Characterizing the Gut Microbiome of Wild-Caught and Lab-Reared Larval Sea Lamprey Under Different Feeding Regimes

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Environment and Geography

University of Manitoba

Winnipeg

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Abstract

Lampricide treatments are one of the most common population control methods for invasive larval sea lampreys in the Great Lakes. The success of current population control methods depends on obliterating larval cohorts. However, most testing of the efficacy of different population control methods are carried out on lab-reared larval sea lampreys. In aquaculture, the gut microbiome plays a key role in fitness, including determining how rearing conditions affect the host. This has been studied in many commercially popular fish, but research on the gut microbiome of larval sea lampreys and its role in host growth and development is limited. This is a disadvantage for studies aimed at developing population control methods that depend on lab-reared larvae having a similar physiological response to wild populations of larval sea lampreys. To help breach this research gap, we characterized and compared the gut microbiome of lab-grown (LG), wild-caught lab-reared (WCLR) and wild-caught (WC) larval sea lampreys. Our main findings are that there was a i) strong seasonal pressure driving diversity in the gut microbiome of WC and WCLR larvae; ii) strong influence of the collection site on the gut microbiome of WC larvae; iii) lower diversity of the gut microbiome of LG compared to WC and WCLR samples. Although, we did not detect a core microbiome among our samples, we found common predicted functional pathways among sample types that suggest there could be a microbial functional core. At the genus level, *Cetobacterium* was the most abundant taxon in WC and WCLR samples. LG samples had unique taxa at higher abundances but shared less abundant taxa with other sample types. Identifying the factors contributing to differences in the microbiome in WCLR or LG samples will inform the development of aquacultural approaches to rearing larval sea lamprey.

Acknowledgements

I would like to thank my advisors Drs. Lisa Loseto, Sara Good and Margaret Docker for their encouragement, support and, most of all, their kindness. I would also like to thank Dr. Fei Wang for his support from the beginning of my master's degree. Thank you for being excellent advisors.

Several years into my master's program, I made a difficult decision that led me to this project, and I would like to thank everyone that helped me though that time including Drs. Bruce Erickson, Mark Hanson and Mary Benbow.

This study would not have been possible without the financial support of the Sea Lamprey Aquaculture and Procurement (SLAP) project funded by the Great Lakes Fishery Commission, and the support of the Hammond Bay Biological Station team Drs. John Hume and Nick Johnson and Trisha Searcy. Additionally, I would like to thank the Clayton H. Riddell Faculty of Environment, Earth, and Resources for stipend support during this project.

A special thanks to Dr. Lara Puetz for her input and expertise and for being an amazing teacher. Finally, thank you to my friends especially Nahomi, Mara, Tonya, Fowzia, Monojit and so many others for being my family away from home; my lab members for being so welcoming and patient; and my family for never leaving me alone.

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Introduction

1.1 Sea Lamprey Biology

Lampreys have a distinctive external anatomy with an eel-like shape that lacks paired appendages and includes 7 gill openings, a large suctorial disk, a neural crest that develops into a cranium after metamorphosis and eyes that evolve from light-detectors to camera-type eyes after metamorphosis (Janvier, 2008; Moser et al., 2019). They have a skeleton-like structure made up of cartilage, a notochord that supports their body and an undifferentiated digestive system that is made up of only a long continuous intestine (XU et al., 2016).

Considered to be ancient true vertebrates, lampreys are agnathans (jawless fishes), a monophyletic group that includes two classes of cyclostomes, the lampreys (Petromyzontida) and hagfishes (Myxini), the latter being deep ocean fish that diverged from the lamprey lineage approximately 400-500 million years ago (Docker et al., 2015; Evans et al., 2018; Yu et al., 2023; Kuraku & Kuratani, 2006; Miyashita et al., 2019). Lampreys and hagfishes share several morphological (including neural crest cells and a lack of paired appendages) and molecular traits (for example, shared microRNA families and a similar immune system) that have been important for evolutionary developmental biology (evo-devo) studies aimed at understanding the evolution of early vertebrates (York et al., 2019;).

Lampreys are resilient fish that have survived 4 to 5 mass extinction events (Evans et al., 2018). There is now strong evidence that agnathans diverged from gnathostomes (jawed vertebrates) after the first round (1R) of whole genome duplication (WGD) that occurred early in vertebrate evolution, but they did not experience the second round (2R) of WGD that occurred in the ancestor of all gnathostomes (Simakov et al., 2020; Yu et al., 2023). However, lampreys underwent two additional rounds of lineage-specific WGD events that shaped their gene

repertoire (Simakov et al., 2020; Yu et al., 2023). Our understanding of the lamprey genome has been greatly informed by the first whole genome assemblies of the somatic (Smith et al., 2013) and germline genomes (Smith et al., 2018; Timoshevskaya et al., 2023), as well as by the revised whole genome sequence of the hagfish genome (Yu et al., 2023).

Currently, there are 44 described lamprey species that inhabit diverse ecological niches both in the Northern and Southern hemisphere, however, the Northern Hemisphere supports greater lamprey diversity (Maitland et al., 2015). The distribution of all lampreys is "antitropical" due to their poor tolerance of high temperatures (Potter et al., 2015). Some lampreys are non-migratory, and others are anadromous or migratory exclusively within fresh water. Anadromous lampreys hatch and rear in freshwater, but migrate downstream to feed at sea, before returning to freshwater to spawn. Certain sea lampreys (*Petromyzon marinus*) are freshwater residents (sometimes referred to as landlocked); they are found within in-land watersheds. These freshwater-residents are ancestrally anadromous but remain in freshwater throughout their life cycle and travel downstream as juveniles to feed on freshwater bony fishes (e.g. lake trout) (Farmer, 1980; Manion & Stauffer, 1970; Youson & Potter, 1979). On the other hand, anadromous sea lampreys hatch and go through metamorphosis during their freshwater residence to prepare for future ecosystem pressures that they will encounter during life in seawater, including a new diet that consists of fish blood (Manzon et al., 2015). Although lampreys are popularly known for being parasitic, some species are non-parasitic and are smaller in size and do not feed after metamorphosis. Non-parasitic lampreys have an atrophied gut and are sexually mature following metamorphosis; they spawn and die within 6–10 months of metamorphosis (Docker & Potter, 2019; Potter et al., 2015).

Anadromous lampreys have a long and complex life cycle. During the first part of their life, embryos become prolarvae approximately 13-15 days post-fertilization (dpf) (Youson, 1985). For wild lampreys, the larval phase lasts from 5 to 7 years and is marked by a filter-feeding lifestyle (Dawson et al., 2015). Larvae have a very slow metabolism due to the low nutritional content of their diet and are mostly sedentary; burrowing in the most suitable substrate (that usually consists of a mixture of sand and silt); and feeding on detritus as their primary food source (Dawson et al., 2015; Evans & Weber, 2020; Sutton & Bowen, 1994). Detritus is ingested by the larva as water flows through the burrow hole in the streambed. Occasionally, burrowing larvae will emerge and feed on benthic algae and diatoms; however, these benthic microorganisms only make up a small portion of the larval diet (mostly due to low nutritional value) (Moore & Mallatt, 1980).

After 5-7 years of the larval stage, when the conditions are suitable (i.e. warm temperatures), larvae undergo metamorphosis. Metamorphosis requires starvation and causes the gut to atrophy; this process lasts around 4 months (Dawson et al., 2015). In lab-reared larvae, growth can be considerably faster (Barron et al., 2016). Metamorphosis begins in early to mid-July and is less likely to occur after mid-August in the wild (Manion & Stauffer, 1970). During early metamorphosis, the anterior and posterior intestine alternate between decreasing and increasing in size to accommodate the new juvenile form of the intestine, which includes a larger surface area of mucosal folds for absorption of nutrients and potential adherence of microbial communities (Li et al., 2019; Youson & Connelly, 1978). After metamorphosis, the gut is ready for a new feeding method and a new diet that consists of attaching to a host to consume their blood and tissue (Shink et al., 2019). Following metamorphosis, larvae transform into parasitic

juveniles that travel downstream to feed and, eventually, become sexually mature adults that travel upstream to spawn and die (Dawson et al., 2015).

The gut develops early in larval development and goes through dramatic changes during metamorphosis. The stomodaeum forms in embryos between 6 to 8 dpf and the oral hood (that later turns into the mouth) forms by day 15 to 17 (Youson, 1985). Prior to burrowing, oral cirri develop in the oral cavity and will be responsible for particle size selection during filter feeding (Youson, 1985). Once the oral cavity and cirri are developed, burrowing occurs. At the larval stage, the pharynx and the esophagus are connected to increase surface area for absorption of nutrients; after metamorphosis, the esophagus and pharynx separate to accommodate the intestine for the absorption of excess iron (from blood consumption) (Youson, 1985). The first form of nutrition in the larval stage is the egg yolk. Prolarvae feed on the egg yolk, which marks the transition to larvae (also known as ammocoetes) at 33-40 dpf (Richardson et al., 2010). In zebrafish (*Danio rerio*), after prolarvae consume the yolk, the intestine becomes exposed to the environment and is colonized by microbes, something that may also be true in larval lampreys (Stephens et al., 2016). During larval feeding, a water current passes through the oral cavity carrying potential food particles (i.e. algae and detritus) to the pharynx, where the particles are trapped in a mucous substance produced by mucous cells in the surrounding tissue (Moore & Mallatt, 1980; Rodríguez-Alonso et al., 2017). The mucous substance, also called the food cord, is transported by cilia to the esophagus and helps with selection and absorption of food particles; inadequate particles, that are too big or do not adhere to the mucus, are “coughed” out (Moore & Mallatt, 1980).

1.2 Why study Sea Lampreys?

Sea lampreys have become a twofold ecological concern as they are both an abundant invasive species in the Great Lakes and an endangered species in their native environments (Maitland et al., 2015). Sea lampreys are native to areas surrounding the Atlantic Ocean, including the North American coast, Europe (e.g. the Iberian Peninsula) and other watersheds that discharge into the Atlantic Ocean (Hume et al., 2020; and references therein). Native sea lampreys have been described as endangered both in the Iberian Peninsula and in North American watersheds, mostly due to anthropogenic causes, such as construction of dams that limit or destroy lamprey habitats, pollution, and warmer habitat temperatures due to climate change (Mateus et al., 2012; Renaud, 1997). Conversely, sea lampreys are invasive species in the Laurentian Great Lakes for similar reasons. The construction of canals and dams provided a gateway for sea lampreys to colonize the Great Lakes and their surrounding waterways since the 1800s, thereby transforming food webs and the fishing industry in surrounding areas (Docker et al., 2021).

Around the mid-1800s, sea lampreys were found in the Lake Ontario drainage basins that includes Lake Oneida and the Finger Lakes (Cayuga Lake and Seneca Lake); they may have naturally migrated to Lake Ontario through the St. Lawrence River from the Atlantic Ocean (Docker et al., 2021). Afterwards, with the completion of the Welland Canal in 1829, sea lampreys were able to swim upstream of Niagara Falls, reaching Lake Erie and eventually getting access to the upper Great Lakes. By the 1930s, Lake Michigan, Lake Huron and Lake Superior had been colonized by freshwater-resident sea lamprey populations (Docker et al., 2021). The freshwater-resident populations were able to colonize new sites leading to the development of genetically differentiated smaller populations throughout the Great Lake

tributaries that adapted to feeding on and depleting commercially important freshwater fish species like Atlantic salmon (*Salmo salar*) and lake trout (*Salvelinus namaycush*) (Bryan et al., 2005). The financial and ecological toll of the invasion of sea lamprey in the Great Lakes led to the development of methods to limit lamprey dispersal (e.g. barriers, lampricides) to curtail lamprey reproduction and stemmed research projects aimed at developing new approaches to aid in sea lamprey population control. The Great Lakes Fishery Commission (GLFC) was formed to address this on-going concern and to manage the impacts of the growing invasive sea lamprey population.

The bi-national GLFC also manages procurement requests for animals used for research purposes by fishing, but the increasing demand of sea lamprey at different life stages has created a need to develop aquaculture approaches to rear sea lampreys in sufficient numbers and throughout multiple life stages. The development of improved methods for sea lamprey aquaculture benefits researchers working on a range of topics from comparative evolutionary development, to understanding how lampricide treatments affect both larval lampreys and other non-target species; and developing genetic control options for sea lamprey control (Ferreira-Martins et al., 2021; Lampman et al., 2021; York et al., 2019).

Several efforts have been successful at preventing sea lampreys from colonizing more watersheds in the Great Lakes, including using physical/mechanical barriers (Smith & Tibbles, 1980). Due to the predisposition of sea lampreys to bioaccumulate mercury and other contaminants from their host; consumption of sea lampreys cannot be used as an option for population control in the Great Lakes (MacEachen et al., 2000). Due to the dramatic impact of lampreys on freshwater teleost fisheries, eventually, lampricide treatments (lamprey-specific toxic treatments) were developed to remove sea lamprey populations by targeting sea lampreys

during their larval phase; this has prevented feeding and spawning in certain rivers, and has been effective in managing populations at certain locations (Smith & Tibbles, 1980; Sullivan et al., 2021; and references therein). However, when testing new lampricide treatments, it is unclear if lab-grown (LG) samples are a good representation of wild-grown samples. If LG samples could be used to assess the effectiveness of lampricide treatments, this would significantly enhance experimental approaches to develop new population control methods.

1.3 Sea Lamprey Rearing and the Gut Microbiome

To date, sea lampreys have been reared at multiple different life stages for both scientific and procurement purposes. Adults are reared to study behavioral or evolutionary traits, while younger lamprey are reared to investigate developmental traits including the effects of lampricide treatments. When captured in the wild and brought into the lab, larval sea lampreys are easy to maintain. They are commonly fed a diet consisting of yeast and can go through metamorphosis if the physiological conditions are suitable. In the wild, mortality at the larval stage is low and mostly consists of external factors like temperature, larval density, sediment quality and predators (Dawson et al., 2015). High mortality rates have been reported for lamprey eggs and downstream migrants; however, high fecundity of sea lampreys ensure the cohort survives (Dawson et al., 2015).

In contrast, when hatched in the lab, larval sea lampreys typically experience a mortality bottleneck at ~3 months old, when they shift from being prolarvae to larvae, which coincides with the transition to burrowing and to a microphagous diet (Lampman et al., 2016; Lampman et al., 2021). Several factors are known to influence larval lamprey survival in cultured settings, including larval density (i.e. >200 g/m^2 density in tanks causes 100% mortality) and changes in water flow (that cause anoxic conditions in rearing tanks) (Lampman et al., 2021). However,

because we now appreciate that microbes can impact host physiological processes such as immunity and nutrient absorption (Perry et al., 2020; and references therein), it is possible that the gut microbiota of sea lamprey play a role in the survival and successful transition from the natural environment to a lab setting. Comparing the gut microbiome from wild-caught lab-reared (WCLR) larval lampreys to LG larval lampreys may provide novel insights into more successful culturing practices.

As described by Mathai et al. (2021), the sea lamprey intestine holds a microbial community that changes throughout its life cycle; the larval stage contains the most diversity. Bacterial families previously found in larval lampreys include *Fusobacteriaceae, Verrucomicrobiales, Bacteroidales, Micrococcales, Rhodocyclaceae* and *Rubritaleaceae*; however, there is no clear evidence of a sea lamprey core microbial community that persists from the larval to the parasitic phase, rather changes in the microbial community appear to mirror changes in lamprey feeding habits or diet (Mathai et al., 2021; Tetlock et al., 2012). Some fish like zebrafish have been described as having a strong core microbiome that remains similar in wild-caught (WC) and reared fish from different aquaculture facilities (Roeselers et al., 2011). Further research is warranted to determine the presence of a microbial core, even if it is not maintained throughout the sea lamprey life cycle.

The abundance and species richness of all taxa in early microbiome studies were exceedingly underestimated due to the high selectivity of culture-dependent methods as well as restrictive methods for bacterial species identification (Rogers et al., 1980). Nevertheless, studies like that of Rogers et al. (1980) proposed the idea that gut microbial communities were predominantly influenced by the environment surrounding the burrow hole in larval lampreys, going so far as collecting small particles above the sediment layer to compare their microbial community to that

of the larval gut. A strong relationship between the local environment and the gut microbiome was also described in the three-spined stickleback (*Gasterosteus aculeatus*), in which comparison between estuary and freshwater populations suggested that the diversity of the gut microbiome was primarily determined by the genetic similarity among fish but was also highly influenced by environment, geography, feed, and other physical factors (Steury et al., 2019). Similarly, studies on Nile tilapia (*Oreochromis niloticus*) found that environmental factors such as seasonal change influenced the relative abundance of gut microbial phyla (Bereded et al., 2021). In keeping with this, a prevailing viewpoint is that the fish gut microbial community assemblage is predominantly influenced by deterministic and not stochastic (random) events (Yan et al., 2016). Deterministic events include biotic and abiotic factors that influence the host like species interactions as well as environmental factors like temperature and water flow (Stegen et al., 2012). This hypothesis further suggests that there is a dynamic interconnection between the environment and the larval gut, which presents a challenge when rearing larvae in a controlled environment.

The role of the microbiome in the larval sea lamprey gut is still unknown. In other aquaculture fish, the gut microbiome has been described as having different functions including assisting in nutrient uptake, growth, immunity, and protection from pathogenic bacteria (i.e. Nie et al., 2023; Tran et al., 2018). Although the gut microbiota of sea lamprey may play a similar functional role, physiological differences between teleost fish and lampreys should be taken into consideration. For example, typically, the mucosal layer in the fish intestine is used by bacteria for adhesion; however, there is no indication whether this phenomena occurs in sea lampreys but it may be important given their thick mucosal layers that play a role in feeding (Banerjee & Ray, 2017; Kuang et al., 2020).

Common microbial applications in fish rearing and aquaculture include the use of prebiotics to stimulate gut microflora and improve the immune system, probiotics to supplement gut microbiota, and use of antibiotics and anti-pathogens to prevent outbreaks in contained experiments (Yukgehnaish et al., 2020; and references therein). Some probiotic bacteria have a high affinity to host-specific mucosal folds and, along with probiotic yeast, tend to adhere to and compete with native microbial communities in the gut (Li et al., 2019). However, probiotics are meant to support the indigenous gut microbiota or aid in the benefits that the indigenous microbiome provides in the natural environment (Olafsen, 2001).

Although rearing methods influence the identity, diversity, and relative abundance of the gut microbial community (making them different to the wild gut microbial community), in successful aquaculture, the gut microbiome is effective in maintaining fish health and immunity enabling survival to adulthood. In successfully reared fish like Atlantic cod (*Gadus morhua*) and fine flounder (*Paralichthys adspersus*), comparing WC and aquaculture reared fish has identified differences in diversity and relative abundance of culturable microbial communities in the gut (Dhanasiri et al., 2011; Salas-Leiva et al., 2017). Similar results have been described in teleost fish like Nile tilapia, using more accurate methods like 16S amplicon sequencing (Bereded et al., 2021). Nevertheless, larvae are more susceptible to environmental changes than adult fish due to their constant interaction with their surroundings. As such, given the limited research on the larval lamprey gut microbiome, it is premature to weigh in on the benefits and/or harms of rearing conditions including additions of probiotics, antibiotics, and different kinds of feed.

Since our knowledge about the larval sea lamprey gut microbiome is limited, it is unsurprising that it has rarely been considered to be a factor in larval rearing. The primary objective of the proposed research includes exploring differences in microbial communities

within the larval sea lamprey gut. Larval sea lampreys collected for this research represent a range of rearing conditions that will help inform the comparison between native and captive larval populations. Within this research, we: 1) characterized the gut microbial communities in WCLR larval sea lampreys that had been exposed to different feeding regimes, including dry activated yeast (Y), yeast and brown rice flour (YR) or yeast and whole wheat flour (YW); 2) characterized the gut microbial community of WC larval sea lampreys harvested from two different rivers in Michigan; 3) characterized the gut microbial community of LG (fertilized and reared in the lab) larval sea lampreys; 4) compared the gut microbial community from WC and LG larval sea lamprey; and 5) explored the effect of physical parameters like location and seasonality on the gut microbial community of larval sea lampreys. It is hoped that the results of this research will serve to better understand the differences between larval sea lampreys in the wild and lab-reared larval sea lampreys. This research will also be valuable for reducing the knowledge gap on the sea lamprey gut microbiome.

Methods

2.1 Sample collection

Larval Intestine Collection –WCLR Samples

Using backpack electrofishing, larval sea lampreys were collected from Baldwin River, Michigan from July 11th to July 15th, 2020. Samples were collected as part of the Sea Lamprey Aquaculture & Procurement (SLAP) project to determine the effect of larval diet on growth rate and onset of metamorphosis. Larvae were transported to Hammond Bay Biological Station (HBBS) until the beginning of the experiment on August 1st, 2020. Larvae were reared from August 1st, 2020, until May 2021, following recommendations detailed in Hanson et al. (1974) and revised in Lampman et al. (2021), including a substrate for burrowing, air stones for aeration and a standpipe that maintained water flow. Larvae were reared in 6 tanks of 711 L with 25 cm of sand collected from Schmidt Creek, Presque Isle County, MI and a water volume of 172 L. During the winter, larvae were transferred to six smaller tanks with 2-4cm of sand and a water volume of 150 L both collected from Lake Huron. Further details about the experimental setup can be found as Experiment 1 in Hume et al. (2024). After this experiment, from May 2021 to August 2021, the larvae were transferred to the University of Michigan Biological Station Stream Lab (UMBS) facility into rectangular tanks with unfiltered water from Maple River and 8 cm of substrate composed of gravel and sand from Lake Huron. At this point, the larvae were exposed to river water for the first time since being collected in July 2020. Every time the larvae were transferred, low voltage direct current and a strainer were used to stimulate and collect the larvae.

During the experiment, larvae were fed three different diets: active dry yeast (Y), active dry yeast and brown rice flour (YR), and active dry yeast and wheat flour (YW), with two replicates

each. The larval lampreys were fed three times per week during the summer (August 1 – October 30, 2020), twice per week in November, once per week during the winter (December 2020 – April 2021), due to low temperatures, and twice per week from May 2021 until the end of the experiment (August 2021). Larvae were kept in tanks until December 2021, larvae that went through metamorphosis in the summer (transformers), were dissected in October 2021. Temperature, dissolved oxygen, water clarity, feed accumulation, biofilm formation and presence of dead larvae were monitored five times per week.

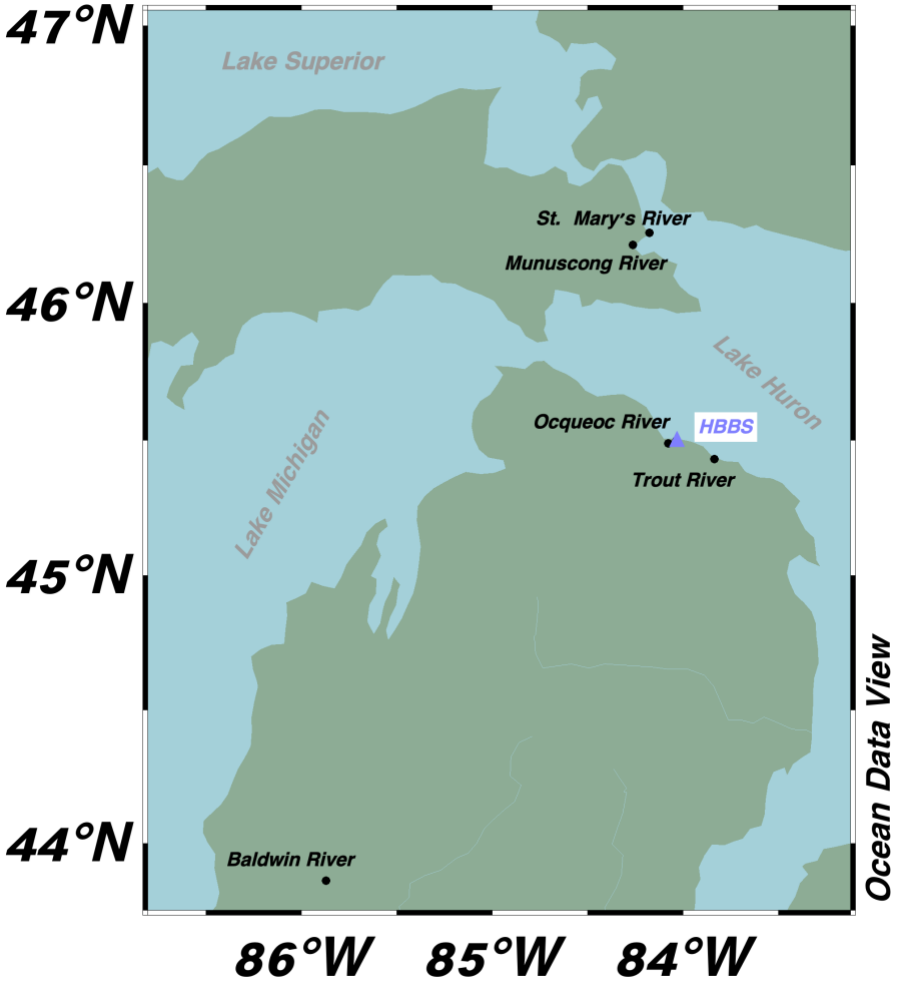


Figure 1: Map of larval sea lamprey collection sites.

Table 1: Summary of relevant physical and biological parameters collected for each rearing site.

Site	Date	Temperature range	Larval density range	Avg. larval length	Avg. larval weight

Outside HBBS	Aug – Oct 2020	0.4 – 19.6°C	30.3 – 33.2 g/m^2	105mm	1.9g
Inside HBBS	Nov 2020 – April 2021	1 – 5 °C	142 – 196 g/m^2	141mm	4.1g
UMBS Facility	May – Aug 2021	9 – 26 °C	83.4 – 162.8 g/m^2	141mm	4.0g

A subset of larval lampreys were sacrificed, measured, weighed, and dissected at approximately three-month intervals from August 2020 to August 2021 (Table 2). Intestines from sacrificed larvae were collected and stored in RNA later® at -80 °C at HBBS for gut microbial analysis. Other organs were also collected and stored for analysis. Larval intestine samples were shipped to the University of Manitoba for gut microbial analysis.

Table 2: Larvae and metamorphosing sea lamprey (transformers) collected from August 1st, 2020 to August 31st, 2021.

Year	Date	Dissection	Total number of sampled larvae
2020	01 Aug	Initial measure and weight	All larvae (n = 120)
2020	01 Nov	Fall measure and weight	All larvae (n = 120)
2021	15 Feb	Winter dissection	5 per treatment (n = 15)
2021	30 Apr	Spring dissection	5 per treatment (n = 15)
2021	01 Jul	Summer dissection	5 per treatment (n = 15)
2021	31 Aug	Summer dissection	Metamorphosing larvae (n = not available)
2021	27 Oct	Transformer dissection	Transformers (n = 34)
2021	08 Dec	Final larvae dissection	All remaining larvae (n = 41)

Larval Intestine Collection – WC Samples

Using backpack electrofishing, larval sea lampreys were collected from Trout River, MI and Ocqueoc River, MI in May and September 2021 (dates detailed in Table 3). Larvae were measured and dissected on the same day. The intestines were stored in RNA Later® at -80 °C at HBBS and later shipped to the University of Manitoba for gut microbial analysis.

Table 3: Larvae collection from Trout River and Ocqueoc River

Year	Date	River	Total number of sampled fish	Range larval length	Range larval weight
2021	13 May	Trout	n = 30	118 – 158mm	2.6 – 5.1g
2021	14 Sept	Trout	n = 30	107 – 168mm	1.7 – 6.9g
2021	14 May	Ocqueoc	n = 30	130 – 161mm	3.5 – 7.0g
2021	14, 16 Sept	Ocqueoc	n = 23	73 – 147mm	0.5 – 4.7g

Transformer intestine collection - Munuscong River and St. Mary's River

Using backpack electrofishing, sea lamprey transformers were collected from the St. Mary's River and Munuscong River, MI (Table 4). Although, the term 'transformer' and 'metamorphosing larva' are used interchangeably, for the purpose of this project, 'transformers' refer to post-metamorphosis sea lampreys. Thereby, in this project, 'transformers' have recently undergone metamorphosis and are in the process of travelling downstream but have yet to feed on blood.

Table 4: Transformer collection from the Munuscong River and St. Mary's River

Year	Date	River	Total number of sampled fish	Transformer length range	Transformer weight range
2021	15 Dec	St. Mary's	n = 8	126 – 156mm	1.9 – 4.5g
2021	15 Dec	Munuscong	n = 8	139 – 187mm	4.3 – 8.4g

Larval Intestine Collection – LG Samples from Hammond Bay Biological Station

LG larvae were hatched, reared in the lab, and collected at 9-months-old on April 27, 2023. These larvae were randomly sampled from a separate project that began in June 2022 until June 2023 (Table 5). This project studied differences in feed including 3 diets: yeast and wheat flour, yeast and fish meal, and yeast and 20% otohime. Larval sea lampreys were maintained at a constant 18°C throughout the project. A cross-section of the larvae was removed and preserved in formalin for histology. The remaining parts of the larvae were stored in RNA Later® and shipped to the University of Winnipeg for further analysis. The larval intestine was dissected using forceps and a sterile scalpel and placed in RNA Later® until DNA extraction.

Table 5: Larval lamprey collection from HBBS

Year	Date	River	Total number of sampled larvae	Range larval length
2023	27 Apr	Not applicable	n = 20	60.2 – 76.9mm

2.2 DNA extraction and Amplicon Sequencing

As previously mentioned, all samples were stored in RNA later® and at 80 °C until they were processed for DNA extraction. A summary of the samples used for DNA extraction and downstream analysis in this project are included in Table 6. Samples were transferred to -20 °C days before DNA extraction took place and were thawed in ice. DNA extraction was performed using the Qiagen DNeasy Blood and Tissue kit. Since some of the intestines were cut into three parts during sample collection, all pieces of the intestine were pooled and used in the protocol to maintain replicability. During pretreatment, each sample was incubated at 56°C, until the tissue was fully dissolved. The samples were vortexed at least twice during the incubation. After DNA extraction, samples were stored at -20 °C until shipped for amplicon sequencing. 16S rRNA long read amplicon sequencing was performed on the intestine samples using PacBio HiFi CCS sequencing. PacBio uses SMRTbell libraries, where adapters are ligated to the DNA molecule before sequencing to make it a circular molecule. Afterwards, each DNA molecule is added to a SMRTwell, which is where the DNA polymerase adds nucleotides that complement the original strain, thereby making copies of the DNA strand (Eid et al., 2009). Using circular consensus sequencing (CSS), the DNA molecule is sequenced several times resulting in a high accuracy long read (Wenger et al., 2019). Although long read amplicon sequencing is an effective method to sequence the 16S gene, increasing amplicon length has been previously described as more prone to per-base error due to a reduced number of passes on each molecule, nevertheless, PacBio Sequel II reads overcomes this issue by using the SMRT technology and providing molecule-specific high accuracy long reads (Kumar et al., 2019). Long, high-quality reads are the most appropriate sequencing option for this project since they provide a better resolution for identifying taxa (Martijn et al., 2019).

Table 6: Samples used for DNA extraction and downstream analysis.

Sample Type	Collection date	Season	Treatment	River	Number of samples	Successful sequencing

WCLR	15 Feb	Winter	Y	Baldwin	3	28
			YW	Baldwin	3	
			YR	Baldwin	3	
WCLR	30 Apr	Spring	Y	Baldwin	3	
			YW	Baldwin	3	
			YR	Baldwin	3	
WCLR	01 July	Summer	Y	Baldwin	3	
			YW	Baldwin	3	
			YR	Baldwin	3	
WCLR	31 Aug	Summer	Y	Baldwin	6	
			YW	Baldwin	3	
			YR	Baldwin	3	
WCLR	08 Dec	Winter	Y	Baldwin	3	
			YW	Baldwin	3	
			YR	Baldwin	3	
WC	13 May	Spring	N/A	Trout	5	23
WC	14 Sept	Autumn	N/A	Trout	5	
WC	14 May	Spring	N/A	Ocqueoc	5	
WC	14 & 16 Sept	Autumn	N/A	Ocqueoc	5	
T	15 Dec	Winter	N/A	St. Mary's	3	0
T	15 Dec	Winter	N/A	Munuscong	3	
LG	27 Apr	Spring	Unknown	N/A	8	8

Processing 16S rRNA Long Reads

16S PacBio long reads were processed following the PacBio CCS Amplicon SOP v2 pipeline (Comeau et al., 2017) using Qiime2 (v. 2023.5.1) (Bolyen et al., 2019). In this pipeline, reads were denoised and demultiplexed using DADA2 (v. 2023.5.0). Due to the high quality of HiFi CCS reads, no quality filtering step was included in this pipeline. However, due to the large range of read lengths (<1000 to >200,000 reads), sequences with less than 2000 reads were removed. During this filtering, contaminants like mitochondria and chloroplasts were also removed. Reads were clustered into Amplicon Sequence Variants (ASVs). ASVs are based on DNA sequences in organisms and allow reproducibility in sequenced data (Callahan et al., 2017). Previously reads were clustered into Operational Taxonomic Units (OTUs), which is still a valid clustering method, however it depends on each individual dataset, and does not account for

biological variation; furthermore OTU classifications cannot be compared to other datasets that use the same method (Callahan et al., 2017). Taxonomy was assigned using a pre-trained Naïve Bayes model against the SILVA (v.138) taxonomic database (Robeson et al., 2020; Bokulich et al., 2018). ASVs that were not present in at least one sample more than once (>1) were removed in an initial filtering step.

Data Analysis

Statistical analyses were performed in R packages (R version 4.1.0) and visualized using ggplot2 (v. 3.4.4) (Wickham, 2016; R Core Team, 2021). The data was exported from QIIME2 and imported into R, followed by another prevalence filtering step, wherein ASVs that were not present in at least 3% of the samples were removed. Due to the different conditions under which samples were collected, several steps were followed to assure that the data was comparable. First, we compared transformed library sizes (number of ASVs per sample) among sample types (Fig. 2). No sample type appeared to be overrepresented in the dataset. Afterwards, the data was imported into the phyloseq package (v. 1.46.0) in R (McMurdie & Holmes, 2013). Rarefied data was compared to unrarefied data; however, the distribution of the data did not change. Downstream analyses were carried out without rarefying the data to prevent possible introduction of errors, including overrepresentation of abundant taxa and false positives during differential abundance analysis (McMurdie & Holmes, 2014).

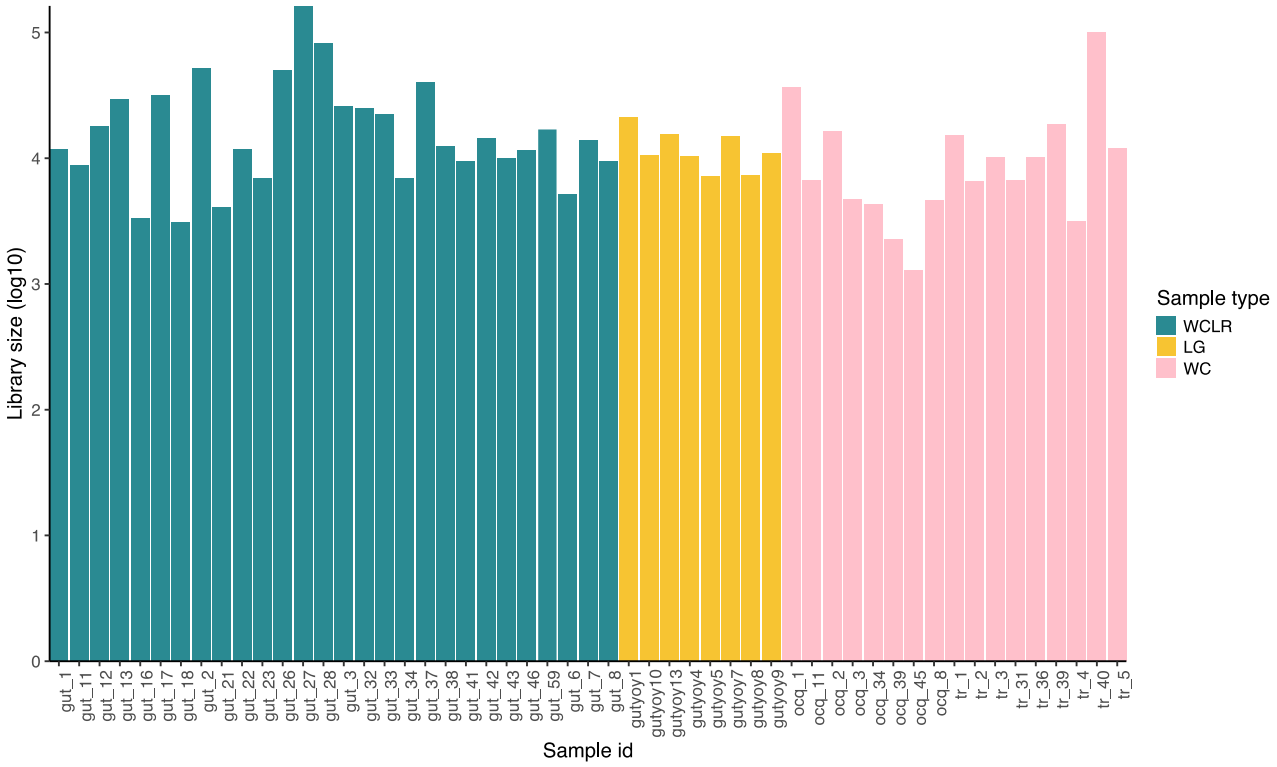


Figure 2: Comparison of sequenced library size per sample type, including samples from WCLR larvae in blue, LG larvae in yellow and WC larvae in pink.

Diversity Analysis

Beta diversity was calculated using a Bray-Curtis dissimilarity matrix to examine the inter-sample distribution to estimate differences and compare the overall taxonomic composition between treatment types. Beta diversity was calculated and visualized using the phyloseq package in R. The output of this analysis was used to perform a Principal Coordinate Analysis (PCoA) and additionally a Permutational Multivariate Analysis of Variance (PERMANOVA) was performed using the function adonis2 in the vegan package (v.2.6-4) in R to compare differences among groups using 999 random permutations (Anderson, 2017; Oksanen et al., 2022)

Alpha diversity was calculated using the DivNet package (v. 0.4.0) in R (Willis & Martin, 2022). DivNet calculates the alpha diversity by taking into consideration unrepresented and

unsampled data. Using relative abundance and the information from the other samples, this approach uses ecological networks (taxon-taxon occurrence) to provide estimates of variance in the alpha diversity (Willis & Martin, 2022). It employs a compositional data analysis approach that has been specifically developed for microbiome data rather than use multinomial modelling approaches (Willis & Martin, 2022). For reproducibility and ease of comparison, we estimated alpha diversity at the genus level, and included feed, sample type, season, and river (when applicable) as covariates. Due to the inclusion of diet and season as variables of interest, WCLR samples were analyzed separately from WC and from LG samples.

Differential Abundance Analysis and Microbial Community Composition

To determine if there were significant taxa associated with variables like location, season, and diet, a differential abundance (DA) analysis was performed. DA analysis compares the individual abundance of each taxon between groups and determines if the proportion of taxon #1 changes with respect to taxon #2, etc. (Cappellato et al., 2022). Taxa are differentially abundant if they fail the null hypothesis, which states that the same parameter setting explains the observed taxonomic distribution (Nearing et al., 2022). In other words, two taxa are differentially abundant if different parameters are needed to account for their distribution (Nearing et al., 2022). This analysis was performed using the corncob package (v. 0.4.1) in R (Martin et al., 2020). This package uses a beta-binomial regression model to estimate relative abundance from absolute abundance of taxa; maximum likelihood of the model and Wald tests were used to determine if any taxon was significantly abundant (Martin et al., 2020). We analyzed WCLR samples using season and feed as covariates. WC samples were analyzed using the corncob package, analyzing the effect of location only. Seasonal patterns in WC samples were analyzed using the metacoder package (v. 0.3.6) in R (Foster et al., 2017). The metacoder package (v.

0.3.6) was also used to highlight differences in the microbial community of LG larvae to WC samples from the spring season, since LG larvae were also sampled in the spring. Finally, the presence of shared taxa among all sample types and the presence of a core microbiome were analyzed and visualized using the phylosmith package (v. 1.0.7) and the microViz package (v. 0.12.1) in R (Smith, 2019; Barnett et al., 2021).

Functional Gene Insights Analysis

To gain insight into the possible functional roles of the gut microbiome across samples, we used the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) package. PICRUSt2 is a software that uses evolutionary modeling (specifically an ancestral-state reconstruction algorithm) to predict metagenomes from 16S rRNA data and reference genomes (Langille et al., 2013). The software follows the assumption that members of a bacterial clade are likely to share a core genome; this is used to infer metabolic pathways based on genes found in the KEGG Orthology (KO) database (Langille et al., 2013; Kanehisa, et al., 2016a; Kanehisa, et al., 2016b). Although PICRUSt2 was tested initially on human genes, mammalian guts, microbial mats and soil samples, none of which overlap with our dataset, this software is capable of providing insights into possible functions that could provide a rationale for further exploration, using metagenomic or metatranscriptomic approaches (Langille et al., 2013). The output from PICRUSt2 was analyzed using corncob and visualized using ggplot2 (Martin et al., 2020; Wickham, 2016).

Results

3.1 Sequencing Output

DNA sequencing failed for 26 of the 82 samples. Samples that failed belonged to either metamorphosing larvae or transformers (larvae that recently went through metamorphosis). For the remaining 56 samples, PacBio CCS 16S amplicon sequencing was successful. From these 56 samples, a total of 1,706,520 long reads with an average of 30,473 reads per sample were obtained. After quality filtering, 1,183,033 reads remained (523,487 reads were removed for denoising), with an average of 22,321 reads per sample, representing a total of 13,932 ASVs. Further pruning was performed to include ASVs that were present in at least 3% of samples, leading to a total of 3,158 ASVs. Per sample type, we identified 1,812 ASVs in WC larvae, 459 ASVs in LG young-of-the-year and 1,910 ASVs in WCLR larvae (Fig. 3).

The data were analyzed based on sample type. Samples belonging to WCLR larvae were analyzed together and separately from WC larvae and LG larvae samples. This was due to the data containing confounding variables such as season, location and diet, which are known to have major impacts on reshaping the gut microbiome. WCLR samples were part of a feeding experiment and were collected at three different seasons. WC samples were also collected at two different seasons and from two rivers. Finally, LG samples were kept at the same temperature throughout their lives and were younger than the other larvae. For this reason, the LG samples were only compared to the WC samples only when the variables were limited to one season and one location. Throughout the results section, WC samples are represented by pink, LG samples by blue and WCLR samples by yellow.

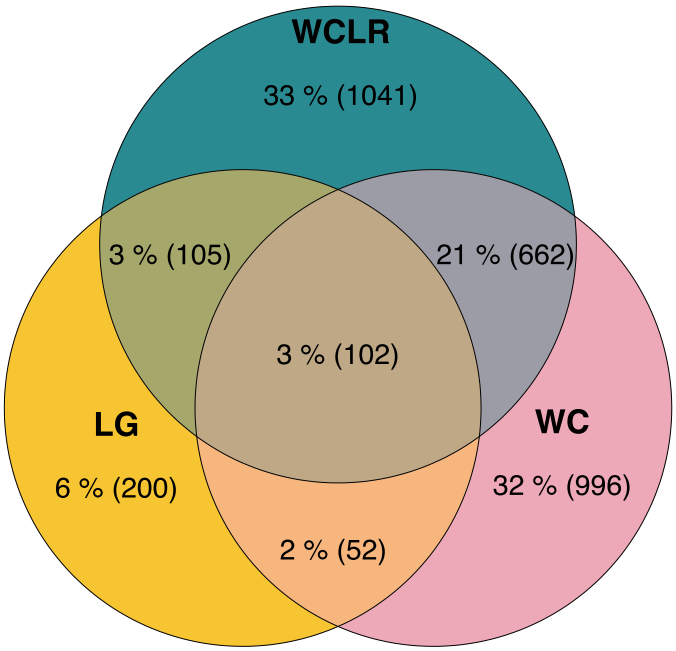


Figure 3: Venn diagram indicating ASVs among sample types: lab-grown (LG), wild-caught (WC), wild-caught lab-reared (WCLR).

3.2 General trends

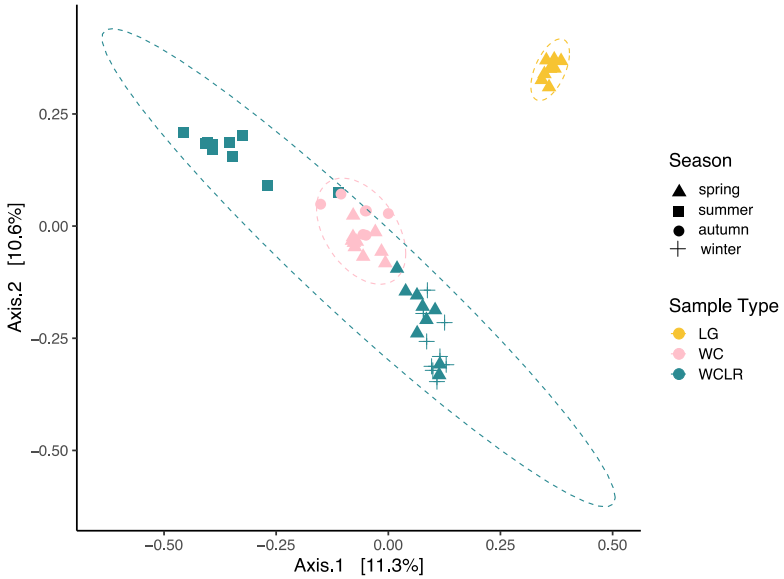


Figure 4: Principal Coordinate Analysis (PCoA) of all samples using Bray-Curtis distance. Samples are grouped by sample type (color) including lab-grown (LG) (yellow), wild-caught (WC) (pink) and wild-caught lab-reared (WCLR) (blue). Every sample is also represented by a shape that represents sample collection season, including spring (triangle), summer (square), autumn (circle) and winter (cross). Ellipses are drawn at a 95% confidence interval.

WCLR, WC and LG samples were very different from each other in collection date, rearing and other available variables. A global analysis of microbial beta diversity was calculated using Bray-Curtis distance which showed that LG samples clustered together but distant from the remaining samples, while WC and WCLR samples fell in overlapping clusters. WC samples clustered in the middle of the plot and WCLR samples diverged from the center, influenced by seasonal patterns (Fig. 4). WCLR samples from warm months (summer and autumn) clustered together, while samples collected in cold months (spring, winter) clustered together and away from the samples collected during the warm months. Seasonality had a significant effect on microbial community variability (adonis2; $p \leq 0.001$). Only WCLR samples were exposed to different feeding regimes. All feeding treatments appeared to overlap, however, results from the PERMANOVA test indicated that different feeding regimes were associated with changes in gut microbiome samples (adonis2; p-value = 0.038, Fig. 5).

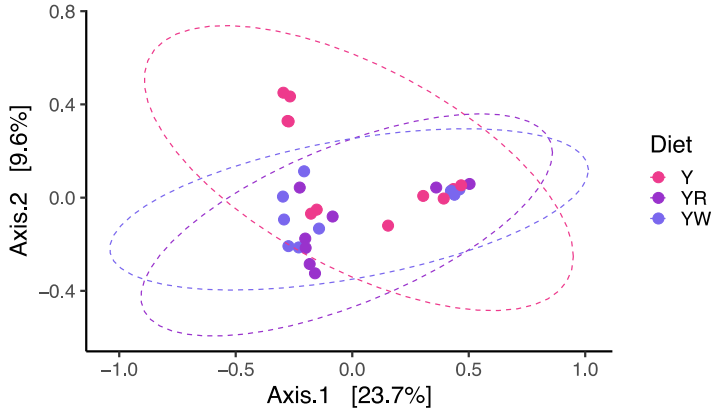


Figure 5: Principal Coordinates Analysis (PCoA) of WCLR samples using Bray-Curtis distance. Samples are grouped by feed (color) including Yeast (Y) (pink), Yeast + Rice flour (YR) (purple) and Yeast +Wheat flour (YW) (blue). Ellipses are drawn at a 95% confidence interval.

Effects of seasonality can also be seen in WC samples. Samples collected in spring had significantly different global diversity than samples collected in autumn (adonis2; $p \leq 0.001$). Additionally, microbial communities in samples collected from Trout River were significantly

different from microbial communities in samples collected from Ocqueoc River (adonis2; $p \leq 0.001$).

The LG samples were reared in tanks with water temperature kept consistent at 18 °C which prevented an analysis of microbial diversity across seasons. However, these samples were compared to WC samples to corroborate results from the PCOA. LG samples were compared to spring samples from Trout River and Ocqueoc River samples, respectively, due to similar temperature profiles at the time of collection. LG samples were significantly different from both Trout River (adonis2; p-value = 0.003) and Ocqueoc River samples (adonis2; p-value ≤ 0.001).

3.3 Alpha Diversity

WCLR samples

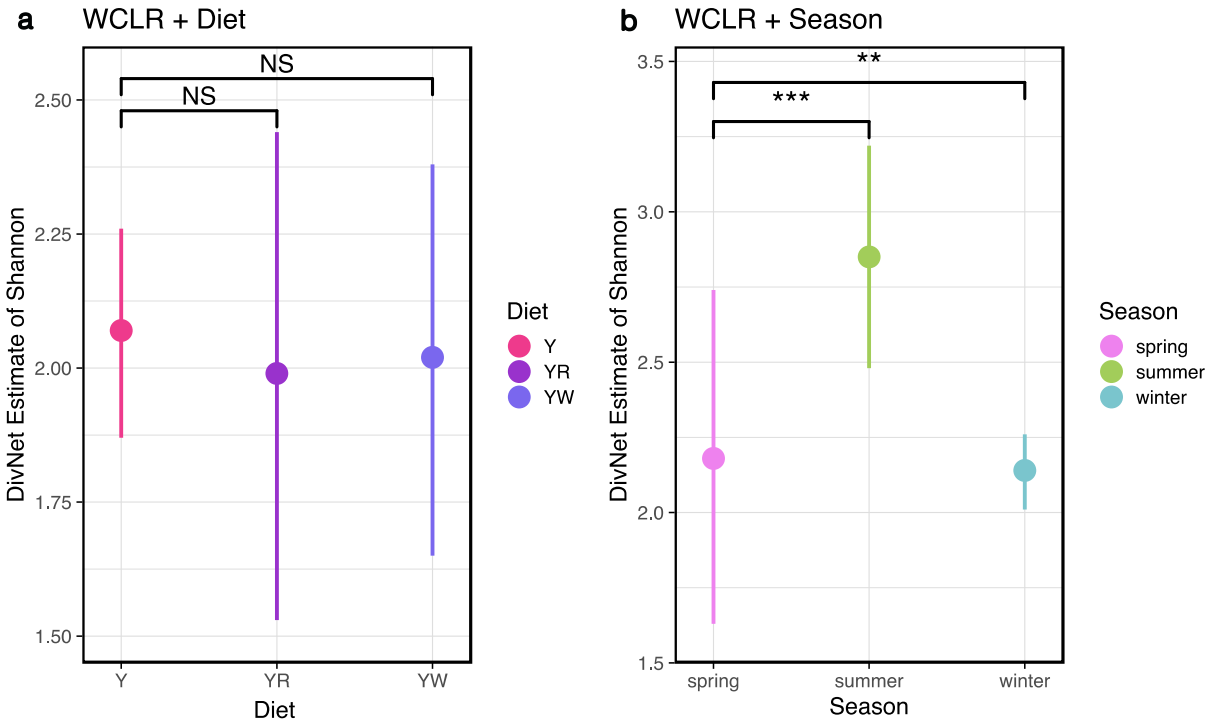


Figure 6: Estimated Shannon diversity for genera in WCLR samples in the context of (a) diet and (b) season, respectively. Diets are identified as Yeast (Y) (pink), Yeast + Rice flour (YR) (purple) and Yeast +Wheat flour (YW) (blue). Seasons are identified as spring (pink), summer (green) and winter (blue). Significance was calculated using the betta package.

Differences in alpha diversity were also assessed by sample type due to the different conditions under which the samples were collected. Using the DivNet package in R, we calculated the Shannon diversity index, using similar variables to those explored in the PCOA plots (Fig. 4 & Fig. 5). Diet did not significantly influence microbial diversity in WCLR samples, as estimates of Shannon diversity were not significantly different among feeding regimes (Fig. 6a). However, diversity analysis at the genus level identified a higher Shannon diversity estimate in the gut microbiota in summer compared to spring (p-value ≤ 0.001) and in winter compared to spring (p-value = 0.032) (Fig. 6b).

WC samples

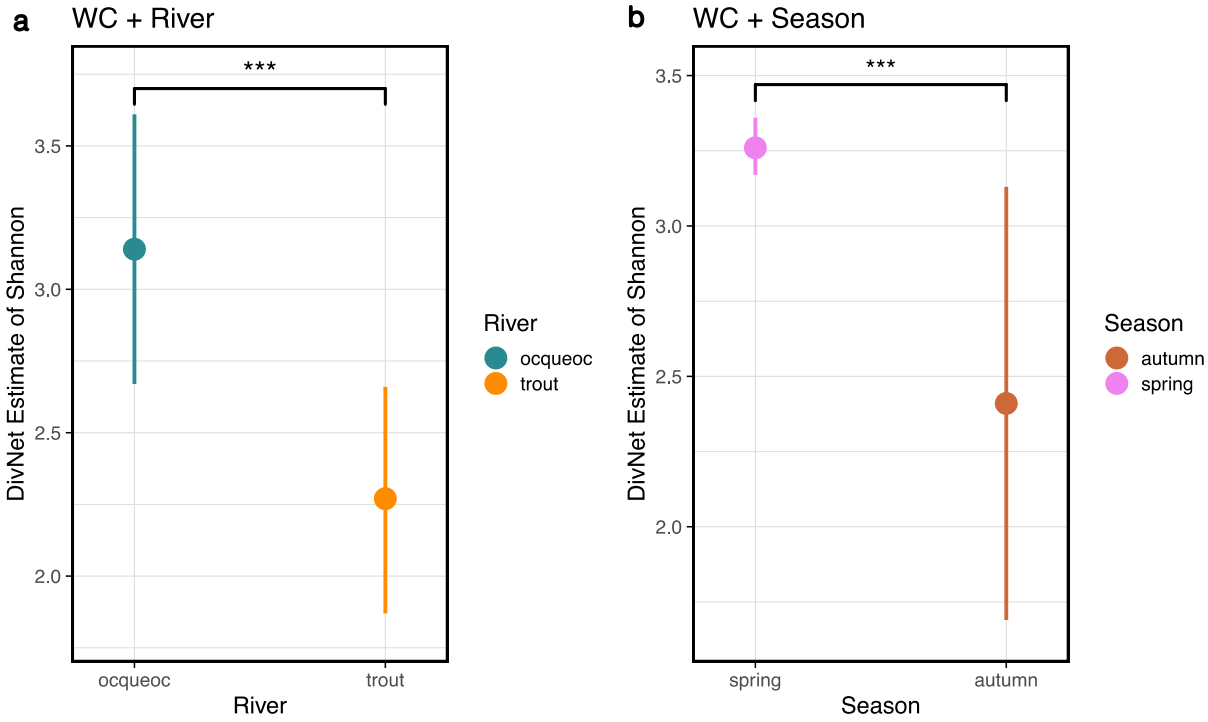


Figure 7: Estimated Shannon diversity for genera in WC samples in the context of (a) location and (b) season, respectively. Each point range includes standard error bars. Location is identified by river Ocqueoc (teal) and Trout (orange). Seasons are identified as spring (pink), autumn (brown). Significance was calculated using the betta package.

Shannon diversity in WC samples was calculated to determine if location (river) and season significantly influenced alpha diversity. Microbial communities in each river were significantly

different from each other (p-value ≤ 0.001) (Fig. 7a). Grouping the samples by season and comparing autumn samples to spring samples determined that seasons in WC samples were also significantly different (p-value ≤ 0.001) (Fig. 7b). Highest diversity was found in Ocqueoc River, when comparing rivers and in the spring, when comparing seasons.

WC & LG samples

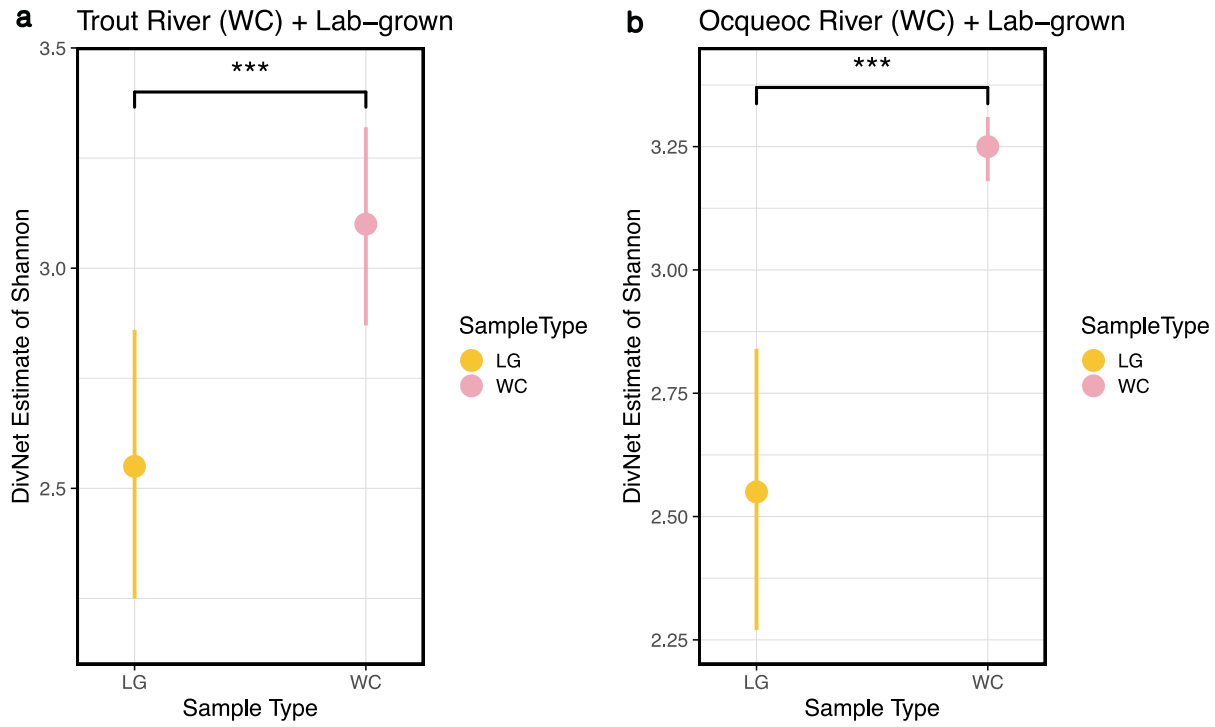


Figure 8: Estimated Shannon diversity calculated at genus level in LG samples compared to spring WC samples by river: (a) Trout River and (b) Ocqueoc River. Sample type is identified as LG (lab-grown) (yellow) and WC (wild-caught) (pink). Significance was calculated using the betta package.

LG samples were compared to both Trout and Ocqueoc rivers, individually. Only spring samples were compared since LG samples were collected only in the spring season. WC samples collected from both Trout (p-value ≤ 0.001) and Ocqueoc (p-value ≤ 0.001) rivers, were significantly different from LG samples, and had a consistently higher Shannon index at the genus level compared to LG samples.

3.4 Characterizing the gut microbial community

The distribution of taxonomic genera across sample types is shown in a Venn diagram (Fig. 3). Although we used full length amplicon sequencing of 16S, genus is the lowest, most accurate taxonomic rank that can be inferred; and estimates about the diversity and function of the gut microbial community will be inferred at this level. Most of the genera identified were found in both WC and WCLR samples, and fewer genera were identified in the LG samples. Overall, 79 genera (24%) were shared among all sample types. The taxonomic composition differed broadly by sample type, but *Proteobacteria* was always the dominant phyla (Table 7).

Table 7. Most abundant phyla and genera by sample type

Sample type	Taxonomy	Percent abundance*
WCLR		
Phylum	*Proteobacteria*	35.09%
	Verrucomicrobia	20.18%
Genus	*Dechloromonas*	10.54%
	Cetobacterium	14.56
	Unclassified	14.72%
WC		
Phylum	*Bacteroidota*	28.09%
	Proteobacteria	23.75%
Genus	*Cetobacterium*	18.33%
	Unclassified	27.82%
LG		
Phylum	*Proteobacteria*	30.81%
	Fusobacteriota	27.31
Genus	*Unclassified*	14.97%
	Candidatus Accumulibacter	8.65%
	Luteolibacter	5.73%

*Percentages represent percent relative abundance per sample type.

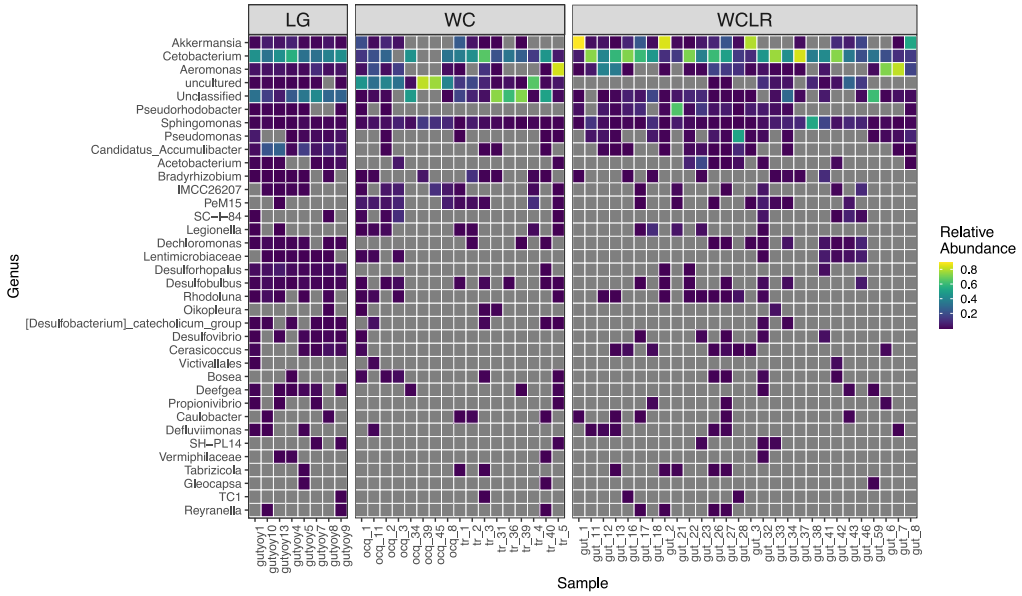


Figure 9: Heatmap showing relative abundance of genera that are present in all three sample types. The presence of each genus is colored by a gradient that represents relative abundance.

Determining how the microbial community in the larval gut of the LG samples compares to the microbial community in the WC larval gut entails several complex analyses that this exploratory project will not be able to cover. In lieu of this, examining the presence and absence of taxa is a good indicator of qualitative differences in the microbiome across different rearing conditions. Across all samples, the most abundant genera included *Akkermansia*, *Cetobacterium, Sphingomonas* and *Aeromonas*, these genera were found in most samples with high relative abundance (Fig. 9). However, as previously described, LG samples had the lowest diversity, common taxa, such as those previously mentioned, as well as *Dechloromonas* and *Desulfobulbus*, among others, were found in all samples. However, LG samples explored in this experiment had less diverse and more uniform microbiomes than WC or WCLR samples (Fig. 10). This supports our initial finding of LG samples being tightly clustered together (Fig. 4). Similarities of genera found in Figure 9 and Figure 10, like *Cetobacterium*, indicate differences

at ASV level. A higher resolution of lower taxonomic levels like species or strain, would provide more information on how this genus differs between common and unique taxa.

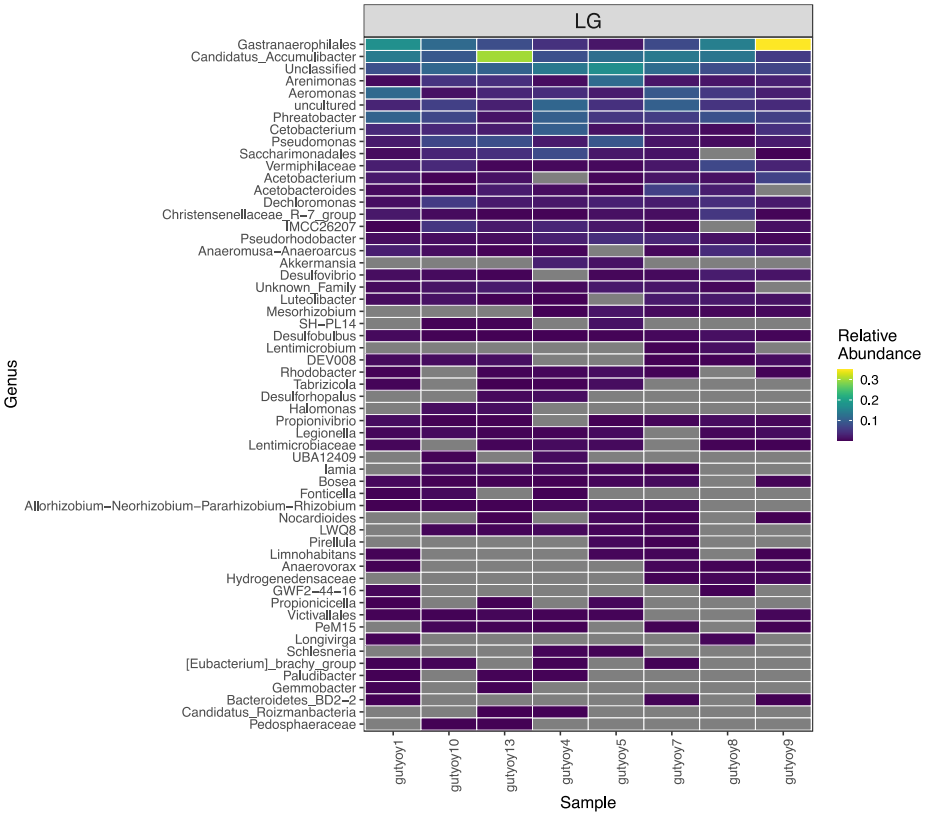


Figure 10: Heatmap showing unique taxa found in lab-grown (LG) samples. Different colors represent differences in relative abundance. Grey spaces represent samples where the correspondent taxa are missing.

WCLR samples

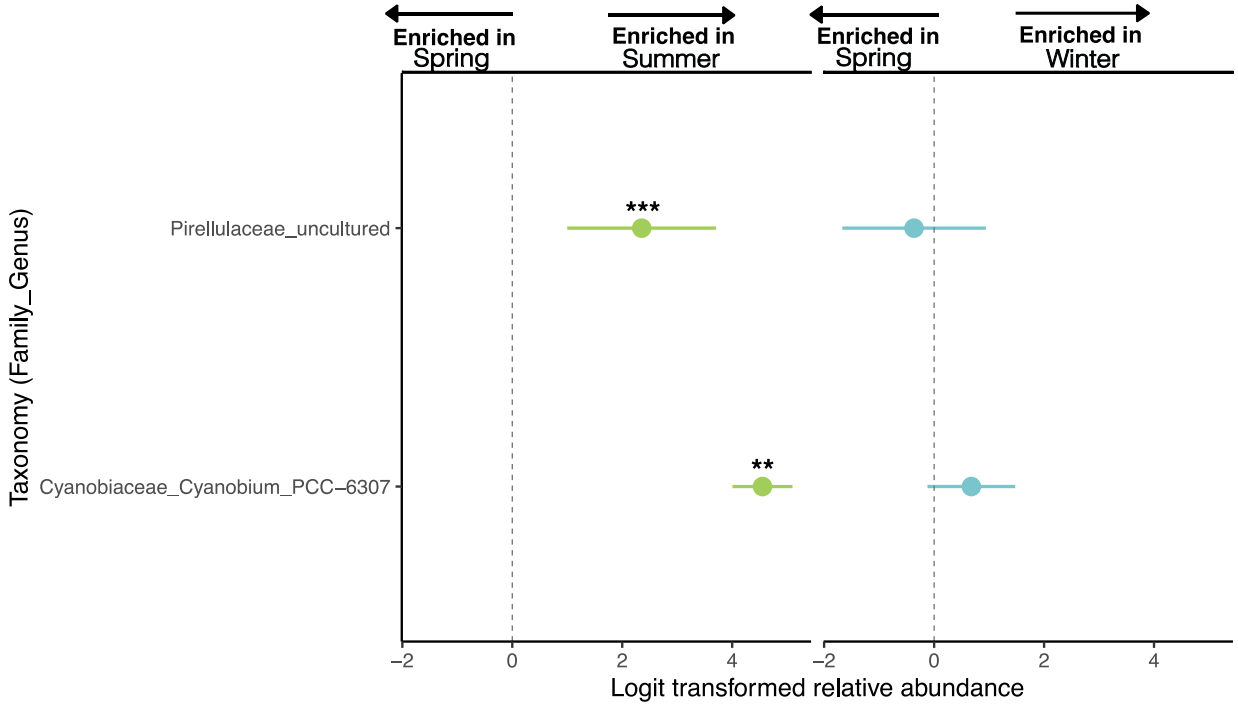


Figure 11: Differential abundance analysis of WCLR samples at genus level. The x-axis represents logit-transformed relative abundance. The dotted line separates enriched (positive numbers) and depleted (negative numbers) taxa using spring as the predictor season that other seasons are being compared to. The point range is determined using the Wald test in the corncob package. The point range is colored based on when it is enriched: pink (spring), blue (winter) and green (summer). Significance of each taxon is calculated between the covariate (summer or winter) and the predictor variable (spring) using the bbdml function in corncob that performs a t-test on each individual taxon.

A differential abundance analysis was performed using the corncob package to test if taxa at the genus level were significantly differentially abundant in some seasons (spring, summer, or winter) using the different diets (Y, YR and YW) as covariates (Wald test; pfdr = 0.03). Significant differences in the abundance of genera from the families *Pirellulaceae* (t-value = 16.335, *p*-value < 0.001) and *Cyanobiaceae* (t-value = 3.409, *p*-value = 0.003) were observed (Fig. 11).

The differential abundance analysis also identified discriminant genera, which were strongly associated with a specific season. In this analysis, we found 210 discriminant genera. Some of these taxa presented seasonal patterns. Several genera like *Methyocystis*, *Roseomonas* and *Terrimicrobium* were present in the summer months. Less diverse seasons like winter also had strongly associated taxa, for example, the genera *Fluviicola* and *Shewanella*; and, in spring, *Rickettsiella* and *Trichococcus* (Fig. S5).

In the discriminant taxa, we observe strong taxonomic association to specific seasons. However, certain genera like *Cetobacterium* and *Akkermansia*, appear to overwinter (Fig. 12). Rare taxonomic groups could also be present year-round. However, the low taxonomic resolution of 16S amplicon sequencing limits the ability to determine the presence of a core microbiome. Nevertheless, the presence of highly abundant taxa, like *Cetobacterium* (Fig. 12) suggest that some taxonomic groups are present at all seasons (at different abundances) and are therefore more resilient.

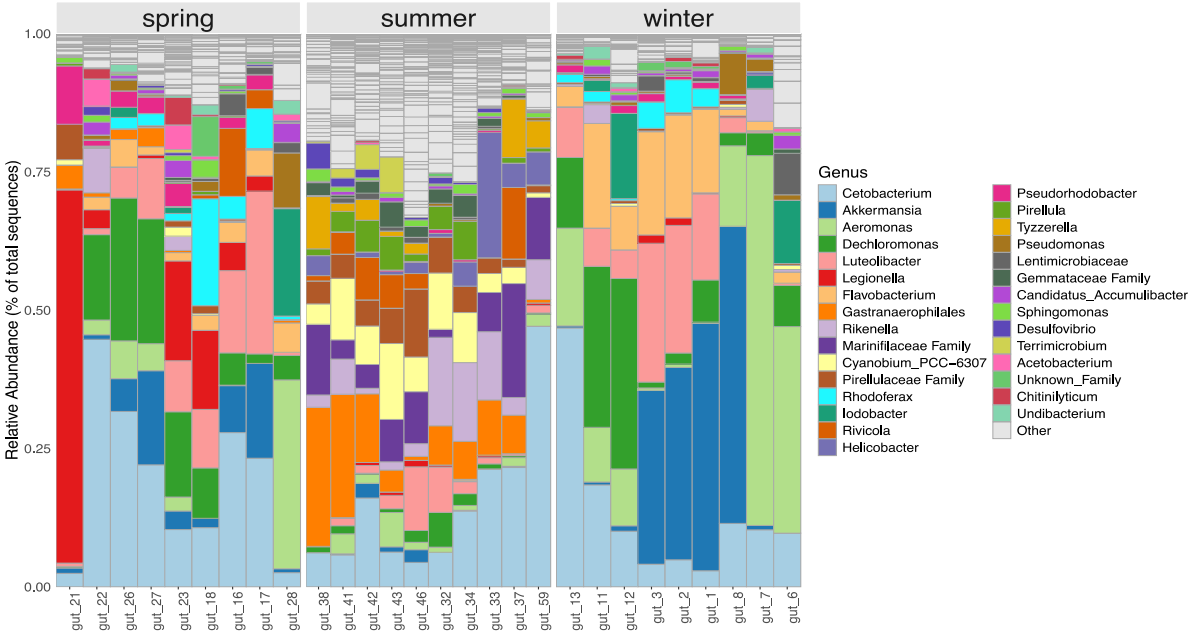


Figure 12: Relative abundance of wild-caught lab-reared (WCLR) samples grouped by season. Only top 30 most abundant genera are shown.

WC samples

Due to the complex nature of our data, WC samples were analyzed using the corncob package, only looking at differences in abundance based on location (river). Seasonality was addressed within a phylogenetic context using the metacoder package in R. The differential abundance analysis determined that there was no significant difference in abundance of WC taxa, however it identified 83 discriminant taxa (Fig. S8). As mentioned before, genera from Trout River and Ocqueoc River exhibited significantly different microbial diversity. The differences between the two rivers were further explored looking at discriminant taxa, to identify taxa that were strongly associated with a specific river. For example, at the genus level, *Mycrocystis* and *Rhodomicrobium* are discriminant to Trout River, while *Desulfomicrobium* and *Pseudoxanthobacter* are unique to Ocqueoc river. At the family level, *Rhodobacteraceae* and *Xanthobacteraceae* were present in both rivers, suggesting that several factors (not only location) determine taxonomic distribution.

To explore the effects of seasonality on the WC larval gut microbiome, we generated a metacoder heatmap tree to visually compare differentially abundant taxa (using a log2 ratio) in autumn vs. spring samples (Fig. 13). A member of the class *Lentisphaeria* was the only taxon that was differentially abundant in autumn. In the spring, enriched members of phyla *Actinobacteriota* (like family *Microtrichaceae* and class *Coriobacteria*) and classes *Gammaproteobacteria* (including *Methylocaldum spp.* and *Rhodoferax spp*.), *Bacteroidia* (like *Flavobacterium spp.*) and *Alphaproteobacteria* (like family *Xanthobacteraceae* and genus *Polymorphobacter*) were abundant in spring. As previously mentioned, autumn and spring seasons in WC samples were significantly different and a low number of differentially abundant taxa could suggest a higher presence of rare taxa in these two seasons.

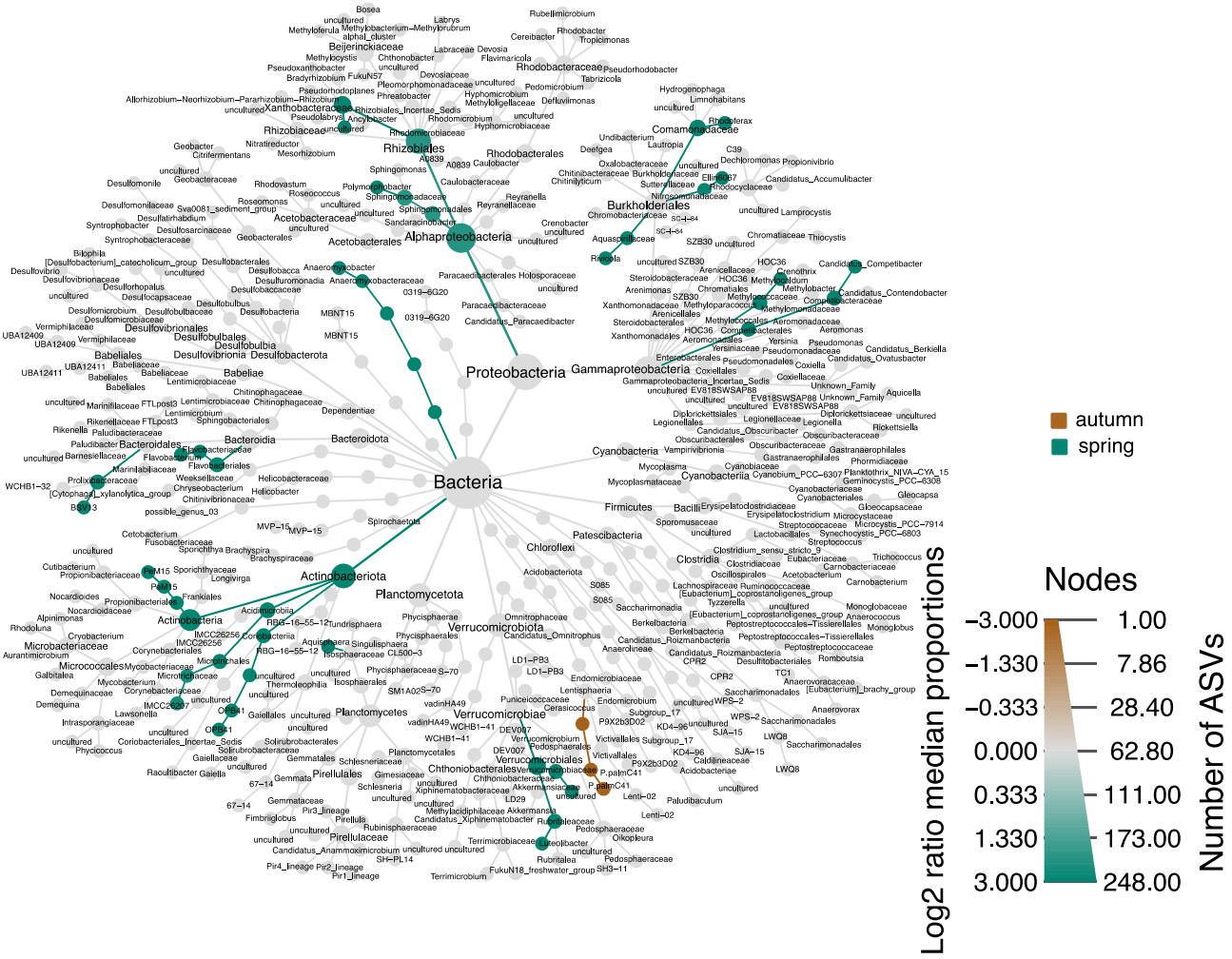


Figure 13: Metacoder heat tree showing log2 ratio of median proportions at all taxonomic levels of wild-caught (WC) samples. Node size is based on number of ASVs. Brown nodes and edges represent taxa that are significantly abundant in autumn and green nodes and edges represent taxa that are significantly abundant in spring. Samples from both Trout River and Ocqueoc River are included in this figure. Grey nodes and edges represent taxa that are not significantly abundant at any season. Wilcoxon Rank Sum test was used to determine significance and taxa with a p-value < 0.05 were removed.

WC + LG samples

The LG samples were younger than the other samples and had never been exposed to a natural river or lake environment. To reduce the number of variables in the analysis, we compared WC samples from each river individually to the LG samples (Fig. 14). Due to a low

number of samples per season, both spring and autumn seasons were grouped together within each river and then compared to LG samples.

The comparison between the two rivers exhibited the fewest number of significantly abundant taxa. Each enriched taxa belonged to a different phylum and no phyla was shared between both rivers. Members of the phulym *Fimicutes* and family *Acetobacteracea* were significantly abundant in the Trout River. Members of the family *Vermiphilaceae*, and genera *Terrimicrobium* and *Methyloparacoccus* were the only taxa enriched in Ocqueoc River. Comparisons between samples from Trout or Ocqueoc Rivers and LG samples had more significantly abundant taxa.

Between Trout River and LG samples, some phyla were significantly abundant only in one type of sample. For example, memebers of phylum Plactomycetes (like families *Isospaeraceae* and *Pirellulaceae*) were only enriched in Trout River samples. Interestingly, a member of *Pirellulaceae* was significantly abundant in WCLR samples, which could suggest that this is a taxon that is acquired in the ntural environment. An example of taxa only enriched in LG samples were members of the phylum *Desulfobacterota* (like *Desulforhopalus* spp. and *Desulfolobus* spp.). Members of the class *Gammaproteobacteria* were enriched in both Trout River and LG samples. Members of the order *Burkholderiales* and *Pseudomonas* spp. were enriched in LG samples, while other *Gammaproteobacteria* like *Halomonas* spp., *Methylocaldum* spp., *Aquicella* spp. and *Rickettsiella* spp. were enriched in Trout River samples. Additionally, different members of the phylum *Firmicutes* were significantly enriched in both LG and Trout River samples, respectively.

When comparing Ocqueoc River to LG samples, the taxonomic distribution is similar to the comparison of LG samples and Trout River. Members of *Desulfobacterota* were exclusively enriched in LG samples, while members of *Plactomycetota* were found only in Ocqueoc River

samples, just like in Trout River samples. Some taxa that differ from the Trout River vs. LG analysis include *Aeromonas* spp.*,* enriched in LG samples and phylum *Chloroflexi*, enriched in Ocqueoc River samples.

Nevertheless, some taxa were present in both groups indicating a more cosmopolitan distribution. For example, *Gammaproteobacteria* were similarly enriched in both rivers. Interestingly, Figure 14 showed that *Gammaprotebacteria* were almost exclusively enriched in the spring, suggesting that perhaps location is a stronger driver of diversity in WC samples. LG samples dominate certain taxa like phylum *Cyanobacteria and Pseudomonas spp.*, that are either not present, or found in low abundance, in WC samples. *Gammaproteobacteria* are present in all three types of samples. Most phyla were strictly enriched in one sample type, which the exception of Proteobacteria.

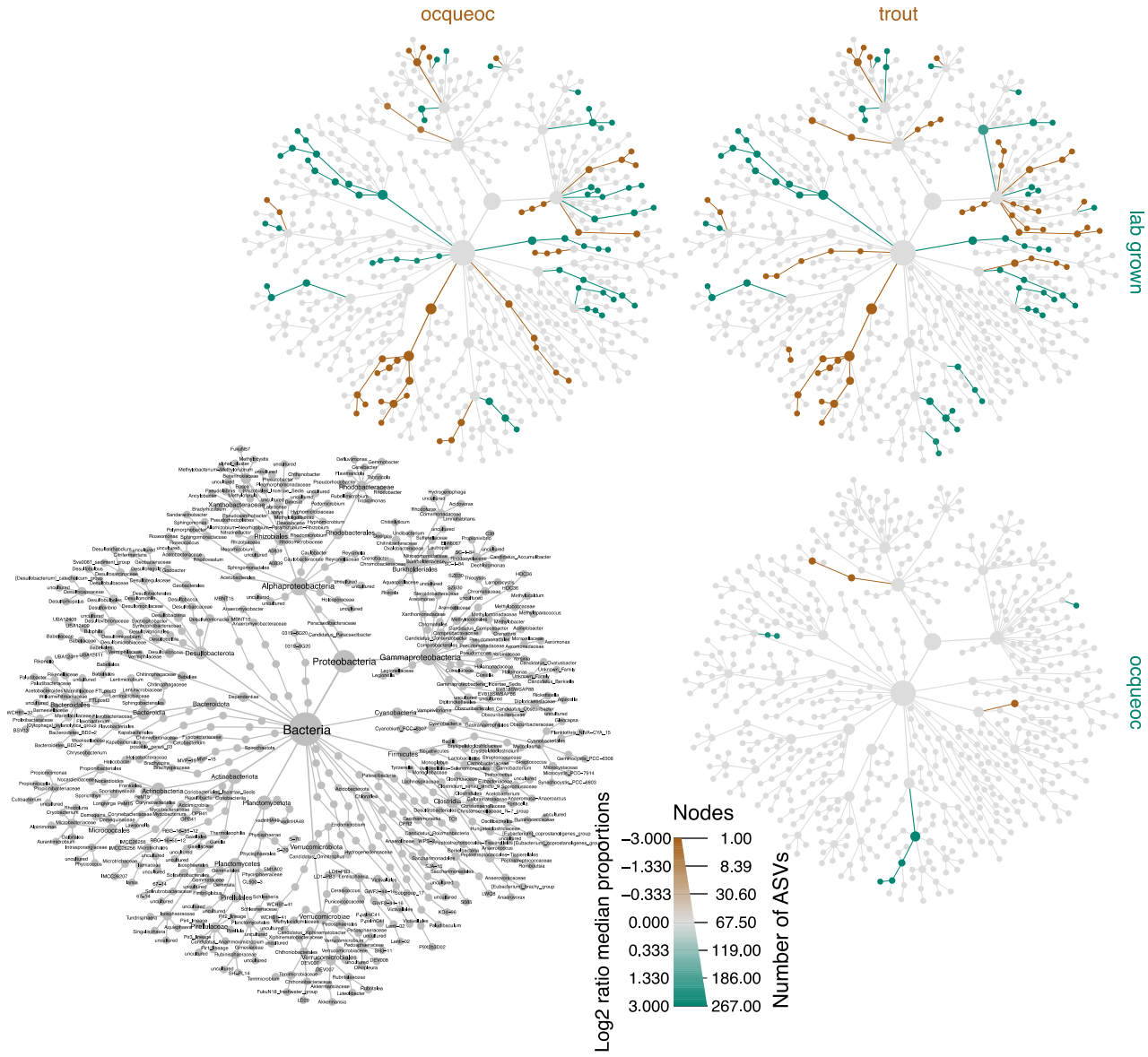


Figure 14: Metacoder heat tree showing log2 ratio of median proportions at all taxonomic levels of wild-caught (WC) and lab-grown (LG) samples. WC samples were grouped by location (Trout River and Ocqueoc River) and compared individually to LG samples. Node size is based on number of ASVs. The large tree at the bottom left is the key tree, remaining trees are "small trees" that show comparisons but do not contain any taxonomic labels. Colors in the small trees represent the comparisons that are indicated at the top and right side of the trees. Comparisons are as follows: Ocqueoc River (brown) vs. LG (green) (top left small tree), Trout River (brown) vs. LG (green) (top right small tree) and Trout River (brown) vs. Ocqueoc River (green) (bottom right small tree). Grey nodes and edges represent taxa that are not significantly abundant. Wilcoxon Rank Sum test was used to determine significance and taxa with a p-value < 0.05 were removed.

3.5 Predicted functional pathways using PICRUSt2

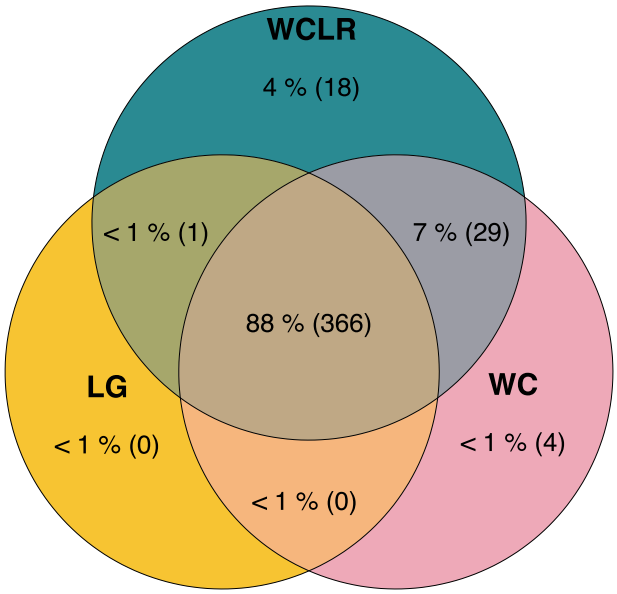


Figure 15: Venn diagram of predicted functional pathways obtained from PICRUSt2.

Using PICRUSt2 we analyzed possible functional pathways. Most identified functional pathways were shared among LG, WCLR and WC (Fig. 15). The WC and WCLR samples shared pathways that were not found in the LG samples; however, the LG samples did not have any unique functional pathways. To further explore functional pathways among different sample types, a differential abundance analysis (DAA) was performed using corncob. The DAA was performed using the PICRUSt2 pathway counts and sample types (LG, WC and WCLR) as covariates.

The DAA identified 135 significant functional pathways (pfdr = 0.007; S10). Most significant pathways correspond to metabolic functions, however, some interesting pathways include "menaquinol biosynthesis" (Vitamin K), "thiamin biosynthesis" (Vitamin B1), "mannan degradation" and "adenosylcobalamin biosynthesis" (Vitamin B12) (Fig. 16). Most of these pathways were significantly enriched in LG samples compared to both WC and WCLR samples. "Mannan degradation" was significantly enriched in WCLR and in WC samples but depleted in

LG samples. Conversely, with the exception of "mannan degradation," most nutritional pathways were significantly enriched in LG samples when compared to WC but not with WCLR.

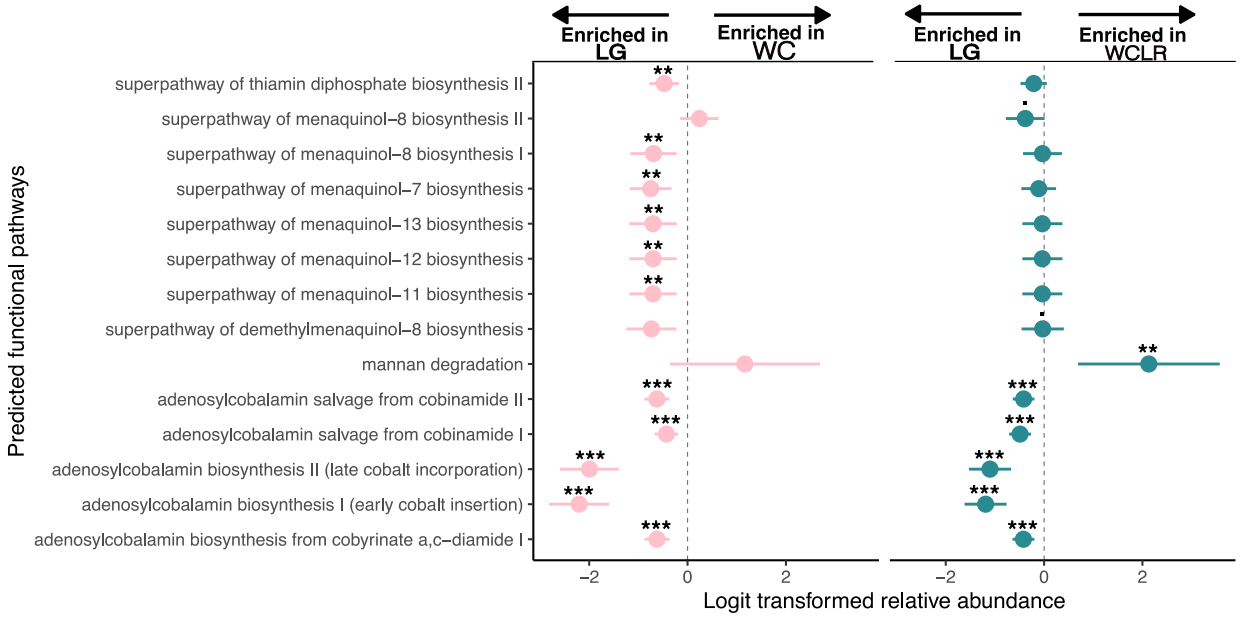


Figure 16: Differential abundance analysis of PICRUSt2 predicted functional pathways using different sample types as covariates. The x-axis represents logit-transformed relative abundance. The dotted line separates enriched (positive numbers) and depleted (negative numbers) taxa using LG as the predictor sample type, that other sample types are being compared to. The point range is determined using a Wald test. Significance of each taxon is calculated using the bbdml function in corncob that performs a t-test on each taxon. The point range is colored based on when it is enriched: pink (WC), teal (WCLR) and yellow (LG).

Discussion

The larval sea lamprey gut microbiome has been relatively understudied, especially compared to other aquaculture fish. Lampreys are unique in their life history and physiological characteristics, which makes closing this knowledge gap especially urgent. Studying the sea lamprey gut microbiome will assist not only in aquaculture application but also in conservation efforts for native sea lampreys. Previous research has explored differences in the gut microbiome across sea lamprey life history stages (Mathai et al., 2021; Tetlock et al., 2012); however, to our knowledge, the comparison of the gut microbiome between different rearing conditions remains unexplored. Understanding the difference in the gut microbiome between wild and lab-reared conditions is essential for developing aquaculture techniques, in particular developing protocols that will sufficiently mimic the growth and health of sea lampreys raised in natural conditions.

We analyzed the gut microbiome of LG, WC and WCLR larval sea lampreys to characterize their gut microbiome. This allowed us to characterize the gut microbiome under different conditions of rearing, feed, river origin and seasonality. We found that seasonality was a major factor in shaping the microbial diversity of WCLR and WC larvae. Since the LG larvae were kept at a constant temperature, we were not able to estimate the effect of seasonality for these samples. For WC samples, we were further able to assess differences in the microbial diversity in larvae from two natural rivers, Trout and Ocqueoc. Despite having different taxonomic profiles, we observed several shared functional pathways across all sample types and propose that the functional potential of the gut microbiome may be conserved in a functional core microbiome.

Environmental factors have a strong impact on diversity of the gut microbiome

Analyzing beta and alpha diversity in WC and WCLR samples, seasonality was determined to be a strong driver of the microbial community. This had not been described before in the sea lamprey larval gut microbiome but was not surprising since the sea lamprey life cycle is

dependent on temperature cues. For example, at lower temperatures in the winter and spring, larvae experience a slower growth rate and metabolism and are therefore fed less when reared in captivity (Farmer et al., 2011; Hume et al., 2024; Moser et al., 2019). In our analysis, WCLR samples collected in the winter and early spring had a lower microbial diversity than in the summer. Savard et al. (2023) observed a similar pattern in the gut microbiome of lake trout (*Salvelinus namaycush*). They suggest that a reduction in diversity in the colder months may be caused by fewer natural disturbances in the colder months for the gut microbiome and the host, leading to a more stable microbial community (Savard et al., 2023). Lower feeding rates, growth rates and metabolism in larval sea lampreys during the winter could also indicate a more "stable" microbial environment that leads to lower microbial diversity.

During the summer and late spring, WCLR and WC samples, respectively, had higher microbial diversity. The high microbial diversity was observed in lampreys reared at higher temperatures, that are closer to the optimal body temperature of sea lamprey larvae (~21°C), when larvae are expected to have high metabolism rates while they prepare (store fats) for possible metamorphosis (Dawson et al., 2015; G. Farmer et al., 2011; Holmes & Lin, 1994). From previous studies by Mathai et al. (2021) and Tetlock et al. (2012), we know that major life history stages (larval, parasitic juvenile, adult) broadly differ in their gut microbiome. For this study, our samples represented two larval stages reared under different conditions; however, the samples collected during metamorphosis and early-juveniles failed to generate sufficient 16S sequencing results with ~1000 sequences per sample obtained. On the other hand, this study provides novel information about younger organisms: our transformers measured 126-171mm and had not fed parasitically yet, while the juveniles included in the Mathai et al. (2021) study were feeding parasites ranging from 185-370mm. We hypothesize that fasting during the 4-

month period of metamorphosis and the atrophy of the gut readjusts the gut-microbiome relationship which results in almost complete loss of microbial biomass both in the wild and in reared larvae. This suggests that the gut microbiome is completely re-set following metamorphosis. In addition to the observation that the gut microbiome is lost during metamorphosis, this study is the first to find that the gut microbiome changes in response to seasonality and temperature throughout the year.

The differential abundance analysis using season as a covariate included an unidentified genus of *Pirellulaceae* and *Cyanobium* spp., both of which were enriched in summer. *Cyanobium* spp. have been detected in both the rearing water of aquaculture white shrimp (*Litopenaeus setiferus*) and channel catfish (*Ictalurus punctatus*) (Li et al., 2022). However, Duan et al. (2020) detected *Cyanobium* spp. both in the rearing water and gut of aquaculture giant tiger prawns (*Penaeus monodon*). On the other hand, *Pirellulaceae* is not as commonly found in the literature as *Cyanobium* spp. In lab-reared white shrimp and Nile tilapia (*Oreochromis niloticus*), *Pirellulaceae* has been observed to be an abundant family (Cheng et al., 2023; Parata et al., 2020). Likely, Nile tilapia acquires *Pirellulaceae* from feed; however, in the gut microbiome of white shrimp and other shrimp species, this is perhaps an indigenous taxon acquired from the environment; and it is often associated with immune responses (Cheng et al., 2023; Parata et al., 2020). Shrimp are filter feeders, and their gut microbiome mirrors their surrounding environment, which implies that their gut microbiome is predominantly shaped by naturally free-living bacteria in their environment. Further testing is needed to confirm this association in reared larval sea lampreys. Furthermore, *Pirellulaceae* and *Cyanobium spp.* had not been described as significant or abundant taxa in previous studies involving the gut microbiome of larval sea lampreys.

Other factors like location in WC and feed in WC and WCLR samples, respectively, were also analyzed. For WC samples, location was a driver of diversity; however, more studies on the physiochemical parameters and the microbiome in the water column and sediments of Trout River and Ocqueoc River are needed to determine what is impacting the larval gut microbiome in these locations. Several studies have determined that the gut microbial community is directly associated to host factors like feeding habits and trophic levels (Huang et al., 2020; Kim et al., 2021). Detritivores, like larval sea lampreys, are expected to have a gut microbiome that is highly influenced by their environment, although this is still only a theory for fish, it has stronger support in some invertebrate filter feeders (Huang et al., 2020). For example, Fan et al. (2019) found that Pacific white shrimp *(Litopenaeus vannamei*) have a similar gut microbial composition to their surrounding environment, even though there were differences in relative abundance between the sediment and the gut. On the other hand, studies in bivalves including Pacific oysters (*Crassostrea gigas*) and Mediterranean oysters (*Mytilus galloprovincialis*) found evidence of seasonal changes in the gut microbiome although the microbial composition of the gut differs in identity and abundance from the surrounding environment (Akter et al. 2023).

The relationship between the gut microbiome and the surrounding environment is, however, complex. For example, Kuang et al. (2020) explored the gut microbiome of two filter-feeding fish, silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Hypophthalmichthys nobilis*), and found that even though the fish belong to the same genus, they have different feeding habits and different gut microbiomes, which also differed from their surrounding environment. This suggests that there are important host-related factors including biotic and abiotic factors that influence the community composition of their gut microbiome. Although the environment-gut microbiome relationship has yet to be explored in larval sea lampreys, larvae spend most of the

duration of this stage burrowed in the sediment, which increases the possibility that their gut microbiome may be similar to the microbial community in the sediment surrounding their burrow hole.

The impacts of diet in the gut microbial community composition

The final factor explored in this study, but only in WCLR samples, was the effect of diet on the gut microbial community. We found little evidence that the gut microbiome was influenced by differences in the diet; however, there are several factors that may have prevented an effect of diet type on the gut microbial community. First, the different feeding regimes in the experiment consisted of a yeast base with the addition of a supplement (rice flour or yeast flour). In this case, since yeast was present in all feeds, there was no significant change in the microbial community with the addition of a supplement.

Diet or feed can play a significant role in shaping fish gut microbial communities in both wild and in aquaculture environments. In aquaculture, Karlsen et al. (2022) reported that the feed itself has a microbiome that directly influences the fish gut microbial community. In other words, the feed microbial community can be found in the fish gut along with resident fish gut taxa. Although there were no significant changes in our samples with the addition of one of the supplements, our data does not allow us to directly assess the effect of a yeast diet on the gut microbiome of larval sea lampreys. Nevertheless, previous studies have analyzed the effect of a yeast diet in captive fish. Typically, the effect of a yeast diet depends on the fish species, the yeast species and the amount of yeast added in the diet (Siriyappagouder et al., 2018). The addition of yeast has been found to increase the number of beneficial bacteria, reduce the gut microbial diversity or to exhibit little impact on the gut microbial community (Hines et al., 2021; Huyben et al., 2017; Liu et al., 2018; Zheng et al., 2021). Regardless of the effect on the gut

microbiome, we know that yeast contributes to fish growth, as has been described in shrimp (Zheng et al., 2021) and in various fish species (Liu et al., 2018; Ran et al., 2015; Tovar-Ramírez et al., 2004), including larval sea lampreys (Moser et al., 2019). More studies are needed to determine if a yeast diet changes the gut microbiome to the extent that it significantly modifies the gut microbiome relative to that of WC larvae.

Rearing affects the gut microbial composition of larval sea lampreys

Various studies have compared the gut microbiome of fish in captivity relative to WC fish in species such as the rainbow darter (*Etheostoma caeruleum*), Nile tilapia and Atlantic cod, among others (Bereded et al., 2021; Dhanasiri et al., 2011; Restivo et al., 2021). There are at least three outcomes that could be expected when comparing the gut microbiome of wild vs. farmed/captive fish: i) the gut microbiome may be the same; ii) one microbial community may be more diverse than the other; or iii) there are changes in the relative abundance (but not in the taxonomy) of the microbial community (Bereded et al., 2021; Dhanasiri et al., 2011; Huang et al., 2024; Lavoie et al., 2018; Lorgen-Ritchie et al., 2021; Ramírez & Romero, 2017; Restivo et al., 2021; Rungrassamee et al., 2014).

Although these studies provide insights into how the captive and natural environments compare to each other, there are differences between teleost fish and sea lamprey larvae that must be considered. One of the factors that differentiates aquaculture teleost fish and larval sea lampreys is the presence of a core microbiome. For example, Wong et al. (2013) found that rainbow trout (*Oncorhynchus mykiss*) have a core microbiome that remains unchanged even when the fish are exposed to treatments; fish that belonged to the same cohort and were reared at the same facility had a stronger core with a similar gut microbiome. Black tiger shrimp (*Penaeus*

monodon) and Nile tilapia also have a core microbiome that remains unchanged in reared and aquaculture conditions (Bereded et al., 2021; Rungrassamee et al., 2014).

Mathai et al. (2021), found 116 core taxa in WC larvae. They defined the core microbiome as taxa found in ≥90% of samples. Based on this criteria, in our samples, we detected shared taxa but no core microbiome. Occurrence and relative abundance (or a combination of both) are commonly used to determine members of the core microbiome, however, factors like seasonality and functional redundancy can also be used to determine a core (Custer et al., 2023; Neu et al., 2021; Shade & Handelsman, 2012).

The core microbial community includes generalists that are prevalent in the gut regardless of environment or diet and, therefore, can be described as the stable microbial community; transient microbial communities are usually found in feces and are described as temporary bacteria that pass through the intestinal tract due to host adaptation to changes in diet or the environment (Kokou et al., 2019). According to Kokou et al. (2019), certain adaptations allow the core microbiome to persist, through different environmental conditions and diets, like niche partitioning, intraspecies variability and synergistic interactions (positive interactions). The core microbiome remains constant even during lab rearing and transitioning to controlled experiments; however, the remaining gut microbial community can be affected by changes in the environment (Roeselers et al., 2011).

Even though we did not find evidence of a core microbiome, it is important to note that 102 ASVs were found in all sample types (Fig. 3). One of the most abundant taxa and consequently the most common shared taxa across samples was *Cetobacterium* spp. *Cetobacterium* spp. is a common bacterium found in the intestine of freshwater fish (Tsuchiya et al., 2008). Notably, it is known for being responsible for most (if not all) production of vitamin B_{12} in fish (Qi et al.,

2023). *Cetobacterium* spp. was present in high abundance in most of our samples, indicating that this could also be an indigenous bacterium in larval sea lampreys. *Aeromonas* spp. was also present in almost all samples. *Aeromonas* spp. is mostly recognized as a pathogen in reared fish, however, it has also been described as a cellulase producer (Jiang et al., 2011; Qi et al., 2023). Its function in the larval sea lamprey intestine is not known. Additionally, using the ≥90% threshold of occurrence with relative abundance ≥1%, our LG samples had 7 ASVs found in all samples within the treatment; this was not the case for the WC or WCLR samples. However, since the LG samples were exposed to the same controlled conditions throughout their lives, it is perhaps unsurprising that their microbiomes were so similar among individuals. Interestingly, the LG samples were part of different feeding regimes; all of which included yeast with the addition of a supplement. This could suggest once more that supplements do not impact the gut microbiome when active dry yeast is the base diet. However, further sampling is needed to determine if different diets caused taxonomic shifts in the microbiome.

One benefit of having a core microbiome is reaching a stable microbial community in the gut that contributes to quicker acclimation in aquaculture. Without a gut microbiome it's difficult to know if there is a baseline community that settles after acclimation, and this makes our sample types truly different from each other. Additionally, a lack of a core makes it harder to determine dysbiosis (shifts in the microbial community) in the gut. Dysbiosis in aquaculture fish is typically characterized by loss of diversity and, is associated with susceptibility to disease (Xavier et al., 2024; and references therein). According to observation at the rearing facility (HBBS), reared sea lampreys rarely show signs of disease, which may indicate that in the larval gut, low diversity or changes in microbial identity do not affect host fitness. This is consistent with the presence of *Aeromonas*, a known pathogen in almost all samples including in LG.

Nevertheless, as mentioned before, there are several ways to determine whether species have a core microbiome. The core microbiome described earlier is based on the presence of common taxa across animals grown in different conditions. However, a second concept of a core microbiome is that the microbiome should exhibit functional redundancy (prevalence of functional pathways of the microbiome); thus, in subsequent analyses we examined whether larval sea lampreys exhibit a functional redundant common microbiome rather than a core microbiome based on taxonomy.

Functional pathways are shared but at different rates

PICRUSt2 has been used in aquaculture research to infer pathways from 16S amplicon sequencing data (e.g. Huang et al., 2024). Surprisingly, the functional pathways predicted from the larval sea lamprey gut microbiome showed a strong pattern with most of the predicted pathways being shared among samples. This was not expected because the taxonomic composition varied tremendously among individuals; however, this could suggest the presence of a functional core. Further research is needed to verify the functional pathways being performed in the larval gut since PICRUSt2 pathways are based on predicted metagenomes.

Several significantly abundant pathways were identified, most of which belonged to biochemical pathways used for metabolic functions. Some interesting pathways that are of interest to the SLAP project included those that might supply important nutritional components to support larval health. Pathways such as: “Adenosylcobalamin biosynthesis,” “superpathway of thiamin diphosphate biosynthesis” and “superpathway of menaquinol” were enriched in LG samples and depleted in WC samples: these pathways produce vitamins B_{12}, B_1 and K, respectively**.** These vitamins are essential for fish survival and deficiency presents as physiological characteristics such as a reduced growth rate (Bentley & Meganathan, 1982; Bito

et al., 2018; Cheng et al., 2023; Li et al., 2014). This could be due to LG samples being exposed to a very nutritious diet for the duration of their life (including their first exogenous feedings), compared to WC samples which were never exposed to a nutritious yeast diet.

Additionally, we found pathways associated with a yeast diet, like "mannan degradation". Mannan is a component of the *Saccharomyces cerevisiae* (yeast) cell wall that has been previously used in aquaculture as a probiotic (Dimitroglou et al., 2010; Ren et al., 2020). This pathway was depleted in LG samples and similarly enriched in both WC and WCLR samples. This could suggest that active dry yeast should be further researched as the chosen diet for larval sea lampreys grown in the lab. Perhaps wild larval sea lampreys that had not been exposed to a highly nutritious diet were better adapted to degrade yeast cells (including their chitin wall) compared to LG larval sea lampreys, which may be feeding on other yeast metabolites or on dead yeast cells. Further research is needed to determine the effect of a yeast diet in the gut microbiome of larval sea lampreys and how this compared to a detritivore diet in the wild.

Future directions

One of the limitations of this study was that the samples had many confounding variables that may drastically influence the gut microbial community composition to fully answer the questions we were proposing. For example, WC and WCLR samples were of similar lengths, however WC samples were sampled at two different rivers and at two different seasons. Similarly, WCLR samples were collected at 3 different seasons and exposed to 3 different diets. This created conflict when running the corncob model to determine differentially abundant taxa. At the same time, LG samples were too young to be robustly comparable to WC or WCLR samples, as WC and WCLR samples were much larger larvae that were closer to metamorphosis.

Comparing the gut of LG young larvae (~60-70 mm) and WC individuals of the same length and that are collected at a similar temperature range, would be helpful to narrow down possible effects on the gut microbiome such as shifts in diet or rearing. Additionally, sampling the water and sediment surrounding the burrow hole of both LG and WC larvae would help determine the effect of the surrounding environment on the larval gut.

In the event of a new feeding experiment where large larvae are exposed to different diets or supplements, having a control group that is either fed a different diet or is starved would be useful to establish a baseline for the gut microbiome (in terms of diet effects). Additionally, active dry yeast is a cheap and efficient way to feed larval sea lampreys, however, it would be interesting to compare the effects of active dry yeast and yeast extract as diets. In our analysis, only WC and WCLR larval sea lampreys had mannan degradation pathways, which could suggest that having a more bioavailable form of yeast could support LG larvae in their growth at faster rates and reducing the amount of biofilm that accumulates in the larval tanks.

Conclusion

Comparing WC and lab-reared larvae remains complex. However, this thesis provides an in-depth analysis of the gut microbiome of larval sea lampreys. Broadly, the gut microbiome in larvae differs based on rearing conditions. Within WC larval communities in our dataset, differences in the microbial community composition appeared to be strongly influenced by seasonality and location. On the other hand, in lab-reared larvae analyzed, several factors contribute to the microbial diversity including seasonality. Diet supplements did not strongly shape the microbial composition in our samples, however the possibility remains that, given different experimental conditions, diet could become a factor of microbial diversity. A common core microbiome based on taxonomy was not detected in our samples, however, we propose the possibility of a functional microbiome that is based on the redundancy of functional pathways. Although taxa in our samples did not meet the prevalence threshold for a core microbiome, certain genera like *Cetobacterium spp.* and *Aeromonas spp.* were present in most of our samples. This could suggest that the redundant taxa in the larval gut microbiome persist at lower abundances. Finally, identified functional pathways mostly performed metabolic or nutritional functions. Further research is needed to explore the proposed presence of a functional core microbiome. Nevertheless, this research provided new insights into environmental factors driving changes in diversity in the gut microbiome of larval sea lampreys and will help inform future research on this topic.

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Supplementary Materials

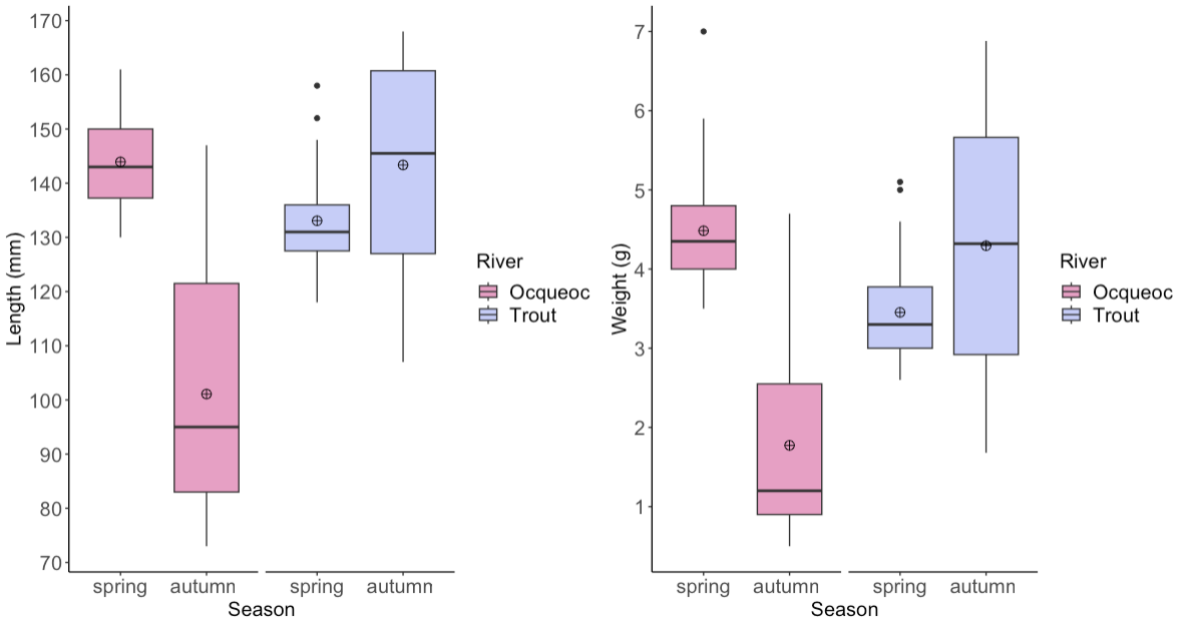


Figure S1. Length and weight distribution for larvae caught in the Trout and Ocqueoc Rivers grouped by season.

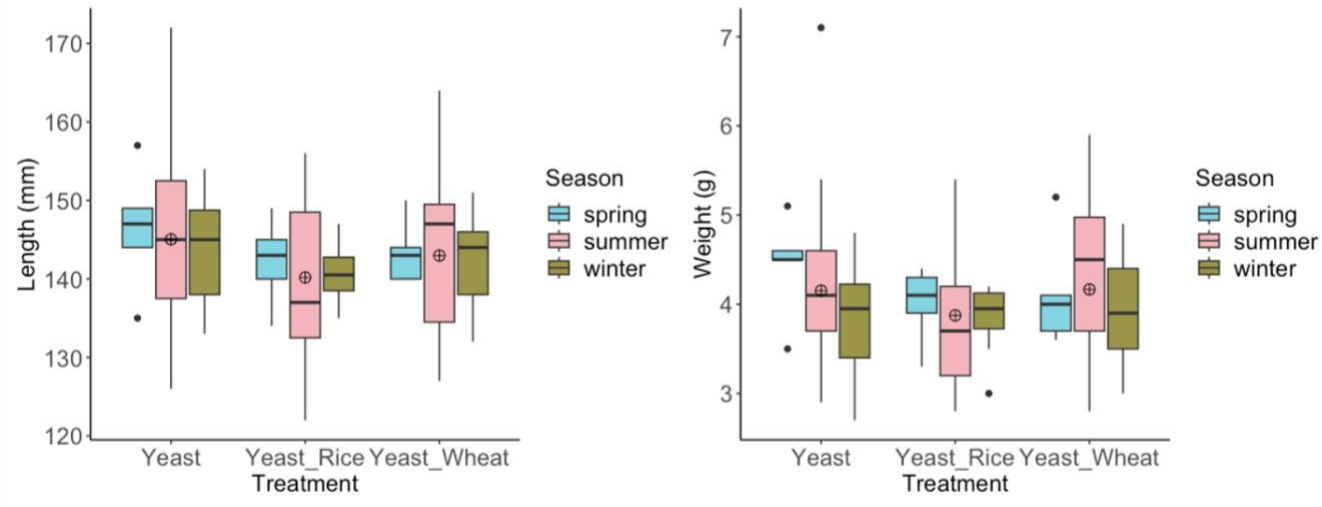


Figure S2. Length and weight distribution for larvae in the feeding experiment, grouped by season.

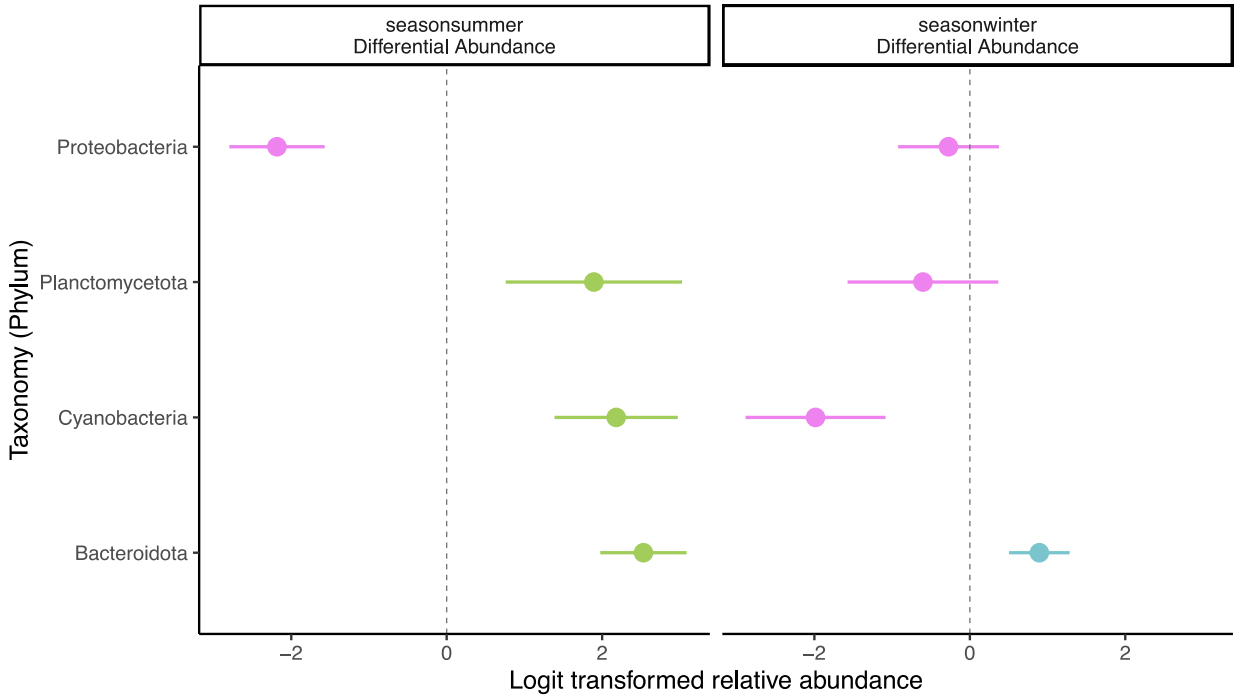


Figure S3. Differential abundance analysis of WCLR samples at phylum level. The x-axis represents logit-transformed relative abundance. The dotted line separates enriched (positive numbers) and depleted (negative numbers) taxa using Spring as the predictor season that other seasons are being compared to. The point range is determined using the Wald test in the corncob package.

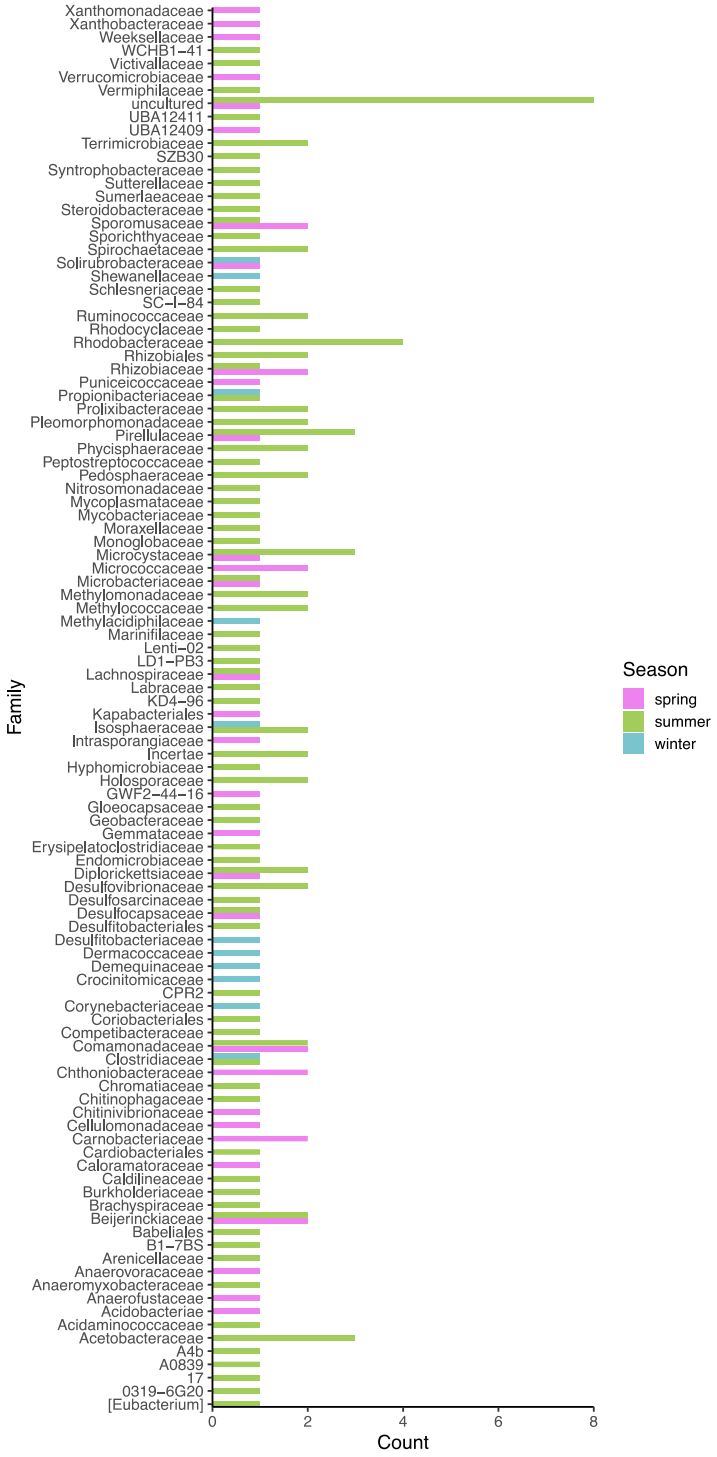


Figure S4: Discriminant taxa determined from the differential abundance analysis WCLR samples at genus level. Families of discriminant taxa in spring (pink), summer (green) and winter (blue) are included. Multiple bars in a same taxon represent discriminant taxa that fit more than one category.



Figure S5: Discriminant taxa determined from the differential abundance analysis WCLR samples at genus level. Genera of discriminant taxa in spring (pink), summer (green) and winter

(blue) are included. Multiple bars in a same taxon represent discriminant taxa that fit more than one category.

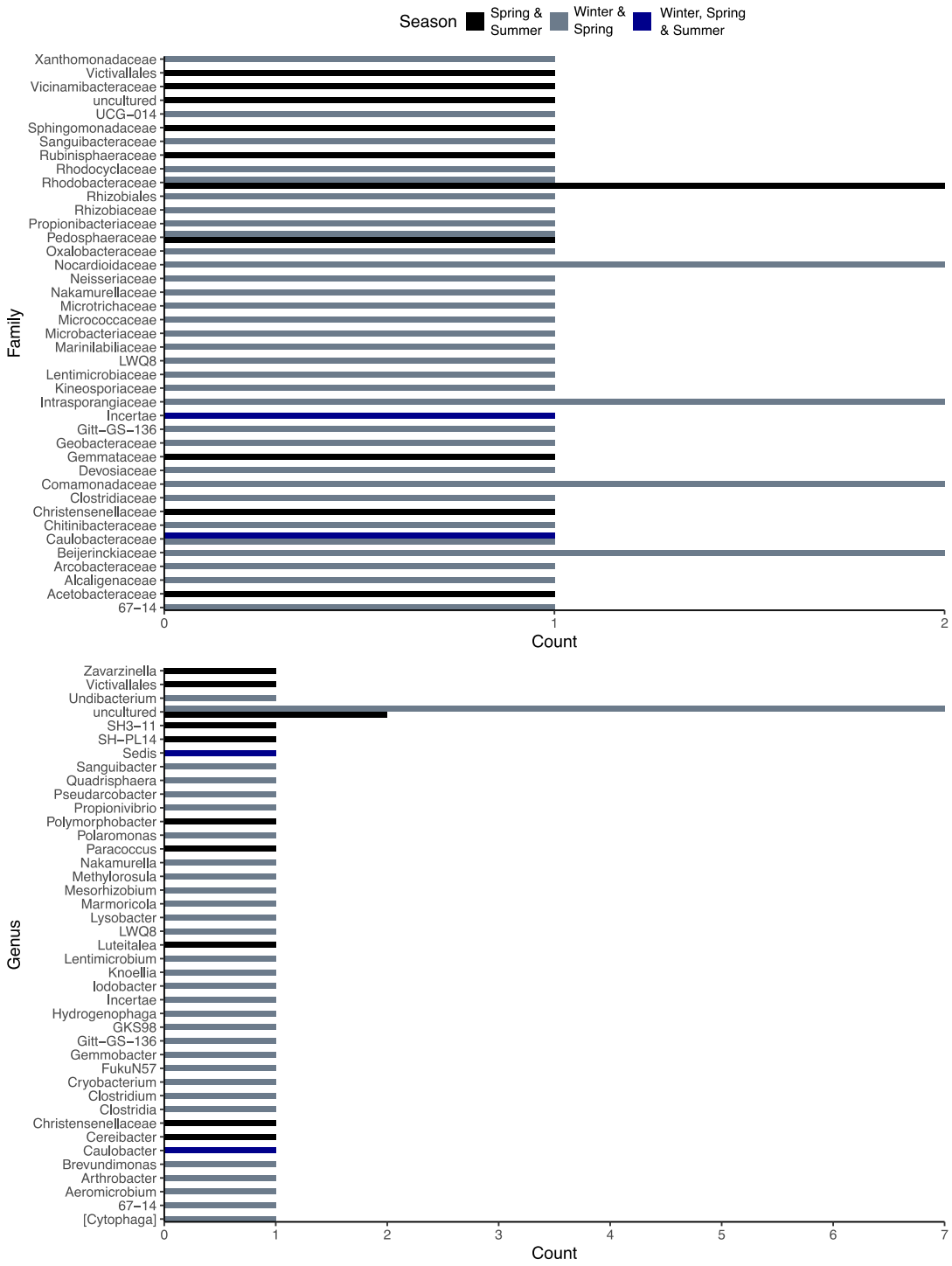


Figure S6: Discriminant taxa determined from the differential abundance analysis WCLR samples at genus level. Genera of discriminant taxa in more than one season are included. Spring & Summer, Spring & Winter and all three seasons (Spring, Summer & Winter). Multiple bars in a same taxon represent discriminant taxa that fit more than one category.

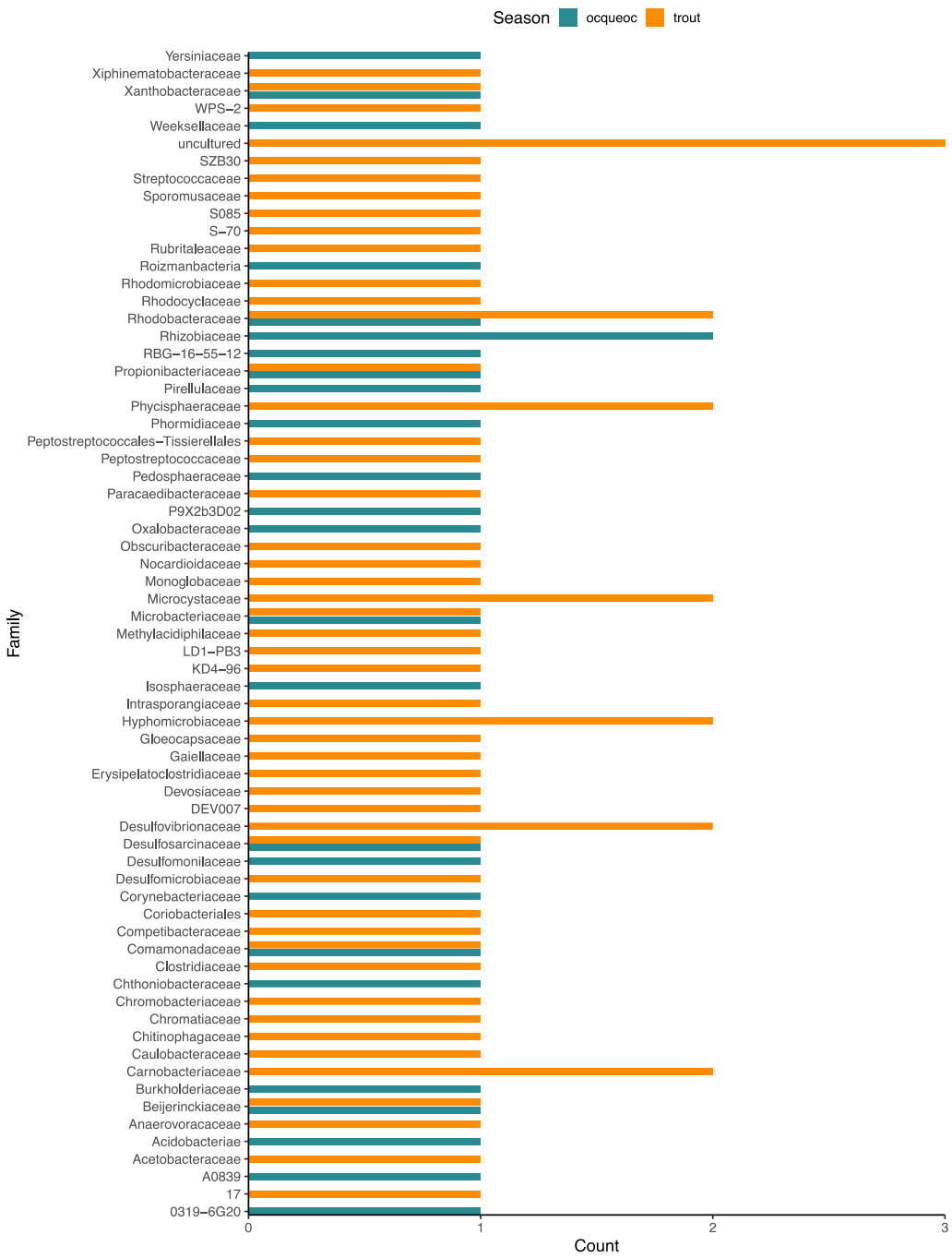


Figure S7: Discriminant taxa determined from the differential abundance analysis WC samples at genus level. Families of discriminant taxa in Trout River (orange) and Ocqueoc River (teal) are included. Some taxa are discriminant to both rivers and are presented as multiple bars in the same taxon.

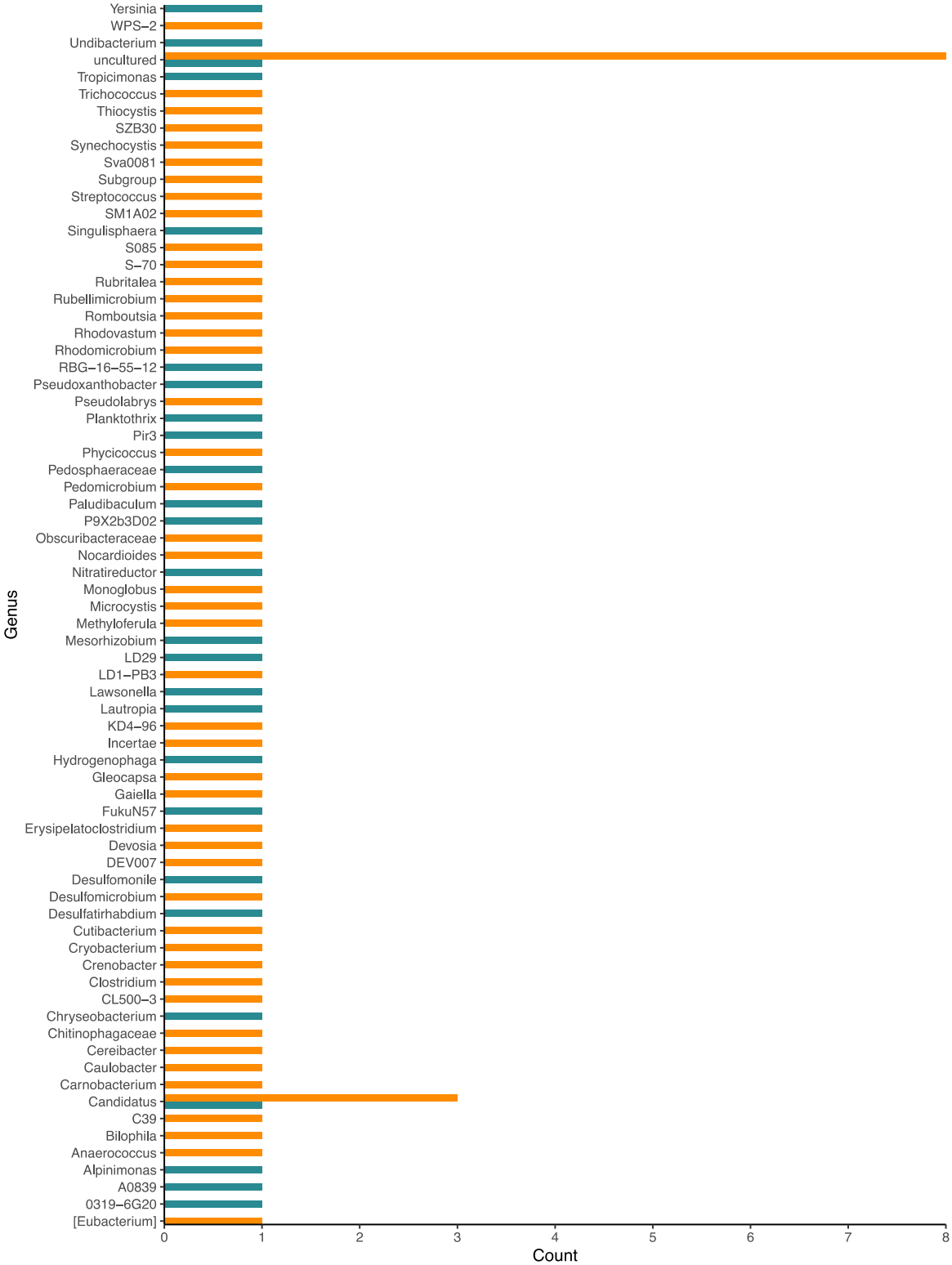


Figure S8: Discriminant taxa determined from the differential abundance analysis WC samples at genus level. Genera of discriminant taxa in Trout River (orange) and Ocqueoc River (teal) are included. Some taxa are discriminant to both rivers and are presented as multiple bars in the same taxon.

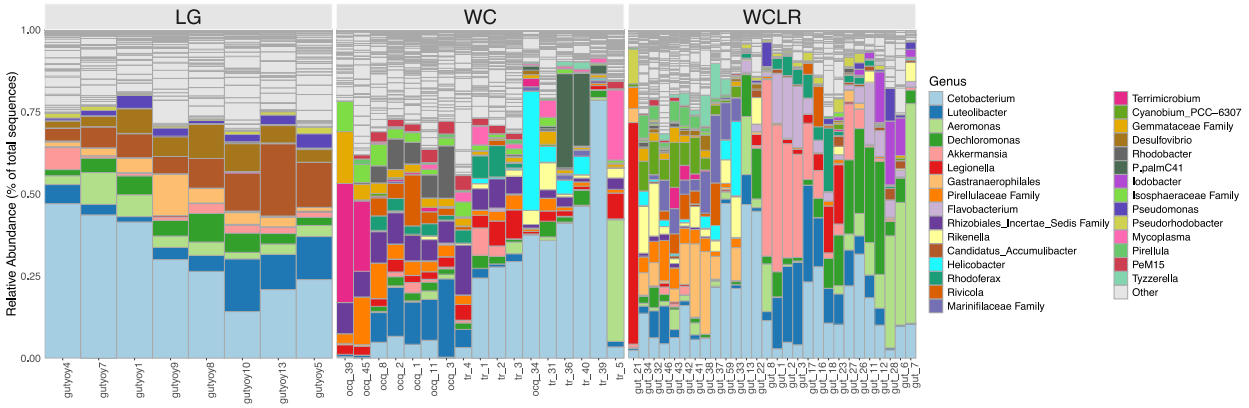


Figure S9: Relative abundance of LG, WC and WCLR samples. Only top 30 most abundant genera are included in this plot.

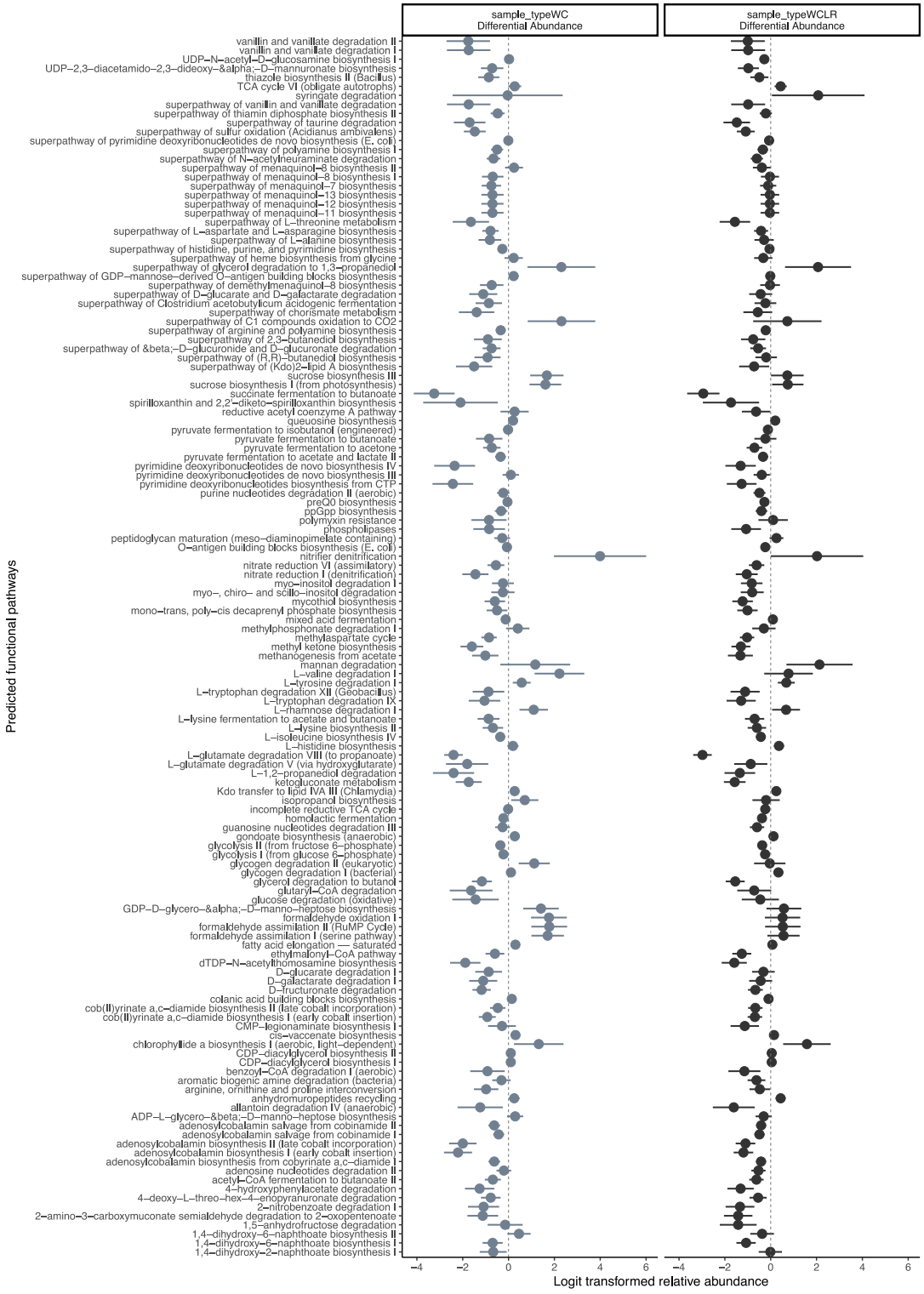


Figure S10: Complete output from differential abundance analysis of PICRUSt2 predicted functional pathways using sample types as covariates. The x-axis represents logit-transformed relative abundance. The dotted line separates enriched (positive numbers) and depleted (negative numbers) taxa using LG as the predictor sample type that other samples are being compared to. The point range is determined using a Wald test in the corncob package.