Uncovering Dietary Diversity Through Gut Microbiomes for Several Species of Elongated Fishes

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NSF REU-Blinks Program 2022
Summer 2022

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Keywords: Elongated fishes, gut microbiome, dietary diversity, host genetics, phylosymbiosis, phylogenetics, aquaculture

Abstract

Host phylogeny, habitat and diet are recognized for their role in shaping the gastrointestinal microbial community, however the extent of their influence on the taxonomic composition of the gut microbiome remains poorly understood within marine ecosystems. We investigated these factors closely by working with nine species of elongated fishes: *Anoplarchus insignis*^{C}, *Anoplarchus purpurescens*^{C}, *Apodicthys flavidus*^{C}, *Lumpenus sagitta*^{C}, *Phytichthys chirus*^{O}, *Pholis laeta*^{C}, *Pholis ornata*^{C}, *Xiphister atropurpureus*^{O}, and *Xiphister mucosus*^{H} (C=Carnivore, H=Herbivore, O=Omnivore) across two families (Stichadae and Pholidae). A four week high-protein feeding trial replaced the natural diets of 28 lab-reared individuals while simultaneously controlling for host habitat. Intestinal microbiota were identified with high-throughput DNA sequencing of 16s rRNA V3-V4 gene amplicons pooled from lab-reared individuals and wild-caught individuals (n=44) that maintained their natural diets. Our results have implications for future research related to pre- and probiotic supplementation of fish feed in aquaculture. As more research illuminates the dynamics of fish gut microbiomes, aquaculture operations worldwide will be enhanced with fish food tailored with probiotics to boost immune response and nutritional uptake too.

Introduction

The gastrointestinal (GI) microbial community is one of the richest and most complex ecosystems on Earth (Tzeng et. al. 2015). An assemblage of intestinal microbes play a central role in the fitness of their host including early development and protection against pathogens, but also enhanced host metabolism, energy utilization, and storage (Nayak 2010). Although the presence of GI microbiota has been recognized in fishes, the taxonomic composition of the gut

microbiome remains poorly understood in its functional roles in fish nutrition and health (Kim et.al 2021). Across mammalian species host genetics and diet have been correlated with the structure and diversity of GI microbiota (Ley et.al. 2008). The concordance of host phylogeny and GI microbiota has been evidenced in germ-free (GF) zebrafish and mice microbiota transplants, in which recolonization of the GI tract occurs in predictable ways (Rawls et.al. 2006). These works suggest specific host-microbe interactions between bacterial clades and distinct organisms known as phylosymbiosis (Rawls et.al. 2006).

We investigated the evolutionary underpinnings of host-microbe gut symbiosis, by studying the gut microbiomes of non-mammalian vertebrate species. Being that fishes demonstrate substantial species diversity, with approximately 33,000 species (Kim et.al 2021), there is a solid foundation for elucidating a range of ecological interactions, particularly dietary adaptations in fishes. Disentangling the potential complexity and functional contributions of the gut microbiome provides the framework for deeper insight into the myriad of dietary specializations observed in hosts (Baldo et al. 2017). Generally, carnivores rely on animal-rich diets (proteins and simple polysaccharides), herbivores on a plant-rich diet (fibers and structural polysaccharides), and omnivores with a mix (Baldo et al. 2017; Stevens and Hume 2004; German et al. 2015). Still, eukaryotic cells alone cannot break down complex polysaccharides and rely on bacterial fermentation to enhance nutrient assimilation (Baldo et al. 2017; Flint et al. 2012). The diversification of gut morphology best exemplifies these metabolic differences in animals with gut length and complexity typically increasing as fiber content increases (German and Horn 2006). Moreover, these differences in digestive physiology translate into increased gut microbial diversity among terrestrial herbivores compared to carnivores (Baldo et. al. 2017; Muegge et.al 2011). Consequently, these trends among several others have led to long-standing

questions and debates about the gut microbiome in fishes. For example, is the gut microbiome influenced by host habitat, if so, to what extent? Are genetic factors, specifically phylogenetic distances of the host species, shaping the gut microbiota in fish? Are microbes represented differently based on diet, especially among taxa with dietary diversity (e.g. carnivores, herbivores, and omnivores)?

A comparative study of several species of elongated fishes from pricklebacks (Family Stichaeidae) and gunnels (Family pholidae) provides an excellent opportunity to explore all three factors, host habitat, diet, and host phylogeny in shaping the gut microbiome. In this study we focus on pricklebacks and gunnels located in coastal waters throughout the northern Pacific Ocean occupying both subtidal and intertidal habitats, therefore additional insight on the impact of host habitat strengthens this investigation. Selected for this study were: *Anoplarchus insignis*^{C}, *Anoplarchus purpurescens*^{C}, *Apodicthys flavidus*^{C}, *Lumpenus sagitta*^{C}, *Phytichthys chirus*^{O}, *Pholis laeta*^{C}, *Pholis ornata*^{C}, *Xiphister atropurpureus*^{O}, and *Xiphister mucosus*^{H} (C=Carnivore, H=Herbivore, O=Omnivore). In this study, we provide the comparisons of gut microbial communities of wild-caught fish to those reared in the lab on a high-protein diet, in order to enhance our understanding of the community composition of the gut microbiota within these fishes. Our primary questions are the following (1) Do gut microbial communities vary by diet or phylogenetic distance among: *Anoplarchus insignis, Anoplarchus purpurescens*^{C}, *Apodicthys flavidus*^{C}, *Lumpenus sagitta*^{C}, *Phythycthis chirus*^{O}, *Pholis laeta*^{C}, *Pholis ornata*^{C}, *Xiphister atropurpureus*^{O}, and *Xiphister mucosus*^{H} ? (2) How does the representation of gut microbes differ for fish fed a high protein lab diet?

The use and study of these diverse microbial communities is underexplored in non-model organisms, thus offering agro-industrial sectors such as aquaculture new strategies for optimizing

fish nutrition (Perry et. al. 2020). In particular, the use of microorganisms in aquaculture as probiotics and a direct food source for cultured species has expanded in the last decade (Martínez-Porchas & Varga-Albores 2017). Next-generation sequencing has created a number of 'Omics' approaches used to explore the structures, functions, relationships and dynamics of various biomolecules regulating cellular processes within an organism (Alfaro & Young 2016). These host-microbe interactions, specifically in the context of fish nutrition and digestion will amplify our understanding of these microbial niches. As fish in marine and freshwater systems are increasingly in demand as global protein sources, the need for innovative feed and consequent feeding technologies for existing and future aquaculture species will be pertinent.

Materials and Methods

Fish Capture

Several species of elongated fishes from the families Stichaeidae and Pholidae were selected for this study, which include *A. insignis*^{C}, *A. purpurescens*^{C}, *A. flavidus*^{C}, *L. sagitta*^{C}, *P. laeta*^{C}, *P. ornata*^{C}, *P.Chirus*^{O}, *X. atropurpureus*^{O}, and *X. mucosus*^{H} (Table 1). Lab-reared individuals ranged from juveniles to adults (79-257mm) as did wild-caught individuals (79-161mm), which were collected from rocky intertidal and subtidal habitats at multiple locations on the San Juan Island during June and July 2022 (Table 1).

For intertidal species, (*A. insignis*^{C}, *A. purpurescens*^{C}, *X. atropurpureus*^{O}, and *X. mucosus*^{H}), we collected during low tide in front of Friday Harbor Laboratories (48.55 °N, 123.01°W) by hand and dipnet. The remaining species, (*A. flavidus*^{C}, *L. sagitta*^{C}, *P. chirus*^{O}, and *P. laeta*^{C})occupy subtidal habitats and were captured either during a beach seine (Jackson Beach, 48.52 °N, 123.01 °W),a tidepool bale (Dead Man Bay, 48.51 °N, 123.14 °W), or on tires attached to the docks at Friday Harbor Laboratories (48.55 °N, 123.01°W). Four individuals of each

species, (*A. insignis*^{C}, *A. purpurescens*^{C}, *A. flavidus*^{C}, *L. sagitta*^{C}, *P. laeta*^{C}, *X. atropurpureus*^{O}, and *X. mucosus* ^{H}), were selected to undergo a laboratory feeding trial, where they remained in a plexiglass aquaria (4 X 6 units, 24 total units) with the exception of four *L. sagitta* individuals that were kept in a separate wet table aquaria, because these individuals are capable of jumping out of the designed units. Both tanks were operating on a flow through seawater system where the water maintained a temperature of approximately 13 °C (Herrera et al. 2022), in which seawater was pumped from the coast of the Friday Harbor Laboratories. The remaining 45 individuals of species, (*A. insignis*^{C}, *A. purpurescens*^{C}, *A. flavidus*^{C}, *L. sagitta*^{C}, *P. laeta*^{C}, *P. ornata*^{C}, *P.Chirus*^{O}, *X. atropurpureus*^{O}, and *X. mucosus*^{H}), comprised of the wild-caught group with their natural diets. All animal care and experiments were conducted under approved IACUC protocols #20-013 developed at California State University, San Bernardino and #4238-16 from the University of Washington.

Feeding Experiment

Twenty-four individuals of *A. insignis*^{C}, *A. purpurescens*^{C}, *A. flavidus*^{C}, *L. sagitta*^{C}, *P. laeta*^{C}, *X. atropurpureus*^{O}, and *X. mucosus*^{H} were randomly assigned a numbered cubicle as part of a wet table which contains 4 X 6 cubicles (24 total) with circulating seawater (approximately 1.5-L in volume of cubicles). A single ~17 cm PVC tube was added into each compartment to mimic the refuge provided by rocks in the wild. Four individuals of *L. sagitta* were separately contained in a flow-through tank system (108.5 X 50.5 cm) with the addition of three PVC tubes to serve as refuge. Each individual fish (n=28) was anesthetized for weight and standard length measurements at the start of the experiment. The natural diets of all individuals of *A. insignis*^{C}, *A. purpurescens*^{C}, *A. flavidus*^{C}, *L. sagitta*^{C}, *P. laeta*^{C}, *X. atropurpureus*^{O}, and *X. mucosus*^{H} were replaced with a high-protein laboratory diet for a total of twenty-eight days. Tetra ® Freeze

Dried Jumbo Krill (Crude Protein 52.0%; Crude Fat 10.0%; Crude Fiber 19%; Moisture 6%; Phosphorus 1.0%; Vitamin E 350 IU/kg) was broken apart and distributed to all individuals twice daily to satiation over 28 days. Leftover food was removed before feedings by dipnet and hand. Feces and other debris was removed from both systems through a simple siphoning technique scheduled every other day.

Preparation of gut microbiome samples

In total, (n=72) fishes were euthanized following an overdose of tricaine methanesulfonate (MS-222 in 1 gl^-1 seawater) and the entire intestinal tract was excised for DNA extraction. Standard length (cm), gut length (cm), and mass (g) were measured for all wild-caught individuals (n=45). In addition, lab-reared individuals were weighed and their standard lengths measured at the start and conclusion of the feeding experiment (n=28).

Dissection tools and the cutting boards were sterilized with a 75% ethanol solution. All dissections were carried out on chilled cutting boards kept over laboratory grade ice (4°C). The digestive system of each fish was removed by cutting at the uppermost esophagus and the anus. Following removal, the gut was uncoiled and the total length (mm) of the gut was measured as the distance from esophagus the pyloric caeca to the distal-most end of the intestine (Herrera et. al. 2022). The intestine was divided into three equal segments representing the proximal, middle, and distal intestine. All undigested food and connective tissue were removed from each segment of the intestinal tract and discarded. The proximal, middle, and distal intestine were transferred into vials and stored in a – 80°C freezer for preservation until microbial DNA extractions.

16s rRNA gene amplicon sequencing

Total microbiome DNA was collected from the gut contents (total DNA extracted) of *A. insignis*[C], *A. purpurescens*[C], *A. flavidus*[C], *L. sagitta*[C], *P. laeta*[C], *P. ornata*[C], *P.Chirus*[O], *X.*

atropurpureus^{O}, and *X. mucosus*^{H} across both lab-reared and wild-caught groups using the ZymoBIOMICS Miniprep Kit (D-4300). All extracted microbiome DNA samples were quantified with a Nanodrop ND-1000 spectrophotometer (3.64-164.30 ng/μL; Heras and Martin 2022). Following extraction, lab-reared and wild-caught DNA samples were sent to the Genomic High-Throughput Facility located at the University of California, Irvine . The V4-V5 regions of the 16S rRNA gene were selected as metabarcoding markers of prokaryotes (archaea and bacteria) for this study.

Results

In this section comparisons of gut morphometrics parameters and standard length are reported amongst the species comprising lab-reared and wild-caught individuals (Figures 1-2). Generally, individuals of the Pholidae family reported lower gut lengths with respect to standard length than Stichaeidae individuals. This trend is observed across both lab-reared and wild-caught groups (Figure 1-2) with triangles denoting members of the Pholidae family. It is likely that differences in gut morphology between the families is driving the relationship between Log_GL (logarithmically transformed gut length; log 10) and Log_SL (logarithmically transformed standard length; log 10). Interestingly, *X. mucosus* individuals demonstrated the greatest Log_GL values with respect to Log_SL across lab-reared and wild-caught groups. Inversely, carnivorous species consistently ranked below *X. mucosus* signifying the influence of host diet on gut morphometrics.

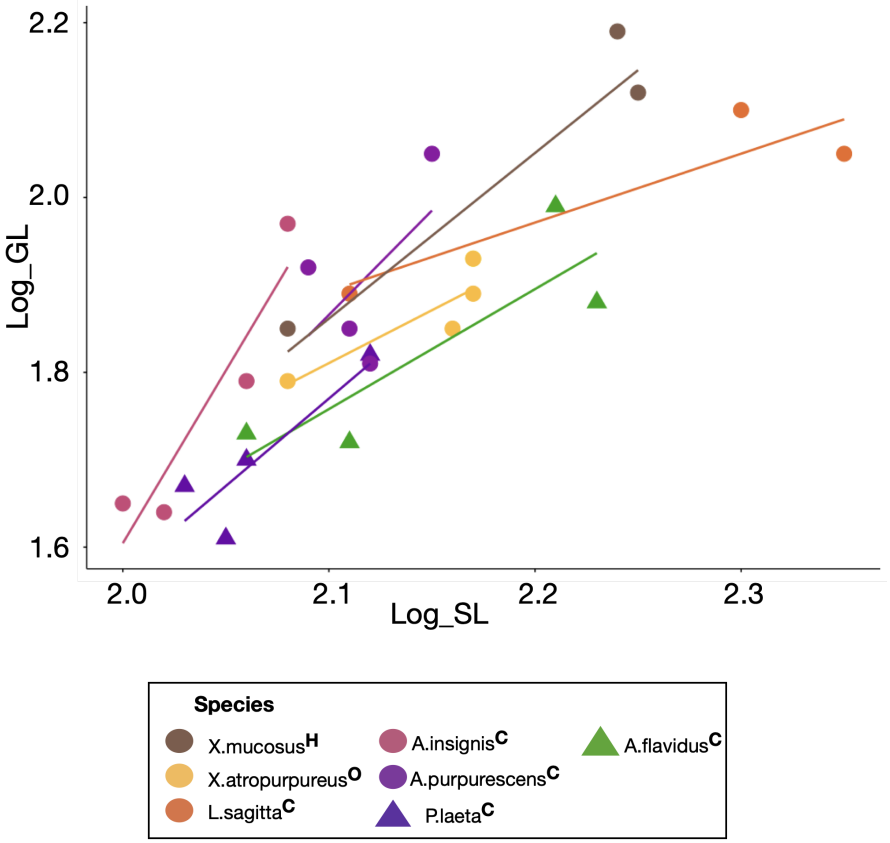

Figure 1. The relationship between standard length (SL) and gut length (GL) using log transformed values for individuals reared on a high-protein (52% by composition) laboratory diet (n=28). Standard lengths were measured before and after the feeding trial, reporting only final standard length in the plot. Fishes were euthanized using MS-222 (1 g / 1 L seawater) for final standard length and gut length measurements.

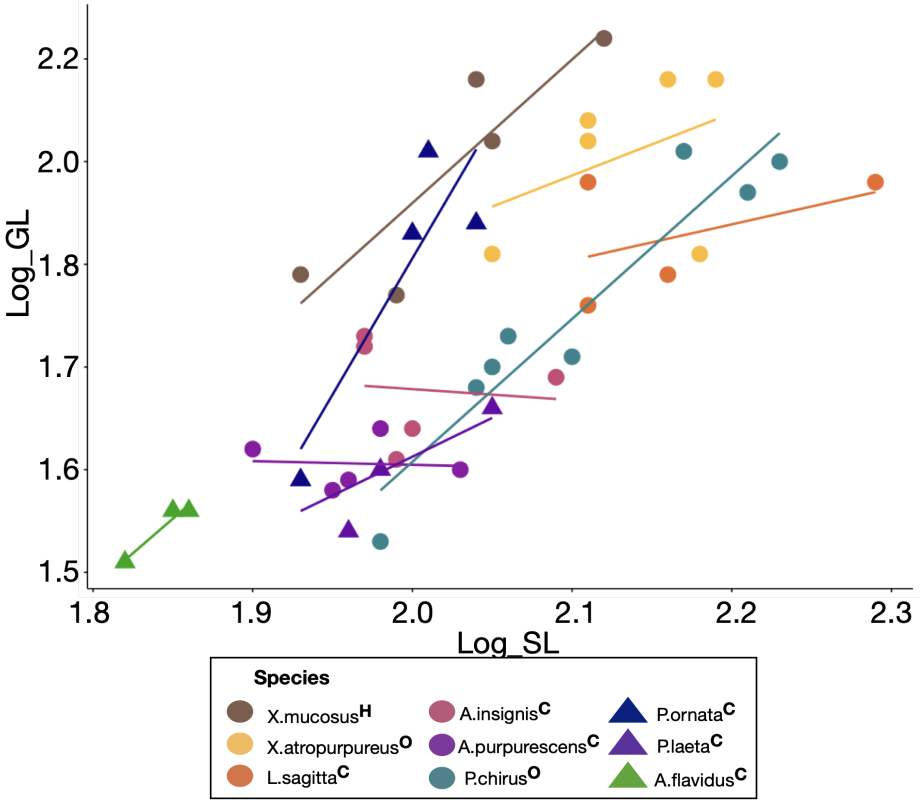

Figure 2. The relationship between standard length (SL) and gut length (GL) using log transformed values for wild-caught individuals (n=44). Fishes were euthanized using MS-222 (1 g / 1 L seawater) and then standard length and gut length were measured for all individuals.

Analysis of the Taxonomic Composition of the Gut Microbiome

In the future, comparisons of gut microbial communities between lab-reared and wild-caught individuals will be evaluated and an assessment will be made in regard to the degree of influence host genetics, host habitat, and diet has over the taxonomic composition of the GI tract of these elongated fishes. The use of taxa plots will show the relative abundance of taxa for all individuals in this study (n=72), and alpha diversity will also be analyzed through Chao1 and Shannon Diversity plots, in order to estimate species richness from the gut microbiome for each individual. Finally, Beta-diversity (non-metric multidimensional scaling;

NMDS) plots will be generated to measure similarity and dissimilarity between individuals across multiple variables, specifically host genetics, host habitat, and diet as a way to discover trends linking individuals to the microbial diversity found within the gut.

Discussion

The trends observed between the relationship of Log_GL and Log_SL are consistent with previous studies that have used gut morphometrics to identify the dietary strategy of a fish based on its gut length (German and Horn 2006). *X. mucosus* was the lone herbivorous species in this study and also exhibited the largest gut dimensions with increase in body size of the six prickleback species and three gunnel species. From an evolutionary perspective, both species of *Xiphisters* showed greater gut lengths with increasing body sizes than the two species of *Anoplarchus*, indicating host genetics is affecting gut morphometrics in these species (German and Horn 2006). Overall, these preliminary findings are consistent with previous works on the digestive physiology of herbivorous, carnivorous, and omnivorous fishes.

As the gastrointestinal microbiome of marine and freshwater are continually sequenced, profiling the functional roles of these microorganisms will involve extensive bioinformatic analyses. In regards to the nutritional and digestive potential of these microbial communities, integrating metabolomics into future studies surrounding aquaculture feeding practices is a promising avenue of research. With metabolites representing the end products of cellular regulatory processes, and being acutely sensitive to environmental changes, their profiles can be regarded as the culmination of genetic or environmental responses at the organismal level (Roques et al. 2020). In this way, host phylogeny, habitat, and diet as the main drivers of gut microbial diversity can be more thoroughly understood with a metabolomics-based approach.

The taxonomic classification of the gut microbiome contributes to our understanding of dietary diversity in marine systems by generating community profiles for these samples. Taxa enrichment across carnivores, omnivores, and herbivores can have great implications in aquaculture. Perhaps certain microbial communities heavily facilitate nutritional uptake and maintain a host diet (Heras & Martin 2022). Together, these approaches will benefit feeding practices and fish nutrition for aquaculture systems.

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