3). It should also be noted that samples LK139 and LK160 did not reach a plateau on the rarefaction curve, indicating that they did not sequence deeply enough to capture all OTUs (Figure 2-

2). Peak migrants were enriched in Acidobacteria, Actinobacteria, and Verrucomicrobia compared to late migrants (18.1X, 7.31X, and 3.29X more than late migrants, respectively), while late migrants were enriched in Tenericutes, Firmicutes, and Proteobacteria compared to peak migrants (no Tenericutes present in peak migrants, 4.14X, and 2.45X more than peak migrants, respectively; Figure 2-3).

At the family level, 13 families accounted for 93.3 % of the OTU sequences (Figure 2-4). The most abundant family was *Aeromonadaceae* (67.86 %), followed by *Fusobacteriaceae* (7.62 %), an unclassified bacteria (4.43 %), *Enterobacteriaceae* (3.64 %), and *Mycoplasmataceae* (2.22 %). Grouping the samples based on migration time, we see that peak migrants were enriched in *Sphingomonadaceae, Gp6_unclassified,* and *Fusobacteriaceae* (33.5X, 16.7X, and 1.53X more than late migrants, respectively), while late migrants were enriched in *Mycoplasmataceae, Clostridiaceae,* and *Enterobacteriaceae* (no *Mycoplasmataceae* in peak migrants, 7.59X, and 4.09X more than peak migrants, respectively; Figure 2-4).

Alpha diversity, including observed species richness, Chao1 diversity, inverse Simpson diversity index (S), and Shannon diversity index (H`), were calculated for each fish sample at the OTU level (Appendix A). Shannon diversity index (H`) was examined under three groupings: migration time, sex, and tumor presence/absence. Shapiro-Wilk tests showed that the assumption of normality was violated ($W=0.69$, $p=0.0003$); therefore, a Wilcoxon rank-sum test was used. Alpha diversity of peak migrants was significantly higher than that of late migrants ($W=42$, $p=0.007$; Figure 2-5) but did not vary significantly by sex ($W=19$, $p=0.57$) or tumor presence/absence ($W=19$, $p=0.57$).

Beta diversity was described using non-metric multidimensional scaling (NMDS), where bacterial communities of fish hindgut samples were visualized based on a Bray-Curtis dissimilarity matrix. Only migration time showed some separation of fish hindgut microbial communities (Figure 2-6). PERMANOVA analyses further supported this conclusion; migration time had significant differences in microbial composition between peak and late migrants (p=0.038); while sex and tumors did not show significant differences (p=0.737 and p=0.259, respectively; Table 2-2). In addition, NMDS analysis showed greater inter-individual variation among peak migrants than late migrants, as represented by the larger 95 % confidence ellipse for peak migrants, indicating the hindgut microbial composition of peak migrants varied more between individual fish (Figure 2-6); however, this dispersion was not significantly different (betadisper; p=0.078; Table 2-3).

The LEfSe analysis further showed that 13 OTUs had significantly higher abundance in peak migrants but only one OTU with significantly higher abundance in the late migrants (Figure 2-7). The overrepresented OTU in the late migrants (Otu0016) belonged to the genera *Mycoplasma*.

2.5 Discussion

The present study characterized the hindgut composition and diversity of the potamodromous fish species *C. commersonii* and, in doing so, emphasized the need to incorporate different life-history traits into microbiome research. To date, relatively few studies examine migratory tendencies on the structure and function of the gut microbiome, and of those studies most focus on anadromous species (Llewellyn et al.

2016; Hamilton et al. 2019; Element et al. 2020a, 2020b). In general, there is a paucity of data on the gut microbiomes of wild fish that lie outside the realm of cultured species (e.g., salmonids, carp, zebrafish), making it somewhat challenging to place the results of the present study into context. However, the importance of the gut microbial community to fish health, nutrition, immunity, and pathogen defense is now well documented (Nayak 2010).

The bacterial communities of peak migrants were significantly different from late migrants, as shown by our diversity and community composition analyses (Figures 2-5, 2-6, 2-7). Spawning white suckers can migrate relatively long distances to their spawning grounds; however, outside of the spawning season, they appear to maintain small home ranges and show strong site fidelity (Doherty et al. 2010). The difference in the composition of the bacterial communities between peak and late migrants may be explained by their non-spawning home range. Local environment and diet have been shown to exhibit a strong selective pressure on fish gut microbial composition within species (Luczkovich and Stellwag 1993; Hansen and Olafsen 1999; Nayak 2010; Smith et al. 2015). Late migrants may be individuals coming from further reaches of Lake Ontario, while peak migrants may be coming from shorter distances. Using stable isotope analysis (Blazer et al. 2014) found that spawning populations of white sucker in the lower St. Louis River were composed of individuals from distant and nearby habitats, indicating convergence.

An alternate explanation could be that late migrants may have had their migration slowed or interrupted due to disease, longer stop-over sites for refuelling, weather, or other reasons. Many factors that could impact migration timing could also have a

correlative impact on that individual's microbiome. The current study found that late migrants had significantly lower alpha diversity in their hindgut microbiomes than peak migrants. Infection status was associated with reduced movement capacity and slightly delayed migration timing in a meta-analysis across migratory taxa (Risely et al. 2018). From a microbiome perspective, pathogens reduce alpha diversity by outcompeting commensals for space within the fish gut environment (Xiong et al. 2019), and low alpha diversity has been correlated with diseased crucian carp (*Carassius auratus*; Li et al. 2017a). Furthermore, the LEfSe analysis indicated that late migrants were significantly enriched in the genus *Mycoplasma*, which was not present in peak migrants. While *Mycoplasma* appears to be a normal component of some fish gut microbiomes (e.g., salmonids; Holben et al. 2002; Llewellyn et al. 2016; Dehler et al. 2017b), some strains within the genus are pathogenic and cause fish disease (Legrand et al. 2020b; Sellyei et al. 2021). Therefore, the low alpha diversity in the late migrants may favour colonization by *Mycoplasma* as there is less competition for space. It should be noted that in the current study, the resolution of bacterial taxa was only to genera and, in some cases, only to family. We cannot say definitively that pathogenic bacteria species were present – only that genera or families that contain pathogenic bacteria members were present. Further studies, including whole-genome sequencing, could help elucidate the pathogenicity within these broader taxa.

The most abundant phyla of gut microbes across the entire sampled migrating population of white suckers were Proteobacteria, Fusobacteria, an unclassified bacteria, Firmicutes, and Tenericutes. This aligns with previous research on freshwater fishes, barring the unclassified bacteria (Sullam et al. 2012; Eichmiller et al. 2016).

Proteobacteria represented almost 78 % of sequences in the current study, which is similar to previous research that found Proteobacteria to be the dominant phyla in fish gut microbiomes (Wu et al. 2010; Roeselers et al. 2011; Sullam et al. 2012). One deviation from the reported common phyla of freshwater fishes was the low prevalence of Bacteroidetes in our study. This could be an artifact of freezing the samples prior to DNA extraction, which has been shown to alter the ratio of Firmicutes to Bacteroidetes (Bahl et al. 2012), or it could be a true representation for this understudied fish species. A smaller proportion of Bacteroidetes was also found in several carp species, which are fellow Cypriniformes (Han et al. 2010; Wu et al. 2010, 2012; Eichmiller et al. 2016). We also found a high prevalence of unclassified bacteria at the phylum level (4.43 % of reads), representing 107 separate OTUs of varying abundances across samples. This indicates that there is still much to be characterized within the fish gut microbiota, and further studies are needed.

The most abundant families and genera of gut microbes across this population were *Aeromonadaceae* (*Aeromonas*)*, Fusobacteriaceae* (*Cetobacterium*), an unclassified bacteria, *Enterobacteriaceae, Mycoplasmataceae* (*Mycoplasma*), and *Clostridiaceae* (*Clostridium sensu stricto*). These taxa are all reported to be common colonizers in the intestinal tract of freshwater fish (Nayak 2010; Llewellyn et al. 2014). *Aeromonas* dominated the sampled population with 67.9 % of the total sequences. This is in line with previous research that found *Aeromonas* to be the dominant genera in fish gut microbiota and freshwater environments (Cahill 1990; Roeselers et al. 2011; Sullam et al. 2012). *Cetobacterium* was the second most abundant genera recovered and contains members that produce vitamin B_{12}, which has been hypothesized to serve a role in synthesizing

vitamins for their host (Romero et al. 2014). Members of *Enterobacteriaceae* and *Clostridiaceae* are also commonly recovered from fish intestinal tracts and can act as both commensals or pathogenic members (Wu et al. 2012, Sevellec et al. 2018). As commensals, *Enterobacteriaceae* and *Clostridiaceae* contain members that aid in the digestion of cellulose and are commonly found in herbivorous and omnivorous fishes such as carp (van Kessel et al. 2011; Wu et al. 2012). White suckers have a very generalist diet, and as detritus-feeding omnivores, they feed on both benthic plants and invertebrates (Ahlgren 1996).

No significant difference in diversity or community composition was found for sex or tumor presence in the sampled population of white suckers, though it should be acknowledged that the sample size was small, and potential differences might have been missed. The relationship between sex and the hindgut microbial community was examined as this was a spawning migration run of white suckers, and major endocrine changes, as well as other sex-related variations in traits, occur as fish prepare to spawn (Scott et al. 1984; Hanson et al. 2008). Estrogens and androgens, as well as cortisol, are known to modulate the immune system of fish (Campbell et al. 2021) and, in turn, could influence the hindgut microbial composition. In humans, sex hormones were associated with gut microbiome diversity and composition (Shin et al. 2019). The incidence of tumor presence on white suckers in Lake Ontario can sometimes be correlated with pollution (Smith and Zajdlik 1987). Chemical pollutants have been shown to disrupt the gut microbial communities of fish and other aquatic organisms and cause dysbiosis (Evariste et al. 2019). While we did not observe any significant effect of tumor prevalence on gut microbial communities, tumor development on white suckers is not

always an indicator of water contaminants. Smith and colleagues found in another study the presence of high tumors in both polluted and non-polluted sites, suggesting a multi-factorial etiology (Smith et al. 1989a, 1989b).

2.5.1 Limitations of study

The current study provided only an overview of the bacterial community of the hindgut of migrating white suckers, and it did not delve into any potential explanatory variables for differences in the observed bacterial communities between peak and late migrants. Stable isotope analysis would add strength to the hypothesis that the late migrants were from a different area of Lake Ontario than peak migrants and help elucidate the population structure of a heterogenous aggregation of spawning individuals. Additionally, the low diversity found in the late migrants may be due to microbial dysbiosis, and so a combination of bacterial culturing and metagenome sequencing could identify if any pathogenic bacteria strains are present. As with all fish-associated microbiota studies, the bacterial composition uncovered through 16S rRNA gene sequencing represents a snapshot picture of the representative diversity of bacteria in that location and time. Different compartments within the intestinal tract have been shown to harbour different microbial assemblages (Gajardo et al. 2016), so care must be taken to avoid extrapolating results of one compartment to the gastrointestinal tract as a whole.

2.5.2 Conclusions

This study characterized the hindgut bacterial community of migrating white suckers as they prepared to spawn in Cobourg Creek, Ontario. While *Aeromonas*

dominated the bacterial community in our sampled population, we noted a significant difference in diversity and composition between white sucker migrants during the peak vs. late run times. Late migrants were less diverse and harboured the potentially pathogenic bacterial genus, *Mycoplasma*. The basis for this difference was unclear, but exploring the prevalence of *Mycoplasma* in other fish species during migration using targeted amplicon approaches to detect and quantify *Mycoplasma* may help elucidate our findings. Metagenomically assembled genomes could also provide resolution to species level and identify if a pathogenic species of *Mycoplasma* is present. Many questions remain, including the extent to which variation in the microbiome community is associated with individual migration success and fitness.

Table 2-1. Description of migrating adult white sucker fish sampled for this study.

Sample	Date	Migration Time	Sex	Reproductive State	Tumor Presence	Fork Length (cm)	Weight (g)
LK139	2017-May-01	Peak	M	Mature	No	36	712
LK160	2017-May-02	Peak	M	Mature	Yes	38	841
LK163	2017-May-02	Peak	M	Mature	Yes	37.5	906.5
LK188	2017-May-06	Peak	F	Immature	No	43	1519
LK191	2017-May-06	Peak	M	Mature	Yes	38	881
LK207	2017-May-18	Late	M	Mature	No	34.5	666.5
LK249	2017-May-24	Late	F	Immature	Yes	39	952.5
LK251	2017-May-24	Late	F	Mature	No	43	1430
LK254	2017-May-24	Late	F	Immature	No	39	1075
LK299	2017-May-26	Late	M	Mature	Yes	38.5	855.5
LK302	2017-May-26	Late	F	Immature	No	42.5	1366
LK306	2017-May-26	Late	F	Immature	No	44	1612
LK310	2017-May-26	Late	F	Immature	No	38	1026
LK312	2017-May-26	Late	F	Immature	Yes	44.5	1766

Table 2-2. Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance of hindgut bacterial communities of white suckers for groupings: Migration time, sex, and tumors. Df = degrees of freedom, SS = sum of squares, MS = mean squares, F. Model = F-value by permutation, R2 = percentage of variance explained by the groups, Pr(>F) = p-value for F-statistic. P-values based on 999 permutations. *Difference is significant at 0.05 level.

	Df	SS	MS	F. Model	R2	Pr(>F)
Migration time	1	0.25573	0.255726	2.21212	0.1579	0.038 *
Sex	1	0.04465	0.044648	0.38622	0.02757	0.737
Tumors	1	0.16313	0.16313	1.41113	0.10073	0.259
Residuals	10	1.15602	0.115602		0.7138	
Total	13	1.61953			1	

Table 2-3. Analysis of multivariate homogeneity of group dispersions (betadisper) based on Bray-Curtis distance of hindgut bacterial communities of late and peak migrating white suckers. Df = degrees of freedom, SS = sum of squares, MS = mean squares, Pr(>F) = p-value for F-statistic.

	Df	SS	MS	F value	Pr(>F)
Groups	1	0.21541	0.215406	3.7099	0.07811
Residuals	12	0.69676	0.058063		

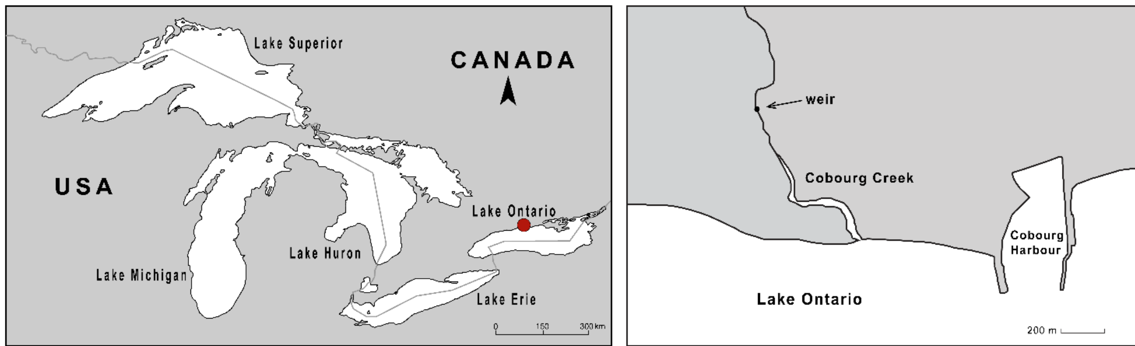

Figure 2-1. Study site map showing A) location of Cobourg within the Great Lakes system, and B) location of the weir where sampling occurred, approximately 800m from the entrance to Lake Ontario. Figure was created using Adobe Illustrator version 25.0; base-map data from Google maps ©2021

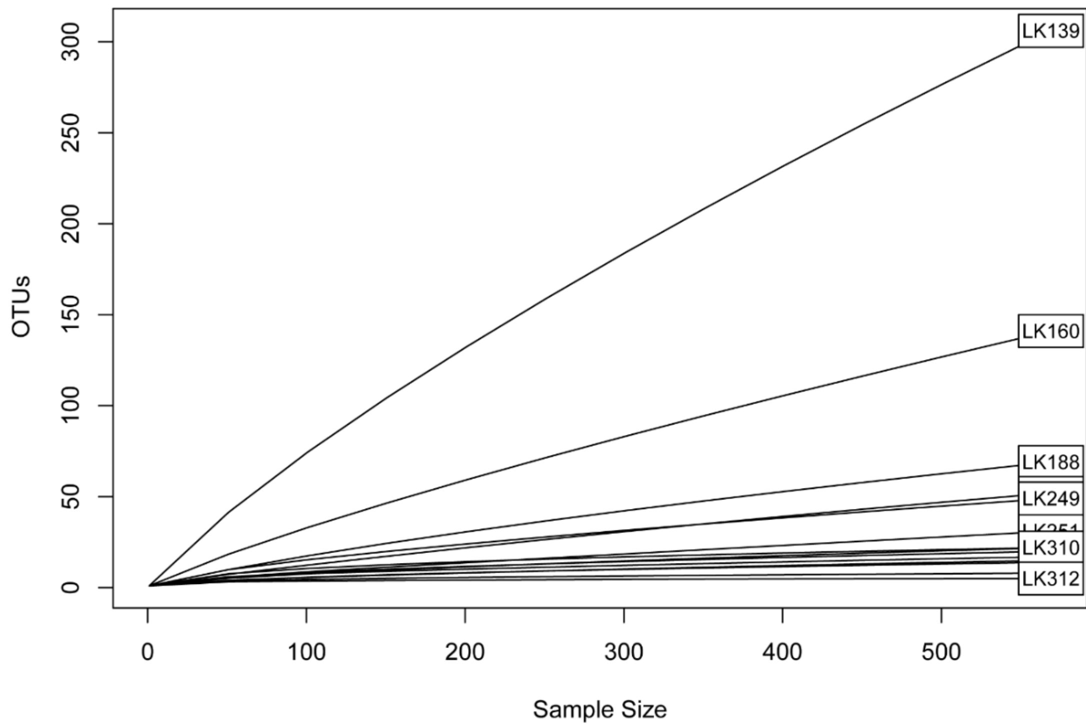

Figure 2-2. Rarefaction curve showing the number of OTUs on the vertical axis against the number of sequences (sample size) on the horizontal axis for the 14 white sucker fish samples.

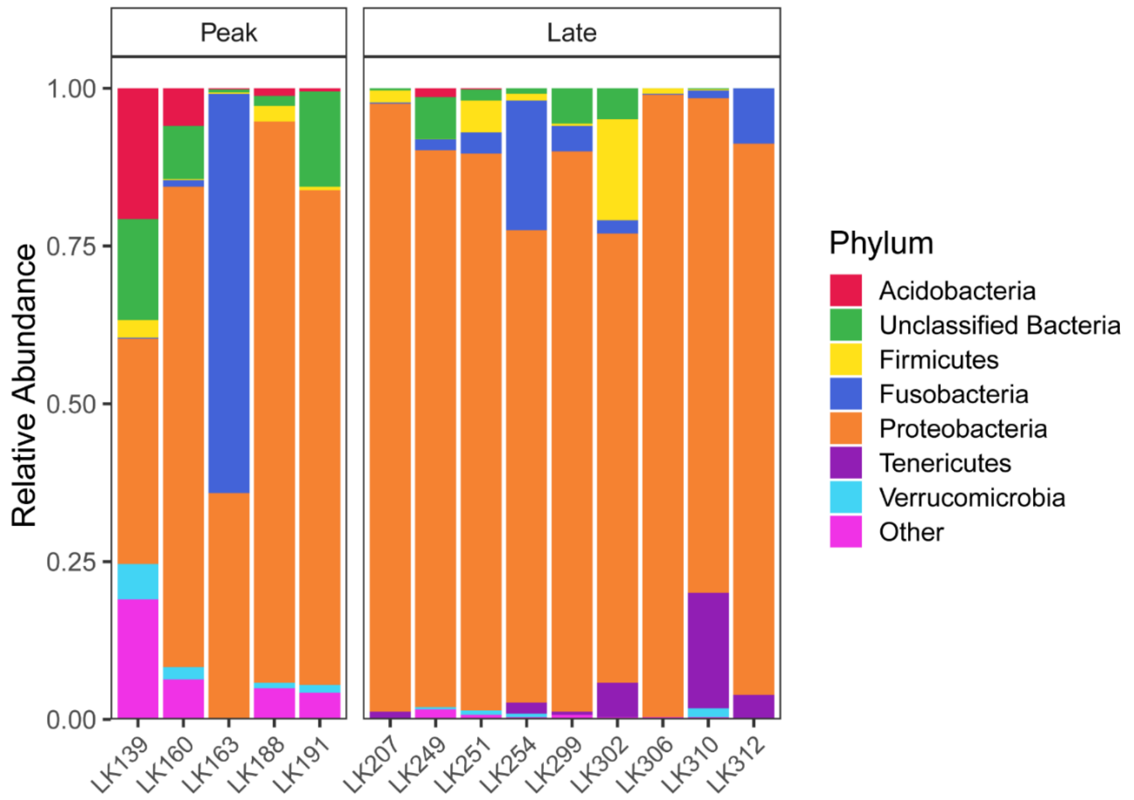

Figure 2-3. Relative abundance of the major phyla present across all samples grouped into peak and late migration timing.

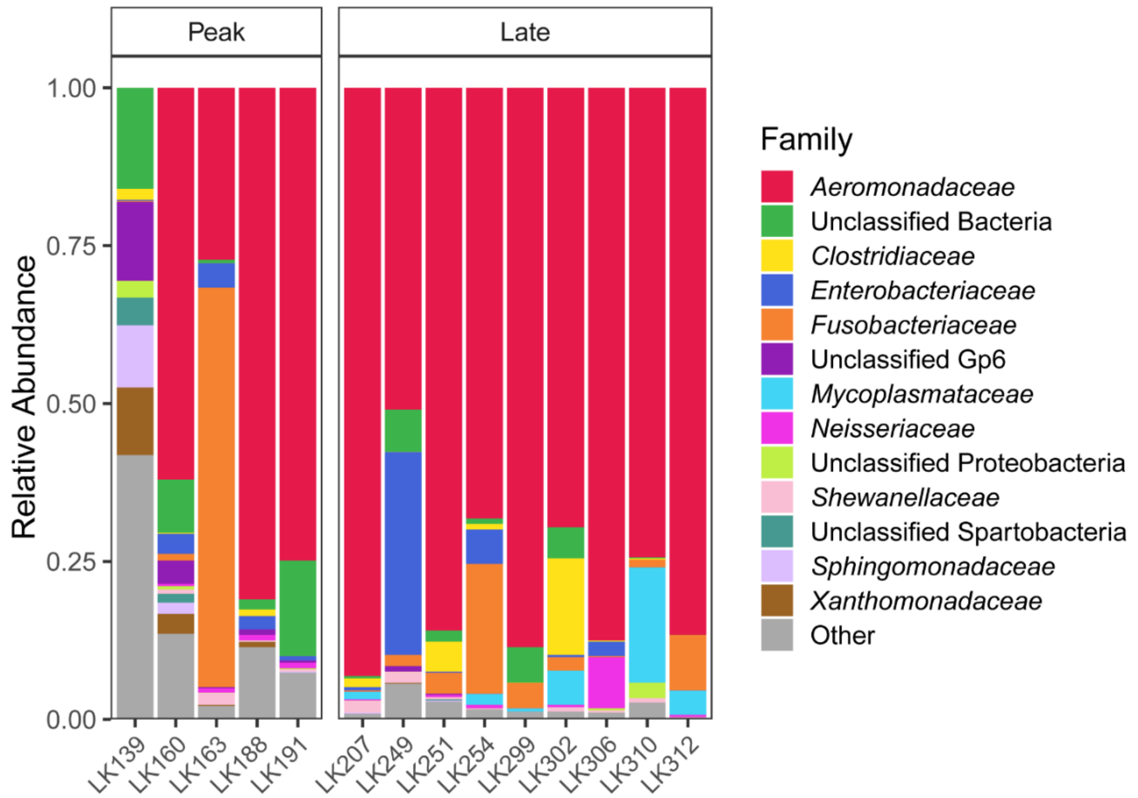

Figure 2-4. Relative abundance of the major families present across all samples grouped into peak and late migration timing.

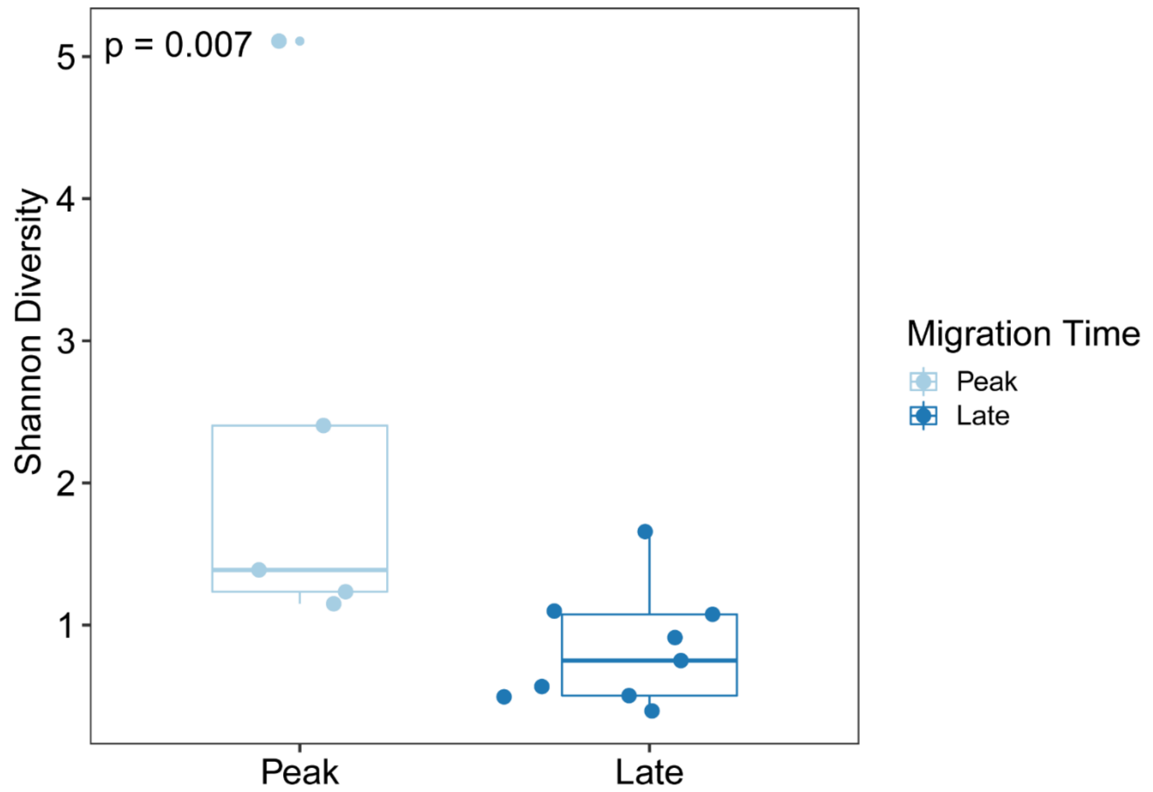

Figure 2-5. Shannon alpha diversity measure on operational taxonomic units (OTUs) of hindgut bacterial community of peak and late migrating white sucker fish.

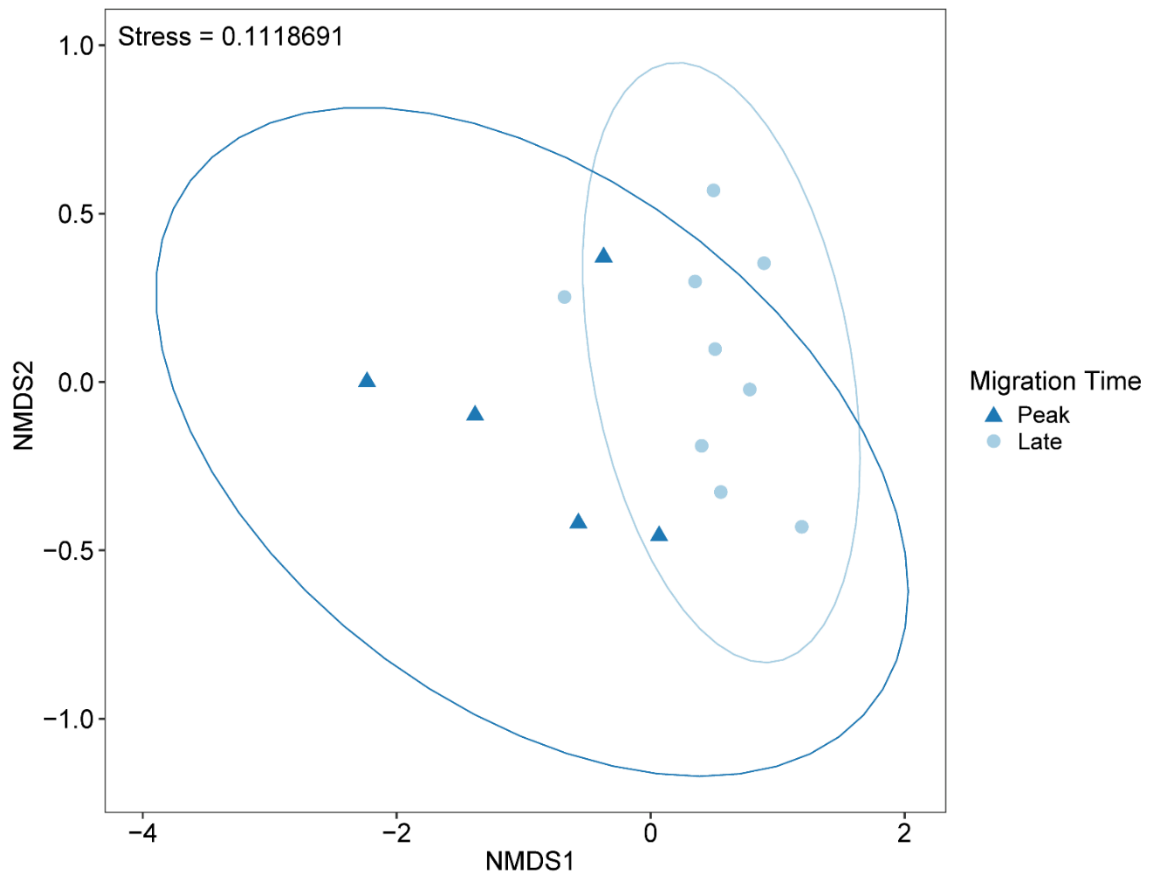

Figure 2-6. Two-dimensional non-metric multidimensional scaling (NMDS) plots with 95% confidence ellipses around each group centroid of white sucker hindgut bacterial communities using a Bray Curtis distance matrix to show dissimilarity between individual fish samples grouped by peak and late migration times.

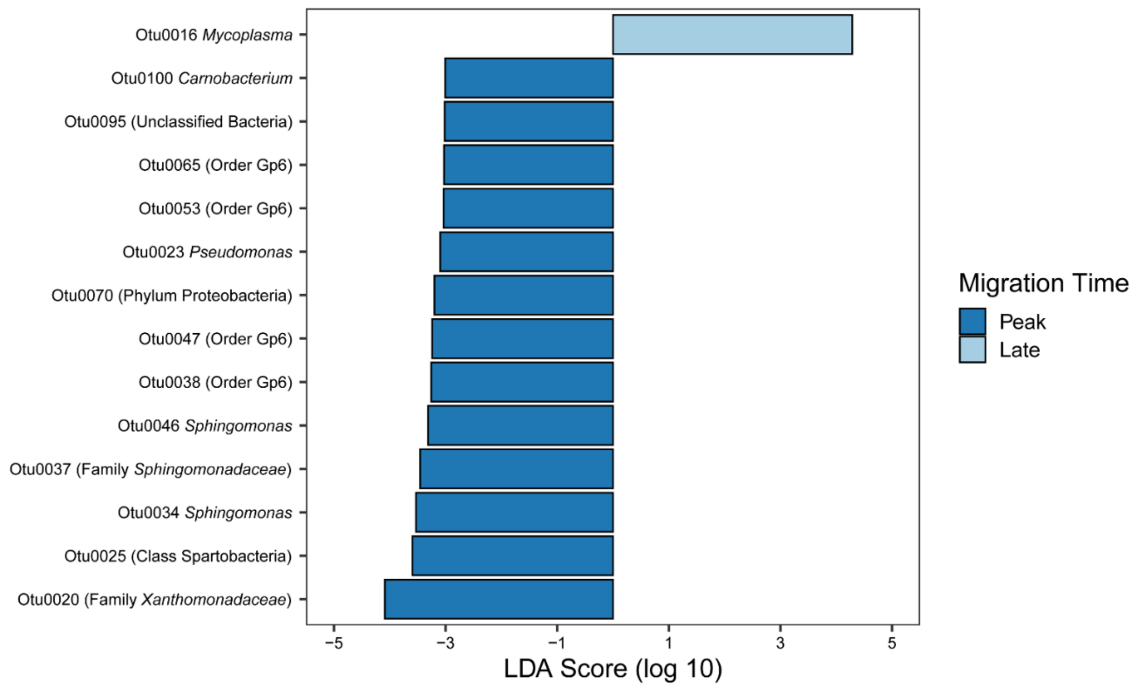

Figure 2-7. Linear discriminant analysis effect size (LEfSe) analysis showing OTUs likely driving the dissimilarity observed in the hindgut bacterial community structure between Peak and Late migrating white suckers. A negative LDA score shows OTUs with significantly higher abundances in Peak migrants, while positive LDA scores show OTUs with significantly higher abundances in Late migrants.

Chapter 3: The hindgut microbial composition, but not diversity, in adult sockeye salmon (*Oncorhynchus nerka*) varies among three spawning populations in the Fraser River Watershed

3.1 Abstract

Sockeye salmon (*Oncorhynchus nerka*) are an anadromous semelparous species that undergo migration journeys that vary in difficulty and effort while relying solely on endogenous energy reserves for fuel to reach their natal spawning grounds. To date, there has been limited research into how the gut microbiota interacts with different aspects of migration biology. However, it is now established that gut microbiota are implicated in host health and fitness and are largely shaped by diet, environment, and to a lesser degree, host physiology. Using 16S rRNA gene amplicon sequencing, we analyzed the diversity and composition of the hindgut microbiota of fish from three spatially distinct populations (i.e., Weaver Creek, Adams River, and Chilko River). Overall, alpha diversity was low and dominated by unclassified bacteria, *Mycoplasma*, *Brevinema*, and *Photobacterium*, and did not vary significantly between the three populations. There was weak evidence that the composition of microbes varied between the Weaver Creek population, which had the easiest and shortest migration route, and the Chilko River population, which had the longest and most challenging route. Weaver Creek fish had a higher abundance of the genera *Brevinema*, *Aliivibio*, and *Cetobacterium*, while Chilko River fish had higher abundances of *Flavobacterium*, *Actinales*, and an unclassified *Comamonadaceae*. Further, we examined bacterial diversity and composition against host physiological metrics associated with stress (plasma lactate, glucose, and hematocrit) and condition factor. Only condition factor showed any association with microbial composition; fish with below-average condition factor scores had higher abundances of

Flavobacterium. The results here suggest that migrating salmon retain many marine-associated microbes, especially for Weaver Creek fish. Further, fish with more difficult migrations and with lower condition factor scores were associated with the genus *Flavobacterium*, which is generally considered an opportunistic pathogen.

3.2 Introduction

Sockeye salmon (*Oncorhynchus nerka*) are a semelparous anadromous species that migrate from their foraging grounds in the ocean back to natal streams to spawn (Dittman and Quinn 1996). The high fidelity to their natal streams (philopatry) has enabled local adaptation, leading to genetically distinct stocks (Beacham et al. 2004, 2006). Capital breeders, such as sockeye salmon, utilize endogenous energy stores for their spawning migration and cease feeding once they enter the river system (Hinch et al. 2006). These limited energy reserves must therefore encompass not only the energy required for the physiological adaptations needed to transfer from salt to freshwater but also for the development of gonads, spawning, and the actual migration run itself, which can span up to 1,000km (Fagerlund 1967; Hinch et al. 2006). Furthermore, populations will likely experience different levels of migration difficulty (e.g., areas of increased flow velocities), with more difficult passages being more energetically costly to individuals (Crossin et al. 2004). Increased flows and migration difficulty also result in more burst swimming and higher levels of plasma lactate, glucose, and cortisol (Hinch and Rand 1998; Hinch and Bratty 2000; Hinch et al. 2006). Premature mortality can occur when energy use is disproportionately high during migration (Rand and Hinch 1998), and because sockeye salmon have a semelparous reproductive strategy, meaning they have a

single reproductive event and then die, this results in negligible lifetime fitness (Dingle 1980).

As a result of a growing body of research, it is now understood that the gut microbiota are implicated in supporting host health and fitness (Walter and Ley 2011; Sommer and Bäckhed 2013). Recent research has highlighted the role gut microbiota play on host metabolism, immune function, and even behaviour (Yi and Li 2012; Visconti et al. 2019; Yoo et al. 2020), indicating a potential role of the microbiota as drivers of hosts' phenotypes and evolution (Alberdi et al. 2016). Factors that are thought to shape the composition of the gut microbial communities of vertebrates include environment, diet, host physiology, and genetics (Ley et al. 2008a; Muegge et al. 2011; Spor et al. 2011; Wong and Rawls 2012; Kashinskaya et al. 2018). Much of this research has been conducted on the gut microbiota of mammals, which generally have a complex and diverse bacterial community (Groussin et al. 2020). Conversely, teleost species typically have gut microbial communities that are less complex and diverse (Lescak and Milligan-Myhre 2017), particularly within piscivorous fish, such as salmonids (Wang et al. 2018). Salmonids are among the most studied in fish gastrointestinal microbiota research due to their importance in aquaculture (Burtseva et al. 2021). Several studies have noted, however, that the gut microbiomes differ between captive and wild stocks, due in part, to changes that occur under captivity (e.g. differences in rearing conditions and diet as well as use of antibiotics; Dhanasiri et al. 2011; Kormas et al. 2014; Eichmiller et al. 2016). Increased attempts at characterizing the microbiomes of wild salmonids have been made in recent years (Llewellyn et al. 2016; Lavoie et al. 2018; Uren Webster et al. 2018, 2020a; Hamilton et al. 2019; Element et al. 2020a, 2020b;

Skrodenytė-Arbačiauskienė et al. 2022). While studying microbiomes in wild populations adds additional complexity due to confounding variables, it is vital to help understand host-gut microbe co-evolution (Amato 2013; Hird 2017).

Salmonids pose numerous challenges as they relate to studying the gut microbiome, as many species are anadromous. Migrating across heterogeneous environments, such as the transition from saltwater to freshwater, not only exposes fish to different environments, but the physiological processes necessary for osmoregulation may also impact the gut microbiota (Dehler et al. 2017b; Hamilton et al. 2019). Moreover, some semelparous species, such as sockeye salmon, cease feeding prior to entering the river system (Hinch et al. 2006). Diet, therefore, would play a less significant role in shaping the gut microbiota during this migratory phase. As the impact of starvation on the gut microbiota has been reported in laboratory studies for diversity in tilapia (Kohl et al. 2014) and composition in Asian sea bass (Xia et al. 2014). Upriver migrations and spawning activities represent physiologically demanding and stressful endeavors (Lucas and Baras 2001). Indicators of stress (such as plasma lactate, glucose, and cortisol) increase during the breeding season until fish senesce and die (Kubokawa et al. 1999; Hruska et al. 2010), though Hruska et al. (2010) found glucose decreased in some fish after they arrived on the spawning grounds, possibly due to the inability to mobilize plasma glucose because of low energy reserves. Particularly noteworthy are the highly elevated levels of plasma cortisol that occur leading up to spawning and senescence (Fagerlund 1967; Carruth et al. 2000). Cortisol elevation has been correlated with changes to the fecal community composition and richness and an increase in opportunistic pathogens in salmonids (Uren Webster et al. 2020b; Couch et al. 2023).

More, Couch et al. (2023) found changes in gut integrity, associated with senescence, were also associated with gut microbial composition.

Most of the salmonid microbiome research to date has been conducted on iteroparous species, such as Atlantic salmon (Llewellyn et al. 2016; Rasmussen et al. 2023). Semelparous species, such as sockeye salmon, can offer novel insights into how physiological challenges, such as fasting, correlate with gut microbial composition and diversity. Further, semelparous species undergo senescence, which has been reported to change gut integrity, with degeneration of the epithelial cells lining the tract (McBride et al. 1965; Couch et al. 2023). The primary goal of the work presented here was to characterize the hindgut bacterial community of adult sockeye salmon on three spatially distinct spawning grounds, representing increasing levels of migratory difficulty and workload for the fish (Weaver Creek, lower Adams River, and Chilko River stocks within the Fraser River Watershed of British Columbia). We also explored if and how physiological metrics, such as plasma glucose, lactate, hematocrit (representing stress indicators), and condition factor, correlated with the hindgut bacterial community. We hypothesized that spawning populations with more difficult and lengthy migrations would be associated with increased levels of plasma stress indicators and reduced condition factor, which would negatively impact the diversity and composition of the hindgut microbiome of sockeye salmon.

3.3 Methods

3.3.1 Study system and sample collection

Adult sockeye salmon were collected in 2018 from the spawning grounds of three populations (Weaver, lower Adams, and Chilko) within the Fraser River Watershed, British Columbia, Canada (Figure 3-1). The Weaver and Adams population belong to the late-summer run populations and were sampled on October 22 and October 18, 2018, respectively. At the Weaver Creek spawning channel, fish were dip-netted at the channel entrance and processed individually. Fish from the lower Adams River were captured by beach seine, and fish were removed using dip nets and held in pens with flowing water before processing. The Chilko population belong to the summer-run population and were sampled on September 29, 2018, using rod and reel and immediately processed. All fish were captured live and humanely euthanized just before sample processing, where a blood sample and hindgut microbial swab sample were taken. Blood sampling of sockeye was conducted as part of the Department of Fisheries and Oceans (DFO) Fraser River Environmental Watch (EWatch) program, and a subset of fish were opportunistically sampled for microbiome characterization concurrently. In total, 20 fish from each of the three spawning populations (n=60) were initially sampled for blood and microbial analysis. Blood samples were collected via caudal puncture using a 3-mL Vacutainer (containing heparin). A heparinized hematocrit tube was immediately filled from this sample. The hematocrit tube was centrifuged for 5 minutes at 8000 RPM to separate contents into red cells, white cells, and plasma, and the percent estimate of hematocrit was obtained by dividing the red cell length against the total length. The remaining blood sample was stored on ice for approximately 20 minutes before being centrifuged for 15

min at 1300 x g. Subsequently, plasma was aliquoted into 1ml Eppendorf tubes and frozen on dry ice before transferring to -80°C until sample processing. Hindgut microbiota samples were obtained by inserting a sterile cotton swab (Puritan, Guilford, ME) approximately 8cm in the hindgut of the fish and rotating, ensuring it made contact with the walls of the intestine. The swab tip was broken off within a sterile 2 ml cryovial (Thermo Fisher Scientific, San Diego, CA) containing 1.5 ml of RNAlater stabilization solution (Invitrogen, Carlsbad, CA). Samples were held overnight at 4°C and then moved to -20°C until transferred to the laboratory for long-term storage at -20°C until processing. Fish fork length (cm), body weight (g), and sex were also taken during processing. Condition factor (k) was calculated using equation 1.

$$(1)\ K = \left(\frac{mass}{length^3}\right) x\ 100$$

3.3.2 Blood plasma processing

Plasma glucose and lactate concentrations were measured using a YSI 2300 Stat Plus Glucose and L-Lactate Analyzer (YSI Inc, Yellow Springs, OH). Samples were removed from the freezer and thawed on ice prior to analysis; each Eppendorf tube was vortexed for 30s before aspirating into the analyzer. The analyzer aspirated 25 µl of plasma and then determined glucose and lactate concentration simultaneously (in mmol/L). All samples were run in duplicate and were within 2% of each other.

3.3.3 DNA extraction and 16S rRNA amplicon sequencing

Swab samples were thawed at room temperature and centrifuged for 7 minutes at 12000 RPM. Because the density of RNAlater was similar to that of the bacterial cells, 1 ml of RNAlater was removed and replaced with PBS buffer, and samples were vortexed

for 10 minutes to produce the necessary bacterial pellet. Swabs were then transferred to the powerbead tube from the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). The original tubes were then centrifuged again for 7 minutes, the PBS buffer was discarded, and the small bacterial pellet was resuspended in 100 µl PBS buffer and transferred to the powerbead tube containing the swab. DNA extractions were performed using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the following modifications: 60 µl of C1 was added to the powerbead tube, vortexed briefly, and then incubated at 65 °C for 15 minutes; In lieu of vortexing for 10 minutes, a Retsch MM 400 Mixer Mill (Thermo Fisher Scientific, MA, USA) was used for one minute of 30 cycles/second to mix and homogenize the samples; and finally, 50 µl of elution buffer was added to the white filter membrane and incubated at room temperature for 5 minutes before centrifuging at 12000 RPM for 1 minute.

DNA was quantified using the Qubit™ dsDNA HR Assay Kit (Thermo Fisher Scientific, MA, USA) and then concentrated using a Savant DNA 120 SpeedVac Concentrator (Thermo Fisher Scientific, MA, USA) for 30 minutes to increase DNA concentrations for downstream sequencing. Sequencing libraries were prepared using a sequencing protocol previously described by Kozich et al. (2013) using PCR primers designed for the V4 hypervariable region of the 16S rRNA gene (Wu et al. 2015), with the following modification; 5 µL from all PCR amplicons were pooled together to create the library and concentrated in a Savant DNA 120 SpeedVac Concentrator (Thermo Fisher Scientific, MA, USA) for approximately 3 hours to reach a volume of 30 µL. PCR amplicons were then sequenced on an Illumina MiSeq system using a MiSeq® Reagent

Kit v3 with 500 cycles (2x250; Illumina, San Diego, USA), following the manufacturer's protocol and using a 15 % spike of PhiX Control v3 (Illumina, San Diego, USA).

3.3.4 Sequencing data processing and statistical analysis

Sequences were processed in mothur (version 1.35.1), as referenced by Schloss et al. (2009). Operational taxonomic units (OTUs) were assigned based on 97 % similarity, and taxonomy was assigned using the SILVA reference database (version 138; Quast et al. 2013). Samples containing less than 800 reads were discarded, and all remaining samples were rarified to 800 reads (Figure 3-2). Normalized OTU tables were used for all downstream analyses in RStudio (v1.4.1106) for R (R Core Team 2021; v4.0.5), barring the LEfSe analysis that used the unrarefied OTU table (Segata et al. 2011).

Relative abundances of the top bacterial genera composing ≥ 1.0 % of the total OTU sequences among the sampled sockeye salmon spawning populations were visualized using phyloseq (McMurdie and Holmes 2013; v1.34.0) and ggplot (Wickham 2016; v3.3.5). Observed species richness and inverse Simpson diversity for adult sockeye microbiomes between the three spawning locations were calculated in phyloseq (McMurdie and Holmes 2013; v1.34.0). A non-parametric Kruskal-Wallis test was then used to test for significant differences ($p < 0.05$) in alpha diversity. Non-metric multidimensional scaling (NMDS) was performed on Bray-Curtis distances in phyloseq (Bray and Curtis 1957; McMurdie and Holmes 2013). Permutational multivariate analysis of variance (PERMANOVA; $p < 0.05$) was performed with 999 permutations on Bray-Curtis distances in vegan (Oksanen et al. 2020; v2.5-7) to assess if there were significant differences in beta diversity for a number of variables, including spawning

location, sex, glucose, lactate, hematocrit, and condition factor. Pairwise comparisons based on significant PERMANOVA results were then computed in the pairwise Adonis package using 999 permutations (Martinez Arbizu 2020; v.0.4; $p < 0.05$). Multivariate homogeneity of group dispersions among samples of sockeye salmon between spawning locations was assessed on Bray-Curtis distances in vegan (Anderson 2006; Oksanen et al. 2020; v2.5-7; betadisper; $p < 0.05$). Differentially abundant OTUs between groups with a significant difference in beta diversity were examined using the linear discriminant analysis effect size (LEfSe) method, with an alpha value of 0.05 and threshold logarithmic LDA score of 3 for spawning location and 2.5 for condition factor (Segata et al. 2011).

Blood and body condition physiological variables were compared between spawning locations using parametric ANOVA ($p < 0.05$) where residuals were normally distributed (i.e., for lactate and condition factor) and non-parametric Kruskal-Wallis ($p < 0.05$) where residuals violated the assumption of normality (i.e., for glucose and hematocrit). Multiple comparisons were made using either the Tukey HSD post-hoc test ($p < 0.05$) for ANOVA (from the package agricolae in R; v1.3-5; De Mendiburu 2009) or the Dunn post-hoc test ($p < 0.05$) for Kruskal-Wallis (from the package rstatix in R; v0.7.0; Dunn 1964).

3.4 Results

3.4.1 Microbiome amplicon sequence quality

A total of 60 live spawning salmon were initially swab sampled for microbiome analysis from 3 spawning locations in the Fraser River watershed (Weaver = 20 fish;

Chilko = 20 fish; and Adams = 20 fish; Figure 3-1; Table 3-1). Before sequencing, the PCR Qubit DNA quantification step revealed that swab samples from 15 fish did not contain sufficient DNA for further sequencing and were removed from the study. Following sequencing, a further three samples did not sequence with enough reads to include in downstream analysis (read range between 159-420 reads). Data were then rarefied to 800 reads per sample for downstream analysis. 800 reads were chosen based on rarefaction curves (Figure 3-2) and the rationale to balance adequate read depth while maintaining enough samples for statistical power. This resulted in a final total of 35 samples (Weaver=13; Adams=14; Chilko=8) and corresponded to 352 unique OTUs.

3.4.2 Characterizing community composition and diversity patterns among different spawning populations

At the phylum level, we identified 17 taxa, with six taxa occurring at a relative abundance greater than 1%. These six phyla accounted for over 99% of the total reads (Table 3-2). The relative abundance of the top phyla were as follows: Unclassified bacteria (51.9%), Proteobacteria (20.0%), Tenericutes (13.2%), Spirochaetes (10.5%), Bacteroidetes (2.4%), and Fusobacteria (1.3%; Table 3-2).

At the genus level, we identified 207 taxa. However, only 9 had a relative abundance of greater than 1%, which accounted for over 97% of the total reads, indicating a large proportion of reads with very low abundance (Table 3-3). The top three most abundant genera were unclassified bacteria (47.1%), *Mycoplasma* (16.3%), and *Photobacterium* (11.1%; Table 3-3). Grouping samples based on spawning location showed some distinct differences in composition between the three spawning

populations. *Vibrio* was present in the Adams fish (4.4%) but absent from Chilko and Weaver individuals (Figure 3-3). *Brevinema* was enriched in the Adams and Weaver populations (10.0% and 17.4%) but rare in the Chilko population (0.3%), while *Flavobacterium* was more highly abundant in the Chilko population (7.9%) but rare in Adams and Weaver populations (0.6% and 0.08%; Figure 3-3). Finally, *Aliivibrio* and *Cetobacterium* were enriched in the Weaver population (19.5% and 3.4%) but rare in the Adams population (0.2% and 0.04%) and absent in the Chilko population (Figure 3-3).

Shapiro-Wilks tests showed that the residuals of both the observed species richness and inverse Simpson alpha diversity metrics violated the assumption of normality (observed species richness; W=0.855, p=0.0003, inverse Simpson; W=0.921, p=0.015). Therefore a non-parametric Kruskal-Wallis test was used for diversity estimates among spawning locations. While observed alpha diversity showed weak evidence that Weaver was associated with lower diversity, it did not vary significantly between spawning locations (Kruskal-Wallis X^2=5.87, p=0.053), nor did the inverse Simpson alpha metric (Kruskal-Wallis X^2=2.71, p=0.255; Figure 3-4).

Beta diversity was described using a non-metric multidimensional scaling (NMDS) ordination with a Bray-Curtis dissimilarity matrix, which revealed weak separation of the Weaver bacterial communities from those at Chilko (Figure 3-5). Statistically testing this visual with a PERMANOVA revealed that this pattern was statistically significant (F.Model=2.311, R^2=0.12, p=0.022; Table 3-4), and PERMDISP revealed that this difference was not due to group dispersions (p=0.697; Table 3-5). However, pairwise comparisons showed only weak evidence that Chilko bacterial communities were distinct from Weaver (F.Model=2.93, R2=0.13, p.adjusted=0.051),

whereas there was no evidence of distinct bacterial communities between the Adams-Chilko pairing (F.Model=2.17, R2=0.09, p.adjusted=0.225) and Adams-Weaver pairing (F.Model=1.42, R2=0.05, p.adjusted=0.636; Table 3-6).

Despite only weak evidence for bacterial community separation between Chilko and Weaver fish, a LEfSe analysis was still undertaken to divulge which OTUs contributed to the slight difference in bacterial composition. LEfSe analysis revealed that four OTUs were over-represented in Chilko, and three OTUs were over-represented in Weaver (Figure 3-6A). In the Chilko group, these four OTUs belonged to genera: *Flavobacterium*, *Actinoplanes*, and an unclassified *Comamonadaceae*. While in the Weaver group, the three OTUs belonged to genera: *Brevinema*, *Aliivibrio*, and *Cetobacterium* (Figure 3-6A).

3.4.3 Physiological status of different spawning populations

Biological monitoring through the Department of Fisheries and Oceans (DFO) Environmental Watch (EWatch) program provided several blood physiology and fish condition metrics, which were taken concurrently with the microbiome sampling. Shapiro-Wilks tests showed that some of these variables did not have normally distributed residuals (Appendix A), so non-parametric Kruskal-Wallis tests were used instead of ANOVA. Several biological variables varied significantly among spawning populations (Figure 3-7). Plasma glucose was significantly higher in Chilko than Adams and Weaver (Fig 3-7A), while plasma lactate was significantly higher in Adams compared to Chilko or Weaver (Fig 3-7B). Further, hematocrit was significantly higher in

Adams compared to Weaver but not Chilko (Fig 3-7C). Finally, condition factor was significantly higher in Adams compared to Chilko but not Weaver (Fig 3-7D).

3.4.4 Integrating physiological metrics and composition of bacterial communities

We examined potential associations between fish physiological variables and hindgut bacterial communities using a PERMANOVA analysis. PERMANOVA revealed that only condition factor was associated with differences in bacterial composition (F.Model=2.90, R2=0.07, p=0.027; Table 3-4). Following this, a LEfSe analysis was conducted to examine which OTUs contributed to this observation. For simplification, condition factor was dichotomized based on whether values were above or below the mean. The LEfSE analysis revealed that community differences were driven by an over-abundance of *Flavobacterium* in fish with below-average condition factors (Figure 3-6B).

3.5 Discussion

The results of this study demonstrated that while there was no significant difference in alpha diversity between spawning populations, there was weak evidence that diversity increased with increasing migration distance in observed species richness (Fig 3-4A). This was consistent with Ying et al. (2020), who found the same trend with alpha diversity and migration distance in *Coilia nasus*, an anadromous fish species in the family *Engraulidae*. However, this pattern was not retained in our study using the inverse Simpson index, which is a more robust measure of alpha diversity as it incorporates both species richness and evenness and is a more appropriate measure of diversity given the unevenness in our dataset (Haegeman et al. 2013). In addition, alpha diversity was low

across our spawning populations and defined by a few abundant OTUs. At the phylum level, this represented only six phyla, with unclassified bacteria accounting for over 50% of the sequence reads. This result is surprising and may be due to the degrading intestinal environment of the host fish due to fasting. As migrating salmon ready themselves for spawning, the intestinal tract atrophies and epithelial cells degenerate (McBride et al. 1965). Autochthonous (resident) bacteria are thought to be tightly associated with intestinal mucosa that overlies the intestinal epithelium (Nava and Stappenbeck 2011). Therefore the degeneration of these cells may result in dysbiosis of the commensal microbiota and the proliferation of currently unknown bacterial phyla that have yet to be characterized. Previous studies that focused on salmonid gastrointestinal microbiomes that included migratory phases tended to sample fish (i.e., Atlantic salmon, Arctic charr, lake whitefish) along their migration route and did not have the prevalence of unclassified bacteria observed here (Llewellyn et al. 2016; Element et al. 2020a, 2020b). However, these species are not semelparous and would not be experiencing gastrointestinal atrophy and degeneration. Therefore, there may be unknown bacteria associated with senescence. High abundances of unclassified bacteria can be relatively common in gut microbiota studies of marine fish species, where up to 70% of sequence reads are unknown at the genera level (Huang et al. 2020). It is hypothesized that this is due to the close association of benthivorous and detritivorous fish with the seafloor and sediments that have yet to be sequenced and classified (Huang et al. 2020). Barring these unclassified sequences, the remaining dominant phyla observed in this study (Proteobacteria, Tenericutes, Spirochaetes, Bacteroidetes, and Fusobacteria) were similar (though less diverse) to other salmonid gut microbiota studies (Llewellyn et al. 2016;

Ciric et al. 2019; Bozzi et al. 2021). Though Llewellyn et al. (2016) remarked in their study on migratory Atlantic salmon that returning adults were much less diverse than other life-stages and physiological changes and fasting could cause reduced community stability. In-line with our results, they also reported Proteobacteria and Tenericutes as the dominant phyla in returning adults.

There was weak evidence that the hindgut microbial composition of sockeye salmon adults varied between spawning populations, specifically between the Weaver and Chilko spawning populations. These two locations were separated by the greatest in-river distance, with Weaver Creek located approximately 150km from the mouth of the Fraser River and Chilko River approximately 650km from the mouth of the Fraser River (Figure 3-1). Gut microbial communities have been shown to be strongly shaped by the local environment and diet (Bolnick et al. 2014b; Kashinskaya et al. 2018). However, migrating adult sockeye salmon cease feeding once they enter the riverine system and depend on endogenous energy resources to fuel their migration run (Hinch et al. 2006). Further, drinking rates also decline in freshwater as fish adjust their osmoregulation in a hypo-osmotic environment (Clarke and Hirano 1995). Therefore, it may stand to reason that local acquisition of microbes may be limited and play a less significant role in shaping gut bacterial communities. This may therefore impact the turnover rate of microbes in sockeye hindguts, preserving the gut microbe composition from previously encountered environments. When looking at the relative abundance of the genera across the three spawning populations, it is evident that the presence of genera that are typically associated with marine environments, including *Photobacterium*, *Aliivibrio*, and *Vibrio,* are dominating the bacterial communities. Returning wild Atlantic salmon also retained

much of their microbiome from the marine environment (Llewellyn et al. 2016). For example, *Photobacterium* has been found in high prevalence in many salmonid microbiome studies (Sullam et al. 2012; Gajardo et al. 2016; Zhao et al. 2020) and can play a role in chitin digestion (Itoi et al. 2006), suggesting it may be part of the normal salmonid gut microbiome. Alternatively, gut microbial communities may also be influenced during the early acclimation period before entering the Fraser River, where fish adapt their osmoregulatory mechanisms for entry into freshwater. The Weaver and Adams populations belong to the late run, who delay for extended periods in the Strait of Georgia before entering the Fraser River. In contrast, the Chilko population belongs to the summer run and typically exhibits little to no delay before entering the river (English et al. 2005). While this could potentially explain why Weaver and Chilko populations had a borderline significant pairwise comparison of bacterial communities and why Weaver and Adams had a nonsignificant pairing, it does not explain why Adams and Chilko also had similar bacterial communities. These two populations comigrate in coastal areas before entering the Fraser River (Crossin et al. 2009) and likely encounter similar microbial environments in seawater. Pairing sockeye population samples with environmental microbe water samples in the Strait of Georgia and the Fraser River would help decipher if the similarity between Weaver and Adams populations and Adams and Weaver populations were due to acquiring microbes locally.

We observed that fish sampled at Weaver had a significantly higher abundances of three OTUs belonging to the genera *Brevinema*, *Aliivibrio*, and *Cetobacterium* relative to the Chilko sampled fish. *Brevinema andersonii* was found to be part of the core microbiome in the marine phase for Atlantic salmon (Gupta et al. 2019a, 2019b),

specifically as part of the distal mucosa microbial community and associated with the expression of genes related to immune responses (Li et al. 2021). As Chilko is the most up-river spawning population, it is perhaps unsurprising that fish would have a significantly lower number of *Brevinema*. Further, looking at relative abundance patterns for the three spawning locations, we see that Adams fish were intermediary in the relative abundance of this genera, suggesting that *Brevinema* are reduced in the distal gut as fish travel further upriver. *Aliivibrio* is also commonly found in the seawater phase of Atlantic salmon and is often part of the core microbiome (Dehler et al. 2017a; Gupta et al. 2019b). *Aliivibrio* can also be an opportunistic pathogen, and Bozzi et al. (2021) found that the relative abundance of *Aliivibrio* sp. was inversely correlated to the relative abundance of *Mycoplasma* sp. in the intestinal microbiota of Atlantic salmon. *Mycoplasma* was a dominant taxon recovered from our samples, however, it was lowest in fish sampled at Weaver, and concomitantly, this was the only group that contained *Aliivibrio* (Figure 3-3). *Mycoplasma* has often been found to be the dominant taxa in salmonid intestinal microbial communities (Heys et al. 2020; Cheaib et al. 2021; Rasmussen et al. 2021, 2023) and is thought to be vertically transmitted from parent to offspring since it has not been identified in the environment (Llewellyn et al. 2016). Using a metagenome approach, Rasmussen et al. (2023) found that the gut microbiota of Atlantic salmon was dominated by a single *Mycoplasma* species associated with the biosynthesis of lysine and threonine amino acids, as well as B1 vitamin. This suggests a key role of *Mycoplasma* in the fitness of wild salmonids. Finally, *Cetobacterium* was found in significantly higher abundance in the Weaver population than in Chilko. *Cetobacterium* is a major component of freshwater fish gut microbiota (Larsen et al. 2014; Ramírez et al. 2018). Therefore it is

interesting that it was most associated with the Weaver population when the previous two significantly different taxa were primarily associated with the marine phase of Atlantic salmon. Conversely, the Chilko population was associated with four OTUs in significantly higher abundance than Weaver, belonging to three different genera. *Flavobacterium* was the most abundant and was not present in the Weaver population. *Flavobacterium* contains pathogenic species that are the cause of multiple different fish diseases that affect both wild and captive individuals, including cold water disease (*Flavobacterium psychrophilum*), columnaris disease (*F. columnare*), and bacterial gill disease (*F. branchiophilum*; Woo 1999). There are no reports of *Flavobacterium* being reported as part of a normal gut microbiome in salmonids (Gajardo et al. 2016; Llewellyn et al. 2016); therefore, it is likely that the prevalence in the Chilko fish represents a signal of disease. It is plausible that reduced immune function due to long-distance migration would allow the proliferation of opportunistic pathogens, such as *Flavobacterium*.

We have described differences in the diversity and composition of the hindgut microbial communities in three spawning populations representing three spatially discrete locations of increasing distances from the mouth of the Fraser River. These locations also represent different levels of migration difficulty that fish must endure to reach the spawning grounds. Weaver Creek has the least difficult migration in terms of shortest distance travelled and lowest elevation gain, while Chilko River has the most difficult, with the longest distance travelled and highest elevation gain (Crossin et al. 2004). Adams River was classified as intermediary in difficulty (Crossin et al. 2004). More difficult migrations typically cause greater physiological stress (e.g., elevated plasma lactate, glucose, and hematocrit) and energy reserve depletion (Brett 1995; Hinch et al.

2006). Host physiology can also contribute to shaping the gut microbiome of animals (Sullam et al. 2015; Baldo et al. 2015; Macke et al. 2017), so there is merit in integrating physiological variables with microbial communities. Our results showed significant differences in blood physiology and body composition parameters between spawning locations, but when integrated with our microbiome dataset, we observed that only condition factor was significantly associated with differences in microbiome composition. The LEfSe analysis showed that it was primarily an OTU belonging to the genus *Flavobacterium* in fish with below-average condition factors that drove the difference in bacterial communities between fish with below- and above-average condition factors. As stated previously, *Flavobacterium* does not appear to be a normal component of salmonid gut microbiomes, but species within this genus are disease-causing in fish (Woo 1999).

3.5.1 Conclusion

Overall, the results presented here indicate that the composition of gut bacterial communities differ somewhat among migrating sockeye salmon populations, with the extent of variation apparently related to the physical distance separating different populations and their associated migratory challenges (i.e., Weaver is a short and relatively easy migration whereas fish in the Chilko population undertake a long and arduous migration). Additionally, alpha diversity did not vary between these locations and was generally low, with a few dominant genera observed that typically reflect taxa associated with seawater. This result was more pronounced in the Weaver population, whose differential abundance analysis showed seawater-associated OTUs, such as

Brevenima and *Aliivibrio*. This result suggests that migrating salmon retain many microbes acquired during their marine phase. Future studies that incorporate sequencing environmental water samples from different locations along salmon migration routes would add further evidence to this theory. Further, analyzing bacterial communities of different fish compartments, such as the gills, may offer novel insights into location-specific microbes as the gills are exposed to the external environment (Reverter et al. 2017) and may be a better site given the atrophied state of the gastrointestinal tract of salmon once they reach the spawning grounds. This may be especially relevant if looking for associations between the microbiome and disease status, as pathogenic bacteria commonly adhere to gill sites (Secombes and Wang 2012). Finally, condition factor was the only measured host physiology metric associated with bacterial composition. The genus *Flavobacterium*, typically associated with disease in other fish body compartments such as the gills (Decostere 2002), was present in significantly higher abundances in fish with lower condition factors. Resolving this genus to species level would help elucidate these findings and indicate the usefulness of the gut microbiota as a biomarker for disease status. This study was the first to assess the gut microbiota of semelparous sockeye salmon on the spawning grounds. Future research should include documenting if and how the gut microbiome correlates with spawning success and host fitness.

Table 3-1. Description of adult sockeye salmon fish sampled for this study with a minimum of 800 reads.

Sample	Date	Location	Sex	Fork Length (cm)	Mass (g)	Glucose (mmol/L)	Lactate (mmol/L)	Hematocrit (% estimate)	Condition Factor (k)
A2129	2018-10-18	Adams	F	58.4	2053	7.29	4.81	40	1.03
A2131	2018-10-18	Adams	F	57.9	2147	5.83	9.27	45	1.11
A2132	2018-10-18	Adams	F	59.7	2501	8.61	6.97	55	1.18
A2133	2018-10-18	Adams	F	59	2650	8.61	10.70	50	1.29
A2134	2018-10-18	Adams	F	60.4	2850	7.73	6.77	50	1.29
A2135	2018-10-18	Adams	F	55.3	1950	7.01	12.20	50	1.15
A2136	2018-10-18	Adams	F	59.6	2630	5.62	3.46	55	1.24
A2138	2018-10-18	Adams	F	57	2170	6.26	6.38	50	1.17
A2139	2018-10-18	Adams	F	62.1	3110	5.06	11.30	45	1.30
A2141	2018-10-18	Adams	F	60.2	2600	4.98	10.10	80	1.19
A2145	2018-10-18	Adams	M	63.4	3210	5.75	9.50	50	1.26
A2146	2018-10-18	Adams	M	52.2	1990	6.98	6.15	45	1.40
A2147	2018-10-18	Adams	M	66.3	3340	6.97	13.20	55	1.15
A2148	2018-10-18	Adams	M	56.5	1840	5.33	2.28	50	1.02
C2126	2018-09-29	Chilko	F	55.1	1400	13.50	3.59	30	0.84
C2128	2018-09-29	Chilko	F	55.1	1650	9.04	2.75	30	0.99
C5016	2018-09-29	Chilko	M	59.6	2280	20.90	7.53	40	1.08
C5019	2018-09-29	Chilko	F	56.4	1630	23.90	8.96	45	0.91
C5020	2018-09-29	Chilko	F	55.5	1690	22.80	7.29	40	0.99
C5025	2018-09-29	Chilko	F	56.4	1650	16.00	4.93	30	0.92
C5027	2018-09-29	Chilko	M	58	2390	14.50	2.25	30	1.22
C5028	2018-09-29	Chilko	M	60.8	2580	8.16	2.08	25	1.15
W18563	2018-10-22	Weaver	F	62.7	2950	5.04	0.89	45	1.20
W18564	2018-10-22	Weaver	F	59.5	2680	4.71	1.92	50	1.27
W18565	2018-10-22	Weaver	F	59.9	2652	4.94	1.34	40	1.23
W18566	2018-10-22	Weaver	M	65.4	3174	4.70	0.75	20	1.13
W18567	2018-10-22	Weaver	F	57.4	2260	3.91	1.32	50	1.20
W18569	2018-10-22	Weaver	M	68.8	3524	4.91	1.00	35	1.08
W18570	2018-10-22	Weaver	F	58.4	2398	4.44	1.26	35	1.20
W18571	2018-10-22	Weaver	M	61.5	2758	4.48	0.77	40	1.19
W18572	2018-10-22	Weaver	M	66.4	3292	5.11	1.37	30	1.12
W18576	2018-10-22	Weaver	F	62	2678	3.74	2.07	50	1.12
W18577	2018-10-22	Weaver	F	58.1	2326	4.74	1.20	45	1.19
W18578	2018-10-22	Weaver	M	62.7	2744	4.33	0.47	40	1.11
W18581	2018-10-22	Weaver	M	69.3	4046	4.28	0.78	35	1.22

Table 3-2. Prevalence of phylum-level taxa across the sampled adult sockeye salmon population with a relative abundance greater than 1% of total OTU sequences.

Phylum	Percentage of total reads (%)
Unclassified bacteria	51.95
Proteobacteria	20.00
Tenericutes	13.20
Spirochaetes	10.53
Bacteroidetes	2.38
Fusobacteria	1.29

Table 3-3. Prevalence of genus-level taxa across the sampled adult sockeye salmon population with a relative abundance greater than 1% of total OTU sequences.

Genus	Percentage of total reads (%)
Unclassified bacteria	51.95
Mycoplasma	13.20
Brevinema	10.53
Photobacterium	8.39
Aliivibrio	7.35
Flavobacterium	2.09
Vibrio	1.75
Cetobacterium	1.29
Shewanella	1.25

Table 3-4. Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance of hindgut bacterial communities of adult sockeye salmon for groupings: location, sex, glucose, lactate, hematocrit, and condition factor. Df = degrees of freedom, SS = sum of squares, MS = mean squares, F. Model = F-value by permutation, R2 = percentage of variance explained by the groups, Pr(>F) = p-value for F-statistic. P-values based on 999 permutations. *Difference is significant at 0.05 level.

	Df	SS	MS	F.Model	R2	Pr(>F)	
Location	2	0.8975	0.44875	2.31099	0.11643	0.022	*
Sex	1	0.4008	0.40084	2.06426	0.052	0.079	
Glucose	1	0.2144	0.21443	1.10426	0.02782	0.372	
Lactate	1	0.2047	0.20472	1.05428	0.02656	0.378	
Hematocrit	1	0.1846	0.18457	0.95052	0.02394	0.449	
Condition Factor	1	0.5637	0.56373	2.90311	0.07313	0.027	*
Residuals	27	5.2429	0.19418		0.68013		
Total	34	7.7086			1		

Table 3-5. Analysis of multivariate homogeneity of group dispersions (betadisper) based on Bray-Curtis distance of hindgut bacterial communities of adult sockeye salmon from three spawning locations. Df = degrees of freedom, SS = sum of squares, MS = mean squares, Pr(>F) = p-value for F-statistic.

	Df	SS	MS	F value	Pr(>F)
Groups	2	0.06231	0.031153	0.3657	0.6966
Residuals	32	2.72605	0.085189		

Table 3-6. Pairwise comparisons based on the location grouping from the PERMANOVA analysis. Df = degrees of freedom, SS = sum of squares, MS = mean squares, F. Model = F-value by permutation, R2 = percentage of variance explained by the groups, Pr(>F) = p-value for F-statistic. P-values based on 999 permutations. *Difference is significant at 0.05 level.

Pairs	Df	SS	F.Model	R2	p.value	p.adjusted
1 Adams vs Chilko	1	0.417143	2.171161	0.097927	0.075	0.225
2 Adams vs Weaver	1	0.316463	1.422105	0.053823	0.212	0.636
3 Chilko vs Weaver	1	0.651938	2.937778	0.133914	0.017	0.051

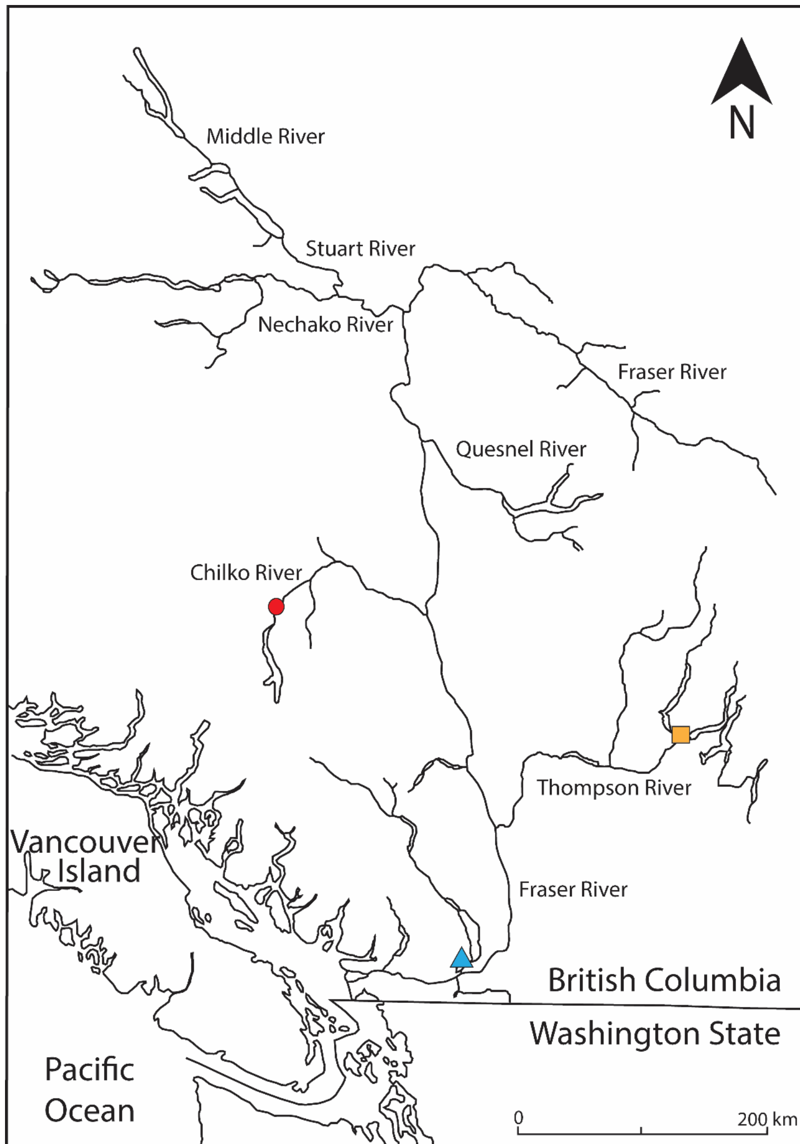

Figure 3-1. Study site locations where adult sockeye salmon were sampled at the terminal end of their migration run representing three separate stocks. Blue triangle is situated at Weaver Creek Spawning Channel, yellow square at the lower Adams River, and red circle at the Chilko River StAD Camp.

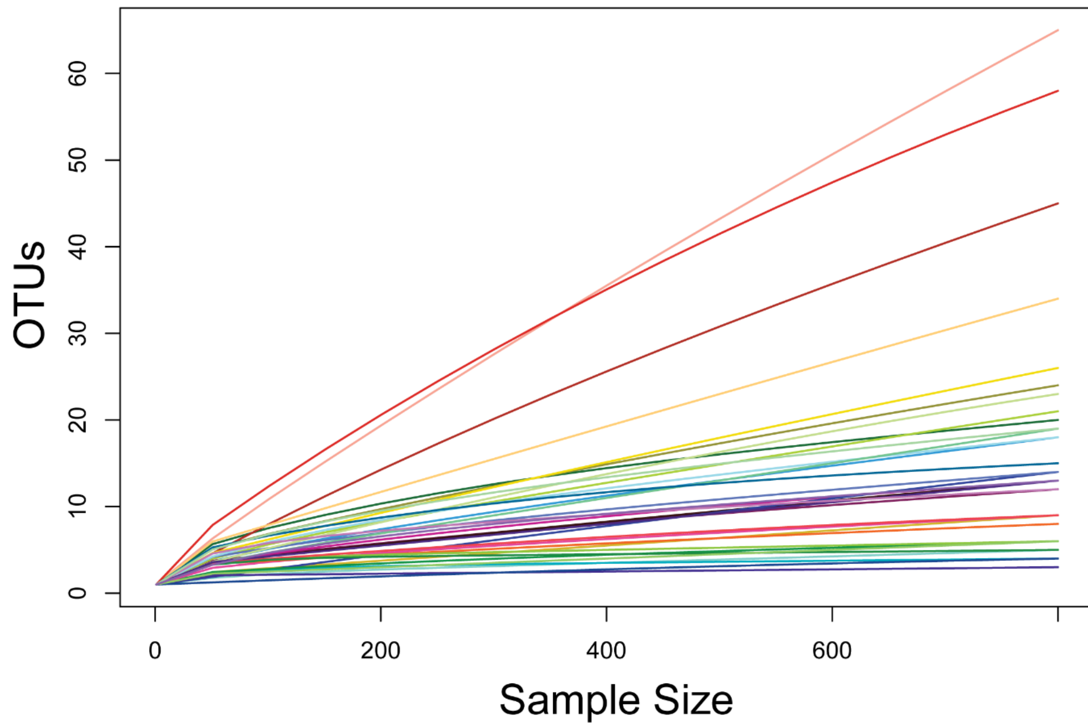

Figure 3-2. Rarefaction curve showing the number of OTUs on the vertical axis against the number of sequences (sample size) on the horizontal axis for all sockeye salmon hindgut swab samples.

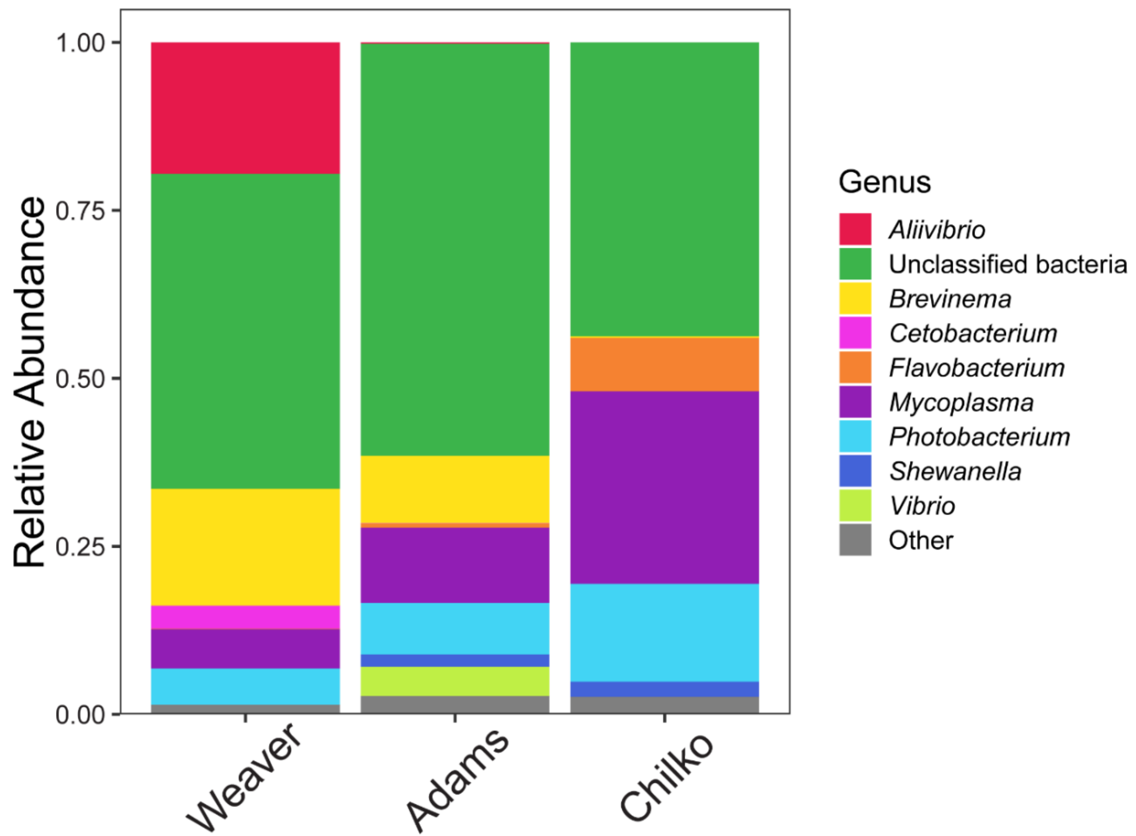

Figure 3-3. Relative abundance of the major genera present across adult sockeye salmon fish from three separate spawning stocks (Weaver Creek, lower Adams River, and Chilko River; representing more than 1.0 % relative abundance).

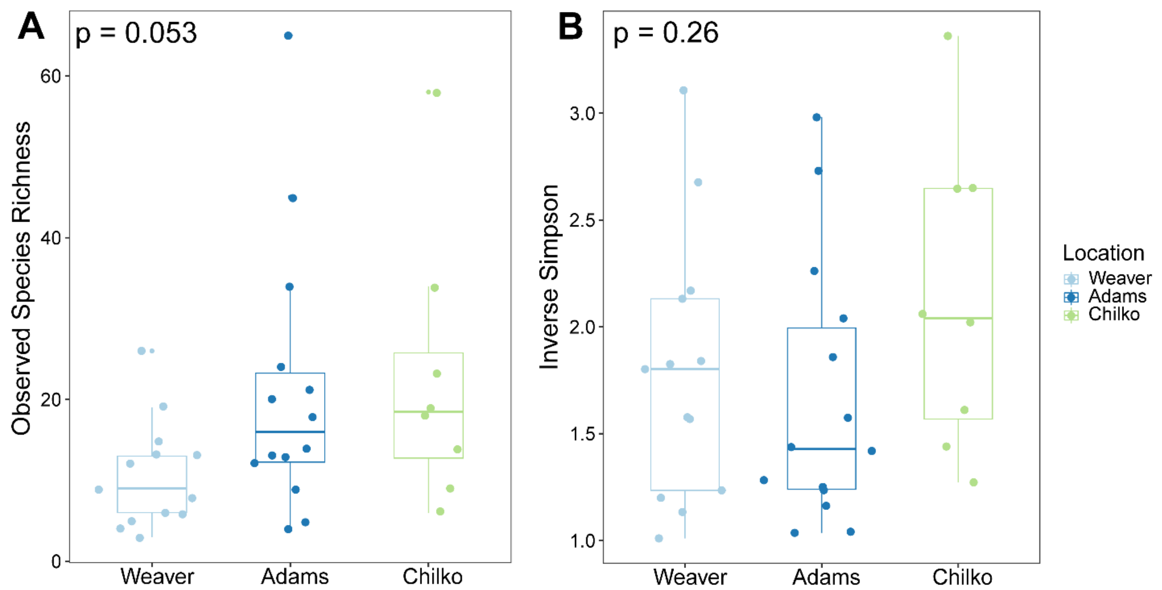

Figure 3-4. Alpha diversity indices showing A) Observed Species Richness and B) Inverse Simpson index on the operational taxonomic units (OTUs) of the hindgut bacterial community of adult sockeye salmon at three spawning sites (Weaver Creek, lower Adams River, and Chilko River).

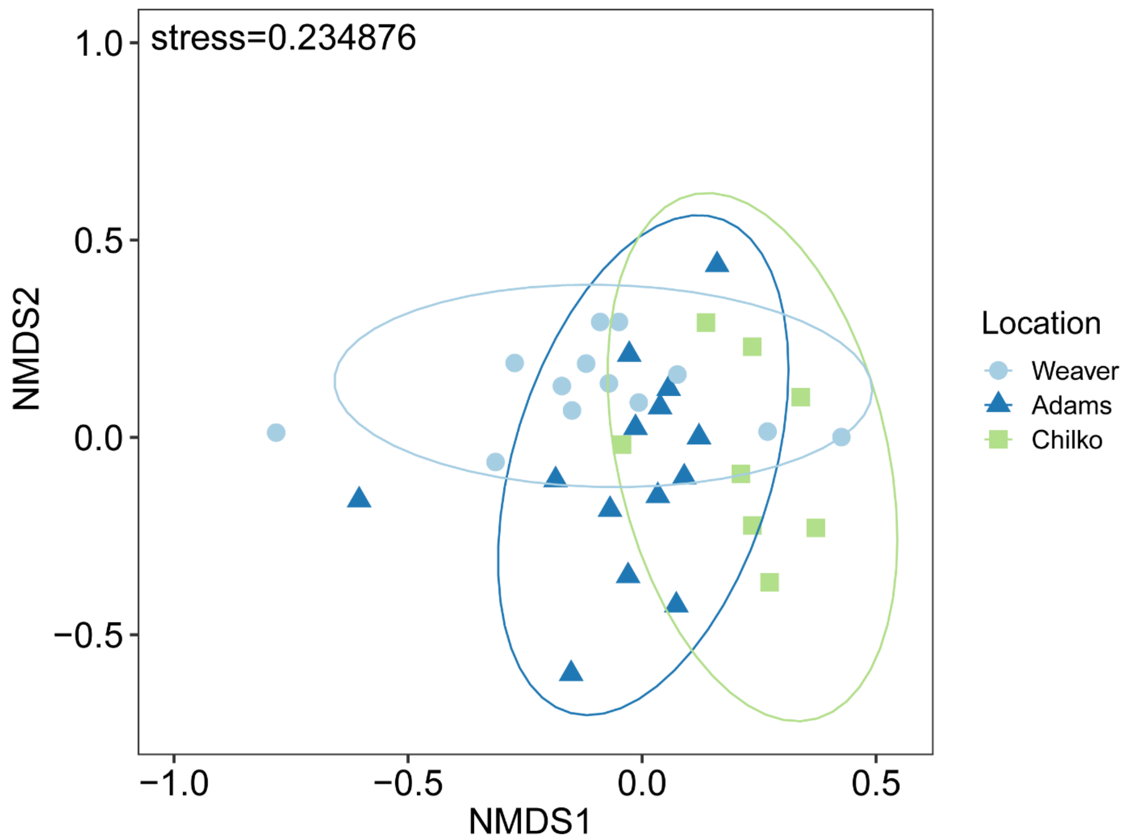

Figure 3-5. Two-dimensional non-metric multidimensional scaling (NMDS) plots with 95% confidence ellipses around each group centroid of adult sockeye salmon hindgut bacterial communities using a Bray Curtis distance matrix to display dissimilarity between individual fish samples grouped by their respective spawning locations.

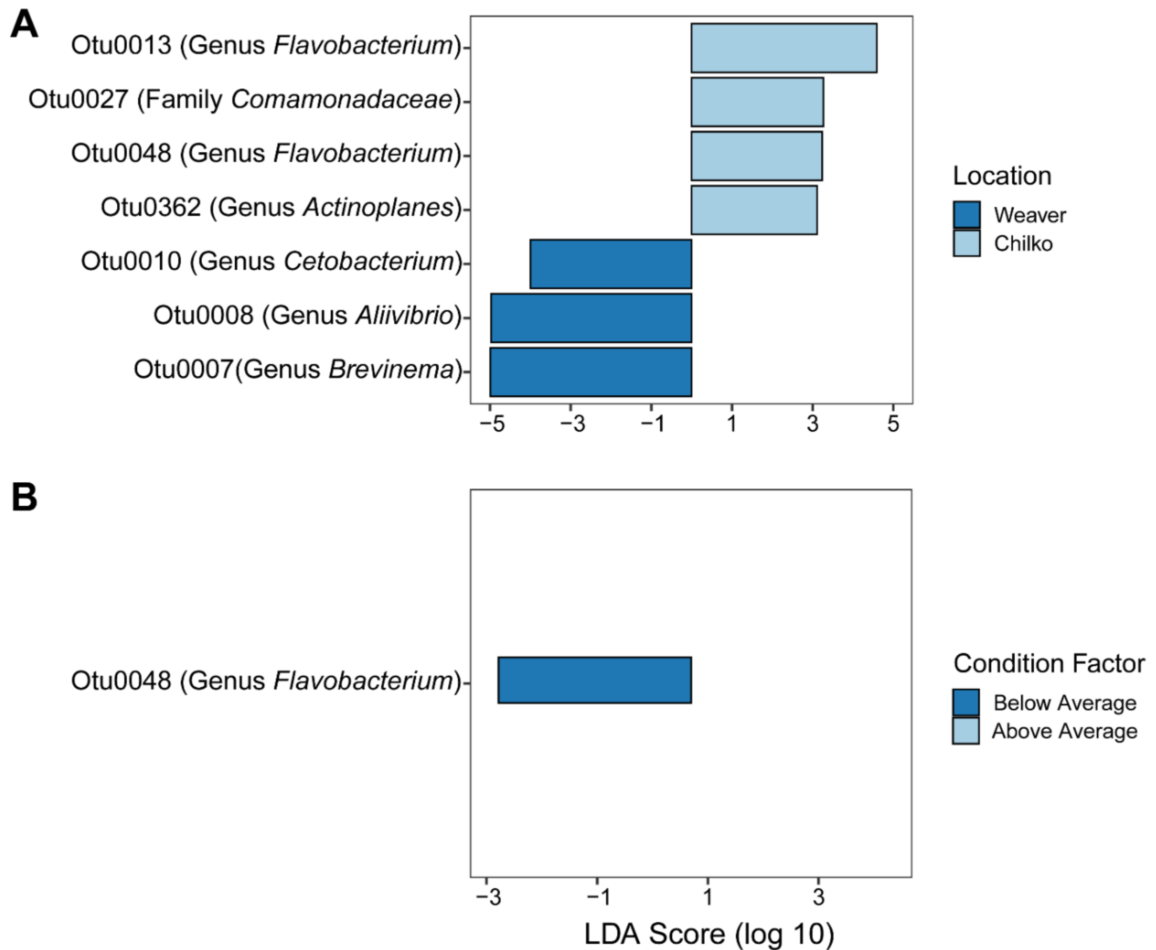

Figure 3-6. Linear discriminant analysis effect size (LEfSe) analysis showing OTUs likely driving the dissimilarity observed in the hindgut bacterial community structure between adult sockeye salmon sampled on the Weaver Creek spawning ground vs. Chilko spawning ground (LDA scores greater than 3; A), and between sockeye salmon with below average and above average condition factors (LDA scores greater than 2.5; B). A negative LDA score shows OTUs with significantly higher abundances in fish from Weaver Creek and fish with below average condition factors, while positive LDA scores show OTUs with significantly higher abundances in fish from Chilko River.

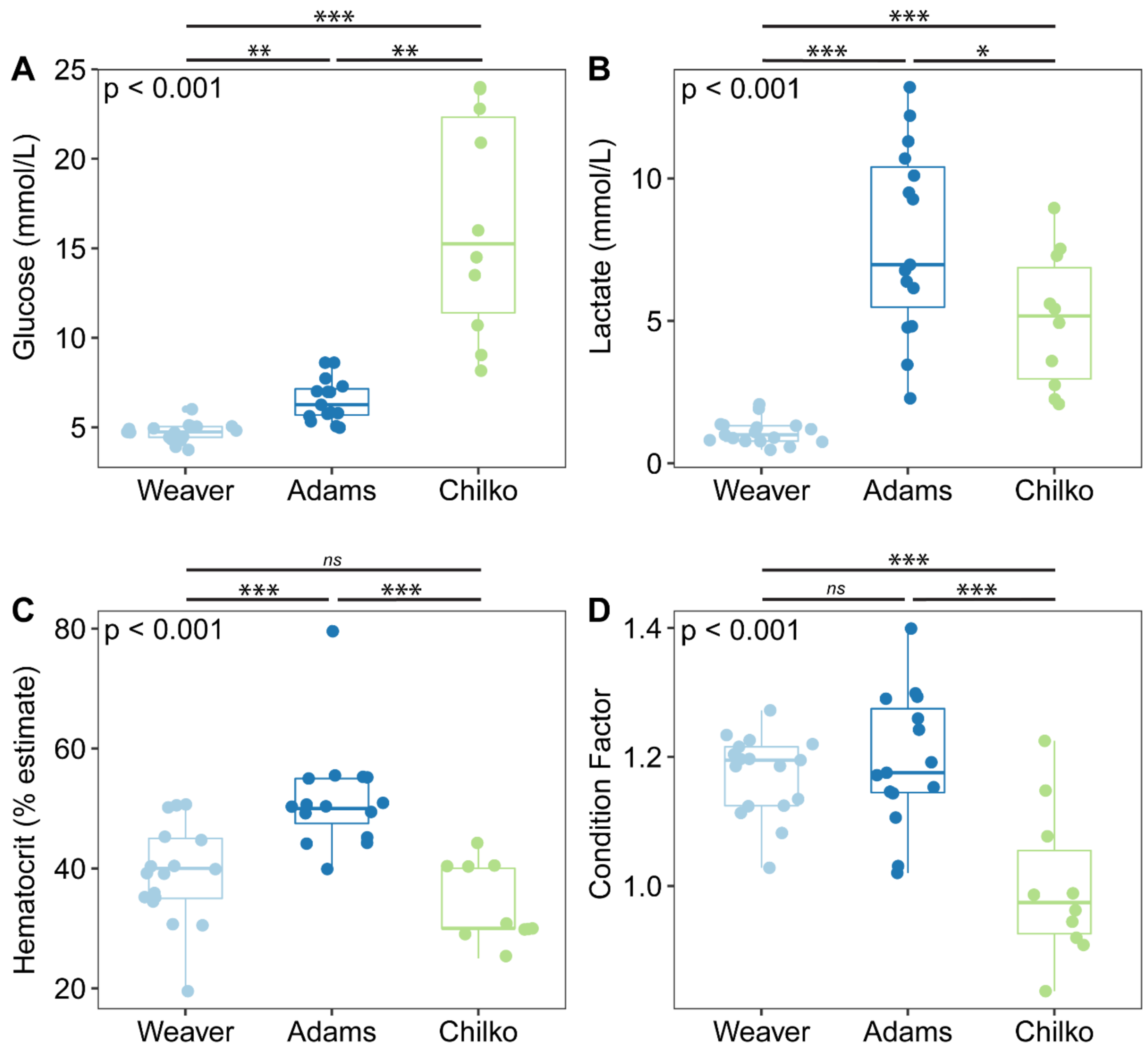

Figure 3-7. Blood and body composition physiological variables taken from adult sockeye salmon on three spawning grounds at the terminal end of their migration run. A) plasma glucose (mmol/L) with overall and pairwise comparison significance values, B) plasma lactate (mmol/L) with overall and pairwise comparison significance values, C) plasma hematocrit (% estimate) with overall and pairwise comparison significance values, and D) condition factor with overall and pairwise comparison significance values.

Chapter 4: Does the fecal microbiome predict migratory status in a population of brown trout (Salma trutta) displaying partial migration?

4.1 Abstract

Brown trout (*Salma trutta*) are a partially anadromous fish species, where spawning populations are comprised of freshwater resident and ocean migratory individuals, the latter of which return to freshwater to spawn. It is thought that the decision to migrate is made by juveniles in the late summer or fall before the following year's spring outward migration and is governed, in part, by an individual's energy status. However, proximate mechanisms underlying migratory decisions are not fully resolved. We analyzed communities of bacteria from non-lethal fecal samples of juveniles in the fall after their presumed decision window and then tracked their migration behaviour using passive integrated transponders to determine if the gut microbial composition and/or diversity was associated with migratory strategy. We found no associations between gut microbiota and migratory status or body condition. We did find moderate evidence that microbial communities were somewhat site-specific, though whether this is due to variations in diet or environmental conditions could not be deciphered. These results show that shortly after the presumed first decision window of juvenile brown trout, there is no differentiation in gut microbial communities in the sampled population with respect to future migratory outcomes. Future research should examine whether this changes once the smoltification phase is underway and stronger physiological forces are at work.

4.2 Introduction

Migration is a life-history strategy used to increase individual fitness by exploiting different habitats for growth and reproduction or avoiding harsh environmental conditions (Alerstam et al. 2003; Dingle and Drake 2007). However, migration is very energetically costly, and predation risks can be high en-route (Alerstam et al. 2003). As such, there are inherent risks and trade-offs of undertaking an arduous migratory journey, and this behaviour has evolved in many species to be facultative, where populations display both migratory and resident phenotypes (Archer et al. 2019). In fishes, particularly salmonids, partial migration is a common expression of life history plasticity (Jonsson and Jonsson 1993; McDowall 1997). The proximate mechanisms underlying the decision to migrate are still under debate but thought to be related to environmental food limitation (Olsson et al. 2006; O'Neal and Stanford 2011), low body condition and/or energetic status (Boel et al. 2014; Peiman et al. 2017; Birnie-Gauvin et al. 2021), and high growth rate (i.e., metabolic rate; Forseth et al. 1999; Cucherousset et al. 2005). The concept of the threshold model suggests that migration is a threshold trait, i.e., one that is expressed when these aforementioned traits exceed a pre-determined genetic threshold, and failure to exceed the threshold leads to migration (Thorpe et al. 1998; Brodersen et al. 2008; Chapman et al. 2011; Ferguson et al. 2019; Birnie-Gauvin et al. 2021). In salmonids, juveniles with low body condition/energetic status and high metabolic rates often smoltify and become migrants (Forseth et al. 1999; Acolas et al. 2012; Peiman et al. 2017; Shry et al. 2019; Birnie-Gauvin et al. 2021). Smoltification is a stressful process (Stefansson et al. 2020), but positive trade-offs include increased growth from overwintering in the productive marine environment (Klemetsen et al. 2003). Individuals

that remain freshwater residents must deal with harsh winter environments and potential low food availability and are thus more prone to overwinter mortality (Shuter et al. 2012).

Smoltification is the parr-smolt transformation that occurs in anadromous salmon, which involves a variety of morphological, behavioural, and physiological changes that occur to prepare juveniles to transition into seawater (Stefansson et al. 2020). Some of the more notable changes include increased Na^+/K^+-ATPase in the gills (Hoar 1988), a streamlined silver body (Zaugg and Wagner 1973), dietary adaptations, and schooling behaviours (McCormick et al. 1998). These physiological disturbances have been associated with reduced community stability in the gut microbiome of juvenile Atlantic salmon (Llewellyn et al. 2016). Furthermore, alteration of the intestinal microbiota has been observed after seawater transfer of juvenile Atlantic salmon in tank experiments (Dehler et al. 2017b). This suggests that physiological and environmental changes directly alter the composition and diversity of the gut microbiome in Atlantic salmon. However, there has been little investigation into whether the inverse relationship occurs, that is, the composition and diversity of the gut microbiome impacts the smoltification process. Smolt development is an energy-demanding process that results in lipid and glycogen depletion, primarily in the liver and muscle tissue (Sheridan 1989). In Coho salmon (*Oncorhynchus kisutch*), this is thought to be due to increased lipolysis and decreased fatty acid synthesis (Sheridan et al. 1985). It is now established that the gut microbiota play a part in lipid metabolism and can aid in the regulation of fat storage in humans (Cani and Delzenne 2009) and fatty acid metabolism in gnotobiotic zebrafish (Semova et al. 2012). However, it is unknown to what extent the gut microbiota influences the smoltification process, or if variation in the gut microbiota can contribute

to plasticity in this life-history trait amongst salmonids. Studies have examined how lipid energy reserves influence life-history decisions in salmonids (Morgan et al. 2002; Jonsson and Jonsson 2005), but none have considered the effect of the gut microbiota on these processes.

Brown trout (*Salmo trutta*) are a partially anadromous species, where populations comprise both an anadromous form (referred to as sea trout) and a resident freshwater form (brown trout; Ferguson et al. 2019). As adults, behavioral, morphological, and physiological differences are often observed between residents and migrants. Body dimorphism is particularly apparent, where migrant adults are typically larger than residents because they can take advantage of the more productive marine habitats (Hendry et al. 2004; Chapman et al. 2011). In terms of a fitness advantage, a larger body size may mean that (female) migrants have a higher fitness, as egg number and egg size correlate with body size (Hendry et al. 2004; Dingle 2006). Sea trout return to freshwater streams to spawn and can interbreed with resident forms and produce offspring capable of either migratory phenotype (Jonsson 1985; Pettersson et al. 2001). The resulting offspring inhabit these streams for a few months or years (depending on location) before the decision to smoltify and migrate or remain resident occurs. It is thought that the decision window for migration occurs in late summer (Metcalfe et al. 1990; Metcalfe and Thorpe 1992), ahead of the spring migration run. However, there is a contingent of individuals that migrate in the autumn season (Birnie-Gauvin et al. 2019), indicating that the timing of migration and the decision window to start the smoltification process is somewhat plastic. Research has been conducted to try to discern the different physiological proximate mechanisms that may play a role in the decision to migrate (e.g.,

cortisol; oxidative stress; energy status; body condition, and growth; see Acolas et al. 2012; Birnie-Gauvin et al. 2017, 2021; Peiman et al. 2017; Shry et al. 2019). However, there have yet to be any studies on whether the gut microbiota may play an underlying role in shaping some of these physiological mechanisms in the context of facultative migration. The gut microbiota is known to influence host physiological functions through the regulation of metabolism and digestive processes (Bäckhed et al. 2004; Turnbaugh et al. 2006; Semova et al. 2012; Ni et al. 2014; Dawood et al. 2016; Janssen and Kersten 2017), and modulation of the immune system (Gómez and Balcázar 2008), and therefore may be indirectly contributing to these proximate mechanisms underpinning migratory decisions.

While gut microbiota can influence host physiology, the converse is also true. Host physiology and genetics play a role in shaping the structure of the gut microbiome in a reciprocal relationship between the host and its microbial consort (Roeselers et al. 2011). In addition to these endogenous factors, exogenous factors, such as diet and the environment, also shape the gut microbiota of the host by providing external colonization pathways (Smith et al. 2015). Environmental inputs of microbes are important early colonizers of the fish gastrointestinal tract (Romero and Navarrete 2006). However, once fish begin feeding, diet is thought to play a more important role in shaping the gut microbiome (Llewellyn et al. 2014; Li et al. 2017b). Juvenile brown trout have an opportunistic diet but feed primarily on macroinvertebrates, such as insects (Degerman 2000; Sagar and Glova 2010). Consuming a diverse prey diet could shape the gut microbiome through exposure to different kinds of prey or eating prey carrying different compositions of microbes, as found in a study on three-spined stickleback (*Gasterosteus*

aculeatus; Smith et al. 2015). Further, macroinvertebrate communities can be quite variable in stream microcosms, such as areas with different land use or the presence or absence of riparian vegetation (Sponseller et al. 2001; Sagar and Glova 2010). As such, it could be possible for the gut microbiome of juvenile fish to vary along a single stream.

In the present study, we captured juvenile brown trout after the presumed first decision window from several reaches of a Danish river and took length and weight measurements, as well as non-lethal fecal microbiome samples for microbial community characterization and diversity measurements. We then released the fish with passive integrated transponders to track their subsequent behaviour and determine if fish were resident or migratory. We tested the hypothesis that microbial communities varied between residents and migrants. We predicted that because the decision to migrate had been made at the time of sampling and internal physiological processes had likely begun, these physiological changes would influence or be influenced by the composition and diversity of the gut microbiome. We also tested the hypothesis that the gut microbial community varied with body condition, and predicted that body condition would also relate to migratory tendency in our study system. Finally, we tested the hypothesis that the gut microbiome of fish from different reaches of the stream would be different due to differences in riparian vegetation, shade, and associated invertebrate communities.

4.3 Methods

4.3.1 Study location

This study was conducted in east-central Jutland, Denmark, within the Gudsø stream, where a population of semi-anadromous brown trout resides (Figure 4-1). Two Passive Integrated Transponder (PIT) antennas, separated by 5m, were located

approximately 500m from the mouth of the stream into the Kolding Fjord, spanning the entire cross-section of the stream, as referenced in Birnie-Gauvin et al. (2017). This setup allowed for the detection of outmigrating tagged fish to be recorded. All protocols were approved by the Danish Experimental Animal Inspectorate (2017-15-0201-01164).

4.3.2 Fish sampling and tagging

Juvenile brown trout greater than 120 mm in length were captured in the main stem of the Gudsø stream between 22 and 25 October 2018, using single-pass electrofishing (Stampes Elektro A/S, Ringkøbing, Denmark). Captured fish were placed in 60L containers with fresh stream water that was changed regularly to maintain adequate oxygenation until sampled. Once a section of the stream had been sampled, fish were placed in a 0.03 g/L benzocaine solution until they became unresponsive to external stimuli. Once adequately anesthetized, total length (±1mm) and wet mass (±0.1g) were measured for individual fish, and condition factor (K) was calculated using equation 1. Fecal samples were collected by gently applying pressure along the posterior ventral portion of individual fish to express any fecal matter contained within the hindgut. A sterile swab was placed at the anus of the fish to collect the fecal matter, and the swab was then transferred to a sterile 2ml tube containing 1.5 ml of RNAlater solution. The swab tip was broken off within the tube, and the tube was placed on ice until transfer to the laboratory, where it was first held at 4°C overnight and then moved to -20°C until transfer back to the laboratory for long-term storage at -20°C until processing. A 23mm PIT tag (RI-TRP-RRHP, 134 kHz, 0.6 g mass in air; Texas Instruments, Plano, TX, USA) was then inserted into the anterior ventral portion of the body cavity. Fish were then

moved to a recovery container with fresh oxygenated water and, once recovered, were put back into the stream.

$$(1)\ \mathrm{K} = \left(\frac{mass}{length^3}\right) x\ 100$$

4.3.3 Evaluation of migration

A fish recapture survey was conducted across the entirety of Gudsø stream between 22 and 23 July 2019 to determine which individuals assumed residency. This period was chosen because it is after the outmigration of brown trout smolts, typically occurring between March and May in that stream. Any tagged fish recaptured within the stream was noted to have assumed residency. To determine which individuals migrated, PIT data were downloaded on 3 July 2019, and all fish that were detected at both the upstream and downstream PIT antenna, in that order, were considered to be migrants. Fish that were neither recaptured during the July survey nor detected at the PIT antennas were classified as unknown. Only fish that were positively identified as residents or migrants were included in subsequent sample processing.

4.3.4 DNA extraction and 16S rRNA amplicon sequencing

Swab samples were thawed at room temperature and centrifuged for 7 minutes at 12000 RPM. Because the density of RNAlater was similar to that of the bacterial cells, 1 ml of RNAlater was removed and replaced with PBS buffer, and samples were vortexed for 10 minutes to produce the necessary bacterial pellet. Swabs were then transferred to the powerbead tube from the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). The original tubes were centrifuged again for 7 minutes to capture any bacteria that remained in the solution, the PBS buffer was discarded, the small bacterial pellet was resuspended

in 100 µl PBS buffer and transferred to the powerbead tube containing the swab. DNA extractions were performed using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the following modifications; Step 2: 60 µl of C1 was added to the powerbead tube, vortexed briefly, and then incubated at 65 °C for 15 minutes. Steps 3 and 4: In lieu of vortexing for 10 minutes, a Retsch MM 400 Mixer Mill (Thermo Fisher Scientific, MA, USA) was used for one minute of 30 cycles/second to mix and homogenize the samples. Step 19: 50 µl of elution buffer was added to the white filter membrane and incubated at room temperature for 5 minutes before centrifuging at 12000 RPM for 1 minute. DNA was quantified using the Qubit™ dsDNA HR Assay Kit (Thermo Fisher Scientific, MA, USA) and then concentrated using a Savant DNA 120 SpeedVac Concentrator (Thermo Fisher Scientific, MA, USA) for 30 minutes to increase DNA concentrations for downstream sequencing. Sequencing libraries were prepared using a sequencing protocol previously described by Kozich et al. (2013) using PCR primers designed for the V4 hypervariable region of the 16S rRNA gene (Wu et al. 2015), with the following modification; 5 µL from all PCR amplicons were pooled together to create the library and concentrated in a Savant DNA 120 SpeedVac Concentrator (Thermo Fisher Scientific, MA, USA) for approximately 3 hours to reach a volume of 30 µL. PCR amplicons were then sequenced on an Illumina MiSeq system using a MiSeq® Reagent Kit v3 with 500 cycles (2x250; Illumina, San Diego, USA), following the manufacturer's protocol and using a 15 % spike of PhiX Control v3 (Illumina, San Diego, USA).

4.3.5 Sequencing data processing and statistical analysis

Sequences were processed in mothur (version 1.35.1), as referenced by Schloss et al. (2009). Operational taxonomic units (OTUs) were assigned based on 97 % similarity, and taxonomy was assigned using the SILVA reference database (version 138; Quast et al. 2013). Samples containing less than 9051 reads were discarded, and all remaining samples were rarified to 9051 reads, which struck a balance between including the maximum number of samples and sufficient read depth. Normalized OTU tables were used for all subsequent analyses in RStudio (v1.4.1106) for R (R Core Team 2021; v4.0.5), barring the LEfSe analysis that used the unrarefied OTU table (Segata et al. 2011).

Relative abundance of the top bacterial phyla and families composing ≥ 1.0 % of the total OTU sequences among the sampled brown trout were visualized using phyloseq (McMurdie and Holmes 2013; v1.34.0) and ggplot (Wickham 2016; v3.3.5). Alpha (Shannon-Weiner index) and beta diversity were calculated in phyloseq (McMurdie and Holmes 2013; v1.34.0). A nonparametric Wilcoxon rank-sum test was used to test for significant differences ($p < 0.05$) in alpha diversity between migrants and residents, and a nonparametric Kruskal-Wallis test was used to test for significant differences ($p < 0.05$) in alpha diversity between body condition factors of $k<1$, $k=1$, and $k>1$ (Table 4-1), as well as between the four different sampling sites. Beta diversity was assessed through non-metric multidimensional scaling (NMDS) ordination performed on a Bray-Curtis distance matrix to visualize the dissimilarity within and between migrants and residents, body condition, and sampling sites, using two dimensions (Bray and Curtis 1957). To assess if there was a significant difference in beta diversity within the three different

groupings, a permutational multivariate analysis of variance (PERMANOVA; $p < 0.05$) was performed with 999 permutations on the Bray-Curtis distance matrix in the R vegan package (Oksanen et al. 2020; v2.5-7), and pairwise comparisons based on significant PERMANOVA results were computed (permutest, $p < 0.05$). The variability of microbial community composition among samples of brown trout for each grouping was assessed using an analysis of multivariate homogeneity of group dispersions (betadisper; $p < 0.05$) on the Bray-Curtis distance matrix (Anderson 2006) in the R vegan package (Oksanen et al. 2020; v2.5-7). Finally, differentially abundant OTUs between groups with a significant difference in beta diversity were examined using the linear discriminant analysis effect size (LEfSe) method, with an alpha value of 0.05 and threshold logarithmic LDA score of 3 (Segata et al. 2011).

4.4 Results

4.4.1 Life-history strategy and sample sequence quality

A total of 523 juvenile brown trout were initially sampled for microbiome analysis and were PIT tagged before release. Analysis of the PIT data revealed that 147 juveniles migrated out into the fjord, and resampling of the stream revealed that 29 juveniles remained resident. A subset of 35 migrant samples from the 147 migrant microbiome samples were chosen at random for sequencing, along with the 29 resident microbiome samples. The initial PCR Qubit DNA quantification step revealed that two migrant and eight resident samples did not contain enough DNA for further sequencing and were dropped from the dataset. Following sequencing, a further 19 samples did not sequence with enough reads to include in subsequent analyses (read range between 2 and

138 reads; 5 resident and 14 migrant microbiome samples). Therefore, the final dataset included 15 resident and 19 migrant microbiome samples, corresponding to 1,265,324 sequences (Table 4-1). Data were rarefied to 9051 reads per sample (to include sample F3469), which resulted in a final total of 307,734 reads from 34 samples for downstream analysis. This translated to 6818 OTUs. The rarefaction curve of the included samples is provided in Figure 4-2. In addition, we did not find any evidence that body condition was associated with migratory tendency in this dataset (Mann-Whitney U-test; W=129, p=0.6562), nor did sample collection site (chi-square test; $\chi(3)=1.2225$, p=0.7476).

4.4.2 Hindgut microbial community composition and diversity by migratory status and body condition

The bacterial composition of the fecal microbiome of brown trout was characterized using the relative abundance of OTUs from all rarefied samples. At the phylum level, this included a total of 32 phyla, with seven phyla occurring at a relative abundance of greater than 1.0 % and accounting for approximately 97% of the OTU sequences (Figure 4-3). The relative abundance of these top phyla across the sample population is as follows: Proteobacteria (54.5 %), Firmicutes (22.9 %), an unclassified bacterial phylum (7.8 %), Actinobacteria (6.2 %), Tenericutes (2.8 %), Verrucomicrobia (1.6 %), and Planctomycetes (1.1 %). Looking at the distribution of phyla across the life-history types, there is a very similar breakdown between migrants and residents (Figure 4-3). The only difference was that the migrant individuals were enriched in Tenericutes compared to residents (4.2 % vs. 1.1 % of OTU sequences), whereas residents were slightly more enriched in Verrucomicrobia than migrants (2.1 % vs.

1.3 % of OTU sequences). Body condition did not appear to have a relationship with microbial composition at the phylum level, and there were reduced numbers of Tenericutes in fish with a normal body condition (k=1; 0.3 %) compared to those in poorer conditions (k<1; 6.4 %) and higher condition (k>1; 2.7 %; Figure 4-4).

There was a high diversity of taxa at the family level, with 269 families observed across the sampled population. However, only 16 families had relative abundances of more than 1.0 % of the total OTU sequences, which accounted for 80.7 % of the OTU sequences (Table 4-2). The most abundant family was *Enterobacteriaceae* (23.6 %), followed by *Clostridiaceae* (16.2 %) and *Coxiellaceae* (9.2 %). Grouping the samples based on migratory status revealed a similar distribution of the top 16 families between migrant and resident samples (Figure 4-5). Some notable differences included an enrichment of *Phyllobacteriaceae* within the migrant individuals compared to residents (2.8 % vs. 0.2 % of OTU sequences), an enrichment of *Rickettsiaceae* within the migrant individuals compared to residents (5.1 % vs. 0.1 % of OTU sequences), and conversely, an enrichment of *Neisseriaceae* among the residents compared to the migrant individuals (7.0 % vs. 1.6 % of OTU sequences). In terms of body condition, microbial composition was again very similar between fish with low, normal, and high body condition, though there were differences in the relative abundance of these families (Figure 4-6). *Mycoplasmataceae* was enriched in poorer body conditions (K<1; 6.4 %) and higher body conditions (k<1; 2.7 %) compared to normal body conditions (k=1, 0.1 %; Figure 4-6). *Rickettsiaceae* and *Phyllobacteriaceae* also appeared to be more prevalent in the normal body condition (6.1 % and 3.2 %) compared to poorer body condition (0.0 % and 0.3 %) and higher body condition (0.0 % and 0.3 %).

Shannon diversity index (H`), an alpha diversity estimate that also considers species evenness in a community, was calculated for each individual and grouped by their life-history strategy. The assumption of normality was violated (Shapiro-Wilk normality test; W=0.93096, p=0.03337), therefore a Mann-Whitney U test was used to examine differences in alpha diversity between migrants and residents, and a Kruskal-Wallis test was used to test for differences among the three body condition categories. To this end, there was no evidence that life-history strategy (W=129; p=0.66; Figure 4-7) nor body condition (H(2)=0.749; Figure 4-8) had an effect on alpha diversity.

Beta diversity was described using non-metric multidimensional scaling (NMDS), which uses a Bray-Curtis dissimilarity matrix to visualize how similar/dissimilar the bacterial communities are in 2D ordination, and PERMANOVA analysis. Neither life-history strategy (Figure 4-9A) nor body condition categories (Figure 4-9B) showed any significant differences in community composition (p=0.569 and p=0.891, respectively; Table 4-3), indicating that the bacterial communities were relatively homogenous across these groupings.

4.4.3 Hindgut microbial community composition and diversity by sample site

Examining microbial composition using an environmental lens, the distribution of the top seven phyla were also very similar between the four sample sites (Figure 4-10). One notable difference was the enrichment of Tenericutes at sites A and C, compared with sites B and D (site A=2.7 %, site B=0.0 %, site C=7.7 %, site D=0.0 %). At the family level, this translated to an enrichment of *Mycoplasmataceae* at sites A and C,

compared with sites B and D (site A=2.3 %, site B=0.0 %, site C=7.7 %, site D=0.0 %; Figure 4-11).

A Kruskal-Wallis non-parametric test evaluated the Shannon diversity index between the four sampled sites. While site B had the highest alpha diversity measurement overall, the data showed only weak evidence that alpha diversity was associated with sampling site (H(3)=6.6034; p=0.086; Figure 4-12). This corresponded to a 175 m stretch of stream with approximately 82 % of the stream with some degree of overhanging vegetation providing shade, a relatively high amount of shade for our sampling sites, with only site A being more shaded as it was fully forested. Beta diversity showed moderate evidence that community composition was different between sampling sites (Figure 4-9C; PERMANOVA, p=0.02; Table 4-3), and this difference was not due to intragroup compositional variance (betadisper =0.1356; Table 4-4). A pairwise comparison indicated that Site D had a significantly different bacterial community from site B (p=0.005; Table 5) and site C (p=0.05; Table 4-5).

The OTUs driving the difference in beta diversity amongst sampling sites were investigated using a LEfSe analysis. Examining the first pairwise comparison of sites B and D, *Cetobacterium* was the single OTU overrepresented in site B, while 9 OTUs were overrepresented in site D (Figure 4-13). Comparisons between sites C and D showed that 6 OTUs were overrepresented in site D (Figure 4-13).

4.5 Discussion

In this study, the fecal microbial composition of juvenile brown trout was found to be relatively homogenous at the phylum and family levels across the sampled

population. Proteobacteria and Firmicutes were the most abundant phyla, and *Enterobacteriaceae*, *Clostridiaceae*, and *Coxiellaceae* were the most abundant families. Proteobacteria, Firmicutes, and Bacteroidetes are often major components in the fish gut microbiome and can represent up to 90 % of sequence reads (Ringø et al. 2006b; Desai et al. 2012; Ghanbari et al. 2015; Givens et al. 2015; Kim et al. 2021). Specifically, among juvenile brown trout, Michl et al. (2019) also found that Proteobacteria and Firmicutes were the dominant phyla across all their experimentally applied diet treatments, which is consistent with our study. While it is difficult to directly compare wild fish gut microbiomes to aquaculture due to changes that occurs under captivity (Dhanasiri et al. 2011; Kormas et al. 2014; Eichmiller et al. 2016), the agreement here suggests that these two phyla play an essential role in juvenile brown trout microbiomes, and are likely part of their core microbiome. Within Proteobacteria, *Enterobacteriaceae* and *Coxiellaceae* were the most abundant families in our study. *Enterobacteriaceae* was found to be predominant in the gut microbiomes of juvenile freshwater salmon and sea trout (Skrodenytė-Arbačiauskienė et al. 2008; Llewellyn et al. 2016) and has been previously found in freshwater fish, mammals, and freshwater (Sullam et al. 2012). *Coxiellaceae* does not appear to be a common component in fish microbiomes, so the relatively high prevalence of 9.3 % of the total reads is interesting. At the genus level, this is primarily represented by *Diplorickettsia*, accounting for 9.18 % of the total reads (Appendix A). *Diplorickettsia* is an obligate intracellular bacteria associated with arthropods, specifically Ixodid ticks (Mediannikov et al. 2010), and has been previously noted in avian gut microbiomes (Kropáčková et al. 2017; Loo et al. 2019). Its presence in the current study may be diet-related and originating from the microbiome of ingested

arthropods, as juvenile brown trout are omnivorous (Sagar and Glova 2010). Finally, *Clostridium* (from the phyla Firmicutes) dominated the gut microbiota of rainbow trout in a study by Kim et al. (2007) and are a common component of herbivorous and omnivorous fish as they are known to be cellulose-degrading bacteria (Liu et al. 2021).

Differences in the relative abundance of taxa at the family level showed an increase in the families *Phyllobacteriaceae* and *Rickettsiaceae* in juveniles that went on to migrate compared to those that remained resident and individuals with a normal body condition. While *Phyllobacteriaceae* is a normal part of the fish microbiome and has been labelled as part of the core microbiome in Atlantic salmon (Gajardo et al. 2016), *Rickettsiaceae* (primarily the genus *Rickettsia*) harbour many pathogenic species and can cause disease in animals and humans (Bermúdez and Troyo 2018). *Rickettsia* are also commonly associated with arthropods (Perlman et al. 2006), and so may be part of the diet of juvenile brown trout. Conversely, *Neisseriaceae* was enriched in resident fish compared to migrants, with the genus *Deefgea* contributing close to half of the sequence reads within this family (Appendix A). There is limited data on this genus, with only five species described, three of those described within the last year (Chen et al. 2022; Gim et al. 2022). This genus is typically isolated from freshwater and fish samples and is considered part of fish gut microbiota, but may be pathogenic (Jeon et al. 2017; Shtykova et al. 2018; Abdul Razak and Scribner 2020; Terova et al. 2021; Gim et al. 2022). Related taxa within the same family have been described as chitin-degrading species, so it has been proposed that members in *Deefgea* may have a similar function, as suggested for the species *Deefgea chitinilytica* (Jung and Jung-Schroers 2011; Abdul Razak and Scribner 2020). This genus may therefore assist juvenile brown trout in digesting arthropods,

whose exoskeletons are made of chitin (Liu et al. 2019). Finally, *Mycoplasmataceae* was enriched in fish with poor body condition scores and slightly enriched in fish with high body condition scores. *Mycoplasmataceae*, particularly *Mycoplasma*, are common colonizers of vertebrate guts, both as pathogens and commensals (Holben et al. 2002; Brown et al. 2004; Neimark et al. 2004). In fact, *Mycoplasma spp*. appears to be a common component of fish microbiomes, particularly salmonids, and has been documented as a dominant taxon in Atlantic salmon (Holben et al. 2002; Zarkasi et al. 2014; Llewellyn et al. 2016; Bozzi et al. 2021), Chinook salmon (Ciric et al. 2019; Zhao et al. 2020), and rainbow trout (Lyons et al. 2017a, 2017b; Rimoldi et al. 2019). Bozzi et al. (2021) found *Mycoplasma* to be associated with higher fish weight and condition factor, while sick fish with low weight and condition factor were enriched with *Aliivibrio spp*., postulating that *Mycoplasma* may be a good biomarker for monitoring the health status of salmonids. We also found *Mycoplasma* to be associated with high fish body condition ($k>1$), but it was more abundant in fish with low body condition ($k<1$). However, in contrast to Bozzi et al. (2021), low body condition in our study was not an indicator of disease status, as all fish sampled appeared healthy with condition factors well within the normal range.

Examining our core hypotheses, the microbial composition and diversity of the hindgut of juvenile brown trout did not differ between life-history strategies nor among body condition categories, though it did vary by sampling site. Juvenile fish were sampled four to six months ahead of migration and likely ahead of the start of smoltification, so it is not entirely surprising that there were no differences in microbiota composition at this timepoint. In a larger-scale study across the salmonid life cycle, life

stages were linked to microbial composition and diversity in the gut (Llewellyn et al. 2016). Specifically, differences were found in the microbial community between freshwater and marine life stages, but no differences were found within freshwater ecotypes (i.e., microbiome community between smolts and parr; Llewellyn et al. 2016). We were interested in determining if the microbial composition and diversity of juvenile brown trout differed at the time of the presumed first decision window between fish that would later assume residency or migrate, but found no evidence to support this. Perhaps sampling closer to their outward migration, where the smoltification process is more advanced, would show different results. Intestinal fluid absorption and drinking rates increase during smoltification, which may disrupt the microbial community (Loretz et al. 1982; Llewellyn et al. 2016). In wild Atlantic salmon, there is a destabilization of the microbiome observed during the migratory phase, resulting in significant changes in microbiome diversity (but not community) between freshwater parr and smolts (Llewellyn et al. 2016). In the current study, we observed no significant difference in alpha diversity between migrants and residents, and there was a large range of alpha diversity estimates between fishes within each group. Again, this is likely due to the sampling period being too early to observe any effects caused by smoltification. Moreover, juvenile fish tend to have a greater species richness than their adult counterparts, which could be explained, in part, by their omnivorous diet (Orlov et al. 2006; Llewellyn et al. 2016).

Body condition did not relate to life-history strategy in the current study. Previous literature has shown that fish with low body condition are more likely to migrate (Boel et al. 2014; Peiman et al. 2017). Body condition is often used as a proxy for energetic state

(Forseth et al. 1999; Wysujack et al. 2009; Persson et al. 2018), and individuals with a poorer body condition are thought to have higher metabolic demands, necessitating migration to the rich ocean grounds to sustain these demands (Forseth et al. 1999; Morinville and Rasmussen 2003; Cucherousset et al. 2005, 2006). It may be that the sample size was too small in this study to show any trend, as we were limited by the number of residents that were recaptured and the number of fecal samples that were successfully amplified and sequenced. We were also interested in investigating the relationship between body condition and fecal microbiota composition and diversity, as the gut microbiota has been implicated in energy homeostasis in fish (Butt and Volkoff 2019). For example, probiotics have been shown to promote feed efficiency and growth in aquaculture fish species (Ye et al. 2011; Rodriguez-Estrada et al. 2013; Dias et al. 2018). If the gut microbiome can influence a fish's metabolic rate, there may be downstream effects on body condition and life-history strategy. However, we found no significant differences in either diversity or composition among fish with poor body condition ($k<1$), normal body condition ($k=1$), or high body condition ($k>1$). A larger sample size with a more definitive divide between fish with poor and high body conditions may provide a more robust test of this hypothesis and warrant further study.

Environment and diet are two major determinants of microbiota composition (Bolnick et al. 2014b; Li et al. 2014; Miyake et al. 2015; Eichmiller et al. 2016; Uren Webster et al. 2018), and we found differences in gut microbiota of fish between some of our study sites. Microbial communities between sites B and D, and C and D were significantly different, and the LEfSe analysis indicated which specific OTUs were driving these differences. One interesting point is that site D had the greatest number of

OTUs that were differentially enriched. This site is adjacent to a pig farm, where just upstream of our sampling site, there appears to be a containment pond that branches off the main part of the stream. This may be contributing to the different microbial compositions observed at this site as nutrient enrichment from farming practices can alter microorganism communities in adjacent waterways (Zhang et al. 2021). Unfortunately, the majority of the OTUs identified in the LEfSe analysis were not well resolved, with only one enriched OTU at site D identified to the genus *Deefgea* and one enriched OTU at site B belonging to the genus *Cetobacterium*. As previously described, *Deefgea* may have chitin-degrading properties (Jung and Jung-Schroers 2011), which may assist juveniles in digesting arthropods. *Cetobacterium* appears to be part of the core microbiome in various fish species, especially herbivores (Tsuchiya et al. 2008; Di Maiuta et al. 2013). Fish from site B had the highest alpha diversity estimates. This site was a mix of partially shaded (82 % of transect) and open (18 %) portions of the stream. The heterogeneity of the environment may have bolstered fish fecal alpha diversity by supporting more diverse arthropod or phytoplankton communities; sites C and D were also partially shaded (26% and 52%, respectively) but were generally much more open environments.

4.5.1 Conclusions

The results presented here indicate that exogenous factors, such as environment and/or diet, more strongly define the fecal gut microbiome of juvenile brown trout than endogenous factors, such as host physiology, at the life-stage assessed here. The fecal microbiome of juvenile brown trout does not appear to be related to their early migration

decision window. Sampling closer to the outmigration period, when would-be migrants are farther along in the smoltification process, may yield different results and show a divergence of the gut microbiome between residents and migrants, providing an interesting avenue for future research. Body condition was also not related to life-history strategy or microbial composition and diversity. A larger fish and fecal microbiome sample size may be beneficial to determine if that pattern remains or if the sample size was too low to capture the physiological variation in our sample population adequately. Finally, we saw a difference in microbiome composition and diversity of juvenile brown trout fecal microbiota across sampling sites. This was likely due to sample site D being adjacent to a pig farm and containment pond, which altered the local microbial communities. This study is the first to assess whether the gut microbiome component was associated with juvenile life-history strategies, and the present results suggest this is not the case. Given that the gut microbiome has been shown to influence host physiology and energy metabolism, future investigations should consider a sampling timepoint closer to the transition from freshwater to the ocean, and use larger starting sample sizes.

Table 4-1. Description of juvenile brown trout fish retained for this study.

Sample	Date	Site	% Transect with Shade	Migration Status	Length (cm)	Mass (g)	Condition Factor (k)
F3032	2018-Oct-22	Site A	100	Resident	12.4	17.9	0.94
F3106	2018-Oct-22	Site A	100	Resident	13.1	22.6	1.00
F3128	2018-Oct-22	Site A	100	Resident	13.9	27.1	1.015
F3131	2018-Oct-22	Site A	100	Resident	15.4	36.4	1.00
F3175	2018-Oct-23	Site B	82	Resident	14.1	27.7	0.99
F3182	2018-Oct-23	Site B	82	Resident	16.5	46.8	1.04
F3227	2018-Oct-23	Site B	82	Resident	14.9	29.2	0.88
F3344	2018-Oct-24	Site C	26	Resident	14.8	31.5	0.97
F3370	2018-Oct-24	Site C	26	Resident	14.4	29	0.97
F3371	2018-Oct-24	Site C	26	Resident	15.7	34.6	0.89
F3406	2018-Oct-24	Site C	26	Resident	13.9	23.1	0.86
F3413	2018-Oct-24	Site C	26	Resident	14.9	29.8	0.90
F3492	2018-Oct-25	Site D	52	Resident	14.9	30.5	0.92
F3495	2018-Oct-25	Site D	52	Resident	12.7	22.2	1.08
F3257	2018-Oct-23	Site B	82	Resident	14.8	27.2	0.84
F3077	2018-Oct-22	Site A	100	Migrant	14.1	26.9	0.96
F3103	2018-Oct-22	Site A	100	Migrant	13.5	25.9	1.05
F3138	2018-Oct-22	Site A	100	Migrant	13.4	21.1	0.88
F3181	2018-Oct-23	Site B	82	Migrant	13.3	20.6	0.88
F3186	2018-Oct-23	Site B	82	Migrant	15.3	31.5	0.88
F3203	2018-Oct-23	Site B	82	Migrant	13	21.2	0.96
F3207	2018-Oct-23	Site B	82	Migrant	13.5	22.2	0.90
F3216	2018-Oct-23	Site B	82	Migrant	15.8	35.8	0.91
F3219	2018-Oct-23	Site B	82	Migrant	12.5	17.5	0.90
F3224	2018-Oct-23	Site B	82	Migrant	13.1	20.9	0.93
F3229	2018-Oct-23	Site B	82	Migrant	13.1	18.7	0.83
F3404	2018-Oct-24	Site C	26	Migrant	14.7	30	0.94
F3410	2018-Oct-24	Site C	26	Migrant	12.9	18.8	0.88
F3427	2018-Oct-24	Site C	26	Migrant	15.7	34.2	0.88
F3435	2018-Oct-24	Site C	26	Migrant	17	55.4	1.13
F3469	2018-Oct-25	Site D	52	Migrant	13.8	23	0.88
F3503	2018-Oct-25	Site D	52	Migrant	15	30.8	0.91
F3507	2018-Oct-25	Site D	52	Migrant	14.3	26.6	0.91
F3446	2018-Oct-24	Site C	26	Migrant	15.7	35.8	0.93

Table 4-2. Prevalence of family-level taxa across sampled population with a relative abundance greater than 1% of total OTU sequences.

Family	Percentage of total reads (%)
Enterobacteriaceae	23.6
Clostridiaceae_1	16.2
Coxiellaceae	9.3
Unclassified bacteria	7.8
Neisseriaceae	4.0
Aeromonadaceae	3.0
Rickettsiaceae	2.9
Mycoplasmataceae	2.8
Microbacteriaceae	1.9
Unclassified *Firmicutes*	1.8
Phyllobacteriaceae	1.7
Unclassified *Rhizobiales*	1.5
Unclassified *Actinomycetales*	1.2
Peptostreptococcaceae	1.1
Rhodobacteraceae	1.1
Planctomycetaceae	1.1

Table 4-3. Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance of fecal bacterial communities of brown trout for groupings: migratory status, study site, and body condition. Df = degrees of freedom, SS = sum of squares, MS = mean squares, F. Model = F-value by permutation, R2 = percentage of variance explained by the groups, Pr(>F) = p-value for F-statistic. P-values based on 999 permutations. *Difference is significant at 0.05 level.

	Df	SS	MS	F.Model	R2	Pr(>F)	
Migratory Status	1	0.297	0.29697	0.86167	0.02528	0.569	
Sampling Site	3	1.6806	0.56019	1.62542	0.14305	0.02	*
Body Condition	2	0.4652	0.2326	0.67491	0.0396	0.891	
Residuals	27	9.3054	0.34465		0.79207		
Total	33	11.7482			1		

Table 4-4. Analysis of multivariate homogeneity of group dispersions (betadisper) based on Bray-Curtis distance of fecal bacterial communities of juvenile brown trout from different sampling sites. Df = degrees of freedom, SS = sum of squares, MS = mean squares, Pr(>F) = p-value for F-statistic.

	Df	SS	MS	F.value	Pr(>F)
Groups	3	0.25731	0.08577	1.9972	0.1356
Residuals	30	1.28836	0.042945		

Table 4-5. Pairwise comparisons: Observed p-value below diagonal, permuted p-value above diagonal.

	Site A	Site B	Site C	Site D
Site A		0.542	0.659	0.331
Site B	0.541943		0.982	0.005
Site C	0.639126	0.982405		0.054
Site D	0.32758	0.005867	0.04782	

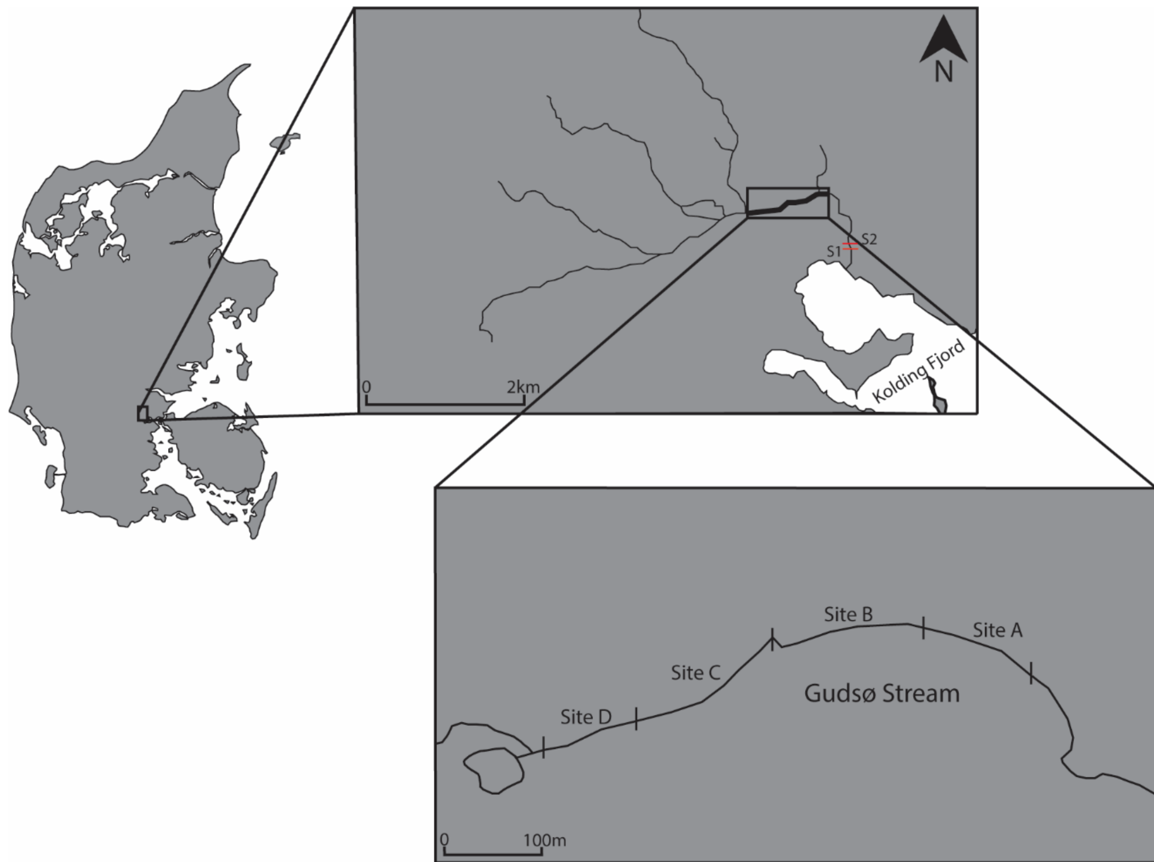

Figure 4-1. Study site location, Gudsø stream, southeastern Jutland, Denmark. Two PIT stations (S1 and S2) are located just upstream of the mouth into Kolding Fjord. The shaded box in the top box represents the area of stream sampled, which is blown up in the bottom box to show the location of the sampling sites (A-D), as well as the containment pond located just upstream from Site D.

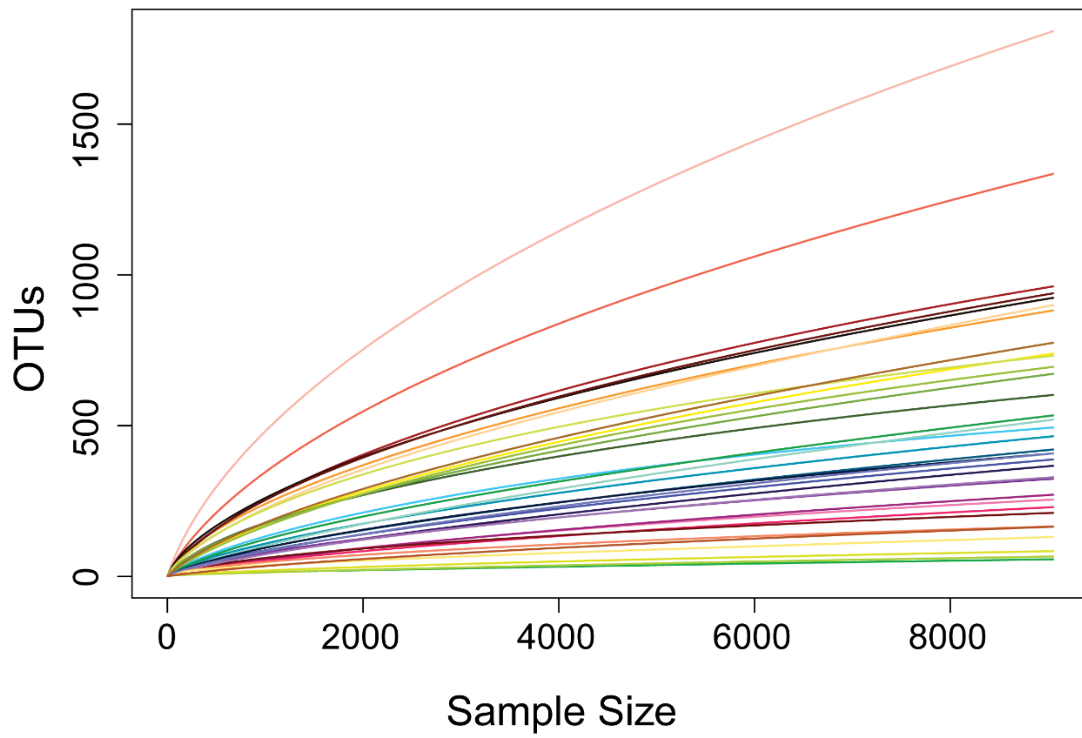

Figure 4-2. Rarefaction curve showing the number of OTUs on the vertical axis against the number of sequences (sample size) on the horizontal axis for all juvenile brown trout fecal samples.

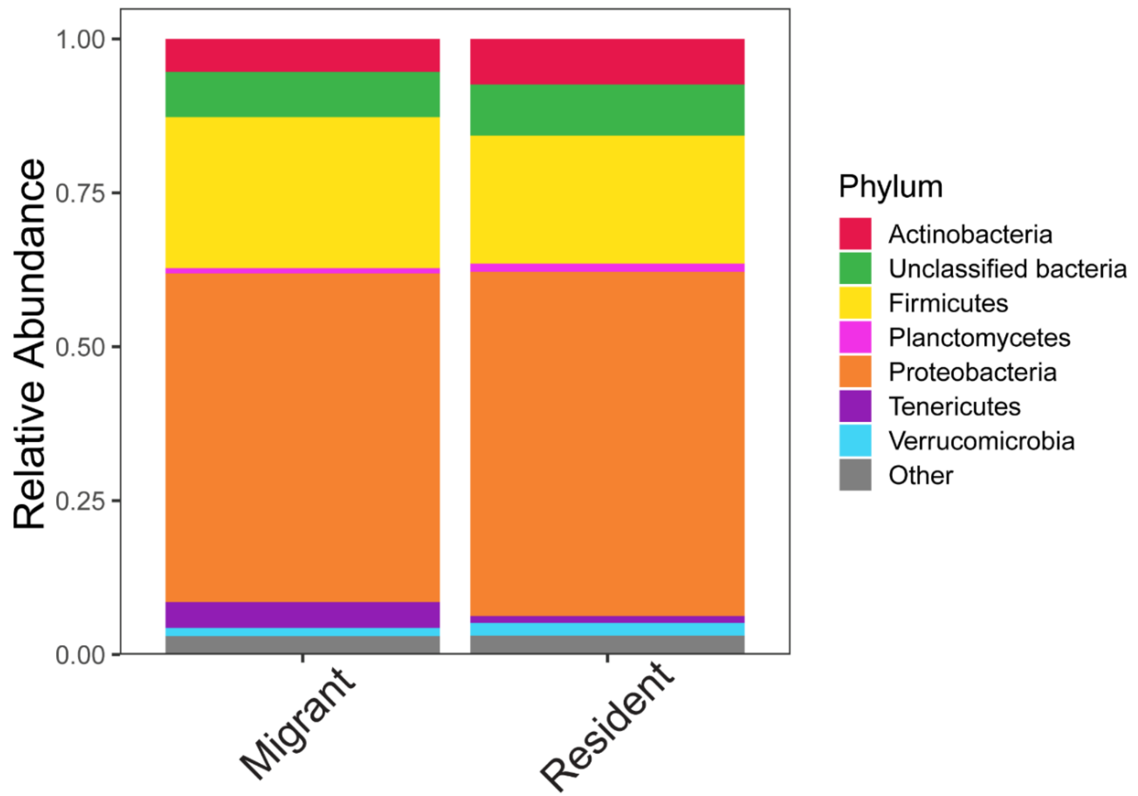

Figure 4-3. Relative abundance of the major phyla present in migrant and resident juvenile brown trout fecal samples (representing more than 1.0 % relative abundance).

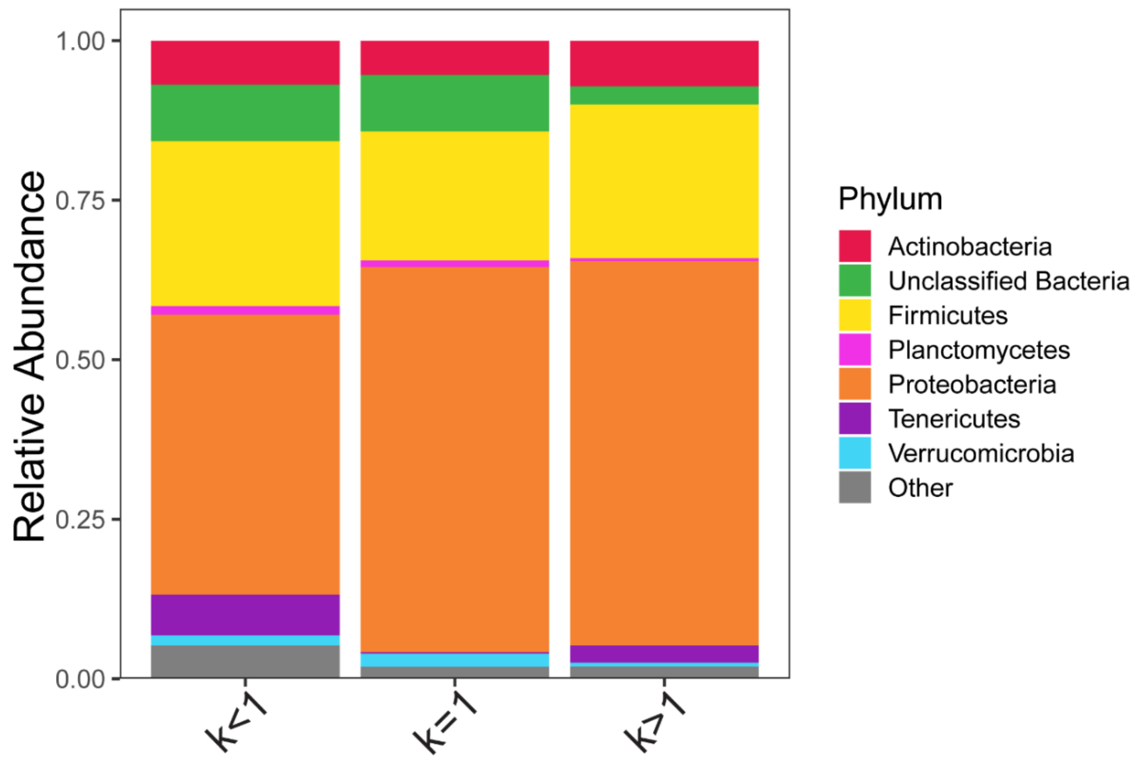

Figure 4-4. Relative abundance of the major phyla present across fish with low body condition (k<1), normal body condition (k=1), and high body condition (k>1; representing more than 1.0 % relative abundance).

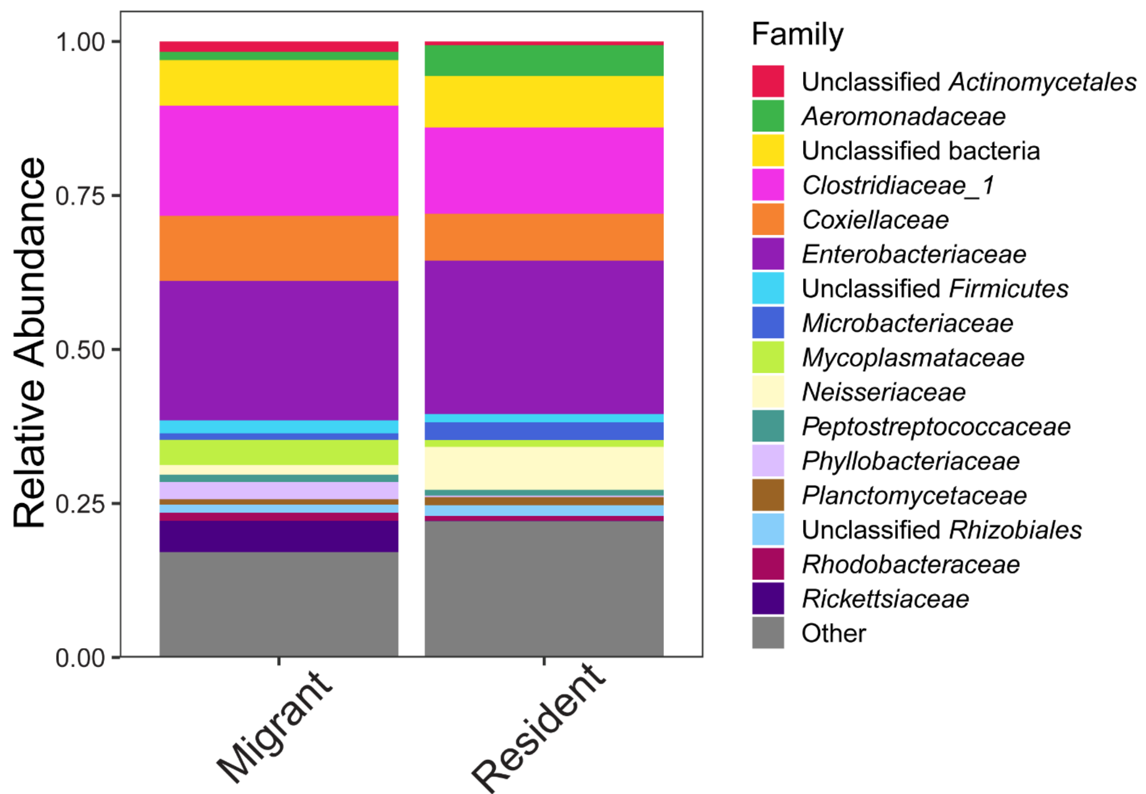

Figure 4-5. Relative abundance of the major families present across migrant and resident fish (representing more than 1.0 % relative abundance).

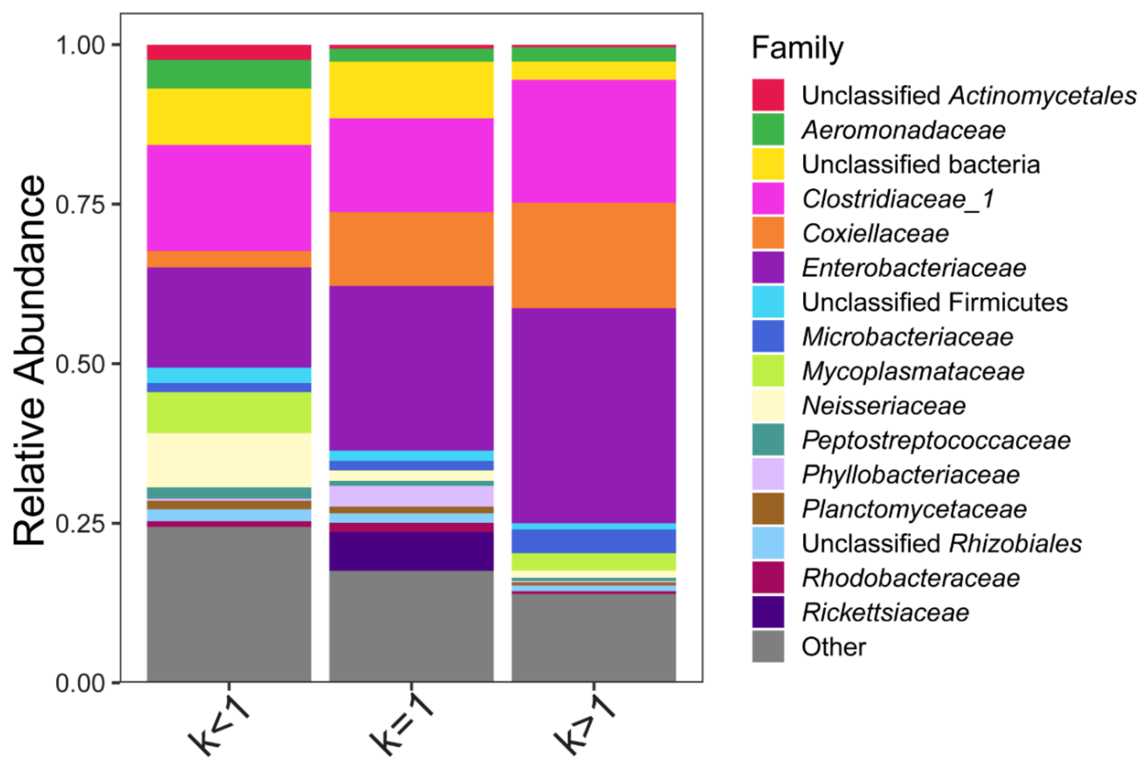

Figure 4-6. Relative abundance of the major families present across fish with low body condition (k<1), normal body condition (k=1), and high body condition (k>1; representing more than 1.0 % relative abundance).

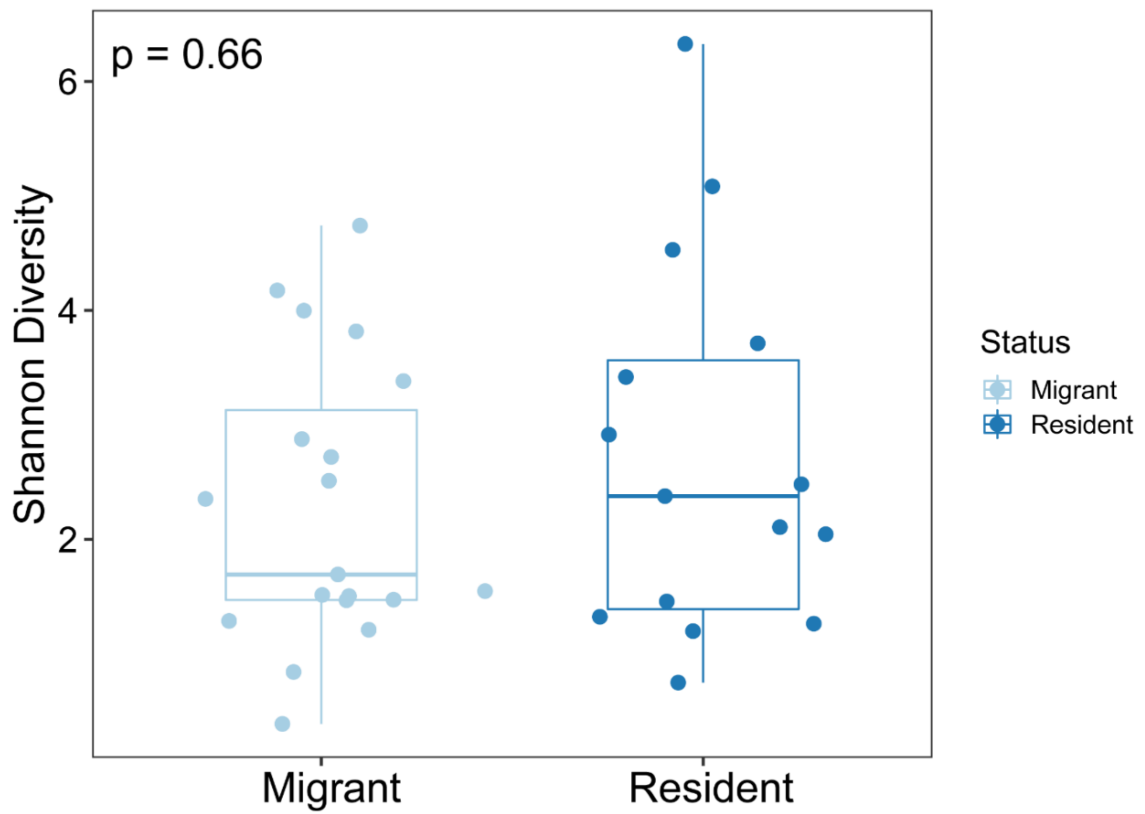

Figure 4-7. Shannon alpha diversity measure on operational taxonomic units (OTUs) of fecal bacterial community of migrant and resident juvenile brown trout.

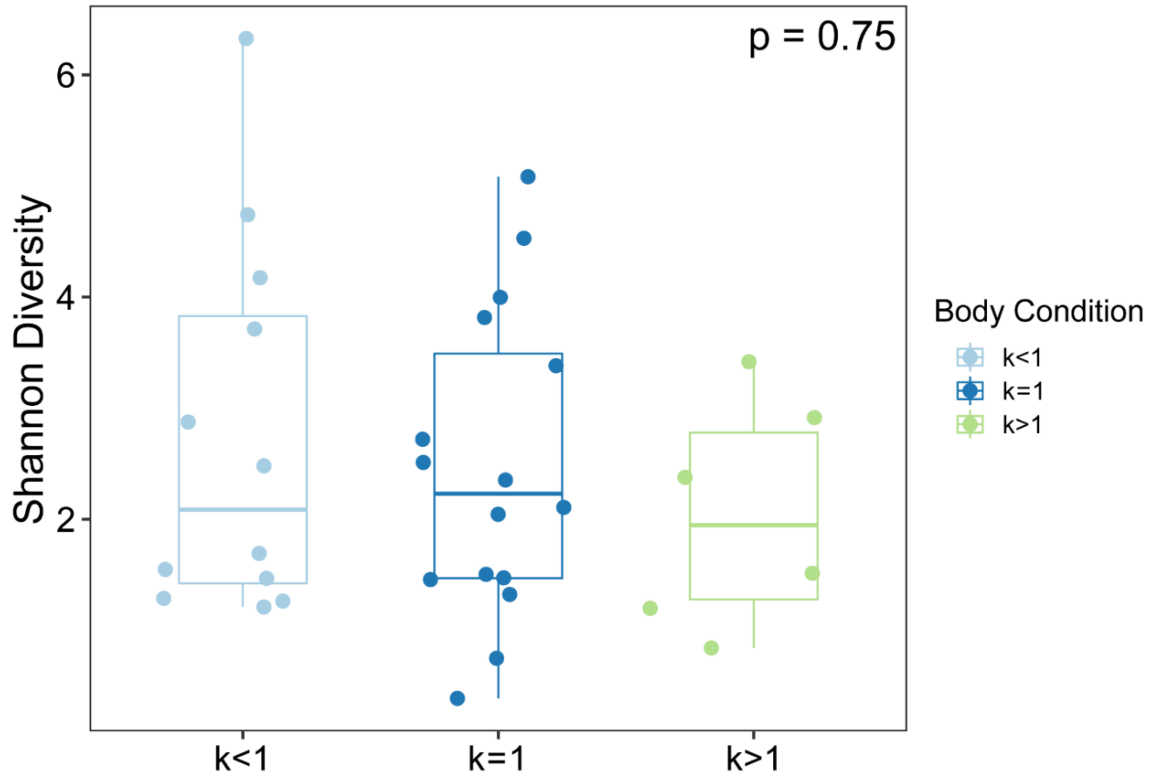

Figure 4-8. Shannon alpha diversity measure on operational taxonomic units (OTUs) of fecal bacterial community of juvenile brown trout with body condition scores of k<1 (low), k=1 (normal), and k>1 (high).

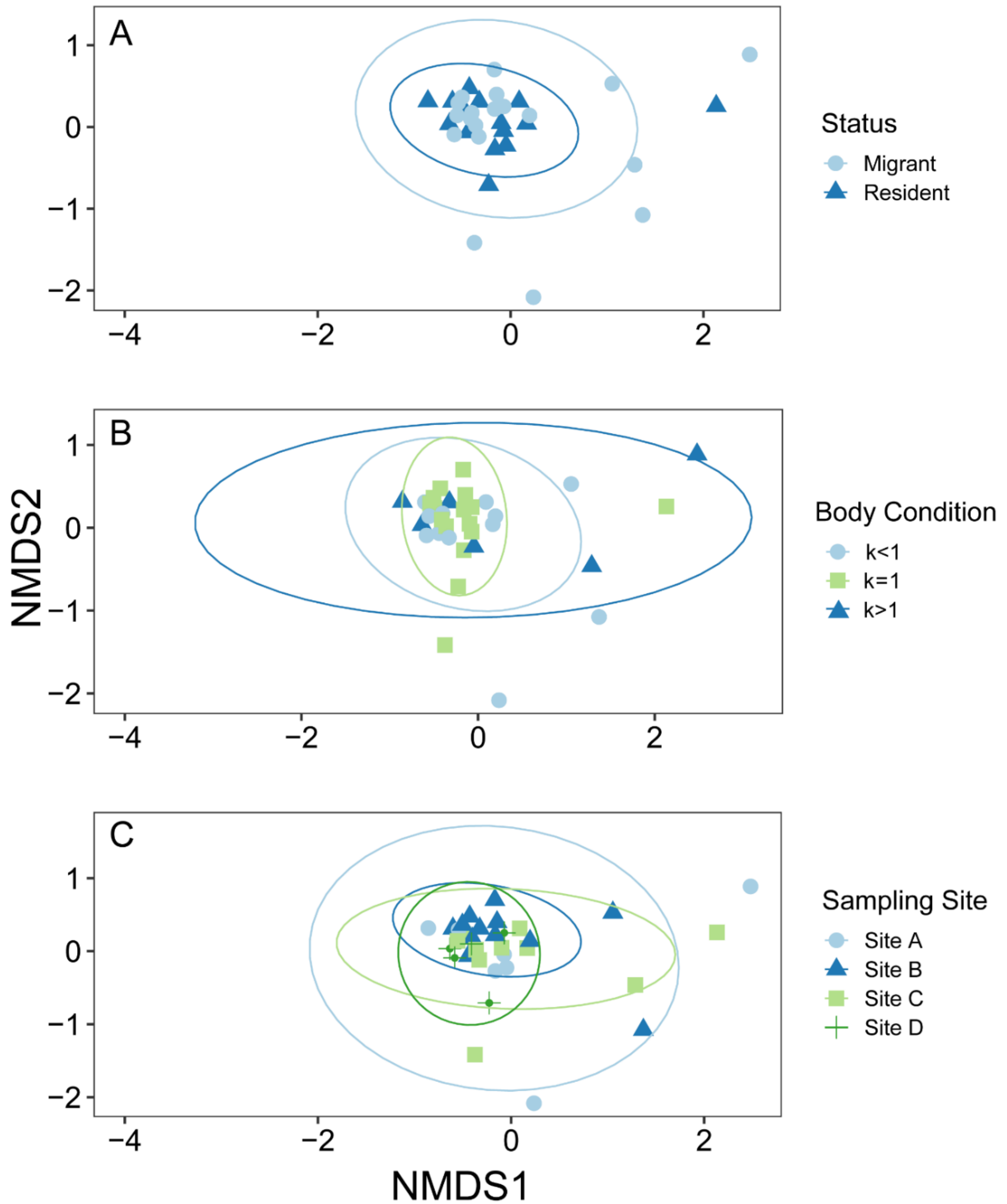

Figure 4-9. Two-dimensional non-metric multidimensional scaling (NMDS) plots with 95% confidence ellipses around each group centroid of juvenile brown trout fecal bacterial communities using a Bray Curtis distance matrix to show dissimilarity between individual fish samples grouped by A) Migratory Status, B) body condition categories, and C) sampling site.

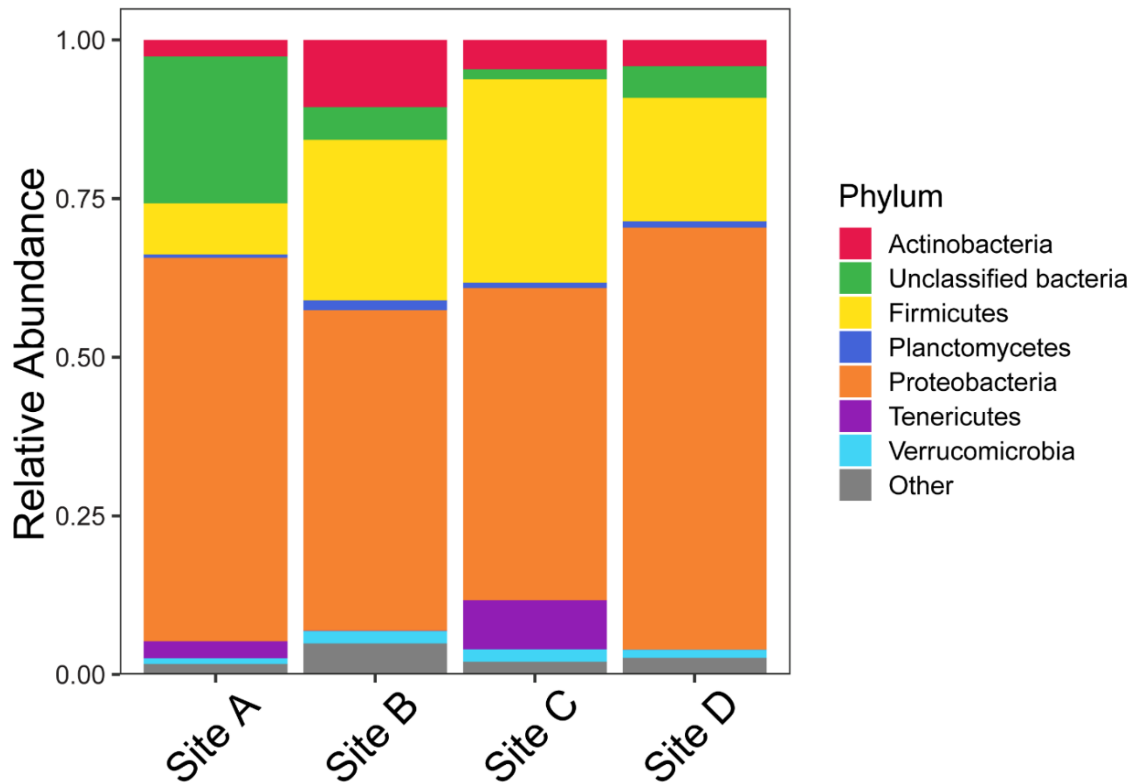

Figure 4-10. Relative abundance of the major phyla present across fish captured at four different sampling sites (representing more than 1.0 % relative abundance).

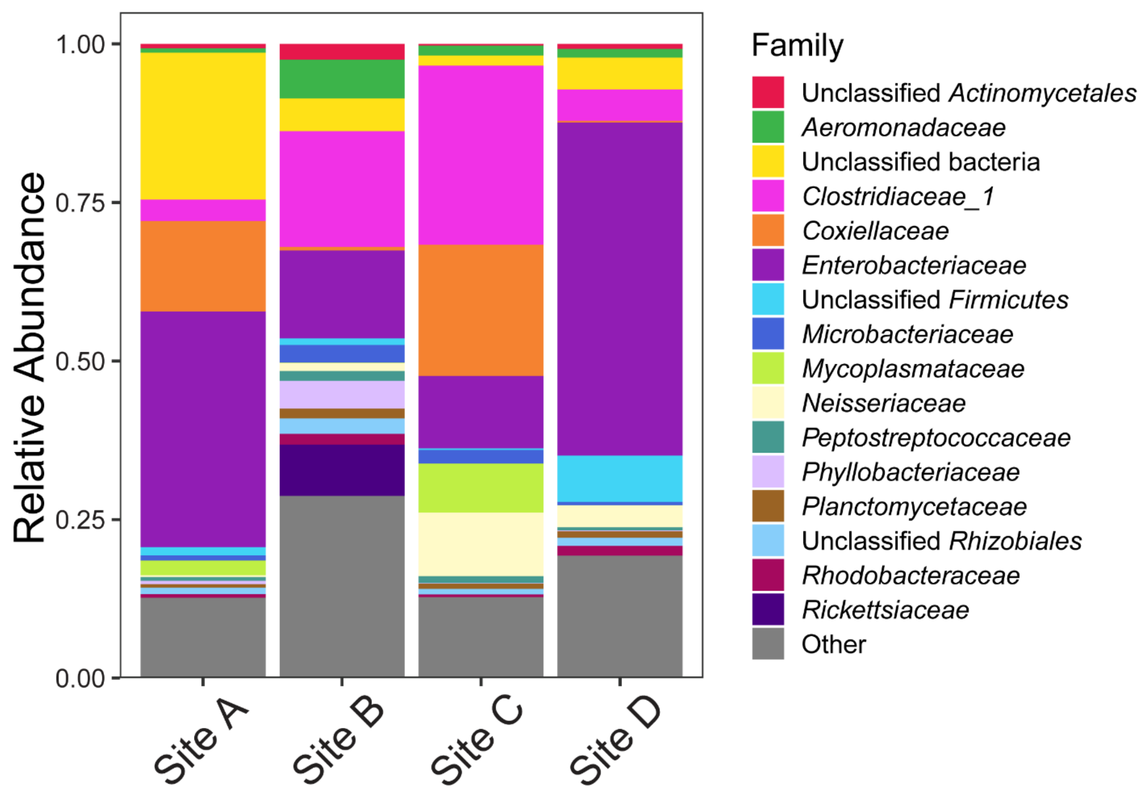

Figure 4-11. Relative abundance of the major families present across fish captured at different sampling sites (representing more than 1.0 % relative abundance).

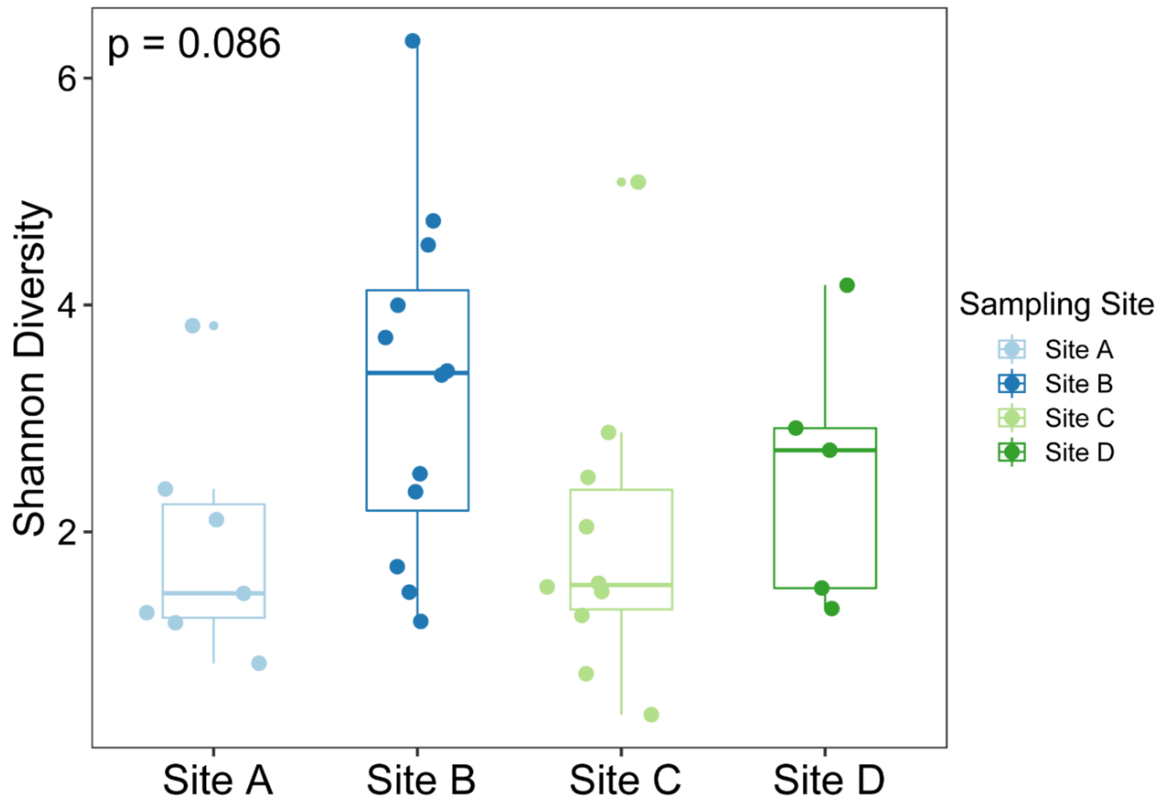

Figure 4-12. Shannon alpha diversity measure on operational taxonomic units (OTUs) of fecal bacterial community of juvenile brown captured at different sampling sites.

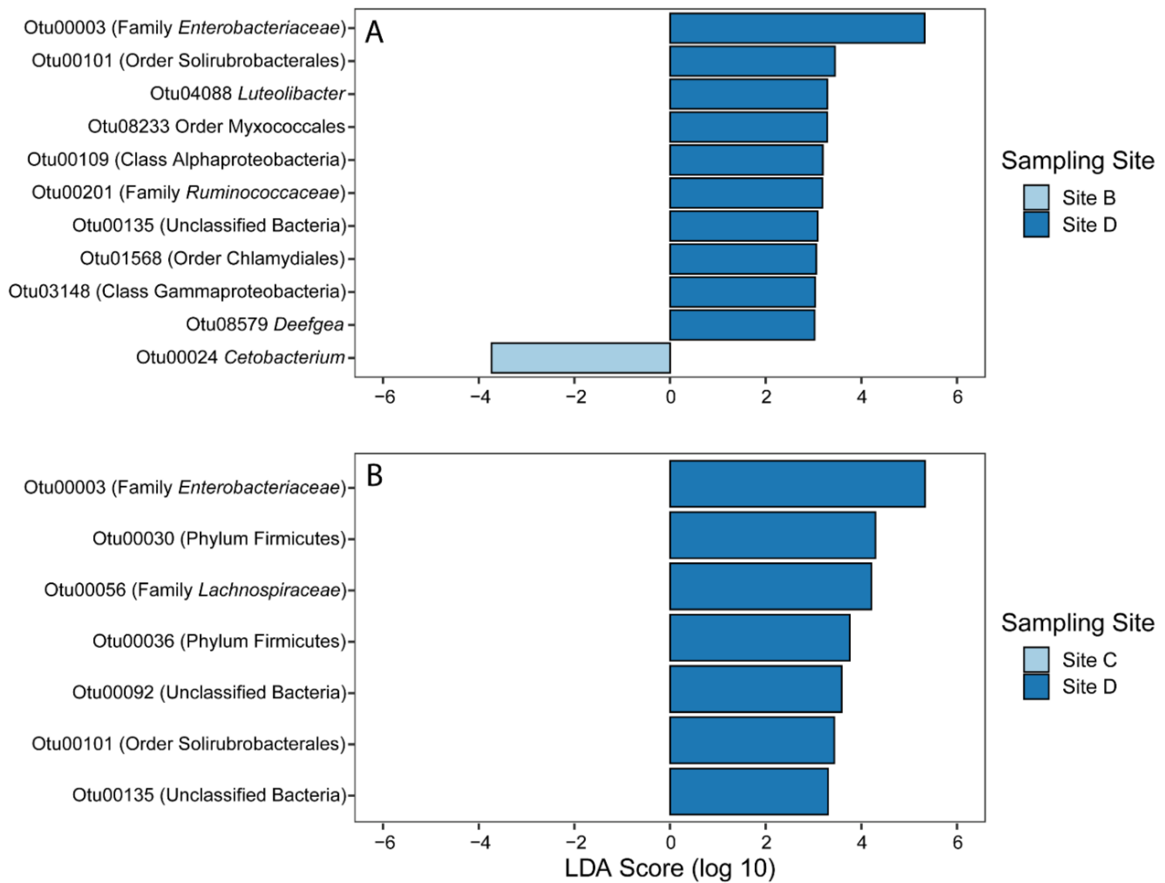

Figure 4-13. Linear discriminant analysis effect size (LEfSe) analysis showing OTUs likely driving the dissimilarity observed in the fecal bacterial community structure between fish captured at sampling sites B and D (top) and C and D (bottom). A negative LDA score shows OTUs with significantly higher abundances in fish from sample sites B (top) and C (bottom), while positive LDA scores show OTUs with significantly higher abundances in fish from site D.

Chapter 5: Challenges and opportunities with transitioning to non-lethal sampling of wild fish for microbiome research

5.1 Abstract

The microbial communities of fish are considered an integral part of maintaining the overall health and fitness of their host. Research has shown that resident microbes reside on various mucosal surfaces, such as the gills, skin, and gastrointestinal tract, and play a key role in various host functions, including digestion, immunity, and disease resistance. A second, more transient group of microbes reside in the digesta, or feces, and are primarily influenced by environmental factors such as host diet. The vast majority of fish microbiome research currently utilizes lethal sampling to analyze any one of these mucosal and/or digesta microbial communities. The present paper discusses the various opportunities that non-lethal microbiome sampling offers, as well as some inherent challenges, with the ultimate goal of creating a sound argument for future researchers to transition to non-lethal sampling of wild fish in microbiome research. Doing so will reduce animal welfare and population impacts on fish while creating novel opportunities to link host microbial communities to an individual's behaviour and survival across space and time (e.g., life-stages, seasons). Current lethal sampling efforts constrain our ability to understand the mechanistic ecological consequences of variation in microbiome communities in the wild such that transitioning to non-lethal sampling will open new frontiers in ecological and microbial research.

5.2 Introduction

The bacterial microbiota of fish are a complex and dynamic community that inhabits the mucosal surfaces of fish, such as the skin, gills, and gastrointestinal tract (Legrand et al. 2020a). These communities interact with the host to provide various beneficial services, including metabolic processes, immune functions, and disease resistance, that are important to fish health and fitness (Llewellyn et al. 2014). The composition and diversity of this collective microbiota are heavily influenced by exogenous factors (i.e., environment, diet) and endogenous factors (i.e., host genetics and physiology; Spor et al. 2011). As such, the microbial community is highly dynamic and varies among individuals and within an individual across time and contexts (e.g., life cycle stages; Boutin et al. 2014; Llewellyn et al. 2016).

Currently, the majority of microbiome studies conducted on fish involve lethal sampling and removal of whole digestive tracts, with a potential secondary sampling of the gill or skin microbiotas (Gajardo et al. 2016; Uren Webster et al. 2018). Sampling will either use the whole digestive tract (Gajardo et al. 2016) or particular sections of the tract, such as the hindgut (Lyons et al. 2017c) or digesta (Eichmiller et al. 2016). Comparative studies between the different gut compartments (i.e., proximal, mid, and distal intestine), as well as the digesta, have revealed that the microbial composition and diversity varied significantly, both between compartments within the intestinal tract, as well as between the intestinal mucosal layer and digesta (Gajardo et al. 2016; Nyholm et al. 2022). Resident (autochthonous) bacteria are found more commonly on the mucosal layer, in close association with the host epithelial cells, and are typically less diverse communities than the transient (allochthonous) community comprising the digesta, which

are more heavily influenced by environmental factors (Gajardo et al. 2016; Legrand et al. 2020a). Therefore, the research questions being asked (i.e., host-associated factors vs. effects of diet, for example) will necessitate which part of the gastrointestinal tract is required for sampling and the necessity for lethal sampling or not.

Lethal sampling is much more commonly used in fish microbiome studies compared to other animal taxa, such as primates and birds, where fecal or cloacal sampling are more often used (Waite et al. 2012; Risely et al. 2017; Björk et al. 2022). Several studies on humans assessed rectal swabs' effectiveness in characterizing the hindgut microbiome, compared to colon biopsy and/or fecal samples (Araújo-Pérez et al. 2012; Budding et al. 2014; Bassis et al. 2017). This has occurred for other vertebrate taxa as well, including bird fecal versus cloacal sampling (Videvall et al. 2018), bat fecal versus intestinal sampling (Ingala et al. 2018), and most recently, fish fecal versus intestinal sampling (Nyholm et al. 2022). The general conclusion is that different sampling methodology captures different parts of the microbiome and should be carefully considered when formulating research questions. However, despite these differences, non-lethal sampling is still highly prevalent among higher vertebrate classes and suggests that lethal sampling in fish microbiome research may be overused and associated opportunities that come from non-lethal sampling missed.

Fish welfare should be prioritized when planning microbiome studies, and lethal sampling should only be conducted when absolutely necessary (i.e., development of robust non-lethal measures would represent a major animal welfare refinement). However, beyond animal welfare arguments, there are research opportunities that arise when able to re-sample individual fish over time and to link individual-level microbial

communities with ecological activities such as behaviour, reproductive success, or survival. The purpose of this perspective article is to highlight some of the opportunities non-lethal microbiome sampling of fish offers, including the ability to work on rare/threatened species, the ability to combine microbiome sampling with other methods (e.g., biotelemetry, biologging) and endpoints (behaviour, reproductive success, survival), as well as the ability to do serial sampling on the same individuals across space and time. Non-lethal microbiome sampling also has its challenges and limitations, which will also be discussed. Our hope is that this paper will stimulate additional validation studies that will determine the contexts in which non-lethal sampling is effective.

5.3 Non-lethal sampling methods

Four main sampling methods are used for non-lethal sampling of fish microbiomes (Figure 5-1). Fecal sampling is the most common of these and involves simply collecting the feces of an animal. This can be done on SCUBA underwater by following a fish until it defecates (Smriga et al. 2010), or fish can be temporarily removed and feces manually expressed by applying gentle pressure along the ventral abdominal wall towards the anus (Eichmiller et al. 2016). Fecal sampling is advantageous as it collects a generous amount of sample, often much more than the minimum requirement for DNA extraction kits, which allows for some redundancy. A disadvantage of fecal sampling is that it collects only fecal matter, which contains bacteria primarily associated with the digesta (allochthonous microbiota; Ringø and Birkbeck 1999). The bacteria associated with the intestinal mucosa (autochthonous bacteria) are largely missed by sampling using this method (Romero et al. 2014).

Hindgut swabbing, where a swab is inserted through the anus and rotated along the intestinal walls of the hindgut (Figure 5-2), is more invasive than collecting fecal matter but offers the advantage of collecting autochthonous bacteria associated with the intestinal mucosa, as well as bacteria associated with the digesta. In theory, it offers a more complete picture of the hindgut microbiota. Hindgut swabbing has not been used substantively in fish studies, but it is fairly common practice among bird and reptile studies, where cloacal swabbing is used as a proxy for the colon or fecal microbiota (Martin et al. 2010; Dewar et al. 2013, 2014; Stanley et al. 2015; Videvall et al. 2018). However, in practice, method papers have found mixed results regarding the validity of this proxy. Videvall et al. (2018) compared the microbiota composition between fecal and cloacal sampling in juvenile ostriches (*Struthio camelus*) and found that fecal samples better represented the bacterial community of the colon than did cloacal swabs. Further, a previous study by Videvall et al. (2017) found that cloacal swabs had lower repeatability compared to fecal samples, and this was likely due to the low biomass nature of swab sampling. Low initial DNA concentration introduces stochasticity, depending on what bacterial taxa are initially amplified (Videvall et al. 2017).

Gill biopsy or gill swabbing are microbiome samples taken from the gill mucus layer and/or tissue. The gills are open to the external environment, which makes them an important site for pathogen entry, and are immunologically active organs (Secombes and Wang 2012). This makes them a good option if the interest is in examining gill microbial communities and the presence of disease, as they have been found to reflect disease states, such as chronic gastroenteritis (Legrand et al. 2018). However, there are limited studies examining gill microbiomes, especially using non-lethal methods such as gill

swabbing (Legrand et al. 2018; Dunn et al. 2020). One study to date has compared gill biopsy and swabbing in Atlantic salmon (*Salmo salar*) and found a divergence in microbial communities obtained using the two sampling strategies (Clinton et al. 2021). Overall, gill swabs were preferable as they isolated a more diverse microbial community and did not have as many issues with host DNA. However, biopsies recovered more cryptic community membership and may be more suitable for sub-surface or intracellular microbes (Clinton et al. 2021). Small, non-lethal gill biopsies are routinely used for other molecular techniques, such as transcriptomics (Jeffries et al. 2014; Drenner et al. 2018).

The final non-lethal sampling method is skin swabbing. The skin of fish is mucosal and exposed to the external environment (Gomez and Primm 2021); this makes it one of the easiest non-lethal methods to use. As such, there is a wide variety of studies that examined the skin microbiota of several fish species, including both wild and aquaculture species (Boutin et al. 2014; Pratte et al. 2018). Sampling typically involves using a swab to sample the skin and mucosa on the lateral side from the back of the operculum to the caudal peduncle, along the lateral line (Uren Webster et al. 2018). There is a risk of disrupting this protective layer, which could lead to disease. Catfish skin microbiomes disrupted by potassium permanganate were found to have increased mortality from the pathogenic bacteria *Flavobacterium columnare*, which causes columnaris disease (Mohammed and Arias 2015). Despite the relative ease of using this sampling method non-lethally, many studies still lethally sample for skin microbiome research (Lowrey et al. 2015; Chiarello et al. 2018). This may be due, in part, to the capture method (e.g., by speargun; Chiarello et al. 2018) or because more invasive samples are being taken in concert (Uren Webster et al. 2018).

5.4 Opportunities of non-lethal microbiome sampling

Non-lethal sampling provides an opportunity to sample rare or threatened populations and species that would otherwise be unattainable due to legal protections or conservation concerns. Having a better understanding of host-microbiome associations and the functional role microbes play in host health and fitness can aid in the conservation of imperiled species (Zhu et al. 2021a). Many threatened species must also contend with anthropogenic disturbances such as habitat degradation and pollution (Arthington et al. 2016), along with climate change factors such as elevated temperatures or changes in salinity (Portner and Peck 2010), which would also negatively impact their microbiome, potentially leading to reduced host fitness and survival that could further depress population numbers or prevent recovery (Zhu et al. 2021a). Conservation reintroduction programs could also benefit from understanding optimal host-microbe associations to maximize fitness after releasing captive individuals back into the wild (Zhu et al. 2021a), as is commonly done for terrestrial organisms (Bahrndorff et al. 2016; West et al. 2019). This is relevant to fish hatcheries that use captive breeding as a means to conserve, reintroduce, or supplement populations in the wild (Rytwinski et al. 2021). Pre-release conditioning of the gut microbial community through diet training was attempted in captive-bred endangered Yangtze sturgeon (*Acipenser dabryanus*) prior to release to increase post-release survival and fitness (Yang et al. 2020). This is a promising area of research (see Jin Song et al. 2019) that would benefit significantly from more research effort.

Another opportunity provided by non-lethal sampling is that it can be integrated with movement research, such as telemetry and mark-recapture, to provide insight into a

fish's behaviour and associated microbiome. For a full review of non-lethal sampling and fish movement research in freshwater fishes, see Thorstensen et al. (2022). In the context of fish movement ecology, non-lethal sampling is necessary as you need to see what the fish are doing after you sample their microbiota to answer your proposed research questions. This has relevance to both migration behaviour and reproductive behaviour studies. Most salmonid migration microbiome studies to date lethally sample fish and provide characterizations of the gut microbiome at different stages of their migration or life-cycle using cross-sectional population-based analyses (Llewellyn et al. 2016; Element et al. 2020a, 2020b; Le et al. 2020; Tosin et al. 2020; Liu et al. 2022), rather than individual-based analysis. However, if host-microbiota research maintains that the commensal microbiome increases host survival and fitness, then there should be relevant studies examining fish under these circumstances. Fish migration and spawning offer an excellent opportunity to intrinsically test these hypotheses. Spawning migration runs are arduous physiologically challenging endeavors, especially among semelparous anadromous species, such as sockeye salmon (*Oncorhynchus nerka*), that rely on endogenous energy reserves to fuel their journey (Brett 1995). Many fish die before reaching the spawning grounds, due to the depletion of energy reserves and disease, amongst other reasons (Hinch et al. 2006). Differential survival among a migratory population would be a prime example to study correlations between successful migrants and the gut, skin, or gill microbiomes. Taking it a step further, spawning success as a proxy for fitness amongst female Pacific salmon can easily be established based on the presence or absence of eggs in the abdominal cavity after death on the spawning grounds and could be correlated to microbiome composition and diversity. Currently, no research

studies utilize non-lethal sampling for wild fish behaviours, such as migration. It is, however, a commonly used method in avian migration studies, where fecal samples are taken at bird stop-over points along their migration route (Lewis et al. 2016; Risely et al. 2017; Skeen et al. 2021).

A final opportunity provided by non-lethal sampling is the ability to collect time-series data, which provides invaluable insight into how microbiome dynamics change over time. It is particularly well-studied in humans, where in-depth research has shown how dynamic microbial communities are during the first years of life (Koenig et al. 2011) and even on shorter timescales, such as after infections (Hoffmann et al. 2009) or antibiotics (Peterfreund et al. 2012). Time-series data has also been studied in wild animal populations, particularly in primates (Björk et al. 2022; Murillo et al. 2022), but also in birds (Skeen et al. 2021). For example, Björk et al. (2022) provided an extensive gut microbial time series from wild baboons and found that despite synchronizing forces in baboon populations (e.g., shared environments and diets), hosts still retained highly idiosyncratic gut microbiomes. Both the studies of primates and birds have important implications in terms of linking microbial dynamics to health outcomes and are, therefore, a topic of interest. Within fish species, microbiome time-series data is important in the aquaculture industry, where health outcomes are also closely monitored in association with microbial dynamics, as well as the effect of different feeding regimes and other pertinent metrics. While repeat fecal microbiome sampling of aquaculture fish does occur (Zarkasi et al. 2014; Neuman et al. 2016), lethal sampling is still largely used where fish are lethally sampled at different time points to examine how microbiomes change over time in response to different treatment regimes (Ringø et al. 2006a; Payne et

al. 2022). Microbiome time series data were also examined using captive clownfish and anemones in a tank experiment, using non-lethal skin mucus swabs to sample the skin microbiome of fish to see how it changes before, during, and after association with an anemone (Pratte et al. 2018); however, time series microbiome studies are rare in wild fish species. We identified two studies that assessed temporal variability (amongst other drivers) of the gut or mucosal microbiota in wild rabbit fish (*Siganus guttatus*; Le et al. 2020) and Pacific chub mackerel (*Scomber japonicus;* Minich et al. 2020). However, these were cross-sectional studies, and fish were killed to collect microbiome samples. That being said, we could not find any studies that non-lethally sampled wild fish microbiomes at more than one time point for temporal analysis of the microbiome. One could argue that it is difficult to recapture the same individual fish in aqueous environments. There are circumstances that would make this task easier. For instance, iteroparous fish that spawn annually could be externally tagged and non-lethally sampled for microbiome analysis over multiple years. On a shorter timeframe, some fish species, such as smallmouth bass (*Micropterus dolomieu*), undertake paternal care during the spawning period and exhibit nest and brood-guarding behaviours for up to four weeks, until offspring are self-sufficient (Cooke et al. 2002). This would also provide an excellent means to examine fish microbiomes in relation to fitness endpoints as well and should be further investigated.

5.5 Challenges of non-lethal microbiome sampling

The greatest challenge concerning non-lethal microbiome sampling is the low biomass often obtained when taking swabs of different fish body compartments. Low-

biomass samples typically have lower repeatability (same results from replicates of the same sample) than higher biomass samples (such as feces; Videvall et al. 2017). This is because low-biomass samples have low initial template DNA concentrations, which increases the likelihood of stochastic noise generated during PCR amplification prior to sequencing (Videvall et al. 2017; Erb-Downward et al. 2020). Further, any small amount of contamination during the sampling stage and/or the DNA extraction stage can result in over-amplification during polymerase chain reaction (PCR), which can critically impact downstream analyses and result in erroneous interpretations (Salter et al. 2014; Eisenhofer et al. 2019). To mitigate this issue, using both positive and negative controls can help recognize contamination signals so that they can then be excluded from the final data set (Kennedy et al. 2023). Contamination can also be removed during the analysis phase using software packages such as *decontam* that removes more abundant contaminants (Davis et al. 2018).

Another challenge in tandem with low-biomass samples is the presence of PCR inhibitors. Inhibitors comprise a variety of organic and inorganic substances and can come from a biological origin (such as the biological materials being sampled) or be introduced during sample processing or DNA extraction (Schrader et al. 2012). Inhibitors function by interfering with cell lysis during DNA extraction, degrading nucleic acid, or inhibiting the amplification of nucleic acids during the PCR process (Wilson 1997). This has downstream effects on the final sequencing libraries produced and overall microbial diversity characterized. Until recently, the majority of gut microbiome optimization method papers have been centered on mammals (Choo et al. 2015; Blekhman et al. 2016; Jin Song et al. 2016) and have targeted protocols to remove PCR inhibitors. Despite an

influx of fish gut microbiota research, fish microbiome optimization has received comparatively less attention (Talwar et al. 2018). Further, fish gut samples have very different chemical and enzymatic profiles, which may result in differing degrees of PCR inhibition (Hildonen et al. 2019). Fish gill samples also prove to be rich in PCR inhibitors; however, gill biopsies are likely more problematic than gill swabs due to being a blood-rich tissue (Clokie et al. 2022). Inhibitors can also be introduced during sample preservation and storage. A comparison study on different storage methods (immediate freezing, 96% ethanol, RNAlater, and DNA/RNA shield) for gut microbiome samples from rainbow trout (*Oncorhynchus mykiss*) showed that different methods were associated with different degrees of PCR inhibition and highlighted the importance of these types of optimization studies when exploring new species systems (Hildonen et al. 2019). The authors found that RNAlater-stored mucosal samples had the lowest levels of inhibition. However, 96% ethanol was the preferred storage method for rainbow trout gut microbiome samples as it yielded higher amounts of DNA, and DNA sequencing libraries were of sufficient quality (Hildonen et al. 2019). However, when working with wild species at remote field sites, especially if air travel is required, some sample storage methods, such as ethanol, are not viable if you plan to fly commercially.

As mentioned previously, a great opportunity provided by non-lethal sampling is the ability to take multiple samples in time-series experiments. However, more research needs to be conducted to determine if, and the impact magnitude, repeat sampling has on the microbiome. This would be particularly relevant to skin and gill swabs, where a thick mucous layer is present, and the disruption of this protective barrier could potentially allow an alternate microbiome to become established, leading to dysbiosis and disease in

the host. To our knowledge, only one study has attempted non-lethal repeat sampling of individual fish. Pratte et al. (2018) examined captive clownfish skin mucosal microbial communities before, during, and after association with sea anemones. However, they did not report on the potentially disruptive effects of repeat sampling (Pratte et al. 2018). More methodological studies are needed to examine if repeat sampling of the mucosal microbiome creates a confounding factor in temporal studies.

5.6 Conclusions

Research has shown the microbiome to be highly dynamic, with large inter- and intra-individual variation. Lethal sampling for microbiome analysis offers only a snapshot of what is present at that specific moment in time. Transitioning to non-lethal sampling can help provide a more in-depth assessment of host-microbe associations as they relate to fitness endpoints and behaviours, both across spatial and temporal scales. Several decades ago, the same discussions occurred in the context of animal physiology where there was a desire to move away from lethal sampling in an effort to understand the physiological basis for individual variation in animal fitness and behaviour (Bennett 1987; Spicer and Gaston 1999). Today, non-lethal sampling enables physiologists to assess the mechanistic physiological basis for variation in fish performance (e.g., Cooke et al. 2006; Chapman et al. 2021), and we submit that the same opportunities exist for non-lethal microbiome studies. Being able to do the same with microbiomes would not only reduce animal welfare and population impacts on fish, but also create novel opportunities to link microbiome communities of fish hosts to the behaviour and survival of individuals across space and time (e.g., life-stages, seasons). Currently, temporal

trends are often assessed in fishes via lethally sampled cross-sectional population studies to examine how the microbiota changes over development stages or time. Indeed, lethal sampling efforts constrain our ability to understand the mechanistic ecological consequences of variation in microbiome communities in the wild such that transitioning to non-lethal sampling will open new frontiers in ecological and microbial research. Moving forward, more fish-specific comparison studies of lethal vs. non-lethal microbiome sampling methods would add value to the literature base and provide evidence that non-lethal sampling has merit. Further refinement of non-lethal methods and validation studies of these methods would also be beneficial. There has been some movement in the last few years toward fish-specific microbiome method evaluations (see Hildonen et al. 2019; Clinton et al. 2021; Clokie et al. 2022; Nyholm et al. 2022). Ideally, the research community will converge on a set of best practices that provide repeatable and reproducible results for different fish body compartments for microbiome analysis. In addition, there is a need to create a robust methodological pipeline for non-lethal microbiome research, from sample collection to data analysis, so that comparisons can be made across studies and meta-analyses and systematic reviews can be conducted to provide more concrete evidence for host-microbe associations and their benefits to host health and fitness in fish research.

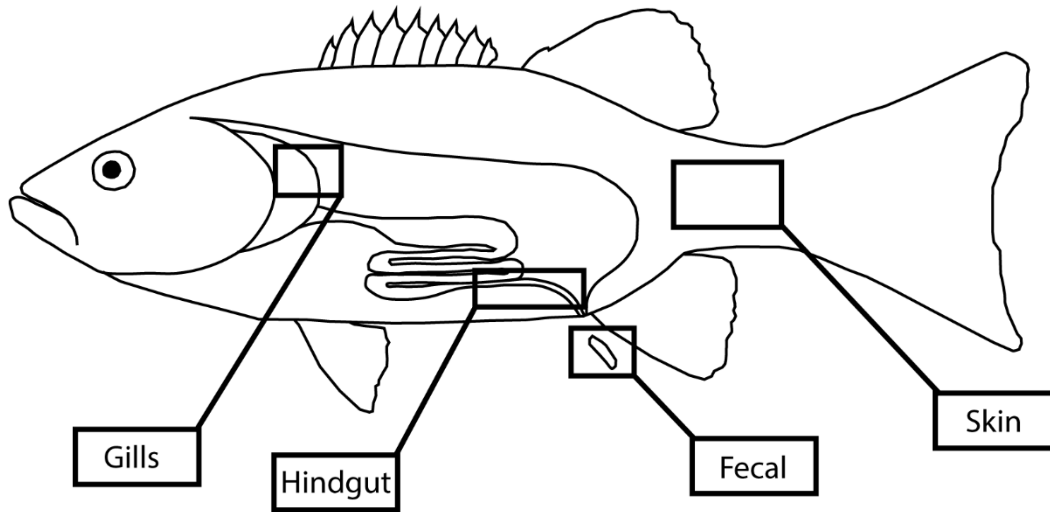

Figure 5-1. The four main non-lethal fish microbiome sample types.

Figure 5-2. Example of hindgut swabbing on a white sucker fish (*Catostomus commersonii*).

Chapter 6: General conclusions and future directions

6.1 General conclusions

Migratory species offer a unique perspective to studying the gut microbiome of fish because dynamic and changing environmental forces are interacting and structuring the gut microbiome, which may, in turn, alter host physiology and impact migratory behaviours, with potential downstream fitness consequences. The overarching aim of this thesis work was to investigate the hypothesis that the gut microbial community is related to the migration behaviour and fate of wild fishes. To this end, I have examined the gut microbiome of three wild fish species with different migratory behaviours using 16S rRNA gene amplicon sequencing techniques. In Chapter 2, I characterized the hindgut microbial composition and diversity of white sucker undertaking a potamodromous spawning migration in relation to migratory timing, sex, and the presence of tumours. In Chapter 3, I characterized the hindgut microbial composition and diversity of semelparous sockeye salmon undertaking anadromous migrations to three separate spawning grounds, representing three levels of migration difficulty. Physiological measurements of stress and body condition were also used in conjunction to assess any relationship with the hindgut microbiota. In Chapter 4, I characterized the fecal microbial composition and diversity of juvenile partially anadromous brown trout prior to the initiation of their outward migration in an attempt to elucidate whether gut microbiota play a proximate role in governing the decision to migrate or remain resident. Finally, in Chapter 5, I offer a field ecologist perspective of transitioning to non-lethal microbiome sampling methods, with a particular focus on fish, to better study behavioural metrics and

other relevant host-associated endpoints in relation to the gut microbiome. In the following sections, I will summarize my key findings and general conclusions for each data chapter. I will then reflect on the general limitations that were present throughout this thesis work. Finally, I will make recommendations for future research.

In Chapter 2, I found that the hindgut microbial community of white suckers differed between fish that arrived during peak migration time versus late-arriving fish. Further, late migrants were found to be less diverse and contained the genus *Mycoplasma*, whereas the peak migrants did not have this taxon present in any individuals. While *Mycoplasma* has been commonly associated with salmonids and thought to be responsible for vitamin B_{12} synthesis (Rasmussen et al. 2023), and indeed, that is what I found as well, particularly within adult sockeye salmon (Chapter 3). *Mycoplasma* has not been commonly reported within other non-salmonid fish species (Eichmiller et al. 2016; Zhu et al. 2021b), though it was found to be more highly abundant in common carp (*Cyprinus carpio*) infected with intestinal tapeworms (Fu et al. 2019). I did not have the genetic resolution to determine if this OTU belonged to a pathogenic species of bacteria. However, some members of this genus are pathogenic and cause fish disease (Legrand et al. 2020a; Sellyei et al. 2021). Future studies using metagenome sequencing combined with bacterial culturing could identify if pathogenic strains were present. I found no other published descriptions of white sucker gut microbiomes in the literature. This study will therefore represent a novel contribution to the field, characterizing the diversity of a common freshwater fish species. White suckers have been previously investigated as ecological indicators due to their propensity for developing epidermal papillomas and lip tumours in polluted waters, possibly due to

immunosuppression (Reizenstein 1983; Smith and Zajdlik 1987; Anderson 1990; Baumann 1992). While we did not find any association between lip tumours and the gut microbiome, future studies examining the skin microbiome using 16S rRNA gene amplicon sequencing and shotgun sequencing could more thoroughly explore any associations. Sample size was a limiting factor influencing the power of my results. Due to the technical limitations encountered in processing fish microbiome samples in the laboratory (see section 6.2 Limitations below), the overall sample size for this chapter was drastically reduced. Future studies with increased sampling power would add validity to the results observed here.

In Chapter 3, I found that adult sockeye salmon had overall low hindgut microbial diversity, which did not vary between spawning populations. However, there was weak evidence that microbial compositions differed between the Weaver and Chilko spawning populations. Weaver Creek represents the easiest migration route and is closest to the ocean, and the microbial community of this group had higher abundances of marine-associated microbes, such as *Aliivibrio*. Conversely, Chilko River had the most difficult and longest migration route, and fish from this spawning population had higher abundances of *Flavobacterium*. In addition, *Flavobacterium* was also present in higher proportions in fish with low body condition. Species within *Flavobacterium*, such as *Flavobacteirum psychrophilum*, are considered an opportunistic pathogen that negatively impacts Fraser River sockeye salmon (Kent 2011). Due to the resolving power of 16S rRNA gene sequencing, I could not ascertain if the species represented within Flavobacterium were pathogenic in nature, though it seems likely. Additional studies using metagenomic approaches could identify the strain or species accurately and provide

insight into the functional diversity of gut microbiota in migrating spawning populations. Given that sockeye salmon rely on endogenous energy sources to fuel their migrations (Brett 1995; Hinch et al. 2006), defining the functional diversity would be a relevant next step to observe if specific gut bacteria assist with energy metabolism and other essential physiological functions that help salmon reach the spawning grounds. Similar to the study conducted by Cooke et al. (2006) that looked at the mechanistic basis of individual mortality during spawning migrations, future studies tagging fish early in their migration run combined with gut microbiome sampling could help elucidate if bacterial taxa or genes provide key functions to the host that may be associated with differential en-route mortality. Conversely, I studied the gut microbiota of salmon on their spawning grounds, at the terminal end of their migration route, to see what bacterial taxa were present. However, this is beneficial as no studies, to my knowledge, have attempted to characterize the gut microbiota of sockeye salmon once they have reached the spawning grounds. Next steps would also incorporate whether the gut microbiota is associated with spawning success outcomes. As I mentioned in Chapter 1 (section 1.5 Challenges), defining the gut microbiota between successful and unsuccessful spawners was one of my thesis chapters that was excluded due to technical issues during sequencing. I discuss this further below in the limitations section.

In Chapter 4, I found that the fecal microbiome did not predict migratory status in a population of juvenile brown trout displaying partial migration. Further, body condition was also not associated with fecal microbial composition or diversity. It may be that sampling occurred too soon after their migratory decision window. Future studies sampling closer to the outmigration period may provide different results from what I have

observed. Examining the functional gene diversity at this time would also be very beneficial. There is evidence that the energetic status of juvenile brown trout modulates the decision to migrate (Shry et al. 2019) and that higher metabolic demands are associated with outmigration (Boel et al. 2014). Therefore, looking at the functional gene diversity closer to the outmigration period may reveal bacterial functions associated with energy metabolism, as the gut microbiota have been shown to modulate energy metabolism in higher vertebrates (Turnbaugh et al. 2009; Donohoe et al. 2011) and postulated to do the same in fish, though research lags behind (Butt and Volkoff 2019). Further, an increased sampling population may also provide a more robust analysis. The use of non-lethal methods to characterize the fecal microbiome and track subsequent behaviour in this chapter has led to novel insights about the gut microbiome of wild fish species and provides a good example of the benefits of transitioning to non-lethal sampling for these types of behaviour studies, which I discussed in depth in Chapter 5. The juvenile population studied in this thesis was more diverse than my corresponding adult population chapters. Though due to different sampling methods, it is not advisable to make direct comparisons between these populations. However, it is something anecdotally notable, and other studies have also found juveniles to have a more diverse gut microbiome compared to the adult phase of the life cycle (Llewellyn et al. 2016). While I did sample resident and migrant adult brown trout found within the study stream, these samples were largely excluded from downstream analysis due to poor DNA yields or sequencing depth. It would be interesting to compare juvenile hindgut microbiotas to both the resident and migrant adult forms to see which microbes are retained into adulthood.

Finally, in Chapter 5, I used a field-ecologist perspective to make a strong case for transitioning to non-lethal sampling of wild fish for microbiome research. It appears especially beneficial for behavioural studies, where tracking downstream behavioural responses may be integral to understanding the ecological consequences of variation in microbial communities. The gut microbiome has been shown to modulate host behaviour in humans and other mammals (Johnson and Foster 2018). Therefore, using non-lethal methods under these circumstances has already been well-optimized. In fish, there is still a need to refine non-lethal methods and subsequent sample processing pipelines to optimize DNA yields for downstream analysis. In the current thesis, fecal sampling appeared to work better compared to swab sampling. However, swabs have been used successfully in other taxa (Vo and Jedlicka 2014). One thing to note is that this study did not use the recommended Mo Bio PowerSoil Kit used by the Human and Earth Microbiome Projects (now Qiagen DNeasy PowerSoil Kit). The authors developed and tested a novel approach for extracting metagenomic DNA using solid phase reversible immobilization (SPRI) beads. This method produced greater DNA quality, quantity, and PCR amplification from swab samples. Therefore, further methodological studies to refine or validate low-biomass samples have merit.

Overall, the chapters in this thesis investigated microbial diversity at two levels, alpha and beta. I found that beta diversity was more strongly associated with migratory behaviour and fate than alpha diversity, meaning that the composition of gut microbial communities typically differed more significantly than the number of bacterial taxa within individuals, against our explanatory variables. However, there is still much unexplained variance, indicating that other unidentified forces are contributing to shaping

the gut microbiome. The results of this thesis somewhat supported the hypothesis that the gut microbial community is related to migratory behaviour and fate in wild fishes. I have found evidence that it may be associated with migratory timing and location, but it does not appear to play a role in the early decision window of facultative migrants, such as juvenile brown trout. Further, exogenous forces, such as environment and/or diet, may have a stronger influence on the gut microbial communities than endogenous forces, such as host physiology. However, condition factor may be an exception in some instances, as it was associated with microbial communities in sockeye salmon but not juvenile brown trout. Therefore, it could depend on the life-cycle stage assessed, or there was not enough variation in condition factors of juvenile brown trout to show an association. The local environment has been shown to be a primary driver in microbial community composition and diversity, so our results align with the current literature (Sullam et al. 2012; Wong and Rawls 2012; Eichmiller et al. 2016; Lokesh and Kiron 2016). We observed differences at large (e.g., sockeye salmon) and small (e.g., juvenile brown trout) spatial scales. The effect of the environment on the gut microbiome highlights the importance of proper watershed management in helping to maintain healthy fish populations.

On a broader scope, the results presented here add to the literature base on wild populations and host-associated microbiota. Wildlife conservation would benefit from the integration of microbiome research, as additional insights from a microbial perspective may help uncover important fitness declines in at-risk populations due to disturbances to the gut microbiota and their associated functions from environmental threats such as land-use change, environmental contamination, climate change, and infectious diseases (Redford et al. 2012; Bahrndorff et al. 2016; Trevelline et al. 2019; West et al. 2019).

This may be particularly relevant for migratory species, as individuals travel across heterogeneous environments and are exposed to a myriad of watershed and land-use changes. Mitigating anthropogenic practices that negatively impact the gut microbiota of migratory species may increase an individual's overall health and fitness.

6.2 Limitations

Apart from this thesis's contributions to the field, some limitations were present across all data chapters. Firstly, and most relevant as it impacted samples spanning the entire thesis, was the issue of DNA yield. PCR results generated samples ranging from no amplification to strong amplification, with a large proportion falling on the weaker side. While we are not sure of the exact cause of PCR inhibition, it is speculated that it may possibly be an issue with how the samples were stored. Remote fieldwork presents many technical challenges that must be overcome, and this is particularly true regarding fieldwork involving molecular biology, as samples are typically stored in liquid nitrogen (Knight et al. 2018; Tripathi et al. 2018). Due to the distance between some of my field sites, access to liquid nitrogen, and the length of time spent in the field, alternate storage methods were sought. RNAlater was chosen as the preferred storage method, as it had been successfully used in our lab in other molecular-based fieldwork (albeit RNA-based tissue collection). I also found successful studies using RNAlater for microbiome-based studies in the literature (Vo and Jedlicka 2014). RNAlater was preferred over ethyl alcohol (EtOH) as I was using commercial air travel with my samples, and ethanol-based preservation, while producing higher quality DNA yields (Hildonen et al. 2019), was not a viable option. Suffice to say that the cause of the low DNA yields following DNA

extraction is unknown. To further concentrate samples to increase DNA yields, a SpeedVac Concentrator was used on both individual samples as well as the pooled sequencing libraries to increase the concentration above the minimum 2nM needed for sequencing. However, the first sequencing run failed (which contained all of the Cobourg white sucker and British Columbia sockeye salmon samples). It was determined that there was a molarity issue (despite having an overall concentration above the minimum threshold of 2nM), likely due to a subset of samples with very low concentrations. Because of these low-concentration samples, the library could not go through the usual dilution steps to dilute the required NaOH concentrations (which can prevent hybridization on the flow cell). As a result, a total of 111 samples from the Cobourg and British Columbia data chapters were excluded (retaining 158 samples). Following the exclusion of these samples, the sequencing run was attempted again and was successful. The Denmark sequencing run followed the same protocol for excluding very low-concentration samples. As mentioned in Chapter 1, these challenges resulted in the exclusion of several field projects and subsequent data chapters. This drastically changed the structure of my thesis and reduced the capacity to fully explore the association of the gut microbiome to the behaviour and fate of wild fishes. In addition, these technical challenges reduced sample sizes for the data chapters that remained as part of this thesis.

In addition, there may have been some DNA degradation in samples, particularly from the British Columbia sockeye salmon samples, as they had the highest percentage of unclassified bacteria. While I discussed this briefly in the discussion of that data chapter, it is unknown if these OTUs represent unknown phyla (perhaps unlikely) or possibly degraded DNA that resulted in shorter sequence reads that could only resolve to bacteria

level and no further. Upon further reflection on this issue, perhaps the gut environment in the migrating salmon may have become too hostile for the microbiota, as salmon cease feeding during the freshwater migration (Hinch et al. 2006). When exposed to hostile environments, bacteria can produce endospores or undertake other forms of dormancy (Nicholson 2002), making typical DNA extraction methods sub-optimal for breaking through the endospore (Delmont et al. 2011; Knüpfer et al. 2020). While it is not known if that is the reason for the high proportion of unclassified bacteria in the sockeye salmon samples, it is an interesting hypothesis, and further research is needed to confirm if hostile host physiological environments could lead to the production of bacterial endospores. I found one study that looked at the gut microbiome of hatchery-raised chinook salmon undergoing senescence, and there was no mention of a high proportion of unclassified phyla. However, study methods indicated that unclassified phyla were removed from downstream analysis, and therefore it is unknown what proportion of the relative abundance this occupied (Couch et al. 2023). Suffice to say that these technical issues resulted in a loss of resolution, rendering the conclusions of my research projects constrained to these limitations.

6.3 Future Directions

Since data collection for this thesis occurred, a few methodological studies have been completed on proper storage preservation methods for fish-specific microbiota studies (Talwar et al. 2018; Hildonen et al. 2019; Gallo et al. 2020). Further validation and methodological studies should continue to occur, especially concerning field-collected and low-biomass samples such as swabs. Producing a set of standard optimized

methodologies for wild fish microbiome research would also allow direct comparisons to be made between studies. This is currently not usually possible due to the wide variety of methods in use (though there are a few meta-analyses in the fish microbiome literature to date; see Sullam et al. 2012; Wong and Rawls 2012). Meta-analysis would be beneficial in this area of research as pooling results across a myriad of studies could quantitatively synthesize the evidence base and improve the power of small-scale studies showing important implications for gut microbiota and host health and fitness. Evidence synthesis would also highlight any relevant knowledge gaps where targeted research could then be applied. Application of a standard operating procedure (SOP) has already occurred at a broader-taxa level with the Earth Microbiome Project, which includes sections for animal surface/corpus/secretion/proximal gut/distal gut (Thompson et al. 2017), which may serve as an appropriate starting guide to creating a fish-specific set of protocols. The Earth Microbiome Project has also published a meta-analysis of their communal catalogue, encompassing both free-living and host-associated microbiomes across the globe (Thompson et al. 2017), further establishing the merit of looking for patterns across studies.

With the advancement of next-generation sequencing technology, we have seen an exponential rise in the number of gut microbiome studies in the last 20 years (Sullam et al. 2012; Chong et al. 2020; Bodawatta et al. 2022). The vast majority of these studies have involved characterizing the diversity of the gut microbiome in a variety of host animal taxa. My thesis further contributed to this goal by characterizing the hindgut microbial composition and diversity in three wild, migrating teleost model species. As with other scientific areas of research, there are often three stages of development: a

descriptive phase, an explanatory phase, and a synthesis phase (Altmann and Altmann 2003). The field of fish gut microbiome research, and the study of vertebrate microbiomes more generally, have now thoroughly investigated the first of these stages, marked by descriptive studies of what taxa are present (Sullam et al. 2012; Tarnecki et al. 2017). We are now moving into the era of whole-genome sequencing (such as shotgun metagenomics) to begin answering questions about what these bacteria are doing, by capturing gene-level functional diversity (Goodwin et al. 2017). Moving to whole-genome sequencing methods will help elucidate the functions of these bacteria in the gut and how they may contribute to host health, behaviour, and fitness (Rasmussen et al. 2023). For example, combining metagenomics with behaviour and physiology studies in the context of wild animals, such as migration behaviour, could help uncover whether the gut microbiome provides essential functions, such as energy metabolism or disease resistance, to migratory individuals. Further, conducting these studies using non-lethal sampling would also open new frontiers in ecological and microbial research.

Appendices

Appendix A Supplementary Tables

Appendix A: Table 1. Alpha diversity metrics – including the observed species richness (of OTUs), Chao1, Inverse Simpson, and Shannan indices for the 14 adult white sucker fish samples in Chapter 2.

Sample	Observed	Chao1	Inverse Simpson	Shannon
LK139	306	1206.00	57.07	5.11
LK160	141	701.67	2.58	2.40
LK163	22	35.50	2.58	1.39
LK188	69	238.00	1.52	1.23
LK191	52	157.06	1.73	1.15
LK207	14	42.00	1.15	0.40
LK249	49	145.33	2.86	1.66
LK251	31	369.00	1.35	0.75
LK254	20	80.50	1.96	1.08
LK299	8	11.00	1.27	0.49
LK302	17	37.25	1.96	1.10
LK306	15	35.25	1.30	0.57
LK310	22	54.67	1.70	0.91
LK312	5	5.00	1.32	0.50

Appendix A: Table 2. Physiological variables for sockeye salmon blood measurements and their associated Shapiro-Wilk test statistic (W) and p-value to ascertain if residuals are normally distributed in Chapter 3.

Physiological Variables	Shapiro-Wilk test statistic (W)	p-value
Lactate	0.98451	0.8919
Glucose	0.82996	0.00008
Condition Factor	0.97883	0.7208
Hematocrit	0.9296	0.02722

Appendix A: Table 3. Prevalence of genus-level taxa across sampled population of juvenile brown trout with a relative abundance greater than 1% of total OTU sequences in Chapter 4.

Genus	Percental of total reads (%)
Unclassified *Enterobacteriaceae*	22.66
Clostridium_sensu_stricto	14.48
Diplorickettsia	9.18
Unclassified bacteria	7.80
Deefgea	3.95
Aeromonas	2.96
Rickettsia	2.85
Mycoplasma	2.78
Unclassified Firmicutes	1.78
Unclassified Clostridiaceae_1	1.71
Unclassified Microbacteriaceae	1.66
Unclassified Rhizobiales	1.52
Aminobacter	1.38
Unclassified Actinomycetales	1.21
Romboutsia	1.06

Appendix B Citations of non-thesis publications during doctoral studies

1. Elmer, L.K., Bass, A.L., Johnston, S.D., Kaukinen, K.H., **Kelly, L.A.,** Li, S., Teffer, A.K., Miller, K.M., Cooke, S.J., Hinch, S.G. (In-Press). Changes in infectious agent profiles and host gene expression during spawning migrations of adult sockeye salmon (*Oncorhynchus nerka*). Canadian Journal of Fisheries and Aquatic Sciences. 00:000-000.

2. Chapman, J.M., **Kelly, L.A**., Teffer, A.K., Miller, K.M., Cooke, S.J. (2021). Disease ecology of wild fish: opportunities and challenges for linking infection metrics with behaviour, condition, and survival. Canadian Journal of Fisheries and Aquatic Sciences. 78(8):995-1007.

3. Rytwinski, T*., **Kelly, L.A***., Donaldson, L.A., Taylor, J.J., Smith, A., Drake, D.A.R., Martel, A.L., Geist, J., Morris, T.J., George, A.L., Dextrase, A.J., Bennett, J.R., Cooke, S.J. (2021). What evidence exists for evaluating the effectiveness of conservation-oriented captive breeding and release programs for imperilled freshwater fishes and mussels? Canadian Journal of Fisheries and Aquatic Sciences. 78(9):1332-1346.
   *Equal author contributions

4. Kemp, J.O.G., Taylor, J.J., **Kelly, L.A**., Larocque, R., Heriazon, A., Tiessen, K.H.D., Cooke, S.J. 2021. Antibiotic resistance genes in the aquaculture sector: global reports and research gaps. Environmental Reviews. 29(2):300-314.

5. Kadykalo, A.N., **Kelly, L.A**., Berberi, A., Reid, J.L., Findlay, C.S. (2021). Research effort devoted to regulating and supporting ecosystem services by environmental scientists and economists. Plos one. 16(5):e0252463.

6. Lamb, J.B., Willis, B.L., Fiorenza, E.A., Couch, C.S., Howard, R., Rader, D.N., True, J.D., **Kelly, L.A**., Ahmad, A., Jompa, J., Harvell, C.D. (2018). Plastic waste associated with disease on coral reefs. Science. 359(6374):460-462.

7. Elmer, L.K., **Kelly, L.A.,** Rivest, S., Steel, S.C., Twardek, W.M., Danylchuk, A.J., Arlinghaus, R., Bennet, J.R., Cooke, S.J. 2017. Angling into the future: ten commandments for recreational fisheries science, management, and stewardship in a good Anthropocene. Environmental Management. 60:165-175.

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