SUCCESSION OF THE YELLOW TANG (*Zebrasoma flavescens*) MICROBIOME FROM HATCH TO SETTLEMENT IN A RECIRCULATING AQUACULTURE SYSTEM

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A Thesis submitted in partial satisfaction of the requirements for

the degree Master of Science

in

Marine Science

College of Natural and Computational Sciences

Hawai‘i Pacific University

Summer 2024

Honolulu, Hawai‘i

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June 27, 2024

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in Marine Science at Hawai‘i Pacific University. We the undersigned have examined this document and have found that it is complete and satisfactory in all respects, and all revisions required by the final examining committee have been made.

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ACKNOWLEDGEMENTS

First, I would like to express my deepest gratitude to my advisor Dr. Olivia Nigro. Over the past three years I have gained an excitement and fascination for microbiology, which I credit in large part to the passion you bring. Despite my minimal experience in microbiology coming into this project, you helped me gain the skills and knowledge I needed and then provided the space for me to spread my wings. I am forever grateful for the skills you have taught me and the knowledge you have imparted on me. I could not have asked for a better mentor and inspiration.

I would like to thank the members of my committee Dr. Michael Rappé, Dr. Matthew Iacchei, and Dr. Chatham Callan. At different points of this project, I needed guidance in each of your areas of expertise. Thank you for always being willing to provide direction, which allowed me to feel supported throughout this entire process. I am grateful to each of you for the time and energy you invested into this research. I would also like to extend my gratitude to our co-author Dr. Sarah Tucker. I could not have accomplished this work without your help and patience. Thank you for the time you invested into me and this research. I can only hope to follow in similar footsteps as you.

Thank you to the entire FinFish team at the Oceanic Institute for your role in this project. I would like to extend a special thank you to Katie Hiew for always being there to answer my urgent fish questions and provide much needed emotional support along the way.

I would like to thank the entire Nigro and Rappé lab for all of your support. I feel very lucky to have been able to conduct my work alongside so many amazing people. A special thank you to Cherise Spotkaeff for being an amazing mentor and role model since the very start. I can't wait to start our PhD programs together.

To my parents, Liz and Tim, and brother, Peter, thank you for always encouraging me to do what I love. Without you, none of this would be possible. To my fiancé Casey, you (along with Mila and Nora) are my rock. Thank you for encouraging me in my moments of self-doubt and always believing in me. To my friends and family near and far, thank you for always supporting me in my pursuit of my dreams.

ABSTRACT

Ornamental fish aquaculture is a developing sustainable alternative to wild collection in meeting aquarium trade demands. In the Hawaiian Islands, *Zebrasoma flavescens*, or yellow tang have been the most highly demanded species for the trade. Characterizing commensal microbial communities, like the gut microbiome, using high-throughput sequencing has had important applications in optimizing host-microbiota symbiosis and improving juvenile recruitment in aquaculture. Here, we first sought to assess the impact of DNA extraction kit, mechanical disruption, and storage technique on the ability to characterize the gut microbiota of adult yellow tang. Of the four commercially available extraction kits evaluated (Omega Bio-Tek E.Z.N.A Soil, Qiagen QIAamp PowerFecal Pro DNA, MasterPure Complete DNA and RNA Purification, and MasterPure Gram Positive DNA Purification), extractions from the two spin column kits, the Omega Bio-Tek E.Z.N.A Soil and Qiagen QIAamp PowerFecal Pro DNA, had the best rate of amplifiability and highest alpha diversity. These kits also showed strong agreement in characterization of microbial community structure. We next described the microbiota assembly of yellow tang larvae, diet, and rearing water from 2 to 76 days post-hatch. We observed Proteobacteria to be the most abundant phylum in larvae samples in 7/8 developmental stages of which the genus *Vibrio* made a notable proportion. Ordination of sample types as well as pairwise comparisons using adonis supported that diet is playing a more significant role in larvae microbiota seeding than rearing water and may be a key vector of *Vibrio*. Resolution of *Vibrio* at the species level within the larvae's different food sources is a necessary next step in elucidating the role this group may be playing in larval yellow tang mortality.

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Chapter 1: Introduction

Ornamental fish aquaculture has garnered scientific interest as many wild fish populations have been negatively impacted by aquarium trade demands. In the Hawaiian Islands, some of the most heavily impacted fish species are for the aquarium trade, including the species *Zebrasoma flavescens*, or yellow tang (Williams et al., 2009; DLNR, 2015). Aquaculture, which is the rearing or husbandry of aquatic organisms for commercial purposes, can be a sustainable alternative to meeting the aquarium trade demands and thus reduce impact on wild reef fish populations (Palmtag, 2017; Calado, 2017). However, rearing ornamental fish in captivity can be challenging with only ~2% of ornamental species offered on a commercial scale (Tlusty, 2002). The challenge of cultivation has been especially arduous for the yellow tang.

Efforts to culture yellow tang began at the Oceanic Institute (OI) in response to their high demand in the aquarium trade and increasing stakeholder opposition to the removal of wild individuals from Hawaii's reefs. After over a decade of effort, this species was successfully cultured to the juvenile stage in 2015, marking an exciting breakthrough in the ornamental aquaculture industry (Holt et al., 2017; Callan et al., 2018). However, high larval mortality rates and low juvenile recruitment has limited the ability to offer this species on a commercial scale. The precise mechanisms responsible for such high mortality rates during the larval stage are poorly understood, although the relationship between yellow tang and the community of symbiotic microbes in the gut, known as the gut microbiome, is hypothesized to play a key role.

The health of vertebrate animals is heavily influenced by commensal microbiota (Turnbaugh et al., 2007; McFall-Ngai et al., 2013). As microbiome exploration has broadened to fields like aquaculture, the critical role of host-microbe symbiosis is increasingly being explored

in juvenile fish recruitment and fishery health (Llewellyn et al., 2014; Hess et al., 2015). The assembly of a favorable microbial community in the gut is vital for the host's assimilation of nutrients, growth, development, and immunity (Turnbaugh et al., 2007, Llewellyn et al., 2014, Talwar et al., 2018). In intensive aquaculture, high larval mortality has been linked to an increase in negative interactions with pathogenic microbes that episodically dominate in aquaculture facilities (Pindling et al., 2018). While this area of research has been rapidly expanding with the development of next generation sequencing (NGS) and standardized bioinformatic tools, our understanding of key ornamental species' gut microbiota development, like the yellow tang's, is still lacking.

1.1 Ornamental Fish Aquaculture

1.1.1 The Aquarium Trade

The trade of marine ornamental fish is a rapidly growing industry. Thought to have originated in Sri Lanka in the 1930s, as demand increased through the 1950s, the industry steadily expanded to places such as Hawaii and the Philippines (Wijesekara & Yakupitiyage, 2001). In recent years, approximately 2,500 species of fish comprising over 46,000,000 organisms are being traded annually with an economic value of over $300,000,000 (Palmtag., 2017; Wabnitz & Taylor, 2003). The hobby of aquarium keeping is the largest driver of the trade with an estimated 1.5-2 million people worldwide keeping marine aquaria (Wabnitz et al., 2003). Marine ornamental fish, make up a large portion of the aquarium trade with 20-24 million individuals collected annually from the ocean worldwide (Wabnitz et al., 2003). While 90% of the traded freshwater fish are captive raised (Chen et al., 2020), in stark contrast, nearly all

(~99%) of tropical marine ornamental fish in the trade are directly sourced from reefs (Palmtag., 2017; Wabnitz & Taylor, 2003).

The heavy reliance on wild collection of marine ornamental fish for the aquarium trade has been subject to controversy. Due to their characteristic vibrant colors and morphological diversity, marine ornamentals are a large part of what makes coral reefs such a lucrative tourist attraction. Coral reef tourism accrues approximately US 35.8 billion dollars globally every year or 9% of all coastal tourism value in the world's coral reef countries (Spalding et al., 2017). Additionally, the cultural significance many marine ornamental species hold in their native habitat ranges has raised concerned among locals regarding the sustainability of commercial collection and the impact these practices may have recreational and subsistence fishing practices (Schaar and Cox, 2021).

The exploitation of marine ornamentals for the aquarium trade has also raised concern for the resilience of vital marine ecosystems like coral reefs. There are mutualistic relationships between many marine ornamental fish with calcifying organisms. The loss of herbivorous grazing fish species is the primary cause of inducing often irreversible phase shifts from coral-dominated reefs to algal-dominated reefs (Mumby P.J. 2006; Tissot & Hallacher, 2003). Loss of these vital mutualistic relationships compounded with other anthropogenic stressors facing coral reefs including, climate change, ocean acidification, eutrophication, and plastic pollution, make the future of the coral reef ecosystems unclear (Carpenter et al., 2008).

In addition to the multifaceted impact the mere loss of ornamental fish has, the techniques employed to collect these fish are among the most destructive practices impacting coral reefs in the Indo-pacific (Olivotto et al., 2017). One notable example is the use of cyanide, a popular chemical spread across reefs as an anesthetic to allow for easier collection of pelagic species (Wabnitz et al., 2003). Cyanide has been well documented to result in non-selective mortality by inducing a weakened physiological state (Olivotto et al., 2017). Another is the use of netting to cover the reef (Fig. 1.1) and then using a blunt object to strike the benthic habitat and scare the fish into the nets. This technique not only results in the direct destruction of the three-dimensional coral structure but also poses hazards if the net becomes entangled and/or is abandoned (Ballesteros et al., 2019). Though these destructive practices have been outlawed, poor enforcement, by both importing and exporting countries, have resulted in these unsustainable practices continuing to this day (Vaz et al., 2017).

Figure 1.1. Net deployed by fisherman in Sulawesi, Indonesia to catch blue tang for the aquarium trade. Hakai Magazine.

The ethical merit regarding the fish's livelihood following collection has also received criticism. Once the fish are captured, they are often subjected to drastic changes in temperature, pressure, and unsatisfactory water conditions (pH, flow, light levels) during transportation (Wabnitz et al., 2003; Olivotto et al., 2017). Subsequently, it is estimated 60-70% of the organisms in the aquarium trade die during capture, handling, and transport (Wabnitz et al., 2003). Such high mortality during all stages of the collection process drives higher collection efforts to account for the significant loss (Wabnitz et al., 2003).

1.1.2 Aquarium Trade Policy in Hawai‘i

Early in the aquarium trade, the Hawaiian Islands were the largest exporter of marine ornamentals in the United States due to the high quality of fish and rare endemic species present (Tissot et al., 2003). However, in the 1970s, collection of ornamentals started to become controversial as concern grew over the sustainability of wild fish stocks. In 1999 Hawaii's Division of Aquatic Resources (DAR) established a network of fish replenishment areas (FRAs) comprising 35% of the Kona coast of Hawai‘i to mediate impacts of collection on fish populations (Stevenson et al., 2013). Within these protected areas collections of heavily collected fish like the yellow tang were restricted through bag limits, size restrictions, and number of permits issued. However, as pressures on wild populations persisted, the Hawai‘i Supreme Court in 2017 halted the renewal of all aquarium collection permits until an environmental review was conducted on the ornamental collection practices and their subsequent environmental impacts (Schaar and Cox, 2021). In 2020, an impact statement produced by a number of aquarium fish collectors was rejected by the Department of Natural Resources (DLNR), serving as a major win for stakeholders against the reinstatement of the trade. Finally, in 2021 DLNR implemented a resounding state-wide ban on the commercial removal of aquarium fish from the wild (Schaar and Cox, 2021). Though this ban on collection permits currently protects wild populations in Hawai‘i, the long-term implications of this legislation are unclear as the aquarium trade of ornamentals remains a contested issue (Schaar and Cox, 2021). Aquaculture of marine ornamentals increasingly has been of interest as a potential longer-term solution to this ongoing issue.

1.1.3 Aquaculture: A Potential Solution

Aquaculture, or the rearing or husbandry of aquatic organisms for commercial purposes (Landau, 1992), is a rapidly growing industry (Fig. 1.2A). Most of the global aquaculture production effort in the past has focused on food production (Naylor et al., 2021) with the global aquaculture yield encompassing more than 50% of all seafood produced for human consumption (FAO, 2018). A novel application of fish aquaculture is marine ornamental fish production, which is steadily growing (Fig. 1.2B) (Naylor et al., 2021). This practice is increasingly being regarded as a potential alternative to meeting the aquarium trade demands, which would help to preserve wild ornamental fish populations (Palmtag 2017; Calado, 2017; Tlusty 2002).

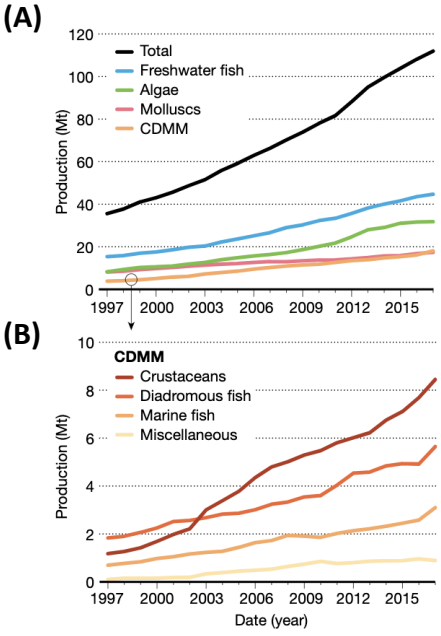


Figure 1.2. (A) Global aquaculture production from 1997 to 2017. **(B)** Production of crustaceans, diadromous fish, marine fish, and miscellaneous sectors of aquaculture from 1997 to 2017. Modified from Naylor et al. (2021).

There are many benefits to the culture of marine ornamental fish in addition to being an alternative to wild collection. By often utilizing a recirculating aquaculture system (RAS), which involves the reuse and treatment of water, the husbandry of marine ornamentals can avoid many of the negative environmental impacts (i.e. effluent disposal, eutrophication, proliferation of antibiotics) associated with traditional flow through aquaculture (Medipally et al., 2016). Additionally, fish reared in captivity exhibit a lower mortality rate and a longer lifespan when living in an aquarium setting than their wild caught counterparts (Palmtag 2017; Wittenrich, 2007). Aquaculture also provides a unique opportunity to study species early life histories, reproductive behavior, and response to human

impacts, many of which are understudied for ornamental species (Wittenrich, 2007; Chen et al., 2020).

Though a growing field, the aquaculture of ornamentals is still largely in its infancy (Chen et al., 2020). Difficulty producing juveniles of the high demand ornamental fish species has limited the number of aquaculture programs able to reach commercial scale (Moorhead & Zeng 2010). Of the 1,471 species traded annually, only ~ 22% have been cultured successfully and only 1.8% enough to be offered on a commercial scale (Tlusty 2002; Wabnitz et al., 2003; Burgess & Callan, 2018). The challenge of marine ornamental husbandry is multifaceted. Originally termed the "critical period" by Johan Hjor in 1914, the transition from endogenous feeding to exogenous feeding, when the larva must pivot from relying on yolk sac reserves to hunting for food, is characterized by a consistent peak in larval mortality (25-50%) (Holt et al., 2017; Callan et al. 2018). Thus, the inability to surpass this developmental milestone is likely a major driver behind the high mortality characteristic of the larval stage. Despite the impact this bottleneck has on production for many ornamental species, the mechanisms responsible for this mass mortality are poorly understood (Olivotto et al., 2011). Transition to exogenous feeding is just one of the challenges limiting the output of commercially available juveniles.

Many of the ornamental fish sought after to culture are pelagic spawners. Pelagic spawners release their eggs, and subsequently larvae upon hatch, into the water column, which are then dispersed by tides and water currents (Walsh 1984). This reproductive strategy features low parental energy and time investment (Holt et a., 2017). Unsurprisingly, in the wild mortality rates of larval cohorts for pelagic spawners are often >99% (China et al., 2017). These high mortality rates are largely attributed to the larvae's small size and underdeveloped physiology upon hatch

(Chen et al., 2020). For example, newly hatched pelagic spawning larvae often have primitive eyesight, closed mouths, and a still developing GI tract (Holt et a., 2017). In aquaculture, these physical characteristics adds difficulty to identifying suitable live feed to newly hatched larvae (Chen et al., 2020; Holt et al., 2017). Additionally, pelagic spawners generally have a long larval duration (often ~2 months) which adds further complexity to the husbandry of these fish (Chen et al., 2020; Holt et al., 2017). In contrast, demersal spawners whose larvae are more developed upon hatch are able to ingest larger prey as first feed items and have higher survival rates throughout the larval stage (Chen et al., 2020).

In aquaculture, by controlling many of the environmental conditions, lack of predation, and optimal supply of food, it is expected the survival rate of pelagic spawners would be much higher than what is observed in the wild. For yellow tang, an average survival in aquaculture of ~1% (Holt et al., 2017) while an order of magnitude larger than what would be expected in the wild, is still dramatically lower than other commercially available species. For example, in the demersal spawning clownfish (*Premnas biaculeatus*), a 78% survival is consistently observed in captive reared larval cohorts (Chen et al., 2020). The high mortality of many marine ornamentals in aquaculture, despite the omission of many limiting factors faced in the wild, indicates other factors are likely playing a key role (Walburn et al., 2018).

1.1.4 The Yellow Tang (*Zebrasoma flavescens*)

Yellow tang *(Zebrasoma flavescens)* is an herbivorous surgeonfish within the Acanthuridae family (Claisse et al., 2009). This species is also known as a teleost fish, which is the most diverse group of vertebrates encompassing about half of all extant vertebrate species (Volff, 2005). Yellow tang can be found throughout the Northwest and Central Pacific Ocean including southwest Japan and Hawai‘i (Allen et al., 2003). Despite beginning as translucent larvae, they are easily identifiable as settled juveniles and adults with vibrant solid yellow coloring (Fig. 1.3) (Claisse et al., 2009). Typical of Acanthuridae and many other marine ornamentals, adult yellow tang are primarily grazers and contribute to coral reef health through their feeding behaviors (Claisse et al., 2009). Yellow tang populations in the wild exhibit a positive correlation with the finger coral *Porites compressa*, indicating this is a key habitat for them (Claisse et al., 2009).

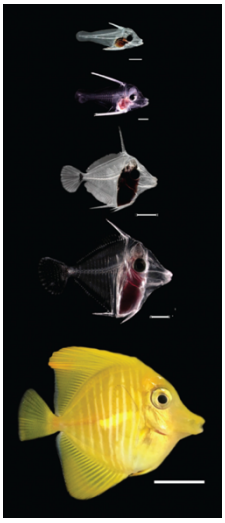


Figure 1.3. Larval yellow tang *(Zebrasoma flavescens)* development from 12 to 69 days post-hatch.

Adult yellow tangs have high reproductive potential. They spawn daily near sunset (peak months are March-July) with females able to produce ~40,000 eggs in a single spawning event (Walsh, 1984). As yellow tang are pelagic spawners, upon hatching the larvae rely on their yolk sac reserves for the first days post-hatch (dph) while their gut and mouth finish fully developing. Around 3 dph the larvae have a completely developed digestive system, open mouth, and begin the transition to exogenous feeding. About 55 dph the planktonic larvae begin to settle on reefs and are thereafter referred to as juveniles (Claisse et al., 2009). In the wild, settled juveniles occupy areas of high coral cover around 10-25m in depth (Walsh, 1984). Adult yellow tang

transition to shallower water (<10m) near turf-algae reef flats during the day to graze and retreat to slightly deeper waters at night (Walsh, 1984).

In addition to being one of Hawai‘i’s most iconic reef fish, yellow tang have historically been the most heavily collected species in the state for the aquarium trade (DLNR, 2015). The number of yellow tang catches on the Kona (west) coast of Hawai‘i, where the heaviest pressure of collection on yellow tang have historically occurred, grew exponentially from the 1980s to 2005 equating to an annual removal of 300,000 to 400,000 yellow tang from the wild (Fig. 1.4, Williams et al., 2009). Unsurprisingly, yellow tang comprises the majority (84.3%) of the total marine ornamental fish collected for the state of Hawai‘i (Stevenson et al., 2013; DLNR, 2015). The high demand of this species in the aquarium trade make yellow tang an ideal candidate for ornamental aquaculture.

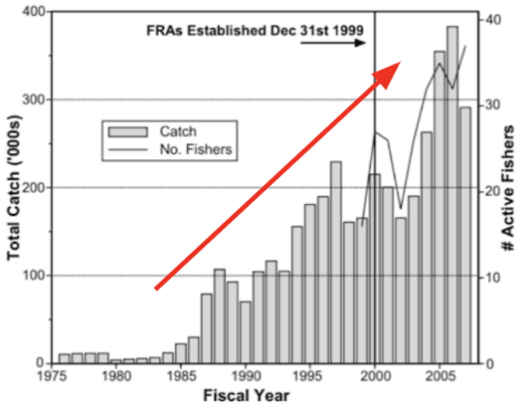


Figure 1.4. Annual total catch (thousands) of yellow tang from the Kona coast of the Big Island of Hawai‘i. Black vertical line indicates formation of fish replenishment areas (FRAs) in 1999. Modified from Williams et al., 2009.

After over a decade of effort, the yellow tang was successfully reared for the first time at the Oceanic Institute (OI) in 2015 making it the first of the Acanthuridae family to be cultured through its full life cycle (Holt et al., 2017; Callan et al., 2018). This accomplishment contributed to other breakthroughs in ornamental aquaculture such as the first successful culture of the Pacific blue tang (*Paracanthurus hepatus*) in 2017 (DiMaggio et al., 2017). However, survivorship of yellow tang from egg to juvenile stage has remained limited to ~1.3% and only

25-50% through the first week of culture (Holt et al., 2017; Callan et al., 2018). High larval mortality rates continue to limit the ability to offer yellow tang on a full commercial scale (Callan et al., 2018). Thus, aquarium trade demands still possess a threat to wild populations.

1.1.5 Previous Investigations into Larval Yellow Tang Mortality

At the Oceanic Institute, factors previously investigated for their role in low juvenile yellow tang recruitment include size of first feed prey, quality of eggs from brood stock, and microbiota associated with the larvae's feed (Pérez-Atehortúa et al., 2023; Moorhead & Zeng, 2010; Olivotto et al., 2011). In 2018, Burgess & Callan investigated how diet and size of live feed may impact survival and development of first feeding yellow tang (Burgess & Callan, 2018). Wild zooplankton were supplemented to the larvae's usual first feed of copepod nauplii (*Parvocalanus crassirostris*) and metabarcoding was used to analyze prey preference. Osteological development and mouth gape, likely key factors in prey preference, were also assessed. Copepod nauplii were found to be the prey of choice and survival rates were lower (6.9% compared to 18.5%) for the larvae fed the supplemented diet containing the wild caught zooplankton. Mouth gap progression indicated currently used cultured copepod nauplii may surprisingly be below the optimal size preferences by 6 dph and thereafter for yellow tang larvae. In both feed groups, most larvae were observed to be feeding by 4 dph and 100% feed incidence was reported by 6 dph. While further investigation is needed to understand the role of prey availability in early yellow tang larval mortality, these data together indicate it is unlikely the lack of prey ingestion/starvation is the sole reason behind the mass mortality observed in newly hatched larval yellow tang.

In 2016, the impact of incorporating probiotics into first feed cultures on larvae survivorship through the first week post-hatch was explored. The probiotic, INVE SanoLife MIC-F (Inve

Aquaculture Inc.), was administered to first feeding larval yellow tang (2-6 dph) through the enrichment of the cultured copepod nauplii (*Parvocalanus crassirostris*) and rearing water. Though a decrease in *Vibrio* spp. abundance was observed within the copepod nauplii cultures, no impact on larval yellow tang survival or ontogenetic development was found. Forbes postulates the short time span (5 days) over which the probiotics were administered may have been an inefficient period to allow for the probiotic microbes to effectively be assimilated into the larvae's commensal microbiota community and have a beneficial effect on survivorship. Probiotic assimilation may also have been confounded by other variables characteristic of this stage including the small larval size and underdeveloped physiology (Forbes, 2016).

Most recently, a study conducted in 2023 investigated if i) altering brood stock density and ii) the addition of the fatty acids docosahexaenoic (DHA) and arachidonic acids (ARA) would impact egg production and quality. Hiew found both ARA and DHA enhanced diets produced significantly higher production and quality of eggs per spawn over the course of a year period. Additionally, it was found that of all sex ratios tested, 1 male to 3 females produced the most viable eggs per female daily (average of 788 +/- 1385) (Hiew, 2023).

1.2 The Gut Microbiome

1.2.1 Studying the Gut Microbiome

Traditionally, labor intensive culture-dependent methods have been used to characterize and understand microbial communities. These techniques led to vast underestimates in microbial diversity as only a small percentage (<1- 2%) of all microbial organisms can be cultured successfully, given current technologies available (Amann et al., 1995; Jiao et a., 2021). In 1977,

dideoxy chain-termination method or Sanger sequencing emerged as a groundbreaking DNA sequencing technology and became the primary method of sequencing for many years after (Sanger et al., 1977; Heather & Chain, 2016). Though Sanger sequencing is still used to this day, next generation sequencing (NGS) technologies have allowed for higher volume and more cost-effective sequencing (Heather & Chain, 2016). A powerful aspect to Sanger and NGS sequencing is the ability to exploit a target gene for taxonomic identification such as the 16S ribosomal RNA (rRNA) gene.

The 16S rRNA gene contains both conserved regions, those maintained by evolutionary pressure, and nine hypervariable regions (V1-9) (Fig. 1.5), allowing for taxonomic identification of bacteria and archaea (McCabe et al., 1999; Van de Peer et al.,1996). By amplifying and sequencing the regions of the gene that display sequence diversity between species, researchers can characterize microbial communities without the bias of culturing (Pace, 1997; Van de Peer et al.,1996). Furthermore, Sanger and NGS technology revolutionized our understanding of the ubiquity of microbial communities and the intimate role they play both as symbionts and pathogens to their host (Talwar et al., 2018).

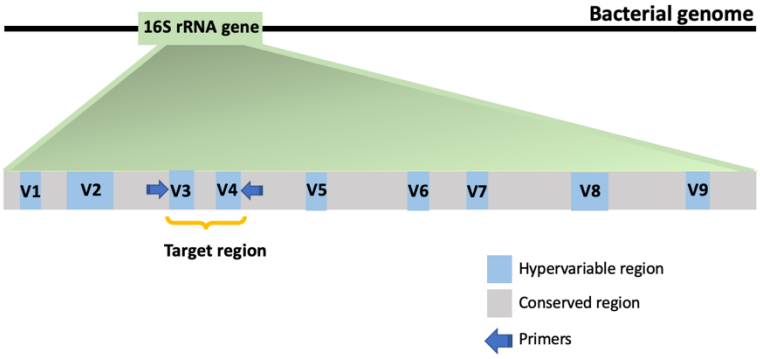


Figure 1.5. 16S rRNA gene. Hypervariable regions V1-9 represented in blue and conserved regions in grey. Orange bracket denotes the popular V3-4 target region. Primers attach to conserved regions flanking desired target region.

In 2007, the National Institute of Health launched the Human Microbiome Project (HMP). The objective of this project was to characterize the human microbiome for the first time and investigate its potential roles in human health and disease. The results revealed humans host

~10,000 microbial species harboring ~8 million genes. Humans, in comparison, possess only 23,000 genes, indicating microbes living on and within the human body may be encoding more genes contributing to our physiological functioning than are self-encoded (The Human Microbiome Project Consortium, 2012). This landmark study also found that despite the human genome being over 99.5% identical among individuals, two individuals can show no overlap in the microbial species detected within their microbiome, indicating profound host specificity (The Human Microbiome Project Consortium, 2012). The findings of the Human Microbiome Project were some of the first major insights into the diversity commensal microbial communities harbor and the impressive functional capacity they may be able to confer to their host.

In parallel with the advancement of human microbiome research, interest in non-human species, including teleost fish, has been steadily increasing. For example, landmark discoveries regarding host-gut microbiota symbiosis have been found using the model vertebrate species, zebrafish (*Danio rerio*) (Rawls et al., 2004; Rawls et al., 2006; Roeselers et al., 2011). However, our understanding of commensal microbial communities like the gut microbiota for non-model fish species is limited (Luan et al., 2023).

1.2.2 Characteristics of the Gut Microbiome

In animals, virtually every biological surface exposed to the external environment supports a complex array of microbial communities with high functional potential to the host (Montalban-Arques et al., 2015). One of the most densely colonized and well-studied of these host surfaces is the gastrointestinal tract. The gut microbiota is the collection of microbes including bacteria, archaea, viruses, and fungi found within the gut, which can interact with the host in a commensal, symbiotic, or pathogen fashion (Hollister et al., 2014). The gut

microbiome, often used interchangeable, refers additionally to the genetic material of these organisms, their functions, and interactions as a system. Marine fish are thought to host approximately 500-1,000 different prokaryotic species within their gastrointestinal tract (Talwar et al., 2018). While this may sound like a large number of species, the gut represents a specialized niche, which selects for a relatively small subset of microbiota introduced through biological and environmental factors (Talwar et al., 2018).

The gastrointestinal tract is considered a specialized niche for several reasons. One is the morphological diversity of the gut which creates steep nutrient and chemical gradients (Donaldson et al., 2015). Additionally, the gut is known to be oxygen depleted as well as highly acidic. These characteristics tend to select for mostly facultative or obligate anaerobes capable of withstanding low pH (Egerton et a., 2018; Donaldson et al., 2015). Of the hundreds of species that colonize the gut, it is often the case these species belong to just several phyla that dominate the community including *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Fusobacteria*, and *Bacteroidetes* (Stephens et al., 2016; Talwar et al., 2018; Butt & Volkoff, 2019; Huang et al., 2020). Despite the gut microbiota being a specialized community, the functional potential harbored within these communities is immense.

1.2.3 Host-Microbiota Symbiosis

Co-evolution of microbial organisms and their hosts over thousands of years has produced an intricate symbiosis (Backhed et al., 2005). The gut provides a particularly intimate environment to curate host specific microbial communities and as opposed to external surfaces that may support more free-living microbes (Ley et al., 2008). Gut symbionts are known to play a critical role in host health through processes including the synthesis of vitamins like B12 (Qi et

al., 2023), production of essential metabolites (Biddle et al., 2013), and immunomodulation (Fu & Jiang et al., 2018). The exchange of these vital physiological services is so significant to host health the gut microbiome has been referred to as its own metabolic organ in the literature (Stephens et al., 2016). One of the most studied symbiotic exchanges of the host-microbe symbiosis is nutrient acquisition.

The enteric microbiota expands the host's metabolic capacity making otherwise inaccessible nutrient sources available (Semova et al., 2012). In teleost fish, bacteria produce an array of enzymes including (carbohydrases, cellulases, lipases, etc.) which aid in the degradation and assimilation of large and complex molecules, greatly increasing the host's capacity for nutrient acquisition (Perry et al., 2020; Cheutin et al., 2021). For example, *Clostridium* and *Photobacterium* spp. can mutualistically digest plant components like cellulose and chitin, which are molecules endogenous digestive enzymes cannot break down on their own, and thus deliver vital short chain fatty acids (SCFA) to their host (Clements et at., 2014). Absorption of short chain fatty acids (SCFA) is critical to herbivorous marine fishes' energy balance and absorption of nutrients and vitamins (Semova et al., 2012). Higher access to SCFAs is also demonstrated to increase host resistance to pathogenic species (Montalban-Arques et al., 2015).

Some fluctuations in the host-microbiota relationship are normal and associated with factors like change in diet or host age (Apprill, 2017). For example, a study on Yellowtail Kingfish (*Seriola lalandi*) reported increasing gut microbiota diversity with fish age (Walburn et al., 2018). However, failure to assemble and maintain a favorable gut microbiota can have detrimental impacts to the health of the host. (Fig. 1.6) (DeGruttola et al., 2016; Apprill, 2017). Dysbiosis is the imbalance of microbial flora, resulting in metabolic and functional changes, which can adversely impact the host's health (Apprill, 2017). Disruption of a healthy gut microbiota can create niches for opportunistic pathogenic bacteria to colonize, which can further compromise the health of the host (Perry et al., 2020). In humans, dysbiosis of the gut microbiota have been correlated to a wide array of health problems including inflammatory bowel disease, diabetes, metabolic disorders, obesity, and nonalcoholic fatty liver disease (DeGruttola et al., 2016; Musso et al., 2011). In larval fish, especially before host innate immunity is fully developed, dysbiosis of the gut microbiota may be a primary driver behind early mortality (Pindling et al., 2018).

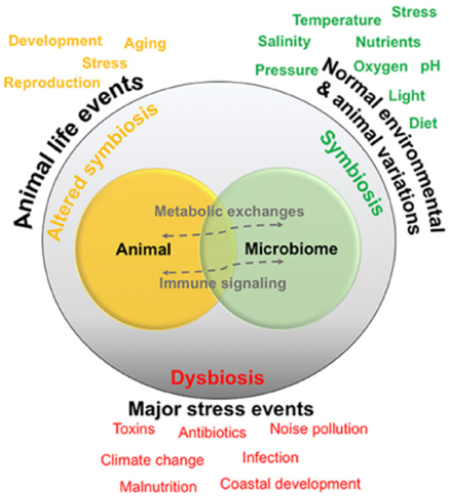


Figure 1.6. Factors that influence host-microbiota symbiosis as well as those that can induce dysbiosis of commensal microbial communities. (Apprill, 2017).

1.2.4 Assembly of the Gut Microbiota in Pelagic Spawning Teleosts

The gut microbiome of teleost fish is most variable and vulnerable during early developmental stages when the gut is not yet fully developed and the immune system is immature (Egerton et al., 2018). Initial colonization processes of the gut post-hatch remain

unclear (Egerton et al., 2018). Some studies indicate the GI tract of newly hatched larvae are first colonized with initial ingestion of water when the mouth opens 2-3 dph (Walburn et al., 2018). Others posit that microbes associated with the chorion, a protective envelope that surrounds the fish embryo until hatch (Pérez-Atehortúa et al., 2023), may be the first colonizers of the still-developing GI tract upon hatch prior to water ingestion (Egerton et al., 2018). It is also possible that initial colonization of the GI tract may be modulated by species-specific factors and thus vary greatly (Egerton et al., 2018, Larsen, 2014). It is well-accepted that substantial diversification of the gut microbiota occurs at the onset of exogenous feeding, where the digestive system's microbiota more closely reflects that of the live feed (Egerton et al., 2018; Walburn et al., 2018). For pelagic spawning teleost species like the yellow tang, ~50-70 dph larvae typically graduate to juvenile status and mortality rates drop significantly (Claisse et al., 2009; Holt et al., 2017). The gut microbiome may continue to exhibit plasticity through adulthood with changes in environment, diet, and life history, though adult individuals show evidence for microbiome stability on the scale of years (Donaldson et al., 2016; Egerton et al., 2018).

The primary mechanisms that modulate the gut microbiota are i) host-related factors (life stage, genetics, phylogeny, fitness) and ii) environmental factors (diet, water chemistry, habitat, rearing conditions etc.) (Cheutin et al., 2021; Lorgen-Ritchie et al., 2021; Giatsis et al., 2015). While it is likely a complex combination of many of the factors listed above, it is not well-understood the extent each of these factors play in shaping the gut microbiome, especially in early developmental stages (Lkhagva et al., 2021). It is also unclear if certain factors exert greater influence during different life stages. Resolution of these knowledge gaps would inform

efforts to modulate the gut microbiome, which could have broad applications both in a clinical setting and in aquaculture (Turjeman & Koren, 2022; Talwar et al., 2018; Perry et al., 2020).

1.2.5 Modulation of the Gut Microbiota

Early landmark studies conducted on the model species, zebrafish (*Danio rerio*), provide strong evidence for the dominant role host-related factors play in modulating gut microbiota composition. For example, in 2006 when reciprocal transplantations were conducted into germ-free zebrafish and mouse recipients, the transplanted microbiota resembled its community of origin in taxonomic lineages present, but the relative abundance of these taxa interestingly shifted to resemble typical gut community signatures of the recipient host (Rawls et al., 2006). The presence of a core microbiota, taxa found across populations and time, also has provided compelling support for the host's role in shaping the gut microbiota (Roeselers et al., 2011; Wong et al., 2013; Mente et al., 2018; Wang et al., 2019; Butt & Volkoff et al., 2019). Phyla commonly reported in marine fish core microbiota are Proteobacteria, Fusobacteria, Firmicutes, and Actinobacteria (Egerton et al., 2018). These phyla contain species known to digest complex carbohydrates, activate the host's inflammatory response, and facilitate colonization of other beneficial microbes, offering compelling support as to why these taxa may routinely be selected for by the host (Egerton., 2018). However, not all studies display evidence for host-related factors being the primary driver of gut microbiota diversity. For example, phylogeny was shown to have a negligible influence on gut microbiota composition when compared with environmental factors like salinity (Kim et al., 2021).

Diet is also well established to be one of the most significant factors modulating the composition and functional potential of the gut microbiota (Egerton et al., 2018). Many studies

have displayed manipulation of diet results in divergent gut microbiota, even among individuals sharing close genetics (Walburn et al., 2018; De Angelis et al., 2020; Sieler Jr. et al., 2023). Moreover, higher bacterial richness and diversity is consistently observed in the intestinal track of fed individuals versus starved (Semova et al., 2012; Sieler Jr. et al., 2023). Diet based shifts in gut microbiota is also well documented to have an impact on microbiota conferred physiological functions. For example, fatty acid absorption is diminished in zebrafish specimens raised under sterile conditions (Semova et al., 2012; Sieler Jr. et al., 2023). Specimens with diet altered gut microbiota also have varying susceptibilities to infection driven dysbiosis. For example, in a study testing exposure to the common aquaculture pathogen *Mycobacterium chelonae*, male zebrafish fed different diet types displayed differential infection rates (Sieler Jr. et al., 2023).

Some studies suggest factors modulating gut microbiota structure shift with host age. In 2011, Stephens et al. found the enteric microbiota to exhibit distinct structure at different developmental stages (Fig. 7). Developmental signatures of the gut microbiota in larval fish have similarly been observed in studies on Yellowtail Kingfish (*Seriola lalandi*) (Walburn et al., 2018). Stephens et al. also found as the larvae aged, the gut microbiota became increasingly distinct from the community associated with both the surrounding environment (i.e. diet and rearing water), and other members of their sibling group. Increasing interindividual variation in the gut microbiota, even under identical rearing conditions and genetic similarity, has been echoed across previous studies on hosts including humans (Turnbaugh et al., 2007) and mice (Rogers et al., 2014). These data support host specific factors, that likely vary between individuals, increasingly plays a key role in shaping the gut microbiota as the larvae grow nearer to the juvenile and adult stages (Stephens et al., 2016). The closer association between the gut microbiota and the environment (rearing water and diet) earlier in development indicates these

factors may play a greater role in initial seeding of the gut microbial composition and dominate its composition in early days of development (Stephens et al., 2016). Studies like Wang et al. display additional evidence for diet playing the most significant role in gut microbiota modulation in the early larval stage for Nile Tilapia (*Oreochromis niloticus*) (Wang et al., 2022).

1.2.6 Microbial Dynamics in Aquaculture

Ornamental aquaculture produces a unique microbial landscape (El-Saadony et al., 2021). By rearing organisms in such high densities, this environment is often characterized by high loads of organic material, which promotes vulnerability to opportunistic pathogens (Ma et al., 2020). A robust microbial community is critical to the health and stability of aquaculture systems as favorable microbes play key roles in metabolizing ammonia, removal of excess organic material, and competitive exclusion of pathogenic species (Ma et al., 2020). As the gut microbiome is well-established to be a key modulator of host health, dysbiosis between host and the microbiota they encounter within the aquaculture environment is suggested to be contributing to high larval mortality rates often observed (El-Saadony et al., 2021). Moreover, understanding the interaction between fish hosts and the microbial landscape they interact with is a vital step in optimizing fish survivorship, particularly in the vulnerable larval stage (Llewellyn et al., 2014).

Improving host survivorship in aquaculture is a top priority for the field as it will improve food security (Anderson et al., 2017) and protection of wild ornamental fish populations (Tlusty 2002). Methods to improve survivorship such as antibiotic use have increasingly garnered criticism due to concerns with antibiotic resistance, microbial dysbiosis, bioaccumulation, and pollution (Pindling et al., 2018; El-Saadony et al., 2021). Instead, optimization of favorable microbiota, through probiotics for example, to remediate poor environmental conditions and

presence of pathogenic microbes has been increasingly viewed as a superior alternative (El-Saadony et al., 2021). Selective introduction of probiotic microbes can prevent and/or resolve pathogen dominance through competitive exclusion, and/or directly producing inhibitory byproducts that kill unfavorable species (Sekirov et al., 2010; Balcazar et al., 2006). Probiotic bacteria can also widen the host's metabolic breath, increasing the host's ability to acquire necessary nutrients and support a more robust immune system (Montalban-Arques et al., 2015; El-Saadony et al., 2021).

Lactic acid bacteria in the genera *Lactobacillus*, *Streptococcus*, and *Lactococcus* in addition to bacteria in the genera *Bacillus* and *Pseudomonas* are considered some of the best candidates for probiotic use in aquaculture (Perry et al., 2020). These taxa promote production of protective mucous in the intestines, trigger host inflammatory response, and increase phagocytic activity (Perry et al., 2020). As many of these bacteria are known to already colonize and survive in healthy fish guts (Wu et al., 2012), seeding larva with the healthy fish gut microbiota is a promising strategy to promote the assembly of a pathogen resistant microbiome (Amenyogbe, 2023). Conducting fecal microbiota transplantation (FMT) is growing in popularity as FMT has had well documented success in humans in treating recurrent Clostridium difficile, among other gastrointestinal disorders (Gupta et al., 2016) and even recently in dolphins (Linnehan et al., 2024). Though directly seeding fish guts with probiotic bacteria is not realistic, introduction of probiotics through the rearing water or diet has been tested as an alternative introduction route (Amenyogbe, 2023). However, this form of probiotic application is far from perfected as in many studies overall fish survivorship is not shown to be improved (Alishahi et al., 2018). Our limited understanding of the mechanisms behind gut microbiome assembly especially in early development for many ornamental fish species has limited the success of this strategy. Closing

knowledge gaps is critical to improving the efficacy of microbiological strategies to increase fish survivorship.

1.3 DNA Extraction Methodology

1.3.1 Extraction Kit, Sample Type, and Sample Storage

It is well described that commercially available DNA extraction kits contain an array of cell lysis and DNA purification strategies that can differentially impact downstream results and thus the efficacy of characterizing gut microbial communities in humans (Elie et al., 2023; Kennedy et al. 2014) and in other vertebrates (Hart et al., 2015; Larsen et al., 2015). In a study conducted by Hart et al. (2015), the performance of five commercially available DNA extraction kits (Qiagen DNeasy kit, MoBio PowerFecal kit, Qiagen QIAamp Cador Pathogen mini kit, Qiagen QIAamp DNA Stool mini kit, and an isopropanol manual extraction method) was tested across five different species (zebrafish, mouse, cat, dog, and horse). The data revealed the number of microbial taxa identified and their relative abundances varied by extraction kit within the same species. For zebrafish samples the isopropanol method and Qiagen's PowerFecal kit indicated the phylum Fusobacteria was the most dominant taxa in the gut microbiome (Fig. 1.7). Contrarily, the Qiagen DNeasy kit

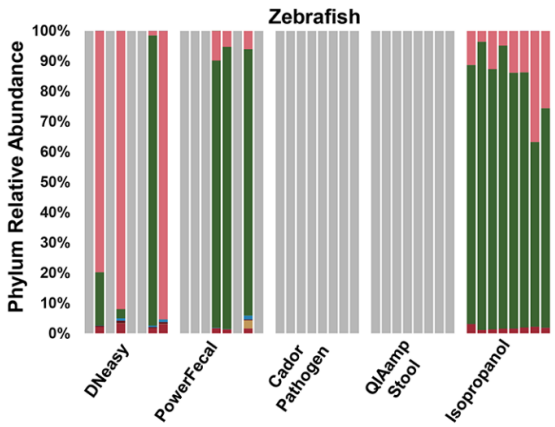


Figure 1.7. Relative abundance of bacteria identified in zebrafish guts across five different extraction methods (n=8 per extraction method). Annotated to the phylum level. Grey bars indicate replicates with a sequencing depth below the defined threshold of 10,000 reads.

characterized proteobacteria to be the most prevalent phylum in 3 of the 4 replicates that successfully amplified (Fig. 1.7).

The results of this study also illustrated the kit's varied ability to produce amplifiable DNA both within and across species. For example, in the mouse extractions, 8/8 samples successfully amplified (> 10,000 reads) with the Qiagen QIAamp kit where only 1/8 samples successfully amplified with this same kit for the cat. In the zebrafish extractions, 8/8 samples successfully amplified for the isopropanol extractions however 0/8 had successful amplification for both the Cador pathogen and QIAamp stool (the DNeasy and PowerFecal performed somewhere in the middle) (Fig. 1.7). Hart el al. hypothesizes the varying ability of certain kits to produce amplifiable DNA across a range of species could be due to variation in PCR inhibitors present within different species. Within-species differences in the kit's ability to produce amplifiable DNA is likely due to a varied capacity to remove certain PCR inhibitors suggesting some kits may be better suited for particular host species over others (Hart et al., 2015).

In the study done by Larsen et al. (2015) the researchers also set out to determine the extent by which variation in methodologies influences downstream analysis. Larsen et al. took both feces and intestine samples from three sympatric freshwater fish species: catfish (*Ictalurus punctatus*), largemouth bass (*Micropterus salmoides*), and bluegill (*Lepomis macrochirus*). These samples were stored under three conditions (fresh, RNAlater preserved, frozen) and DNA was extracted using the two commercially available kits: (DNeasy Blood and Tissue Kit and the QIAamp DNA Stool Mini Kit). In agreement with Hart et al. (2015), Larsen et al. (2015) also found downstream analysis to vary with extraction kit. Data collected by Larsen et al. indicated QIAamp DNA Stool Mini Kit outperformed the DNeasy Blood and Tissue Kit. In all cases where band number differed significantly between treatments (RISA), samples

extracted with the QIAamp DNA Stool Mini Kit showed higher alpha diversity. The authors hypothesized the higher levels of bacterial diversity detected with the stool kit may be due to more efficient cell lysis and/or PCR inhibitor removal. Interestingly, Hart et al. found the QIAamp stool kit to be one of the least successful kits for zebrafish samples with 0/8 replicates being successfully amplified. These data, taken together, reinforce that the performance of commercially available extraction kits may vary based on host species. Thus, preliminary testing of extraction kits is advisable to determine a robust extraction method based on the study species.

Larsen et al. also found distorted bacterial communities associated with frozen samples for both feces and intestine samples. The use of RNAlater prior to freezing seemed to mitigate this bias and the community profiles of these samples more closely resembled those in the fresh samples (Fig. 1.8). Larsen et al. hypothesizes the "Fresh + RNAlater" samples maintained integrity of easier to lyse cells (likely gram-negative) where in the frozen samples the DNA structure was disrupted due to shearing caused by the freeze/thaw. Furthermore, bacterial communities themselves likely influence the efficacy of different storage treatment options. More research is needed to investigate which bacterial taxa are most sensitive to storage temperature and freeze/thaw cycles. Larsen et al. conclude extracting samples when fresh or combined with a solution like RNAlater is preferable to frozen storage prior to extraction (Larsen et al., 2015).

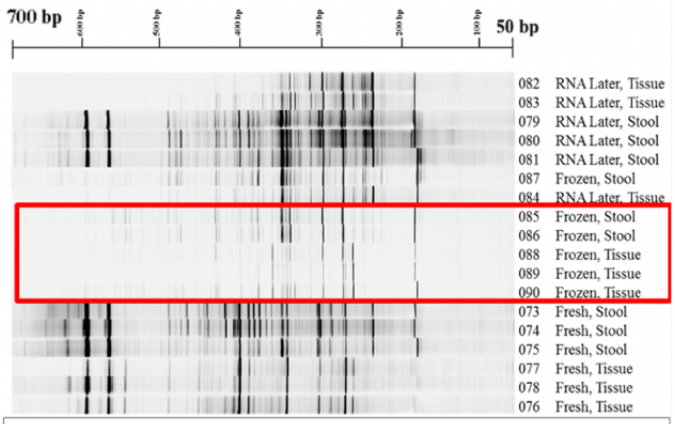


Figure 1.8. RISA (ribosomal intergenic spacer analysis) fingerprint for channel catfish *(Ictalurus punctatus)* feces sample. Banding pattern can be interpreted like a community-specific profile. Red box highlights distorted bacterial community in frozen samples.

1.3.2 Methods of Cell Lysis

Bacteria can be classified into two distinct groups based on the properties of their cell wall: gram-negative and gram-positive (Fig. 1.9). Gram-positive bacteria have a thick layer of peptidoglycan in their cell walls making them more difficult to lyse than their gram-negative counterparts (Fig. 1.9). This characteristic makes it likely gram-positive bacteria are generally underrepresented in microbiome studies (Larsen et al., 2015, Zhang et al., 2020). Three widely used methods of cell lysis are chemical, enzymatic, and mechanical (Shehadul Islam et al., 2017). Commonly, a combination of the three are employed in a single extraction to target different molecular components of the cell wall and membrane to achieve complete lysis (Shehadul Islam et al., 2017). Chemical lysis involves the use of a substance, like detergent for example, that breaks down the hydrophobic-hydrophilic interactions within the cell membrane (Shehadul Islam et al., 2017). Enzymatic lysis involves using an enzyme, such as proteinase K, to break down the glycosidic bond of the peptidoglycan layer (Shehadul Islam et al., 2017). Mechanical disruption, such as bead-beating, employs the use of shear force to disrupt the cell wall non-selectively (Zhang et al., 2020). Bead-beating has been demonstrated to be an efficient method of increasing microbial diversity and gram-positive representation in gut samples when incorporated into commercial protocols (Lim et al., 2018; Zhang et al., 2020).

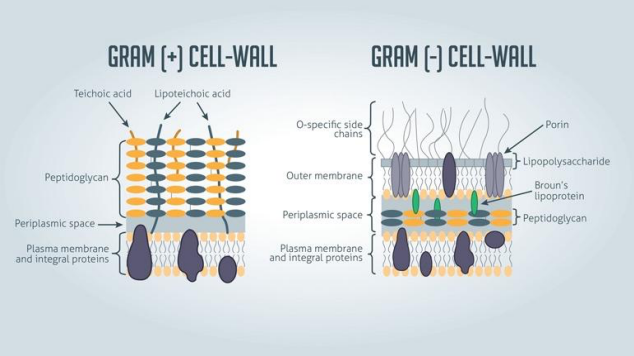


Figure 1.9. Gram-positive versus gram-negative cell wall. Gram-positive cell shows a thick layer of peptidoglycan. Technology Networks.

In a study conducted by Zhang et al. in 2020, higher DNA yields, better DNA integrity, higher Shannon's entropy, and higher Simpson's index were found in samples bead-beaten for 4 to 9 minutes as compared to unbeaten samples. Overall, a 1.1-fold increase in phylogenetic diversity was detected in samples subjected to bead-beating (Zhang et al., 2020). However, Zhang et al. found some microbes showed sensitivity to extensive mechanical lysis (> 5 minutes) such as the gram-negative genera *Stutterella* and *Veillonella*. Zhang et al. concluded 4-5 minutes of bead-beating may be appropriate to process samples where the composition of microbiomes is unknown (Zhang et al., 2020). The results of Zhang et al. are echoed by other studies including Lim et al. (2017) which tested three commercially available extraction kits with and without the step of bead-beating on a human fecal sample. They found that the addition of bead-beating had the greatest impact on the gut microbial community characterized. Samples that were bead-beaten consistently produced higher degrees of microbial diversity than the samples not subjected to bead-beating (Lim et al., 2017).

1.3.3 Summary

As next generation sequencing has become increasingly accessible and widely used, proliferation in host study species and pre-analytical methods have similarly diversified. It is now widely accepted confounding factors like extraction kit and method of cell of lysis, among many other methodological choices, can be highly influential on downstream analysis. Variability in extraction kit and methods of cell lysis performance is thought to be influenced by several factors including host species and even the composition of the gut microbiota itself. More research is needed to further understand the mechanisms behind the inconsistency of these extraction methods. The influence of pre-analytical steps in on downstream results raises concern regarding the reproducibility and validity of gut microbiome data. Optimizing extraction

methodology is critical to obtaining high quality microbial data, reducing methodological induced bias, and increasing comparability among studies.

1.4 Thesis overview

The threat the aquarium trade poses to iconic reef species like the yellow tang make aquaculture an appealing alternative. To improve juvenile recruitment in ornamental fish aquaculture, there is a need to deepen our understanding of microbiological processes like gut microbiome development in this unique environment.

Thesis Goal: The overarching goal of my thesis research is to better inform strategies to optimize host-microbiota symbiosis for larval yellow tang and improve juvenile output, which has been a key bottleneck of ornamental aquaculture production.

Objectives:

1. Chapter 1: Synthesize current knowledge on the aquarium trade, yellow tang aquaculture, the gut microbiome, and methodological challenges to studying gut microbiomes.
2. Chapter 2: Investigate how methodological factors, namely extraction kit, mechanical disruption, and sample storage, influence downstream results and identify a robust extraction protocol for yellow tang gut microbiota.
3. Chapter 3: Characterize the gut microbiota assembly of larval yellow tang in an aquaculture environment and assess how factors like diet and rearing water modulate this community.

These objectives were addressed with two inter-related projects described in the following chapters:

Chapter 2: Comparative Analysis of DNA Extraction Methodology on Yellow Tang (*Zebrasoma flavescens*) Gut Microbiota.

Chapter 3: Succession of the Yellow Tang (*Zebrasoma flavescens*) Gut Microbiome From Hatch to Settlement in a Recirculating Aquaculture System.

1.5 Citations

Alishahi, M., Tulaby Dezfuly, Z., Mohammadian, T., & Mesbah, M. (2018). Effects of Two Probiotics, Lactobacillus Plantarum and Lactobacillus Bulgaricus on Growth Performance and Intestinal Lactic Acid Bacteria of Cyprinus Carpio. *Iranian Journal of Veterinary Medicine*, 12(3), 207–218. https://doi.org/10.22059/ijvm.2018.235444.1004816

Allen, G., R. Steene, P. Humann and N. Deloach. 2003. Reef Fish Identification. Tropical Pacific. New World Publications, Inc., Jacksonville, Florida and Odyssey Publications, El Cajon, California.

Amenyogbe, E. (2023). Application of probiotics for sustainable and environment-friendly aquaculture management—A review. *Cogent Food & Agriculture*, *9*(1), 2226425. https://doi.org/10.1080/23311932.2023.2226425

Amann, R. I., Ludwig, W., & Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, *59*(1), 143–169. https://doi.org/10.1128/mr.59.1.143-169.1995

Anderson, J. L., Asche, F., Garlock, T., & Chu, J. (2017). Aquaculture: Its role in the future of food. In World agricultural resources and food security (Vol. 17, pp. 159-173). Emerald Publishing Limited.

Apprill, A. (2017). Marine Animal Microbiomes: Toward Understanding Host–Microbiome Interactions in a Changing Ocean. *Frontiers in Marine Science*, 4. https://www.frontiersin.org/articles/10.3389/fmars.2017.00222

Atkins, P. 1981. Behavioral determinants of the nocturnal spacing pattern of the yellow tang *Zebrasoma flavescens* (Acanthuridae). *Pacific Science* 35: 263-264.

Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., & Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science (New York, N.Y.)*, *307*(5717), 1915–1920. https://doi.org/10.1126/science.1104816

Balcázar, J. L., Blas, I. de, Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., & Múzquiz, J. L. (2006). The role of probiotics in aquaculture. *Veterinary Microbiology*, 114(3), 173–186. https://doi.org/10.1016/j.vetmic.2006.01.009

Burgess, A. I., Callan, C. K. (2018). Effects of supplemental wild zooplankton on prey preference, mouth gape, osteological development and survival in first feeding cultured larval yellow tang (*Zebrasoma flavescens*). *Aquaculture*, 495, 738–748.https://doi.org/10.1016/j.aquaculture.2018.06.046

Butt, R. L., & Volkoff, H. (2019). Gut Microbiota and Energy Homeostasis in Fish. *Frontiers in Endocrinology*, 10, 9. https://doi.org/10.3389/fendo.2019.00009

Calado, R. (2017). The Need for Cultured Specimens. In *Marine Ornamental Species Aquaculture* (pp. 15–22). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119169147.ch2

Callan, K, C., Burgess, A., Rothe, C., Touse, R. (2018). Development of Improved Feeding Methods in the Culture of Yellow Tang, *Zebrasoma flavescens. Journal of the World Aquaculture Society*, 49. https://doi.org/10.1111/jwas.12496

Carpenter, K. E., Abrar, M., Aeby, G., Aronson, R. B., Banks, S., Bruckner, A., Chiriboga, A., Cortés, J., Delbeek, J. C., DeVantier, L., Edgar, G. J., Edwards, A. J., Fenner, D., Guzmán, H. M., Hoeksema, B. W., Hodgson, G., Johan, O., Licuanan, W. Y., Livingstone, S. R., … Wood, E. (2008). One-Third of Reef-Building Corals Face Elevated Extinction Risk from Climate Change and Local Impacts. *Science*, *321*(5888), 560–563. https://doi.org/10.1126/science.1159196

Chen, J. Y., Zeng, C., Jerry, D. R., & Cobcroft, J. M. (2020). Recent advances of marine ornamental fish larviculture: Broodstock reproduction, live prey and feeding regimes, and comparison between demersal and pelagic spawners. *Reviews in Aquaculture*, *12*(3), 1518–1541. https://doi.org/10.1111/raq.12394

Cheutin, M.-C., Villéger, S., Hicks, C. C., Robinson, J. P. W., Graham, N. A. J., Marconnet, C., Restrepo, C. X. O., Bettarel, Y., Bouvier, T., & Auguet, J.-C. (2021). Microbial Shift in the Enteric Bacteriome of Coral Reef Fish Following Climate-Driven Regime Shifts. *Microorganisms*, 9(8), Article 8. https://doi.org/10.3390/microorganisms9081711

China, V., Levy, L., Liberzon, A., Elmaliach, T., & Holzman, R. (2017). Hydrodynamic regime determines the feeding success of larval fish through the modulation of strike kinematics. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1853), 20170235. https://doi.org/10.1098/rspb.2017.0235

Claisse, J., McTee, S., & Parrish, J. (2009). Effects of age, size, and density on natural survival for an important coral reef fishery species, yellow tang, *Zebrasoma flavescens*. *Coral Reefs*, 28, 95–105. https://doi.org/10.1007/s00338-008-0447-7

Clements, K., Angert, E., Montgomery, L., & Choat, J. (2014). Intestinal microbiota in fishes: What's known and what's not. *Molecular Ecology*, 23. https://doi.org/10.1111/mec.12699

De Angelis, M., Ferrocino, I., Calabrese, F. M., De Filippis, F., Cavallo, N., Siragusa, S., Rampelli, S., Di Cagno, R., Rantsiou, K., Vannini, L., Pellegrini, N., Lazzi, C., Turroni, S., Lorusso, N., Ventura, M., Chieppa, M., Neviani, E., Brigidi, P., O'Toole, P. W., … Cocolin, L. (2020). Diet influences the functions of the human intestinal microbiome. *Scientific Reports*, 10(1), Article 1. https://doi.org/10.1038/s41598-020-61192-y

De Gruttola, A. K., Low, D., Mizoguchi, A., & Mizoguchi, E. (2016). Current understanding of dysbiosis in disease in human and animal models. *Inflammatory Bowel Diseases*, *22*(5), 1137–1150. https://doi.org/10.1097/MIB.0000000000000750

De Schryver, P., & Vadstein, O. (2014). Ecological theory as a foundation to control pathogenic invasion in aquaculture. *The ISME Journal*, *8*(12), Article 12. https://doi.org/10.1038/ismej.2014.84

DiMaggio, M. A., Cassiano, E. J., Barden, K. P., Ramee, S. W., Ohs, C. L., & Watson, C. A. (2017). First Record of Captive Larval Culture and Metamorphosis of the Pacific Blue Tang, *Paracanthurus hepatus*. *Journal of the World Aquaculture Society*, *48*(3), 393–401. https://doi.org/10.1111/jwas.12426

DLNR (Department of Land and Natural Resources). 2015. Report on the findings and recommendations of effectiveness of the West Hawai'i regional fishery management area. Report of the Thirtieth Legislature. Accessed January 31st, 2024 at chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://dlnr.hawaii.gov/dar/files/2015/01/ar_hrs188_2015.pdf

Egerton, S., Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). The Gut Microbiota of Marine Fish. *Frontiers in Microbiology*, 9. https://www.frontiersin.org/article/10.3389/fmicb.2018.00873

Elie, C., Perret, M., Hage, H., Sentausa, E., Hesketh, A., Louis, K., Fritah-Lafont, A., Leissner, P., Vachon, C., Rostaing, H., Reynier, F., Gervasi, G., & Saliou, A. (2023). Comparison of DNA extraction methods for 16S rRNA gene sequencing in the analysis of the human gut microbiome. *Scientific Reports*, *13*, 10279. https://doi.org/10.1038/s41598-023-33959-6

El-Saadony, M. T., Alagawany, M., Patra, A. K., Kar, I., Tiwari, R., Dawood, M. A. O., Dhama, K., & Abdel-Latif, H. M. R. (2021). The functionality of probiotics in aquaculture: An

overview. *Fish & Shellfish Immunology*, *117*, 36–52. https://doi.org/10.1016/j.fsi.2021.07.007

Forbes, E. C. (2016). Examination of the effects of bacteria and probiotics on survival through first feeding of cultured Yellow Tang (*Zebrasoma flavescens*) [Hawai'i Pacific University].

Giatsis, C., Sipkema, D., Smidt, H., Heilig, H., Benvenuti, G., Verreth, J., Verdegem, M. (2015). The impact of rearing environment on the development of gut microbiota in tilapia larvae. *Scientific Reports*, 5, 18206. https://doi.org/10.1038/srep18206

Gupta, S., Allen-Vercoe, E., & Petrof, E. O. (2016). Fecal microbiota transplantation: In perspective. *Therapeutic Advances in Gastroenterology*, 9(2), 229–239. https://doi.org/10.1177/1756283X15607414

Han, S., Liu, Y., Zhou, Z., He, S., Cao, Y., Shi, P., … Ringø, E. (2010). Analysis of bacterial diversity in the intestine of grass carp (*Ctenopharyngodon idellus*) based on 16S rDNA gene sequences. *Aquaculture Research,* 42(1), 47–56. https://doi:10.1111/j.1365-2109.2010.02543.x

Hart, M. L., Meyer, A., Johnson, P. J., & Ericsson, A. C. (2015). Comparative Evaluation of DNA Extraction Methods from Feces of Multiple Host Species for Downstream Next-Generation Sequencing. *PLoS ONE*, 10(11), e0143334. https://doi.org/10.1371/journal.pone.0143334

Heather, J. M., & Chain, B. (2016). The sequence of sequencers: The history of sequencing DNA. *Genomics*, *107*(1), 1–8. https://doi.org/10.1016/j.ygeno.2015.11.003

Hiew, K. E. (2023). Optimization of Yellow Tang (*Zebrasoma flavescens*) Broodstock Management Through Diet and Sex Ratio [Hawai'i Pacific University].

Hjort, J. (1914). Fluctuations in the great fisheries of northern Europe viewed in the light of biological research. ICES.

Hollister, E. B., Gao, C., & Versalovic, J. (2014). Compositional and Functional Features of the Gastrointestinal Microbiome and Their Effects on Human Health. *Gastroenterology*, *146*(6), 1449–1458. https://doi.org/10.1053/j.gastro.2014.01.052

Holt, J. G., Leu, M. Y., Callan, C. K., & Erisman, B. (2017). Large Angelfish and Other Pelagic Spawners (pp. 251-278). John Wiley & Sons Ltd. https://doi.org/DOI:10.1002/9781119169147

Huang, Q., Sham, R. C., Deng, Y., Mao, Y., Wang, C., Zhang, T., & Leung, K. M. Y. (2020). Diversity of gut microbiomes in marine fishes is shaped by host-related factors. *Molecular Ecology*, *29*(24), 5019–5034. https://doi.org/10.1111/mec.15699

Jiao, J.-Y., Liu, L., Hua, Z.-S., Fang, B.-Z., Zhou, E.-M., Salam, N., Hedlund, B. P., & Li, W.-J. (2021). Microbial dark matter coming to light: Challenges and opportunities. *National Science Review*, 8(3), nwaa280. https://doi.org/10.1093/nsr/nwaa280

Kennedy, N. A., Walker, A. W., Berry, S. H., Duncan, S. H., Farquarson, F. M., Louis, P., Thomson, J. M., UK IBD Genetics Consortium, Satsangi, J., Flint, H. J., Parkhill, J., Lees, C. W., & Hold, G. L. (2014). The impact of different DNA extraction kits and laboratories upon the assessment of human gut microbiota composition by 16S rRNA gene sequencing. *PloS One*, 9(2), e88982. https://doi.org/10.1371/journal.pone.0088982

Kim, P. S., Shin, N.-R., Lee, J.-B., Kim, M.-S., Whon, T. W., Hyun, D.-W., Yun, J.-H., Jung, M.-J., Kim, J. Y., & Bae, J.-W. (2021). Host habitat is the major determinant of the gut microbiome of fish. *Microbiome*, 9(1), 166. https://doi.org/10.1186/s40168-021-01113-x

Landau, M. (1992). Introduction to Aquaculture (1st ed.). New York: Wiley. https://www.wiley.com/en-us/Introduction+to+Aquaculture+-p-9780471611462

Larsen, A. M., Mohammed, H. H., Arias, C. R. (2015). Comparison of DNA extraction protocols for the analysis of gut microbiota in fishes. *FEMS Microbiology Letters*, 362(5). https://doi.org/10.1093/femsle/fnu031

Lee, W.-K., Juniper, S. K., Perez, M., Ju, S.-J., & Kim, S.-J. (2021). Diversity and characterization of bacterial communities of five co-occurring species at a hydrothermal vent on the Tonga Arc. *Ecology and Evolution*, 11(9), 4481–4493. https://doi.org/10.1002/ece3.7343

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., Gordon, J. I. (2008). Worlds within worlds: Evolution of the vertebrate gut microbiota. *Nature Reviews Microbiology*, 6(10), 776–788. https://doi.org/10.1038/nrmicro1978

Lim, M. Y., Song, E.-J., Kim, S. H., Lee, J., & Nam, Y.-D. (2018). Comparison of DNA extraction methods for human gut microbial community profiling. *Systematic and Applied Microbiology*, 41(2), 151–157. https://doi.org/10.1016/j.syapm.2017.11.008

Linnehan, B. K., Kodera, S. M., Allard, S. M., Brodie, E. C., Allaband, C., Knight, R., Lutz, H. L., Carroll, M. C., Meegan, J. M., Jensen, E. D., & Gilbert, J. A. (2024). Evaluation of the safety and efficacy of fecal microbiota transplantations in bottlenose dolphins (*Tursiops*

truncatus) using metagenomic sequencing. *Journal of Applied Microbiology*, lxae026. https://doi.org/10.1093/jambio/lxae026

Lkhagva, E., Chung, H.-J., Ahn, J.-S., & Hong, S.-T. (2021). Host Factors Affect the Gut Microbiome More Significantly than Diet Shift. *Microorganisms*, *9*(12), 2520. https://doi.org/10.3390/microorganisms9122520

Llewellyn, M. S., Boutin, S., Hoseinifar, S. H., Derome, N. (2014). Teleost microbiomes: The state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in Microbiology*, 5, 207. https://doi.org/10.3389/fmicb.2014.00207

Lorgen-Ritchie, M., Clarkson, M., Chalmers, L., Taylor, J. F., Migaud, H., & Martin, S. A. M. (2021). A Temporally Dynamic Gut Microbiome in Atlantic Salmon During Freshwater Recirculating Aquaculture System (RAS) Production and Post-seawater Transfer. *Frontiers in Marine Science*, 8. https://www.frontiersin.org/articles/10.3389/fmars.2021.711797

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature*, 489(7415), 220–230. https://doi.org/10.1038/nature11550

Luan, Y., Li, M., Zhou, W., Yao, Y., Yang, Y., Zhang, Z., Ringø, E., Erik Olsen, R., Liu Clarke, J., Xie, S., Mai, K., Ran, C., & Zhou, Z. (2023). The Fish Microbiota: Research Progress and Potential Applications. *Engineering*. https://doi.org/10.1016/j.eng.2022.12.011

Ma, Y., Du, X., Liu, Y., Zhang, T., Wang, Y., & Zhang, S. (2021). Characterization of the bacterial communities associated with biofilters in two full-scale recirculating aquaculture systems. *Journal of Oceanology and Limnology*, *39*(3), 1143–1150. https://doi.org/10.1007/s00343-020-0120-8

McCabe, K. M., Zhang, Y. H., Huang, B. L., Wagar, E. A., & McCabe, E. R. (1999). Bacterial species identification after DNA amplification with a universal primer pair. *Molecular Genetics and Metabolism*, *66*(3), 205–211. https://doi.org/10.1006/mgme.1998.2795

Medipally, S. R., Yusoff, F. M., Sharifhuddin, N., & Shariff, M. (2016). Sustainable aquaculture of Asian arowana—A review. *Journal of Environmental Biology*, *37*(4 Spec No), 829–838.

Montalban-Arques, A., De Schryver, P., Bossier, P., Gorkiewicz, G., Mulero, V., Gatlin, D. M., & Galindo-Villegas, J. (2015). Selective Manipulation of the Gut Microbiota Improves Immune Status in Vertebrates. *Frontiers in Immunology*, 6. https://www.frontiersin.org/articles/10.3389/fimmu.2015.00512

Moorhead, J. A., & Zeng, C. (2010). Development of Captive Breeding Techniques for Marine Ornamental Fish: A Review. *Reviews in Fisheries Science*, *18*(4), 315–343. https://doi.org/10.1080/10641262.2010.516035

Mumby, P. J. (2006). The impact of exploiting grazers (Scaridae) on the dynamics of Caribbean coral reefs. *Ecological Applications: A Publication of the Ecological Society of America*, *16*(2), 747–769. https://doi.org/10.1890/1051-0761(2006)016[0747:tioegs]2.0.co;2

Musso, G., Gambino, R., & Cassader, M. (2011). Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. *Annual Review of Medicine*, *62*, 361–380. https://doi.org/10.1146/annurev-med-012510-175505

Naylor, R. L., Hardy, R. W., Buschmann, A. H., Bush, S. R., Cao, L., Klinger, D. H., Little, D. C., Lubchenco, J., Shumway, S. E., & Troell, M. (2021). A 20-year retrospective review of global aquaculture. *Nature*, *591*(7851), Article 7851.https://doi.org/10.1038/s41586-021-03308-6

Pace, N. R. (1997). A molecular view of microbial diversity and the biosphere. *Science (New York, N.Y.)*, *276*(5313), 734–740. https://doi.org/10.1126/science.276.5313.734

Palmtag, M. R. (2017). The Marine Ornamental Species Trade. In Marine Ornamental Species Aquaculture (pp. 3–14). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119169147.ch1

Pérez-Atehortúa, M., Hernández, A. J., Dantagnan, P., Silva, M., Risopatrón, J., Farías, J., Figueroa Villalobos, E., & Valdebenito, I. (2023). Chorion in fish: Synthesis, functions and factors associated with its malformations. *Aquaculture Reports*, *30*, 101590. https://doi.org/10.1016/j.aqrep.2023.101590

Perry, W. B., Lindsay, E., Payne, C. J., Brodie, C., & Kazlauskaite, R. (2020). The role of the gut microbiome in sustainable teleost aquaculture. *Proceedings of the Royal Society Biological Sciences*, 287(1926), 20200184. https://doi.org/10.1098/rspb.2020.0184

Pindling, S., Azulai, D., Zheng, B., Dahan, D., & Perron, G. G. (2018). Dysbiosis and early mortality in zebrafish larvae exposed to subclinical concentrations of streptomycin. *FEMS Microbiology Letters*, *365*(18), fny188. https://doi.org/10.1093/femsle/fny188

Olivotto, I., Chemello, G., Vargas, A., Randazzo, B., Piccinetti, C. C., & Carnevali, O. (2017). Marine ornamental species culture: From the past to "Finding Dory." *General and Comparative Endocrinology*, 245, 116–121. https://doi.org/10.1016/j.ygcen.2016.03.004

Rawls, J. F., Samuel, B. S., & Gordon, J. I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(13), 4596–4601. https://doi.org/10.1073/pnas.0400706101

Rawls, J. F., Mahowald, M. A., Ley, R. E., & Gordon, J. I. (2006). Reciprocal Gut Microbiota Transplants from Zebrafish and Mice to Germ-free Recipients Reveal Host Habitat Selection. Cell, 127(2), 423–433. https://doi.org/10.1016/j.cell.2006.08.043

Rubec, P. J. (1988). The need for conservation and management of Philippine coral reefs. *Environmental Biology of Fishes*, 23(1), 141–154. https://doi.org/10.1007/BF00000745

Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Cavanaugh, C. M., Guillemin, K., Rawls, J. F. (2011). Evidence for a core gut microbiota in the zebrafish. *The ISME Journal*, 5(10), 1595–1608. https://doi.org/10.1038/ismej.2011.38

Rogers, G. B., Kozlowska, J., Keeble, J., Metcalfe, K., Fao, M., Dowd, S. E., Mason, A. J., McGuckin, M. A., & Bruce, K. D. (2014). Functional divergence in gastrointestinal microbiota in physically-separated genetically identical mice. *Scientific Reports*, 4(1), Article 1. https://doi.org/10.1038/srep05437

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the national academy of sciences*, *74*(12), 5463-5467.

Schaar, S. I., & Cox, L. J. (2021). The future for Hawai‘i’s marine aquarium fishery: A cost benefit analysis compared to an environmental impact assessment. *Marine Policy*, 127, 104429. doi:10.1016/j.marpol.2021.1044

Sekirov, I., Russell, S. L., Antunes, L. C. M., & Finlay, B. B. (2010). Gut microbiota in health and disease. *Physiological Reviews*, *90*(3), 859–904. https://doi.org/10.1152/physrev.00045.2009

Semova, I., Carten, J. D., Stombaugh, J., Mackey, L. C., Knight, R., Farber, S. A., & Rawls, J. F. (2012). Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. *Cell Host & Microbe*, 12(3), 277–288. https://doi.org/10.1016/j.chom.2012.08.003

Sieler, M. J., Al-Samarrie, C. E., Kasschau, K. D., Varga, Z. M., Kent, M. L., & Sharpton, T. J.

(2023). Disentangling the link between zebrafish diet, gut microbiome succession, and Mycobacterium chelonae infection. *Animal Microbiome*, 5(1), 38. https://doi.org/10.1186/s42523-023-00254-8

Shehadul Islam, M., Aryasomayajula, A., & Selvaganapathy, P. R. (2017). A Review on Macroscale and Microscale Cell Lysis Methods. *Micromachines*, 8(3), 83. https://doi.org/10.3390/mi8030083

Stephens, W. Z., Burns, A. R., Stagaman, K., Wong, S., Rawls, J. F., Guillemin, K., & Bohannan, B. J. M. (2016). The composition of the zebrafish intestinal microbial community varies across development. *The ISME Journal*, 10(3), 644–654. https://doi.org/10.1038/ismej.2015.140

Stevenson, T. C., Tissot, B. N., & Walsh, W. J. (2013). Socioeconomic consequences of fishing displacement from marine protected areas in Hawai'i. *Biological Conservation*, 160, 50–58. https://doi.org/10.1016/j.biocon.2012.11.031

Talwar, C., Nagar, S., Lal, R., Negi, R. K. (2018). Fish Gut Microbiome: Current Approaches and Future Perspectives. *Indian Journal of Microbiology*, 58(4), 397–414. https://doi.org/10.1007/s12088-018-0760-y

Tarnecki, A. M., Burgos, F. A., Ray, C. L., Arias, C. R. (2017). Fish intestinal microbiome: Diversity and symbiosis unraveled by metagenomics. *Journal of Applied Microbiology*, 123(1), 2–17. https://doi.org/10.1111/jam.13415

Tissot, B. N., & Hallacher, L. E. (2003). Effects of Aquarium Collectors on Coral Reef Fishes in Kona, Hawai'i. *Conservation Biology*, 17(6), 1759–1768. https://doi.org/10.1111/j.1523-1739.2003.00379.x

The Human Microbiome Project Consortium (2012) A framework for human microbiome research. *Nature* 486: 215–221.

Tlusty, M. (2002). The benefits and risks of aquacultural production for the aquarium trade. *Aquaculture*, 205(3), Article 3. https://doi.org/10.1016/S0044-8486(01)00683-4

Tlusty, M. F., Rhyne, A. L., Kaufman, L., Hutchins, M., Reid, G. M., Andrews, C., Boyle, P., Hemdal, J., McGilvray, F., & Dowd, S. (2013). Opportunities for public aquariums to increase the sustainability of the aquatic animal trade. *Zoo Biology*, 32(1), 1–12. https://doi.org/10.1002/zoo.21019

Turjeman, S., & Koren, O. (2021). Using the microbiome in clinical practice. *Microbial Biotechnology*, 15(1), 129–134. https://doi.org/10.1111/1751-7915.13971

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., Gordon, J. I. (2007). The Human Microbiome Project. *Nature*, 449(7164), 804–810. https://doi.org/10.1038/nature06244

Valderrama Ballesteros, L., Matthews, J. L., & Hoeksema, B. W. (2018). Pollution and coral damage caused by derelict fishing gear on coral reefs around Koh Tao, Gulf of Thailand. *Marine Pollution Bulletin*, 135, 1107–1116. https://doi.org/10.1016/j.marpolbul.2018.08.033

Van de Peer, Y., Chapelle, S., & De Wachter, R. (1996). A quantitative map of nucleotide substitution rates in bacterial rRNA. *Nucleic Acids Research*, *24*(17), 3381–3391. https://doi.org/10.1093/nar/24.17.3381

Vaz, M., Esteves, V. I., & Calado, R. (2017). Live reef fish displaying physiological evidence of cyanide poisoning are still traded in the EU marine aquarium industry. *Scientific reports*, 7(1), 1-5.

Volff, J.-N. (2005). Genome evolution and biodiversity in teleost fish. *Heredity*, *94*(3), Article 3. https://doi.org/10.1038/sj.hdy.6800635

Wabnitz, C., Taylor, M., Green, E., Razak T., 2003. From Ocean to Aquarium UNEP. World Conservation Monitoring Centre, Cambridge. pp. 1–64.

Walsh WJ (1984) Aspects of nocturnal shelter, habitat space, and juvenile recruitment in Hawaiian coral reef fishes, Ph.D. Thesis. University of Hawai‘i at Mānoa, Honolulu, HI. 474 pp

Williams, I. D., Walsh, W. J., Claisse, J. T., Tissot, B. N., & Stamoulis, K. A. (2009). Impacts of a Hawaiian marine protected area network on the abundance and fishery sustainability of the yellow tang, *Zebrasoma flavescens. Biological Conservation*, 142(5), 1066–1073. https://doi.org/10.1016/j.biocon.2008.12.029

Wijesekara, R. G. S., & Yakupitiyage, A. (2001). Ornamental Fish Industry in Sri Lanka: Present Status and Future Trends. *Aquarium Sciences and Conservation*, 3(4), 241–252. https://doi.org/10.1023/A:1013154407298

Wilkes Walburn, J., Wemheuer, B., Thomas, T., Copeland, E., O'Connor, W., Booth, M., Fielder, S., & Egan, S. (2019). Diet and diet-associated bacteria shape early microbiome development in Yellowtail Kingfish (*Seriola lalandi*). *Microbial Biotechnology*, *12*(2), 275–288. https://doi.org/10.1111/1751-7915.13323

Wu, S., Wang, G., Angert, E. R., Wang, W., Li, W., & Zou, H. (2012). Composition, Diversity, and Origin of the Bacterial Community in Grass Carp Intestine. *PLoS ONE*, *7*(2), e30440. https://doi.org/10.1371/journal.pone.0030440

Ye, L., Amberg, J., Chapman, D., Gaikowski, M., & Liu, W.-T. (2014). Fish gut microbiota analysis differentiates physiology and behavior of invasive Asian carp and indigenous American fish. *The ISME Journal*, 8(3), 541–551. https://doi.org/10.1038/ismej.2013.181

Zhang, B., Brock, M., Arana, C., Dende, C., Hooper, L., & Raj, P. (2020). Impact of bead-beating intensity on microbiome recovery in mouse and human stool: Optimization of DNA extraction (p. 2020.06.15.151753). bioRxiv. https://doi.org/10.1101/2020.06.15.151753

Chapter 2: Comparative Analysis of DNA Extraction Methodology on Yellow Tang (*Zebrasoma flavescens*) Gut Microbiota.

2.1 Abstract

Characterizing commensal microbial communities, like the gut microbiome, through high-throughput sequencing is a powerful method that can be applied to optimize host health. However, DNA extraction methodology can greatly impact the composition of recovered DNA and thus the results of downstream sequencing. Here, we assessed the impact of DNA extraction kit, mechanical disruption, and sample storage on the gut microbiota characterized in captive-reared yellow tang (*Zebrasoma flavescens*), a highly sought after species in the aquarium trade. Four commercially available extraction kits (Omega Bio-Tek E.Z.N.A Soil, Qiagen QIAamp PowerFecal Pro DNA, MasterPure Complete DNA and RNA Purification, and MasterPure Gram Positive DNA Purification) were compared utilizing gut samples from naturally deceased adult fish by quantifying DNA yield and subsequently assessing the relative abundance of microbial taxa, alpha diversity, and beta diversity after high throughput sequencing of bacterial 16S rRNA gene amplicons. The four extraction kits yielded a wide range of resulting DNA (< 0.001-10.15 ng/µL) that, in some instances, yielded significantly different microbial communities. At one extreme, the MasterPure Gram Positive DNA Purification kit yielded a highly divergent microbial community consisting of nearly 100% of Firmicutes bacteria. In contrast, strong agreement between microbial communities recovered from the Omega and Qiagen kits were observed. No significant difference was detected from the addition of mechanical disruption prior to extraction or an additional freeze-thaw cycle. This study offers first insights into how methodological choices impact downstream characterization of the yellow tang gut microbiota

and supports the use of either the Omega Bio-Tek E.Z.N.A Soil kit or Qiagen QIAamp PowerFecal Pro DNA kit for characterizing this community.

2.2 Introduction

Vertebrate guts contain dense populations of microbes play a significant role in host digestion (Wu et al., 2012), immunity (Montalban-Arques et al., 2015), disease (DeGruttola et al., 2016), and physiological development (Talwar et al., 2018). With culture-independent approaches that incorporate next generation sequencing (NGS) becoming increasingly accessible, the number of studies investigating the microbiology of host guts has expanded greatly (Talwar et al., 2018; Perry et al., 2020). Microbiome research has important applications in modulating human health in a clinical setting (Turjeman & Koren, 2022) and more recently for fish in the aquaculture industry (Talwar et al., 2018; Perry et al., 2020). For example, probiotics have been incorporated into fish husbandry to optimize growth, nutrient conversion, disease resistance, and survivorship (Mohammadi et al., 2021; Gaffar et al., 2023). However, while NGS technology has facilitated rapid growth in microbiome research, differences in methodology across studies have raised concern about the generalized applicability of results (Hornung et al., 2019; Elie et al., 2023).

It is well established that methodological aspects including the DNA extraction process (Elie et al., 2023; Hart el al., 2015) and associated methods of cell lysis (Zhang et., 2020), can over- or under-represent certain microbial taxa, producing an inaccurate representation of microbial communities (Martin-Laurent et al., 2001). Moreover, steps prior to DNA extraction, such as sample storage, can also influence resulting microbial communities (Larsen et al. in 2014; Tarnecki et al., 2018). The diversification of NGS based research beyond humans and model

organisms (Talwar et al., 2018) generates a need to contrast methodological approaches and assess their suitability for novel host species.

The yellow tang (*Zebrasoma flavescens*) is an iconic reef fish found throughout the Indo-Pacific and is one of the most popular targets of the aquarium trade in Hawai‘i (DLNR, 2015). Aquaculture could provide a sustainable alternative to wild collection to meet aquarium trade demands, however high larval mortality in captive reared yellow tangs represents a major bottleneck in production (Holt et a., 2017). Microbial based exploration may provide critical insights into improving juvenile recruitment (Llewellyn et al., 2014; Perry et al., 2020). Here, we sought to investigate the performance of commercially available DNA extraction kits as well as assess the impact of mechanical disruption and storage technique on the characterization of yellow tang gut microbiota by 16S rRNA gene amplicon sequencing.

2.3 Methods

2.3.1 Specimen Acquisition

Two fully mature broodstock yellow tang specimens (hereafter referred to as YT001 and YT002, respectively) maintained within the Finfish department of the Oceanic Institute of Hawai‘i Pacific University were collectd this study. Within three hours of natural mortality (cause of death uncertain), the adult specimens were removed from the tank, placed in a sterile plastic bag, and frozen at -20°C.

2.3.2 Dissection

Dissection of YT001 and YT002 were conducted on February 4, 2022, and April 13, 2022, respectively (Fig. 2.1 A&B). After thawing at 25°C for approximately 2.5 hours, the

outside of the fish was rinsed thoroughly with 70% ethanol to minimize contamination from the skin associated microbiota. Caution was used to not get ethanol into the fish mouth and gills. The entire gastrointestinal (GI) tract (esophagus to anus) was then dissected out, including both the autochthonous (microbiota associated with gut contents) and allochthonous (those colonizing the mucosal surface on the gut lining itself) microbial communities (Hao et al., 2004). Aseptic dissecting techniques were used, including employing separate dissection tools for the outside of fish and direct contact with GI tract to further reduce contamination. Additionally, all dissection tools were rinsed in 70% ethanol, autoclaved prior to dissection, and frequently flame sterilized throughout the dissection. Following dissection, the guts of YT001 and YT002 were separately homogenized using a handheld Polytron PT 1200 E homogenizer (Kinematica, Bohemia, NY, USA) for approximately 10 min on high speed. Sterile TE (Tris-EDTA) buffer (~ 1mL) was added to aid with homogenization.

2.3.3 DNA Extraction Kits

Four commercially available DNA extraction kits were evaluated in this study: E.Z.N.A Soil kit (Omega Bio-Tek, Norcross, GA, USA), QIAamp PowerFecal Pro DNA kit (Qiagen, Germantown, MD, USA), MasterPure Complete DNA and RNA Purification kit (LGC Biosearch Technologies, Hoddesdon, UK), and the MasterPure Gram Positive DNA Purification kit (LGC Biosearch Technologies, Hoddesdon, UK). The extraction kits were selected to contrast different aspects of the extraction protocol. Most broadly the kits can be separated into two categories based on their strategy of PCR inhibitor removal: spin-column (Qiagen PowerFecal and Omega E.Z.N.A Soil) versus centrifugation and pipetting (MasterPure Complete and MasterPure Gram Positive). The MasterPure Gram Positive kit also differs from the other kits as it contains an additional enzyme, Lysozyme. This enzyme breaks down the glycosidic bonds in the cell wall,

which is thought to increase yield of often under-represented gram-positive bacteria (Primo et al., 2017). All extractions were conducted in triplicate, and the resulting DNA was fluorometrically quantified using the Qubit dsDNA BR and HS kits (Qubit 2.0, Life Technologies, Foster City, CA, USA).

2.3.4 Experimental Design

The two specimens were extracted separately to test several variables thought to contribute to the efficiency of DNA extraction for likely components of the yellow tang gut microbiome. Specimen YT001 was used to compare the efficacy of mechanical disruption via bead-beating on samples ultimately extracted with the MasterPure Gram Positive kit, and to compare DNA extraction performance among all four extraction kits (Fig. 2.1). Specimen YT002 was used to test the impact of sample storage and comparison of DNA extraction kits once again (Fig. 2.2).

Following homogenization, the YT001 gut sample was pipetted into eighteen 150µL aliquots. A subset of six were used to test the effect of mechanical disruption prior to DNA extraction with the MasterPure Gram Positive Extraction kit. Triplicate subsamples of YT001 were extracted following the MasterPure Gram Positive's manufacturer protocol (YT1A, YT2A, and YT3A), or with the addition of bead-beating (YT4B, YT5B, and YT6B). Bead-beating was conducted following the overnight Ready-Lyse Lysozyme incubation step and prior to the addition of Proteinase K. Mechanical disruption was conducted using the Savant FastPrep FP120 Cell Disrupter (Cambridge Scientific, Watertown, MA, USA) for 45 sec. The twelve remaining

subsamples stored at -80° C were extracted in triplicate using the four DNA extraction kits and included a bead-beating step following the protocol described above.

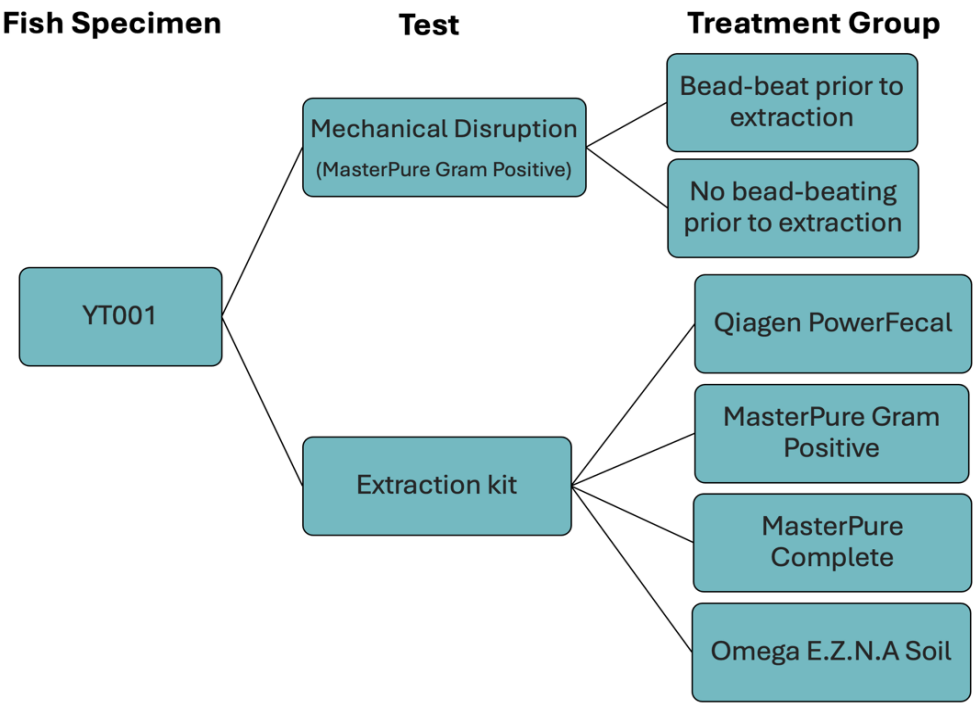


Figure 2.1: Flowchart of methodology testing for YT001 subsamples (n= 18).

After homogenization, the YT002 gut sample was aliquoted into twelve 300µL subsamples, supplemented with 200µL of TE buffer, and mechanically disrupted via bead-beating as described above. Nine of the subsamples were then extracted immediately after bead-beating with the Omega Bio-Tek E.Z.N.A Soil kit, Qiagen QIAamp PowerFecal Pro DNA kit, and the MasterPure Complete DNA and RNA Purification kit. The MasterPure Gram Positive Kit was not tested for YT002 due to insufficient separation of cell debris and DNA observed in previous extractions, and difficulty with PCR amplification from genomic extracts of YT001. In order to test the effect of subsample storage, the three remaining samples were subjected to an additional freeze-thaw cycle at -80°C for 48 hours prior to extraction with the Qiagen PowerFecal kit. “Fresh” samples were those only subjected to the initial freeze/thaw at -20°C of

the entire fish specimen prior to gut dissection. “Frozen” samples were those subjected to an additional freeze/thaw cycle at -80°C prior to extraction.

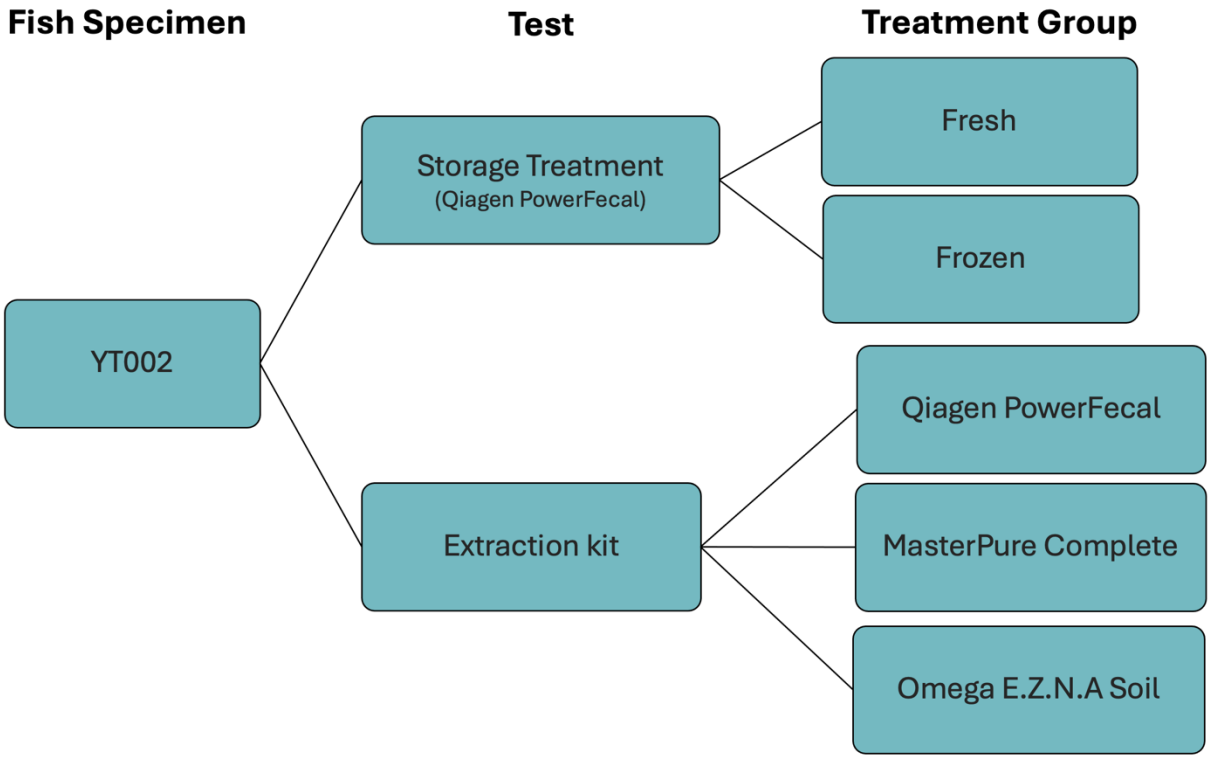


Figure 2.2: Flowchart of methodology testing for YT002 subsamples (n= 12).

2.3.5 Polymerase Chain Reaction and Sequencing

For each of the 31 extractions, polymerase chain reaction (PCR) was used to amplify full-length 16S rRNA gene fragments using the primers 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3' (Lane, 1991). Each 25µL reaction contained 12.5µL of GoTaq Green master mix (Promega, Madison, WI, USA), 0.3µM forward and reverse primers, and 1µL of template genomic DNA (concentrations in Table 2.1 and 2.2 below). The PCR reactions consisted of a 2-minute initial denaturation step at 95° C, followed by 35 cycles of

denaturation for 30 seconds, annealing at 60° C for 45 seconds, extension at 72° C for 1 minute 30 seconds, and a final extension at 72° C for 5 minutes. Amplified full-length 16S rRNA genes were visualized via electrophoresis on a 1.5% agarose gel made with TAE buffer and SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA).

The gDNA from all extractions that showed successful amplification (n=24) were then amplified with the same primers as above, but this time using a high-fidelity polymerase. For this reaction, each 25µL mixture contained 12.5 µL of Platinum SuperFi II PCR Master Mix (Invitrogen, Waltham, MA, USA), 0.3µM forward and reverse primers, and 1µL of template DNA (concentrations in Table 2.1 and 2.2 below). The high-fidelity PCR included an initial denaturation step at 98° C for 30 seconds, followed by 25 cycles of denaturation at 98° C for 10 seconds, annealing at 60° C for 10 seconds, extension at 72° C for 1 minute 30 seconds, and a final extension at 72° C for 5 minutes. Amplified PCR products were visualized via electrophoresis on a 2% agarose gel made with TAE buffer, SmartGlow Pre-Stain (Accuris Instruments, Edison, NJ, USA), and a 1Kb DNA ladder (Invitrogen, Carlsbad, CA, USA) (Fig. 2.1).

The remaining amplification product from the 25-cycle high-fidelity PCR was prepared for sequencing by amplification via a nested eight-cycle, 5µL reaction-volume PCR using the EMP primers 515F GTGYCAGCMGCCGCGGTAA (Parada et al. 2016) and 806R GGACTACNVGGGTWTCTAAT (Apprill et al. 2015) to target the V4 hypervariable region of the 16S rRNA gene (Caporaso et al. 2011). All samples showed successful amplification following the nested PCR and were sequenced on an Illumina MiSeq using v2 2x250 base pair kit (Illumina, San Diego, CA, USA).

2.3.6 Bioinformatics and Statistical Analysis

The raw sequences were demultiplexed using Mothur v. 1.36.1 (Kozich et al., 2013) and the demultiplexed pair-end reads were assessed for quality in Qiime2 v2022.11 (Bolyen et al., 2019) using the DADA2 pipeline (Callahan et al. 2016). Based on quality plot analysis, forward and reverse reads were truncated to 250bp to maintain high read quality (> 30 Phred quality score). Reads were then denoised, screened for chimeras, and merged into distinct amplicon sequence variants (ASVs), defined as sequences with 100% sequence similarity. ASVs were subsequently classified taxonomically using the Silvia v138.1 database. Following taxonomic classification, features with less than 10 reads across all samples or appearing in less than two samples were removed (n= 2 filtered) to minimize impact of sequencing artifacts (Cao et al., 2021). Finally, features unable to be taxonomically identified at the domain and phylum level were removed. Sample MP3YT2 was removed from analysis due to low read depth (<1,000 reads) resulting in a minimum read depth for this study of 7,666.

Statistical analyses and data visualizations were conducted in RStudio v2022.12.0+ using phyloseq (McMurdie & Holmes, 2013), ggplot2 (Wickham, 2016), and vegan (Oksanen et al., 2022). Point estimates (mean of all replicate estimates) of Shannon diversity were generated at the ASV level using the R package DivNet (Willis & Martin, 2022). To determine dissimilarity among samples for subsequent ordination and clustering, Aitchison's distance (the Euclidian distance between log-ratio transformed data points) was calculated (Aitchison et al., 2000). This strategy was chosen for its ability to account for the compositional nature of the dataset as well as address challenges characteristic of amplicon datasets such as zero inflation (Aitchison et al., 2000; Gloor et al., 2017). For multivariate analysis across the entire dataset the non-parametric

strategy, PERMANOVA (permutational multivariate statistical analysis of community separation) was implemented using the Adonis function within the vegan package. PERMANOVA tests were only conducted following confirmation of minimal dispersion within groupings using the 'betdisper' function, also within the vegan package. Community patterns were also visualized using non-metric multidimensional scaling (NMDS) plots, implemented using the vegan package.

2.4 Results

2.4.1 GI Tract Dissection

At 4.22g and 4.40g, dissections yielded similar GI tract samples by weight for YT001 and YT002, respectively (Fig. 2.3). Differences between the overall presentation of the GI tract in the two fish, namely the color, was noted. The gut of YT002 was full of a green/brown substance, likely undigested food. Surrounding tissue appeared a healthy pink for YT001 as opposed to pale grey for YT002.

(A) **(B)**

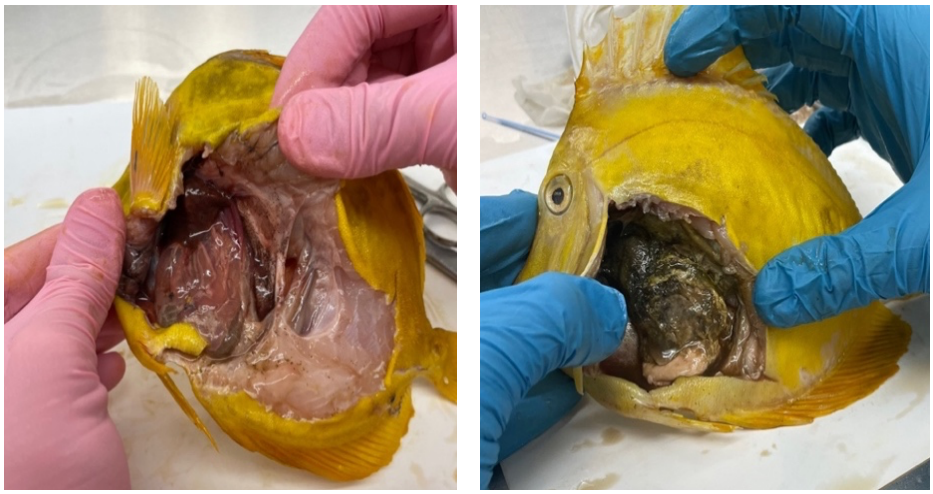


Figure 2.3: Dissection of YT001 and YT002. Exposed gastrointestinal (GI) tract of **(A)** YT001 and **(B)** YT002 prior to dissection.

2.4.2 Trends in DNA Sequencing

Across both YT001 (n=12) and YT002 (n=11) 23 samples were retained for analysis. Following denoising and quality control, 76.7% of sequences were retained with a total read depth across the 23 samples of 478,650. Sequencing depth of retained samples ranged from 7,666 (MP2YT2) to 36,798 (Q3YT2) (Table 2.1 & 2.2). Mean read count for YT001 and YT002 were similar at 22,761 ± 9,370 and 21,880 ± 10,340 respectively. A total of 324 unique ASVs were identified, all of which belonged to the domain Bacteria (Appendix Table 1).

Table 2.1: DNA extractions for yellow tang specimen YT001 using the Omega Bio-Tek E.Z.N.A Soil, Qiagen QIAamp PowerFecal Pro DNA, MasterPure Complete DNA and RNA Purification, and MasterPure Gram Positive kits. Read depth column contains retained reads following all quality control.

Sample ID	Extraction Kit	Mechanical Disruption	DNA Yield (ng/µL)	PCR Amplification	Read Depth
YT1A	MasterPure Gram Positive	No	10.15	+	11,487
YT2A	MasterPure Gram Positive	No	8.44	+	9,973
YT3A	MasterPure Gram Positive	No	5.14	+	7,689
YT4B	MasterPure Gram Positive	Yes	5.82	+	14,970
YT5B	MasterPure Gram Positive	Yes	8.32	+	20, 522
YT6B	MasterPure Gram Positive	Yes	6.40	+	12,431
N/A	MasterPure Gram Positive	Yes	0.02	-	*
N/A	MasterPure Gram Positive	Yes	0.05	-	*
N/A	MasterPure Gram Positive	Yes	0.01	-	*
N/A	MasterPure Complete	Yes	< 0.001	-	*
N/A	MasterPure Complete	Yes	0.02	-	*
N/A	MasterPure Complete	Yes	0.07	-	*
S1YT2	E.Z.N.A Omega Soil	Yes	0.53	+	17,163
S2YT2	E.Z.N.A Omega Soil	Yes	0.67	+	33,812
S3YT2	E.Z.N.A Omega Soil	Yes	0.54	+	27, 564
YTQ1	Qiagen PowerFecal	Yes	2.08	+	14,967
YTQ2	Qiagen PowerFecal	Yes	2.36	+	30,009
YTQ3	Qiagen PowerFecal	Yes	2.68	+	33,411

*Not sent for sequencing due to unsuccessful amplification.

Table 2.2: DNA extractions for yellow tang specimen YT002 using the Omega Bio-Tek E.Z.N.A Soil, Qiagen QIAamp PowerFecal Pro DNA, and MasterPure Complete DNA and RNA Purification kit. Read depth column contains retained reads following all quality control.

Sample ID	Extraction Kit	Mechanical Disruption	DNA Yield (ng/µL)	PCR Amplification	Read Depth
S1YT2	E.Z.N.A Omega Soil	Yes	0.02	+	28,734
S2YT2	E.Z.N.A Omega Soil	Yes	0.02	+	20,416
S3YT2	E.Z.N.A Omega Soil	Yes	0.01	+	12,566
MP1YT2	MasterPure Complete	Yes	0.81	+	12,266
MP2YT2	MasterPure Complete	Yes	1.17	+	7,666
MP3YT2	MasterPure Complete	Yes	1.21	+	3,220*
Q1YT2	Qiagen PowerFecal	Yes	0.08	+	10,221
Q2YT2	Qiagen PowerFecal	Yes	0.07	+	34,616
Q3YT2	Qiagen PowerFecal	Yes	0.07	+	36,798
PF2YT2	Qiagen PowerFecal	Yes	0.02	+	19,004
PF3YT2	Qiagen PowerFecal	Yes	0.03	+	28,023
PF4YT2	Qiagen PowerFecal	Yes	0.03	+	30,375

*Excluded from analysis due to low read depth.

2.4.3 Assessment of Mechanical Disruption

To compare the effect of mechanical disruption on microbiota community structure, subsamples from fish specimen YT001 were compared by bead-beating triplicate samples and comparing them to non-bead-beaten but otherwise identically treated triplicates. Relative abundance plots annotated to genus level revealed a decrease in the relative abundance of the gammaproteobacterial genus *Vibrio* from 11.6-27.5% in samples without mechanical disruption to below detection levels in bead-beaten samples (Fig. 2.4). This shift was accompanied by an increase in the relative abundance of the gram-positive genera *Enterococcus* and *Clostridium* in

bead-beaten samples. However, no significant difference was ultimately detected between these groupings (p-value= 0.18).

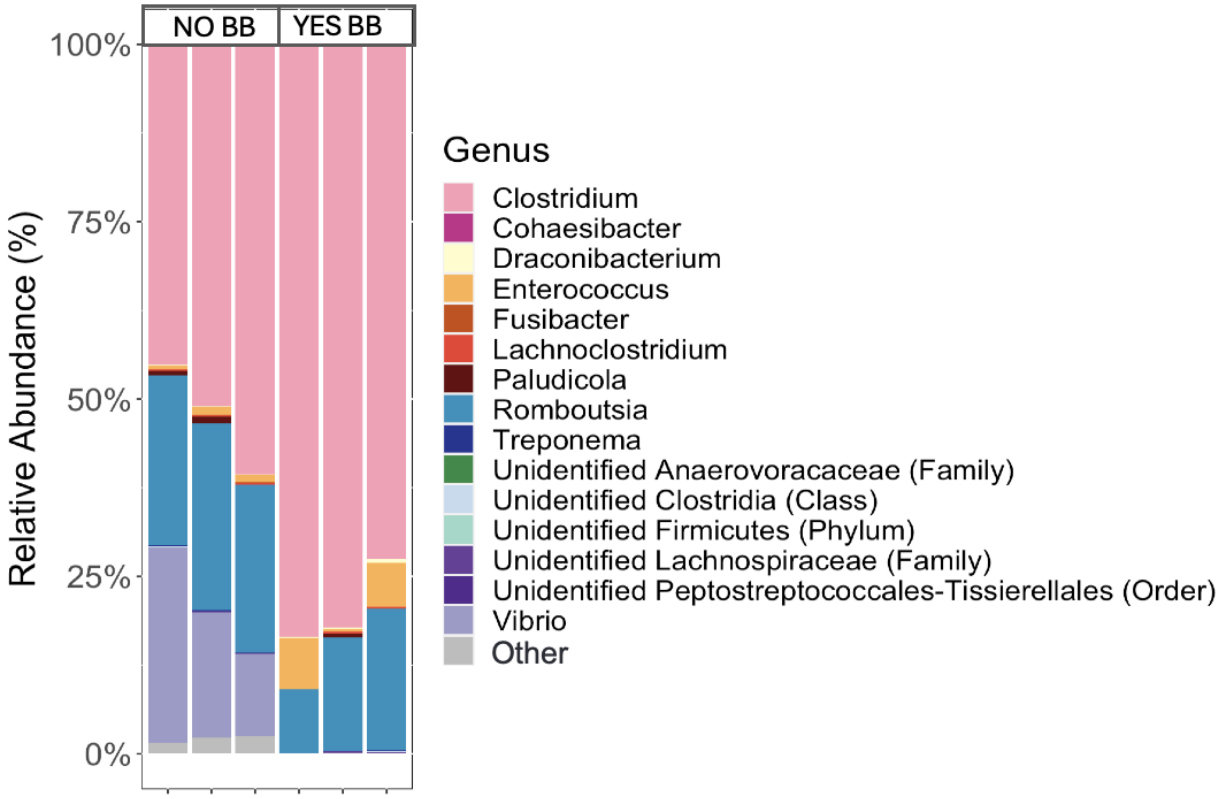


Figure 2.4: Relative abundance of microbiota for bead-beaten and non-bead-beaten samples extracted with the MasterPure Gram Positive kit. Annotated to the genus level (top 20 must abundant genera). If taxonomy was not able to be resolved at the genus level, the next most specific taxonomic classification provided.

2.4.5 Comparison of Extraction Kits on the Microbiome of Fish Specimen YT001

To compare the effect of different extraction kits on amplifiability, DNA yield, and sequence composition, the Omega E.Z.N.A Soil, Qiagen PowerFecal, MasterPure Complete, and MasterPure Gram Positive DNA extraction kits were used to evaluate their performance on identically treated triplicate subsamples from fish specimen YT001. All three MasterPure Complete replicates did not amplify for YT001 extractions (Table 2.1). Additionally, due to the

MasterPure Gram Positive kit testing replicates not successfully amplifying (Table 2.1), MasterPure extractions YT4B, YT5B, and YT6B from the mechanical disruption evaluation were used for comparison to the other kits.

DNA yield across extraction kits for YT001 samples ranged from < 0.001 to 10.15 ng/µL (Fig. 2.5A). The MasterPure Gram Positive Kit had notably higher DNA yields than both spin column kits ranging from 5.14 to 10.15 ng/µL (Fig. 2.5A). Despite having the highest DNA yield, the MasterPure Gram Positive kit had the lowest alpha diversity at 0.68 (Fig. 2.5B). The Omega E.Z.N.A Soil and Qiagen PowerFecal kits had similar diversity estimates at 3.35 and 3.42 respectively (Fig. 2.5B).

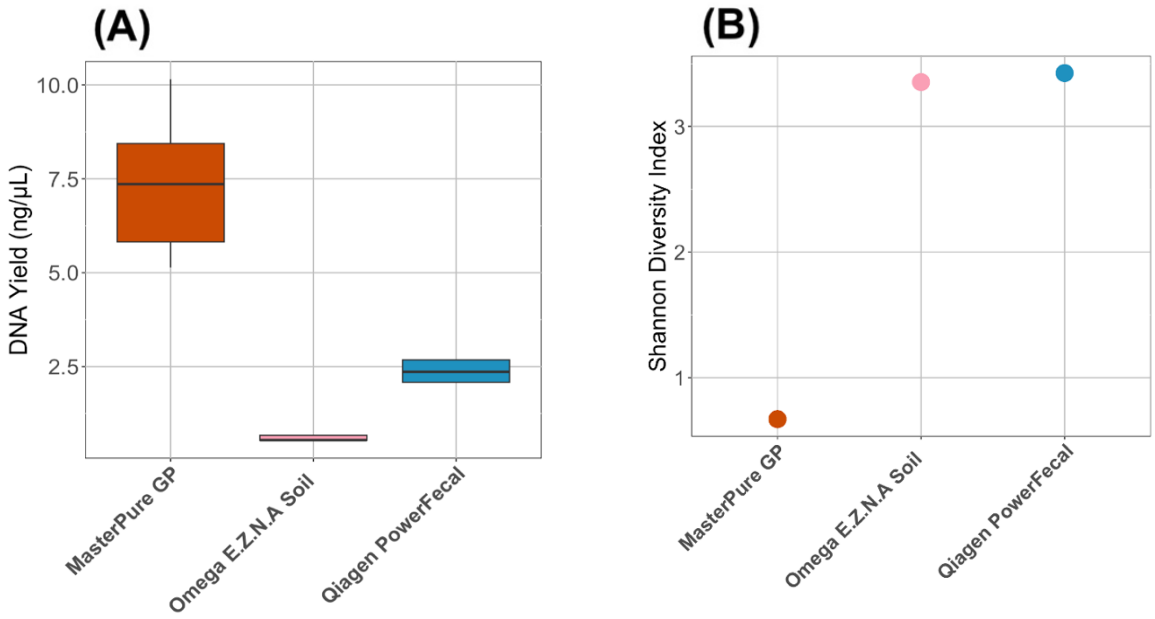
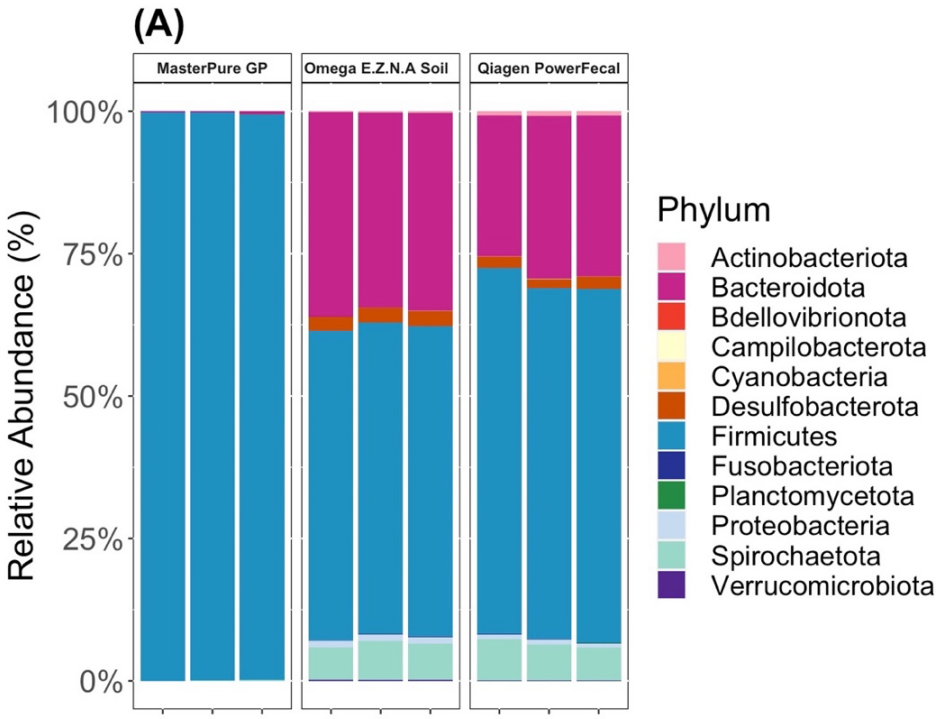


Figure 2.5: DNA yield (ng/µL) and Shannon Diversity Index for YT001. **(A)** DNA yield (ng/µL) for extractions with the Omega Bio-Tek E.Z.N.A Soil kit (n=3), MasterPure Gram-Positive DNA Purification kit (n=6), and Qiagen's QIAamp PowerFecal Pro DNA kit (n=3). Box plots show mean, first, and third quartile. **(B)** Point estimates of Shannon Diversity Index generated at the ASV level for extractions with the Omega Bio-Tek E.Z.N.A Soil kit (n=3), MasterPure Gram-Positive DNA Purification kit (n=6), and Qiagen's QIAamp PowerFecal Pro DNA kit (n=3). Point estimate represents mean Shannon Index across replicates.

Homogenous dispersion among replicates (p-value= 0.41) allowed for pairwise testing between extraction kit replicates based on the microbial community each identified. The Qiagen PowerFecal and Omega E.Z.N.A kits significantly differed from the MasterPure Gram Positive kit (p-value = 0.02). Relative abundance graphs annotated to the phylum level, in consensus with the pairwise tests, revealed strong agreement between the Omega E.Z.N.A Soil kit and Qiagen PowerFecal kit with both identifying Firmicutes, Bacteroidota, and Spirochaetota to be the three most abundant phyla (Fig. 2.6A). In contrast, the MasterPure Gram Positive kit, showed Firmicutes to make up nearly 100% of the relative abundance (Fig. 2.6A). Further investigation at the genus level showed the dominant Firmicutes phylum characterized by the MasterPure Gram Positive kit to consist primarily of the genera *Clostridia* followed by *Romboutsia* and *Enterococcus* (Fig. 2.6B). For the E.Z.N.A Soil and PowerFecal kits, a myriad of genera was identified including *Clostridium*, *Draconibacterium*, *Paludicola*, *Romboutsia*, among others shown below (Fig. 2.6B).



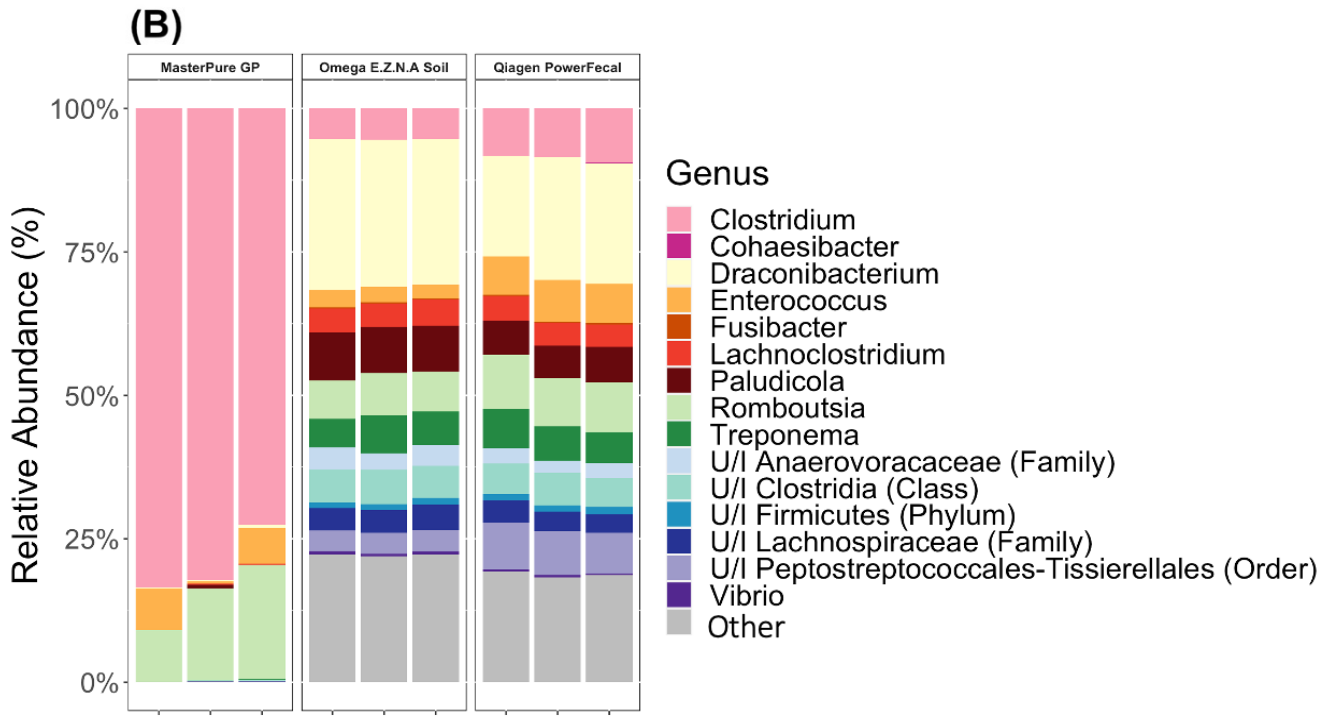


Figure 2.6: Relative abundance of gut microbiota from yellow tang specimen YT001. **(A)** Relative abundance plot annotated to the phylum level for Omega Bio-Tek E.Z.N.A Soil kit, MasterPure Gram-Positive DNA Purification kit, and Qiagen's QIAamp PowerFecal Pro DNA kit. **(B)** Relative abundance plot of the 15 most abundant genera for Omega Bio-Tek E.Z.N.A Soil kit, MasterPure Gram-Positive DNA Purification kit, and Qiagen's QIAamp PowerFecal Pro DNA kit. If taxonomy was not able to be resolved at the genus level, the next most specific taxonomic classification provided.

2.4.6 Comparison of Extraction Kits on the Microbiome of Fish Specimen YT002

The Omega E.Z.N.A Soil, Qiagen PowerFecal, and MasterPure Complete extraction kits were compared for amplifiability, DNA yield, and sequence composition on identically treated subsamples from fish specimen YT002. All samples for YT002 successfully amplified however, one MasterPure Complete extraction was excluded from analysis due to low read depth (Table 2.2). DNA yield for YT002 samples ranged from 0.01-1.17 ng/µL (Fig. 2.7A). The MasterPure Complete kit had the highest DNA yield of the three kits ranging from 0.81-1.21 ng/µL, but

lowest alpha diversity at 2.52 (Fig. 2.7A&B). The spin column kits, Omega E.Z.N.A Soil and Qiagen PowerFecal, once again had similar alpha diversity values at 3.31 and 2.26.

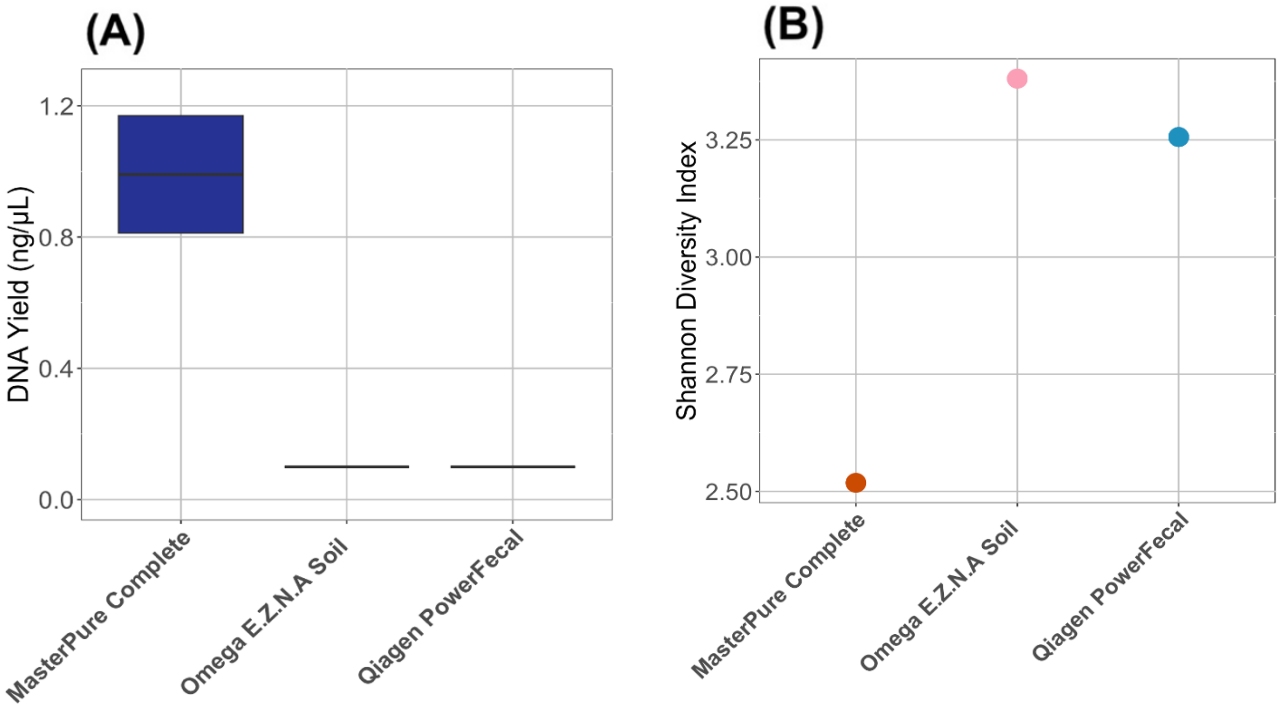
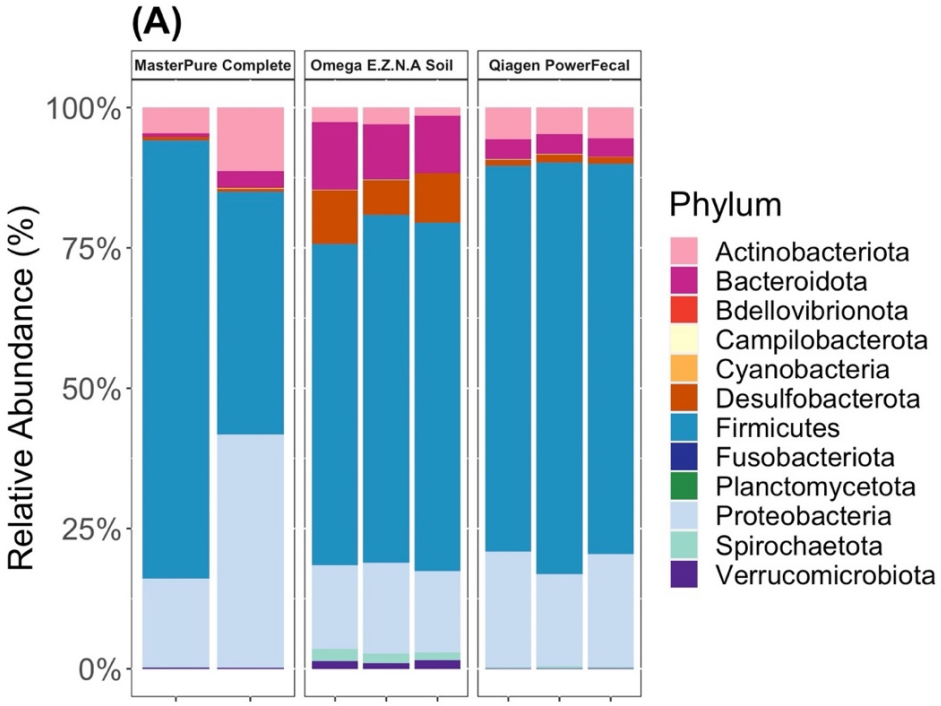


Figure 2.7: DNA yield (ng/µL) and Shannon Diversity Index for YT002. **(A)** DNA yield (ng/µL) for extraction with the Omega Bio-Tek E.Z.N.A Soil kit (n=3), MasterPure Complete DNA and RNA Purification kit (n=3), and Qiagen's QIAamp PowerFecal Pro DNA kit (n=6). Box plots show mean, first, and third quartile. **(B)** Point estimates of Shannon Diversity Index generated at the ASV level for extractions with the Omega Bio-Tek E.Z.N.A Soil kit (n=3), MasterPure Complete DNA and RNA Purification kit (n=6), and Qiagen's QIAamp PowerFecal Pro DNA kit (n=3). Point estimate represents mean Shannon value across replicates.

Heterogeneous dispersion among replicates (p-value= 0.001) did not allow for pairwise testing between extraction kit replicates for YT002 samples. However, relative abundance graphs illustrate strong agreement again between the Omega E.Z.N.A Soil kit and Qiagen PowerFecal kit in gut microbiota community structure (Figs. 2.8A&B). For these two kits, the three genera with the highest relative abundance include *Fusibacter*, *Cohaesibacter*, and *Lachnoclostridium* (Fig. 2.8B). The MasterPure Complete kit on the other hand displayed considerable variability between the two retained replicates. For example, the genus *Romboutsia* accounts for 62.57% of the relative abundance in MP1YT2 and only 0.9% in MP2YT2. In agreement with YT001, Firmicutes was identified as the most dominant phylum for all YT002 samples except one (MP2YT2). The most abundant phyla characterized in the relative abundance plots for YT002 include Proteobacteria, Actinobacteriota, Desulfobacterota, and Bacteroidota (Fig. 2.8B).



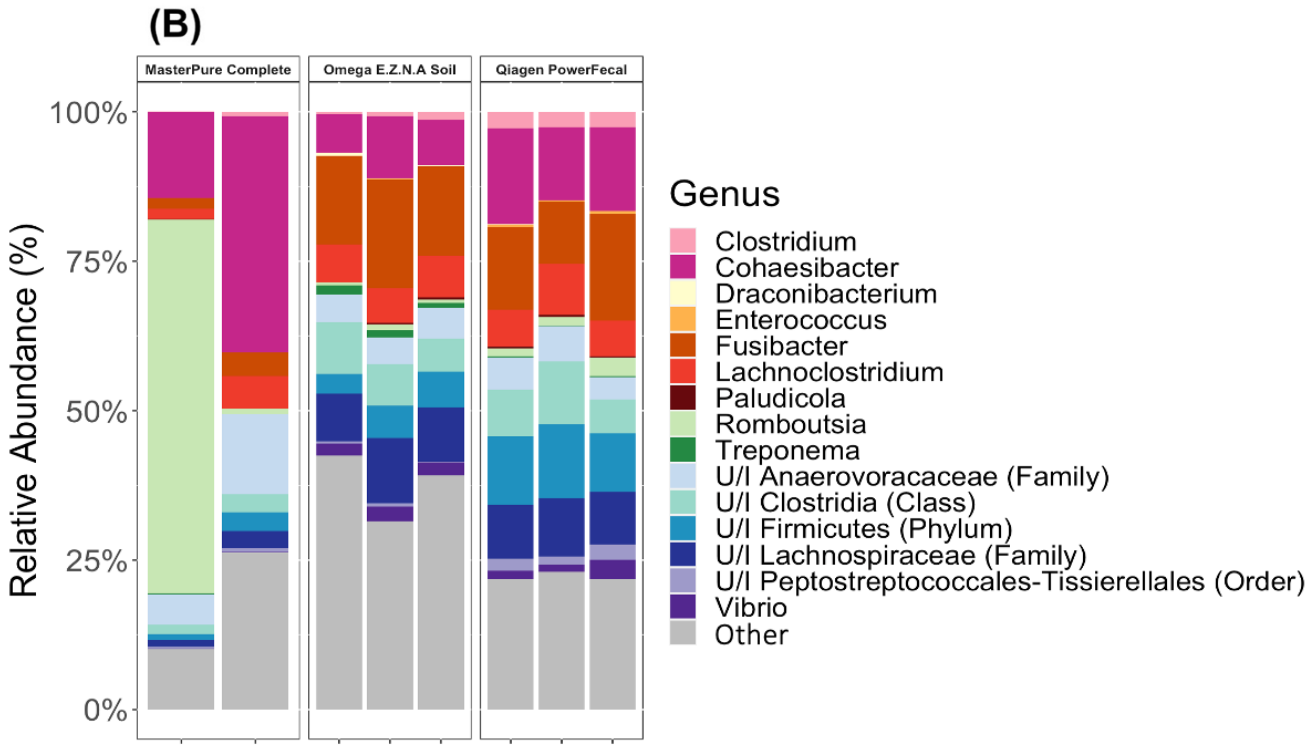


Figure 2.8: Relative abundance of gut microbiota from yellow tang specimen YT002. **(A)** relative abundance annotated to the phylum level for Omega Bio-Tek E.Z.N.A Soil kit, MasterPure Complete DNA Purification kit, and Qiagen's QIAamp PowerFecal Pro DNA kit. **(B)** relative abundance of the 15 most abundant genera for Omega Bio-Tek E.Z.N.A Soil kit, MasterPure Complete DNA Purification kit, and Qiagen's QIAamp PowerFecal Pro DNA kit. If taxonomy was not able to be resolved at the genus level, the next most specific taxonomic classification provided.

2.4.7 Assessment of GI Tract Storage Prior to DNA Extraction

To compare the effect of freezing at -80°C on GI tract subsamples prior to DNA extraction, triplicate subsamples from fish specimen YT002 were extracted using the Qiagen PowerFecal kit immediately following dissection (fresh), as well as from subsamples subject to an additional freeze/thaw cycle at -80°C (Table 2.2). DNA yield for frozen extractions were lower than fresh ranging from 0.02 to 0.03 ng/µL and 0.07 to 0.08 ng/µL respectively. In

agreement with relative abundance graphs illustrating very similar community structure (Fig. 2.9), pairwise comparisons revealed no significant difference between treatment groups based on microbial community identified.

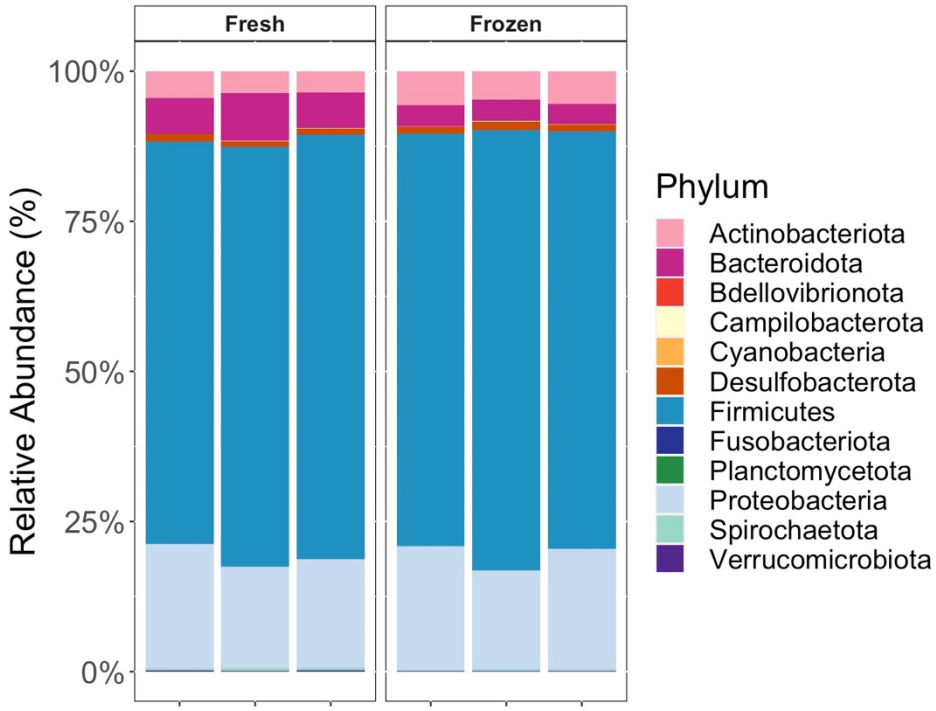


Figure 2.9: Relative abundance plot of gut microbiota (YT002) in fresh vs frozen samples. Both treatment groups extracted using the Qiagen PowerFecal Pro DNA extraction kit.

2.5 Discussion

2.5.1 Introduction

The ability to interrogate gut microbial communities and their intricate relationship with a host rest on the capability of the tools employed to accurately assess microbial community structure. It has been well described that DNA extraction methodology, and associated methods

of cell lysis, can significantly impact the downstream sequencing output and thus the efficacy of characterizing gut microbial communities in humans (Kennedy et al. 2014; Wu et al. 2010) and in various animal species (Hart et al 2015). Given the already complex nature of the gut microbiota and the relationship it has with the host, bias introduced throughout the DNA extraction and sequencing workflows further complicates resolving knowledge gaps in this field (Wu et al., 2010). Here, we compared four commercially available extraction kits on adult yellow tang gut samples, by quantifying their impact on genomic DNA yield and microbiota composition. Additionally, we assessed the impact of mechanical disruption and sample storage, two key aspects of the DNA extraction workflow.

2.5.2 DNA Yield Proves Poor Indicator of Microbiota Diversity

Thirty-one DNA extractions were ultimately completed from YT001 and YT002 yellow tang specimens. We observed variability across the four DNA extraction kits both in terms of DNA yield and alpha diversity. Interestingly, we found higher DNA yield to not be a reliable indicator of greater microbial diversity. For example, across the four kits tested, the Master Pure Gram Positive kit had by far the highest DNA yields (5.14-10.15 ng/µL), but lowest alpha diversity (Fig. 2.4A&B). The extraction kit used as opposed to DNA yield appeared to be the primary driver behind diversity patterns. For example, the Qiagen PowerFecal and Omega E.Z.N.A Soil extractions consistently had higher alpha diversity values (3.35-3.42) than both MasterPure kits. Several recent studies comparing extraction methods are in agreement with the lack of correlation strictly between DNA yield and alpha diversity (Hou et al., 2021; Videnska et al., 2019). Another study concluded while greater DNA yield may indicate more efficient cell lysis of bacterial cells, it does not necessarily equate to greater yields of DNA usable in molecular analysis or even community representation (Stach et al., 2001). Purity of the DNA and

rigor of cell lysis, both of which will be impacted by method of DNA extraction, are examples of other important factors that will impact the diversity able to be identified within a sample (Hou et al., 2021; Videnska et al., 2019). Thus, we conclude DNA yield should be considered in concert with other performance metrics, such as PCR amplification, when evaluating extraction methodologies.

2.5.3 Amplification Varies with DNA Extraction Kit

Producing a sufficient amount of high-quality amplifiable DNA is one of the most important performance metrics when assessing DNA extraction kits (Schrader et al., 2012). The Qiagen PowerFecal and Omega E.Z.N.A Soil kits produced amplifiable DNA in all replicates across both YT001 and YT002 specimens. In contrast, the MasterPure Gram Positive and MasterPure Complete kits showed less reliability with only 3/9 and 3/6 extractions respectively demonstrating successful amplification (Table 2.1 and 2.2). The observed pattern of a varied ability across extraction protocols to produce amplifiable DNA is echoed in other studies. In Hart et al. in (2015) found the ability of different extraction kits to produce sufficient yields of amplifiable DNA (defined at >10,000 reads) to vary depending on the host species. For example, only 3/8 zebrafish samples extracted with the Qiagen PowerFecal kit (tested in this study) amplified successfully where 8/8 samples amplified for the mouse samples using the same kit (Hart et al., 2015).

Findings of previous studies in combination with the data presented here, provides evidence that commercially available extraction kits vary in their ability to produce amplifiable DNA based on host-dependent PCR inhibitors. Inhibitors like proteins and lipids interfere with the amplification of DNA through several mechanisms including degradation of the template

RNA or DNA, competitive binding to the template, and inhibition of enzymes like DNA polymerase (Schrader et al., 2012). Thus, cleaning the sample of these inhibitors is crucial to efficient amplification of DNA for sequencing (Schrader et al., 2012). The higher rate of positive amplification and greater overall read depth of the Qiagen PowerFecal and Omega E.Z.N.A Soil kits, indicate the spin column was more efficient at DNA purification in yellow tang gut samples than the centrifugation and pipetting strategy featured in the two MasterPure kits tested.

2.5.4 Mechanical Disruption and Sample Storage

Efficient cell lysis is another element critical to the accurate characterization of microbial communities. Mechanical disruption, a non-selective form of cell lysis, often increases nucleic acid yield and microbial diversity detected (Zhang et al., 2021). However, mechanical disruption, in the form of bead-beating for example, can also shear microbial DNA in easier to lyse cells when used in excess, introducing undesired bias (Zhang et al., 2021). The ideal bead-beating protocol that provides complete coverage of complex microbial communities, like the gut microbiota, while preserving the integrity of easier to lyse cells remains elusive (Zhang et a., 2021). In this study, the decrease in relative abundance of the gram-negative genus V*ibrio* in mechanically disrupted samples may indicate sensitivity to bead-beating within this genus. It is also possible due to the compositional nature of the dataset the addition of the bead-beading may have instead been more effective at lysing the gram-positive cell walls of the genus *Enterococcus*, resulting in its greater relative abundance. The genera *Clostridium* and *Romboutsia* on the other hand were not observed to be largely impacted by the addition of bead-beating. However, ultimately no significant difference was detected between these treatment groups which is in contrast to previous studies that have shown a significant increase in microbial diversity following the addition of bead-beating (Zhang et al., 2021).

Another aspect to the pre-sequencing pipeline that we explored was sample storage. Though it is well-established that methods for DNA extraction influence downstream results, sample storage may be equally important to the accurate representation of microbial communities (Rubin et al., 2013). For example, freeze-thaw cycles promote cell lysis, which can aid in the representation of difficult to lyse gram-positive species, but also diminish that of easier to lyse gram-negative cells (Larsen et al., 2015). Here, the gut microbiota community was shown to be robust to the additional freeze/thaw cycle at -80°C.

This finding contrasts with previous studies, which have documented storage temperature (Larsen et al., 2015) and duration (Rubin et a., 2013) to drive change in microbial community structure. However, in these studies samples are often frozen for a longer duration (15 days) (Larsen et al., 2015) as opposed to the relatively short 48 hours duration tested here. It is also possible the initial freezing of the fish, which technically serves as the first freeze/thaw cycle on these samples introduced bias to the community that was not further exacerbated by the second freeze/thaw cycle. Previous studies ultimately recommend extracting samples when fresh whenever possible to preserve the integrity of the microbiota within the sample (Larsen et al., 2015; Mølbak et al., 2006). Though a significant impact of an additional freeze/thaw cycle was not detected here, extracting sampling when fresh was prioritized for extraction workflow in chapter 3.

2.5.6 Extraction Kit's Varied in Gut Microbiota Characterization

Another way in which the extraction kits differed was in their community characterization. Discrepancies in downstream sequencing output produced by different commercially available extraction kits is in agreement with a plethora of previous studies on humans (Kennedy et al., 2014; Wu et al., 2010) and other vertebrate hosts (Hart et al., 2015;

Larsen et al., 2015). Relative abundance graphs annotated to the phylum and genus level showed strong agreement between the Omega E.Z.N.A Soil kit and Qiagen PowerFecal kits for both YT001 and YT002. However, the MasterPure Gram Positive kit characterized a largely different community. While it is possible the gut microbiome of aquaculture raised yellow tang to be almost entirely dominated by Firmicutes as the MasterPure Gram-Positive kit displayed in YT001 (Fig. 2.3A), previous literature would support it is unlikely one phylum would dominate so exclusively. In addition to Firmicutes, widely reported phyla characteristic of other marine fish gut microbiota include Proteobacteria, Bacteroidetes, Cyanobacteria, and Actinobacteria (Talwar et al., 2018; Llewellyn et al., 2014; Roeselers et al., 2011). The observed result for the Master Pure Gram-Positive kit instead is likely better explained by methodological bias.

It is possible the overnight enzymatic incubation step featured in the MasterPure Gram Positive protocol resulted in the digestion of a large amount of host tissue accounting for the heightened DNA quantity in these samples. It is well-established a high ratio of host to microbial DNA can cause amplification of non-target sequences (host DNA) due to similarity between prokaryotic and eukaryotic primer-annealing sites (Allen et a., 2015; Lundberg et al., 2013). Furthermore, we hypothesize the abundance of host DNA caused inefficient production of prokaryotic amplicons, resulting in the low microbial diversity detected.

2.5.7 Taxonomic Assessment

Though not the primary research objective of this chapter, the taxonomic data presented here serves as the first insight into the composition of the adult yellow tang gut microbiome. Firmicutes was ubiquitously characterized as the most dominant phyla among all extraction kits for both fish sampled. Bacteroides and Proteobacteria were identified as the second most

abundant phyla in YT001 and YT002 respectively. These findings are similar to previous reports for other adult marine fish. For example, Proteobacteria and Fusobacteria have been previously reported to be dominant phyla represented in the zebrafish gut microbiome (Roeselers et al., 2011; Hart et al., 2015; Stephens et al., 2016). Additionally, Talwar et al. in 2018, reported the phyla Proteobacteria, Firmicutes, and Bacteroidetes encompass 90% of the reported marine fish intestinal microbiota surveyed through 2017 (Talwar et al., 2018). While bacterial taxa in this study are not notably divergent from previously reported gut microbiota for other fish, it is important to note both yellow tang were sampled opportunistically upon natural mortality. Thus, the gut microbiota characterized may not accurately represent a healthy gut microbiota for adult yellow tang maintained at the Oceanic Institute.

One way in which the taxonomy characterized here did diverge from other studies is the lack of archaea. Though the V4 region targeted in this study includes coverage of archaea (Wasimuddin et al., 2020), only ASVs assigned to bacteria were identified. While typically a smaller component of the gut microbiota, archaea is still commonly reported in other marine fish (Egerton et al., 2018). It is likely archaea were less abundant within samples in this dataset and required a greater read depth for detection. Thus, in chapter 3 higher mean read depth was targeted for more complete community characterization.

Relative abundance of taxa also revealed interindividual variation, particularly at the genus level, between the two adult yellow tang sampled despite identical rearing conditions. Interindividual variation, despite identical rearing conditions, is unsurprising based on previous literature. For example, the human microbiome project revealed that despite the host genome of humans being over 99.5% identical, two adult individuals can show no overlap in the microbial

species detected within their microbiome indicating a strong role of host-modulation in gut microbiota structure (The Human Microbiome Project Consortium, 2012). These findings have been echoed in studies conducted on non-human vertebrates including zebrafish (Stephens et al., 2016) and mice (Rogers et al., 2014). In zebrafish, interindividual variation was found to increase with host age, despite the maintenance of identical rearing conditions (Stephens et al., 2016). These data suggest the ability of the host to regulate and optimize their own gut microbiome based on their physiological needs, may increase with development. It is also possible differing causes of death for YT001 and YT002 could be contributing to the observed differences of the gut microbiota.

2.6 Conclusions

The results of this study contribute to the library of knowledge on how extraction methodology influences downstream sequencing results. However, unique to other studies, this study exploits the difficult to culture host species, the yellow tang. Holding significant economic and cultural importance, developing sustainable ways to meet the demand of yellow tang in the aquarium trade is of significant value in Hawai‘i. Devising a robust methodology to be applied in studying critical microbial dynamics in aquaculture is a first step in this objective. The agreement in microbiota community structure among the Qiagen PowerFecal kit and Omega E.Z.N.A Soil kits, low replicate variation, and consistent successful amplification suggested these kits produced a more robust representation of the yellow tang gut microbial community than the MasterPure Gram Positive and MasterPure Complete kits. These data in combination with the time effective nature of the Qiagen PowerFecal kit, resulted in this kit being chosen for application in Chapter 3.

2.7 Citations

Allen, J. F. (2015). Why chloroplasts and mitochondria retain their own genomes and genetic systems: Colocation for redox regulation of gene expression. *Proceedings of the National Academy of Sciences*, *112*(33), 10231–10238. https://doi.org/10.1073/pnas.1500012112

Apprill, A., McNally, S., Parsons, R., & Weber, L. (2015). Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic Microbial Ecology*, 75(2), 129–137. http://doi.org/10.3354/ame01753

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., ... & Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, 37(8), 852-857.

Calado, R. (2017). The Need for Cultured Specimens. In *Marine Ornamental Species Aquaculture* (pp. 15–22). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119169147.ch2

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, 13(7), 581-583.

Cao, Q., Sun, X., Rajesh, K., Chalasani, N., Gelow, K., Katz, B., Shah, V. H., Sanyal, A. J., & Smirnova, E. (2021). Effects of Rare Microbiome Taxa Filtering on Statistical Analysis. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.607325

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., & Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences*, *108*(supplement_1), 4516–4522. https://doi.org/10.1073/pnas.1000080107

DeGruttola, A. K., Low, D., Mizoguchi, A., & Mizoguchi, E. (2016). Current understanding of dysbiosis in disease in human and animal models. *Inflammatory Bowel Diseases*, *22*(5), 1137–1150. https://doi.org/10.1097/MIB.0000000000000750

Egerton, S., Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). The Gut Microbiota of Marine Fish. *Frontiers in Microbiology*, 9. https://www.frontiersin.org/article/10.3389/fmicb.2018.00873

Elie, C., Perret, M., Hage, H., Sentausa, E., Hesketh, A., Louis, K., Fritah-Lafont, A., Leissner, P., Vachon, C., Rostaing, H., Reynier, F., Gervasi, G., & Saliou, A. (2023). Comparison of DNA extraction methods for 16S rRNA gene sequencing in the analysis of the human gut microbiome. *Scientific Reports*, *13*, 10279. https://doi.org/10.1038/s41598-023-33959-6

Gaffar, M. A., Zaman, M. K., Islam, M. S., Islam, M., Hossain, M. K., Shahriar, S. I. M., &

Shahjahan, M. (2023). Effects of probiotics on growth, survival, and intestinal and liver morphometry of Gangetic mystus (*Mystus cavasius*). *Saudi Journal of Biological Sciences*, *30*(7), 103683. https://doi.org/10.1016/j.sjbs.2023.103683

Hart, M. L., Meyer, A., Johnson, P. J., & Ericsson, A. C. (2015). Comparative Evaluation of DNA Extraction Methods from Feces of Multiple Host Species for Downstream Next-Generation Sequencing. *PloS One*, 10(11), e0143334. https://doi.org/10.1371/journal.pone.0143334

Holt, J. G., Leu, M. Y., Callan, C. K., & Erisman, B. (2017). *Large Angelfish and Other Pelagic Spawners* (pp. 251-278). John Wiley & Sons Ltd. https://doi.org/DOI:10.1002/9781119169147

Hornung, B. V. H., Zwittink, R. D., & Kuijper, E. J. (2019). Issues and current standards of controls in microbiome research. *FEMS Microbiology Ecology*, *95*(5), fiz045. https://doi.org/10.1093/femsec/fiz045

Hou, X., Pan, S., Lin, Z., Xu, J., & Zhan, X. (2021). Performance comparison of different microbial DNA extraction methods on bird feces. *Avian Research*, *12*(1), 19. https://doi.org/10.1186/s40657-021-00254-9

Kennedy, N. A., Walker, A. W., Berry, S. H., Duncan, S. H., Farquarson, F. M., Louis, P., Thomson, J. M., UK IBD Genetics Consortium, Satsangi, J., Flint, H. J., Parkhill, J., Lees, C. W., & Hold, G. L. (2014). The impact of different DNA extraction kits and laboratories upon the assessment of human gut microbiota composition by 16S rRNA gene sequencing. *PloS One*, 9(2), e88982. https://doi.org/10.1371/journal.pone.0088982

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology*, 79(17), 5112-5120.

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics. New York: Wiley. pp. 115–175.

Larsen, A. M., Mohammed, H. H., & Arias, C. R. (2015). Comparison of DNA extraction protocols for the analysis of gut microbiota in fishes. *FEMS Microbiology Letters*, 362(5). https://doi.org/10.1093/femsle/fnu031

Llewellyn, M. S., Boutin, S., Hoseinifar, S. H., Derome, N. (2014). Teleost microbiomes: The state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in Microbiology*, 5, 207. https://doi.org/10.3389/fmicb.2014.00207

Lundberg, D. S., Yourstone, S., Mieczkowski, P., Jones, C. D., & Dangl, J. L. (2013). Practical innovations for high-throughput amplicon sequencing. *Nature Methods*, *10*(10), 999–1002. https://doi.org/10.1038/nmeth.2634

Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J. C., Soulas, G., & Catroux, G. (2001). DNA Extraction from Soils: Old Bias for New Microbial Diversity Analysis Methods. *Applied and Environmental Microbiology*, 67(5), 2354–2359. https://doi.org/10.1128/AEM.67.5.2354-2359.2001

McMurdie PJ, Holmes S (2013) phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One* 8(4): e61217. https://doi.org/10.1371/journal.pone.0061217

Mohammadi, G., Rafiee, G., Tavabe, K. R., Abdel-Latif, H. M., & Dawood, M. A. (2021). The enrichment of diet with beneficial bacteria (single-or multi-strain) in biofloc system enhanced the water quality, growth performance, immune responses, and disease resistance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture*, *539*, 736640.

Mølbak, L., Sommer, H. M., Johnsen, K., Boye, M., Johansen, M., Møller, K., & Leser, T. D. (2006). Freezing at -80 degrees C distorts the DNA composition of bacterial communities in intestinal samples. *Current Issues in Intestinal Microbiology*, *7*(1), 29–34.

Montalban-Arques, A., De Schryver, P., Bossier, P., Gorkiewicz, G., Mulero, V., Gatlin, D. M., & Galindo-Villegas, J. (2015). Selective Manipulation of the Gut Microbiota Improves Immune Status in Vertebrates. *Frontiers in Immunology*, 6. https://www.frontiersin.org/articles/10.3389/fimmu.2015.00512

Oksanen, J., Simpson, G., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P., ... & Weedon, J. (2022). Vegan Community Ecology Package Version 2.6-2 April 2022. The Comprehensive R Archive Network. Available online: http://cran. r-project. org (accessed on 15 August 2022).

Palmtag, M. R. (2017). The Marine Ornamental Species Trade. In Marine Ornamental Species Aquaculture (pp. 3–14). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119169147.ch1

Parada, A. E., Needham, D. M., & Fuhrman, J. A. (2016). Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental Microbiology*, 18(5), 1403-1414.

Perry, W. B., Lindsay, E., Payne, C. J., Brodie, C., & Kazlauskaite, R. (2020). The role of the gut microbiome in sustainable teleost aquaculture. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1926), 20200184. https://doi.org/10.1098/rspb.2020.0184

Primo, E. D., Otero, L. H., Ruiz, F., Klinke, S., & Giordano, W. (2018). The disruptive effect of lysozyme on the bacterial cell wall explored by an in-silico structural outlook. *Biochemistry and Molecular Biology Education*, *46*(1), 83–90. https://doi.org/10.1002/bmb.21092

Rintala, A., Pietilä, S., Munukka, E., Eerola, E., Pursiheimo, J.-P., Laiho, A., Pekkala, S., &

Huovinen, P. (2017). Gut Microbiota Analysis Results Are Highly Dependent on the 16S rRNA Gene Target Region, Whereas the Impact of DNA Extraction Is Minor. *Journal of Biomolecular Techniques* : JBT, 28(1), 19–30. https://doi.org/10.7171/jbt.17-2801-003

Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Cavanaugh, C. M., Guillemin, K., Rawls, J. F. (2011). Evidence for a core gut microbiota in the zebrafish. *The ISME Journal*, 5(10), 1595–1608. https://doi.org/10.1038/ismej.2011.38

Rogers, G. B., Kozlowska, J., Keeble, J., Metcalfe, K., Fao, M., Dowd, S. E., Mason, A. J., McGuckin, M. A., & Bruce, K. D. (2014). Functional divergence in gastrointestinal microbiota in physically-separated genetically identical mice. *Scientific Reports*, *4*(1), 5437. https://doi.org/10.1038/srep05437

Rubin, B. E. R., Gibbons, S. M., Kennedy, S., Hampton-Marcell, J., Owens, S., & Gilbert, J. A. (2013). Investigating the Impact of Storage Conditions on Microbial Community Composition in Soil Samples. *PloS One*, *8*(7), e70460. https://doi.org/10.1371/journal.pone.0070460

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors – occurrence, properties and removal. *Journal of Applied Microbiology*, *113*(5), 1014–1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x

Stach, J. E., Bathe, S., Clapp, J. P., & Burns, R. G. (2001). PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. *FEMS Microbiology Ecology*, 36(2-3), 139-151. https://academic.oup.com/femsec/article/36/2-3/139/542189

Talwar, C., Nagar, S., Lal, R., Negi, R. K. (2018). Fish Gut Microbiome: Current Approaches and Future Perspectives. *Indian Journal of Microbiology*, 58(4), 397–414. https://doi.org/10.1007/s12088-018-0760-y

Tarnecki, A. M., Burgos, F. A., Ray, C. L., Arias, C. R. (2017). Fish intestinal microbiome: Diversity and symbiosis unraveled by metagenomics. *Journal of Applied Microbiology*, 123(1), 2–17. https://doi.org/10.1111/jam.13415

The Human Microbiome Project Consortium (2012) A framework for human microbiome research. Nature 486: 215–221.

Tlusty, M. (2002). The benefits and risks of aquacultural production for the aquarium trade. *Aquaculture*, 205(3), 203–219. https://doi.org/10.1016/S0044-8486(01)00683-4

Turjeman, S., & Koren, O. (2021). Using the microbiome in clinical practice. *Microbial Biotechnology*, 15(1), 129–134. https://doi.org/10.1111/1751-7915.13971

Videnska, P., Smerkova, K., Zwinsova, B., Popovici, V., Micenkova, L., Sedlar, K., & Budinska, E. (2019). Stool sampling and DNA isolation kits affect DNA quality and bacterial

composition following 16S rRNA gene sequencing using MiSeq Illumina platform. *Scientific Reports*, *9*(1), 13837. https://doi.org/10.1038/s41598-019-49520-3

Wasimuddin, Schlaeppi, K., Ronchi, F., Leib, S. L., Erb, M., & Ramette, A. (2020). Evaluation of primer pairs for microbiome profiling from soils to humans within the One Health framework. *Molecular Ecology Resources*, *20*(6), 1558–1571. https://doi.org/10.1111/1755-0998.13215

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis, Springer-Verlag New York. Available at: https://ggplot2.tidyverse.org.

Williams, I. D., Walsh, W. J., Claisse, J. T., Tissot, B. N., & Stamoulis, K. A. (2009). Impacts of a Hawaiian marine protected area network on the abundance and fishery sustainability of the yellow tang, *Zebrasoma flavescens*. *Biological Conservation*, 142(5), 1066–1073. https://doi.org/10.1016/j.biocon.2008.12.029

Willis, A. D., & Martin, B. D. (2022). Estimating diversity in networked ecological communities. *Biostatistics*, 23(1), 207-222.

Wu, S., Wang, G., Angert, E. R., Wang, W., Li, W., & Zou, H. (2012). Composition, Diversity, and Origin of the Bacterial Community in Grass Carp Intestine. *PLoS One*, *7*(2), e30440. https://doi.org/10.1371/journal.pone.0030440

Wu, G. D., Lewis, J. D., Hoffmann, C., Chen, Y.-Y., Knight, R., Bittinger, K., Hwang, J., Chen, J., Berkowsky, R., Nessel, L., Li, H., & Bushman, F. D. (2010). Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. *BMC Microbiology*, *10*(1), 206. https://doi.org/10.1186/1471-2180-10-206

Zhang, B., Brock, M., Arana, C., Dende, C., Hooper, L., & Raj, P. (2020). Impact of bead-beating intensity on microbiome recovery in mouse and human stool: Optimization of DNA extraction (p. 2020.06.15.151753). *bioRxiv*. https://doi.org/10.1101/2020.06.15.151753

Chapter 3: Succession of the Yellow Tang (*Zebrasoma flavescens*) Microbiome From Hatch to Settlement in a Recirculating Aquaculture System

3.1 Abstract

Ornamental fish aquaculture has increasingly garnered scientific interest as many wild fish populations have been negatively impacted by aquarium trade demands. Aquaculture may be a sustainable way to address these demands however, low juvenile recruitment remains a key bottleneck for many high-demand species. Here, the assembly of the gut microbiome of a single yellow tang (*Zebrasoma flavescens*) larval cohort from hatch to the juvenile stage (76 days) in a recirculating aquaculture system was investigated using a 16S rRNA gene high throughput sequencing approach. The taxonomic composition of bacteria and archaea were assessed from the gastrointestinal tract of larval yellow tang, their diet, and their recirculating rearing water at eight key developmental timepoints. We observed a dynamic gut microbiome that displayed developmental stage-specific signatures. In larvae samples, Proteobacteria was the most abundant phylum in 7/8 developmental stages, with the genus *Vibrio* representing a notable portion of the reads within this phylum. Other taxa were found to be more stage specific. Though below detection levels from 2-55dph, Fusobacteriota encompassed 52.72 ± 15.15% of the relative abundance in larvae samples at 76 dph. Pairwise comparisons of microbial communities via adonis across all sampling points revealed greater homogeneity between the larvae associated microbiome and the diet microbiome (p-value= 0.33) than that of the larvae and rearing water (p-value= 0.02), suggesting diet plays a stronger role in enteric microbiota seeding. Further investigation into the role diet may be playing in seeding larvae microbiota with potentially pathogenic *Vibrio* is a necessary next step in determining if this is a pathway contributing to larval yellow tang mortality in aquaculture.

3.2 Introduction

Removal of organisms in large quantities from the wild to satisfy aquarium trade demands continues to drive innovation in the developing ornamental aquaculture industry (Perry et al., 2020). Yellow tang (*Zebrasoma flavecens*), one of the most heavily exploited species for the aquarium trade in Hawai‘i, is an ideal candidate for commercial aquaculture production (Williams et al., 2009; DLNR, 2015). This species was first cultured in an aquaculture setting in 2015, marking a landmark achievement for the ornamental aquaculture field (Holt et al., 2017; Callan et al., 2018). However, high hatch-to-juvenile mortality rates of nearly 99% persist, limiting the availability of aquaculture-reared yellow tang on a commercial scale (Holt et al., 2017). In other marine teleosts, high larval mortality has been linked with microbial dysbiosis (Llewellyn et al., 2014). Gut microbiota assembly, a critical pathway of host-microbe interaction, is increasingly of scientific interest for improving larval survivorship in aquaculture (Perry et al., 2020).

The gut microbiome includes bacteria, archaea, viruses, and fungi, many of which play an important role in host immunity, digestion, and overall survivorship (Turnbaugh et al., 2007, Llewellyn et al., 2014, Talwar et al., 2018). Knowledge of the symbiosis between the gut microbiota and host has been primarily derived from human studies (Turnbaugh et al., 2007), as well as model laboratory species including mice (Goodman et al., 2011) and zebrafish (*Danio rerio*) (Roeselers et al., 2011; Rawls et al., 2004). In recent decades, increased access to next generation sequencing (NGS) and continued development of bioinformatic tools have driven the diversification of gut microbiome studies (Perry et al., 2020). However, our understanding of

non-model teleost fish gut microbiomes, including many commercially significant species, is limited (Llewellyn et al., 2014).

Like most other pelagic spawning teleost fish, larval yellow tang hatch with an underdeveloped physiology including a still growing gastrointestinal tract and closed mouths (Holt et al., 2017). Thus, the newly hatched larvae rely on endogenous feeding for the first days of life, where their yolk sac continues to be absorbed for nutrition. Around three days post-hatch (dph) yolk reserves are exhausted, and the larvae must adapt to hunting for food from their surrounding environment (termed exogenous feeding) (Olivotto et al., 2005). In yellow tang, this critical transition occurs during the first week post-hatch and is characterized by a peak in larval mortality (25-50%) that is a bottleneck for aquaculture production (Holt et al., 2017; Callan et al. 2018). This transition is a critical window for the long-term survival of the larvae, as the initial shift to exogenous feeding can play a large role in initial gut microbiota seeding and diversification (Egerton et al., 2018). In addition to feed, rearing water has also been shown to play a role in initial seeding of the enteric microbiota, possibly even prior to exogenous feeding (Giatsis et al., 2015).

While it is generally thought that the assembly of a favorable gut microbiome is vital to the development and survivorship of vulnerable yellow tang larvae (El-Saadony et al., 2021), little is known regarding the process by which they develop a gut microbiome through their early stages of development (Egerton et al., 2018). Here, we sought to investigate these processes by monitoring the assembly of the microbiota of a single yellow tang larval cohort over 76 days from hatch to the juvenile stage of development.

3.3 Methods

3.3.1 Yellow Tang Husbandry and Experimental Design

On May 30, 2023, a 200 L rearing tank was stocked with ~40,000 fertilized eggs from a single spawning event of adult brood stock yellow tang (Callan et al., 2018). Sampling occurred from June 1 to August 13, 2023 (Fig. 3.1). Rearing methods have been described in detail previously (Holt et al., 2017; Callan et al., 2018). Briefly, larvae were raised in a recirculating aquaculture system (RAS) that reuses rearing water after mechanical and biological filtration that incorporates 50µm and 100µm bag filters, a protein skimmer, ultraviolet light, a biofilter, and a bead filter. Larvae were fed overlapping periods of several different live feed organisms (Fig. 3.2) (DiMaggio et al. (2017).

Eight timepoints were selected to strategically sample significant developmental milestones and major changes in diet (Fig. 3.1, Fig. 3.2): (1) larvae rely on endogenous feeding prior to mouth opening (two dph); (2) larvae have begun transition to exogenous feeding on supplied copepod nauplii, and gut microbiome seeding has initiated (5 dph); (3) all larvae alive have successfully transitioned to exogenous feeding (8 dph); (4) rotifers (*Brachionus rotundiformis*) have been introduced as feed to supplement the copepod nauplii diet (13 dph); (5) dry feed and newly hatched *Artemia franciscana* have been introduced into the yellow tang diet, and copepod nauplii have been removed (20 dph); (6) larger *Artemia* enriched with Larviva Multigrain

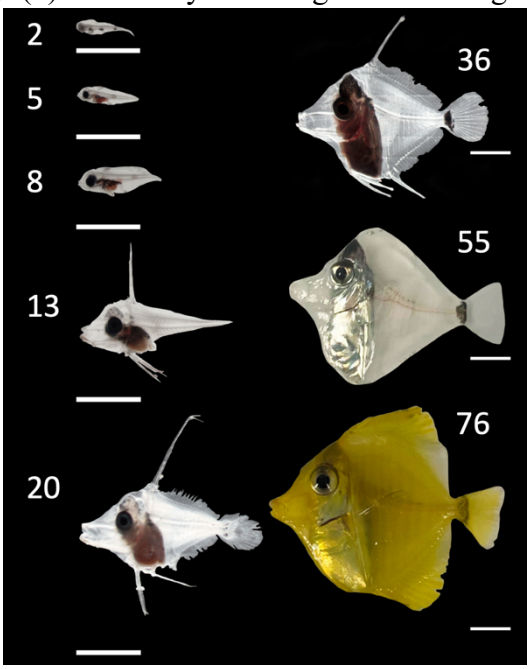


Figure 3.1: Larval yellow tang development from 2 days post-hatch (dph) to settlement (76 dph). Numbers indicate dph and scale bar indicates a length of 1 mm.

have been introduced into the yellow tang diet, and rotifers have been removed (36 dph); (7) newly hatched *Artemia* have been removed from the diet (55 dph); and (8) yellow tang have settled and are now considered juveniles (76 dph).

At approximately 18 dph, cultures of the *Artemia* feed stock were enriched with the additive Larviva Multigrain (BioMar, Aarhus, Denmark). This additive is designed to enrich the *Artemia* with essential nutrients required by marine larvae such as the yellow tang, including highly unsaturated fatty acids, vitamin C, and immunostimulants. The enrichment is also reported to contain the commercial probiotic Bactocell (Lallemand, Montreal, Canada) which is based on the lactic acid bacterium *Pediococcus acidilactici*. Additionally, micro-algae (*Isochrysis galbana*) were added to the rearing tanks starting ~ 2 dph at a concentration of ~ 300,000 cells/mL.

Four sample types were collected at each of the eight timepoints: (1) whole larvae or dissected guts, (2) water from the rearing tank, (3) particulate matter from the rearing tank (referred to as "tank food"), and (4) direct food samples prior to tank addition (referred to as "food stock"). Samples were collected in the morning approximately two hours after the first feed of the day, when applicable. At each time point, larval samples were collected in triplicate while rearing water, tank food, and food stock were collected as single samples. Samples were not collected for food stock at timepoints 1 and 2.

To collect "tank food" and rearing tank water samples, approximately one liter of rearing tank water was collected at each timepoint and filtered through a 40 µm mesh-sized nylon filter to capture larger particles. The nylon filter was then suspended in 800 µl of Qiagen's PowerFecal extraction kit's lysis buffer, vortexed on high speed for three minutes to release captured particles, and then pipetted into a screw-cap bead-beating tube for subsequent mechanical

disruption and extraction. This material served as the “tank food" sample type. Rearing water that passed through the the 40 µm filter was subsequently filtered through a 0.2µm pore-sized sterivex filter (company, city, state) using a peristaltic pump. The sterivex filter was removed from the filter casing as previously described (Cruaud et al., 2017) and stored in a QIAamp PowerFecal Pro bead-beating tube for subsequent mechanical disruption and extraction. The larvae readily avoided the beaker used to collect tank water; however, tank water and filters were visually inspected to ensure larvae were not captured in this process.

Food stock samples consisted of 250 mg of food collected directly from the source stock and stored in bead-beating tubes. In cases where multiple food items were being offered at a single timepoint, equal parts were added and mixed via vortex. All samples were extracted fresh immediately following collection.

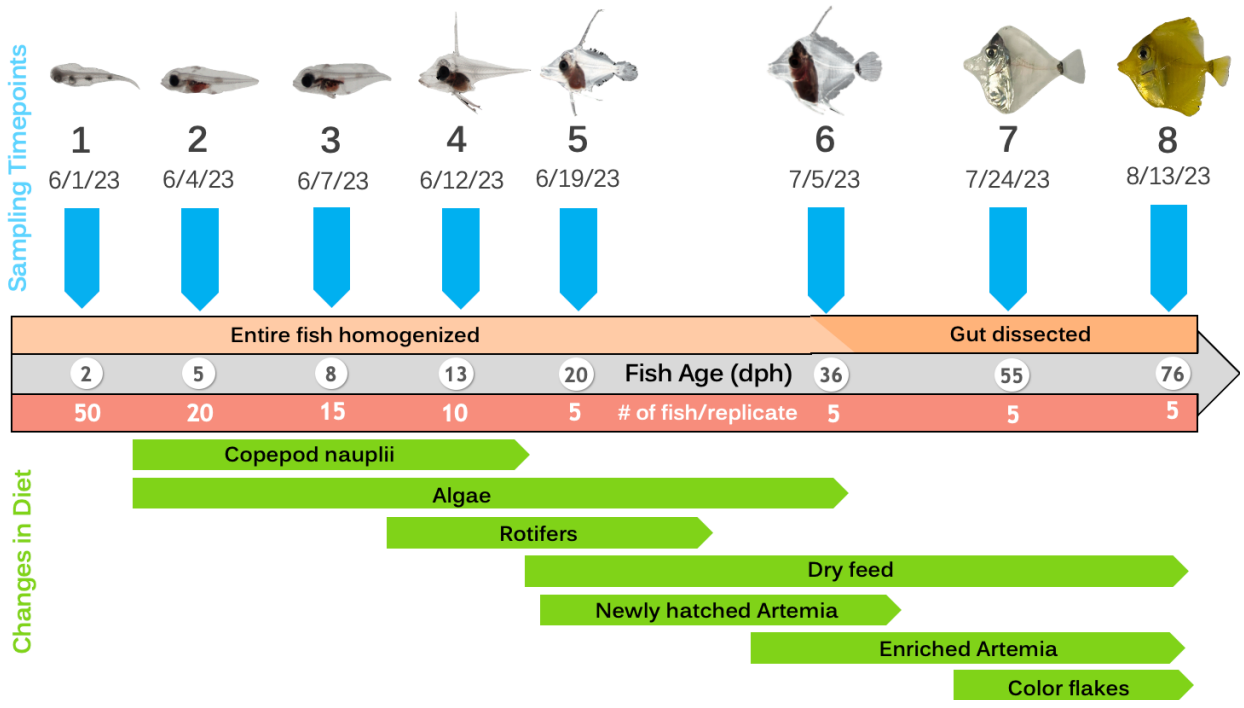


Figure 3.2: Timeline illustrating sampling design, notable developmental milestones, and major changes in diet. At each timepoint yellow tang gut or whole larvae, food, and tank water samples were collected.

Yellow tang sampling:

Yellow tang larvae were collected from the rearing tank using a fine nylon net and microscopically counted into three replicate samples. The number of larvae that were pooled for each replicate decreased across the study period to account for increasing body size over time (Fig. 3.2). Following enumeration, larvae were euthanized by rapid chilling following procedures outlined and approved by the Institutional Animal Care and Use Committee (IACUC). Larval specimens were thoroughly rinsed with 70% ethanol followed by three rinses with sterile water. For timepoints 1-5, larvae were too small for reasonable dissection of the gut and so the entire larval pools were homogenized (three replicate pools per timepoint) (Wilkes Walburn et al., 2018). For timepoints 6-8, the yellow tang gut was aseptically dissected prior to mechanical disruption following previously published procedures (Wilkes Walburn et al., 2018), pooled together, and homogenized (three replicate pools per timepoint).

3.3.2 DNA Extraction and Quantification

DNA extractions were conducted using the QIAamp PowerFecal Pro DNA kit (February 2020 protocol) (Qiagen, Germantown, MD, USA) following the manufacturer protocol and including the addition of a bead-beating step as recommended for gram-positive bacteria. Following extraction, DNA yield was fluorometrically quantified using the Qubit dsDNA BR (Broad-Range) and HS (High- Sensitivity) Assay Kits (Qubit 2.0, Life Technologies, Foster City, CA, USA).

3.3.3 Polymerase Chain Reaction and DNA Sequencing

Library preparation and sequencing were performed at the Microbial Genomics and Analytical Laboratory (MGAL) at the University of Hawai‘i at Mānoa. There, the gDNA was amplified using the Environmental Microbiome Project (EMP) primers 515F

GTGYCAGCMGCCGCGGTAA (Parada et al. 2016) and 806R GGACTACNVGGGTWTCTAAT (Apprill et al. 2015) to target the hypervariable V4 region of the 16S rRNA gene (Caporaso et al. 2011). Following amplification, Nextera sequencing libraries were prepared and sequenced on an Illumina Miseq at the Advanced Studies in Genomics, Proteomics, and Bioinformatics lab at University of Hawai‘i at Mānoa.

3.3.4 Bioinformatics and Statistical Analysis

Demultiplexed pair-end reads were assessed for quality in Qiime2 v2022.11 (Bolyen et al., 2019) using the DADA2 pipeline (Callahan et al. 2016) as described previously in Chapter 2. Plastid sequences were removed from the dataset prior to analysis. Statistical analyses and data visualizations were conducted in RStudio v2022.12.0+ using phyloseq (McMurdie & Holmes, 2013), ggplot2 (Wickham, 2016), and vegan (Oksanen et al., 2022). Point estimates (mean of all replicate estimates) of Shannon diversity were generated at the ASV level using the R package DivNet (Willis & Martin, 2022). For multivariate analysis across the entire dataset, the non-parametric strategy PERMANOVA (permutational multivariate statistical analysis of community separation) was implemented using the Adonis function within the vegan package. PERMANOVA tests were only conducted following confirmation of minimal dispersion within groupings using the ‘betdisper’ function, also within the vegan package. To further analyze community differences between timepoints, while considering the high-dimensional low sample size nature of the dataset, a hierarchical clustering strategy was implemented with the SigClust2 function (Kimes et al., 2017).

For phylogenetic analysis, all amplicon sequence variants (ASVs), defined as sequences with 100% nucleotide similarity, collected in this study and assigned to the genus *Vibrio* were

imported into the ARB software package (Ludwig et al., 2004). The sequences were aligned using SINA v.1.2.12 (Pruesse et al., 2012) to a curated database of all taxonomically described Vibrio based on 'The All-Species Living Tree' Project version LTP_08_2023 (Ludwig et al., 2021). Phylogenetic analyses were performed using the RAxML maximum likelihood method with the GTR model of nucleotide substitution under the gamma and invariable models of rate heterogeneity (Stamatakis, 2006), using a sequence mask of 248 nucleotide positions.

3.4 Results

3.4.1 Sequencing Summary

While DNA yield ranged from below the limit of detection of 0.001 ng/ul to 280 ng/µL for the 46 samples, all samples produced PCR amplification products and subsequent 16S rRNA gene amplicon sequence data (Table 3.1). Following quality control, 62,681 ± 25,962 reads per sample were retained for downstream analysis (Table 3.1 & Appendix Table 1). ASVs affiliated with the domain Bacteria dominated the dataset (1,495 bacterial vs. 16 archaea). Across the 46 samples, 246 genera were assigned to 23 phyla.

Table 3.1: Summary of samples collected from larval yellow tang, their food, and rearing water over the first 76 days of development. Read depth column contains retained reads following all quality control.

Sampling Event	Days Post-Hatch	Sample Type	No. of Larvae	Weight (mg)	DNA Yield (ng/µL)	Read Depth
1	2	Larvae	50	13	87.8	52,071
1	2	Larvae	50	13	89.2	74,986
1	2	Larvae	50	13	79	76,981
1	2	Tank Food			< 0.001	65,813
1	2	Water			4.36	61,993
2	5	Larvae	40	13	103	105,845
2	5	Larvae	40	16	89.2	91,586
2	5	Larvae	40	24	133	61,520
2	5	Tank Food			6	44,891
2	5	Water			108	6,728
3	8	Larvae	20	13	82	46,304
3	8	Larvae	20	8	73.2	65,773
3	8	Larvae	20	9	107	70,057
3	8	Tank Food			0.406	70,179
3	8	Food Stock		250	< 0.001	84,521
3	8	Water			122	7,060
4	13	Larvae	15	20	280	50,885
4	13	Larvae	15	21	238	84,074
4	13	Larvae	15	23	254	71,905
4	13	Tank Food			4.36	56,814
4	13	Food Stock		20	27.4	59,754
4	13	Water			112	5,487
5	20	Larvae	10	34	183	82,370
5	20	Larvae	10	52	173	72,958
5	20	Larvae	10	51	177	22,612
5	20	Tank Food			0.894	60,815
5	20	Food Stock		250	15	31,566
5	20	Water			69	13,623
6	36	Larvae	5	317	84.2	79,772
6	36	Larvae	5	138	137	103,823
6	36	Larvae	5	140	166	44,237
6	36	Tank Food			19.9	63,896
6	36	Food Stock		250	43.6	52,766
6	36	Water			30.4	36,230

7	55	Larvae	4	280	98	86,759
7	55	Larvae	4	262	64.4	87,566
7	55	Larvae	4	310	90.8	117,890
7	55	Tank Food			5.48	94,254
7	55	Food Stock		250	126	100,810
7	55	Water			14.1	67,009
8	76	Larvae	3	250	13.7	47,650
8	76	Larvae	3	250	11.8	63,231
8	76	Larvae	3	250	46	63,298
8	76	Tank Food			0.848	50,499
8	76	Food Stock		250	< 0.001	67,655
8	76	Water			1.49	56,827

3.4.2 Temporal Trends in the Development of the Yellow Tang Microbiome

Ordination using non-metric multidimensional scaling (NMDS) illustrated tighter clustering between replicate larval microbiomes from the same developmental stage than with those of other stages of development (Fig. 3.3A). This is apart from 36 and 55 dph, which showed notable overlap (Fig. 3.3A). Hierarchical clustering was then utilized to statistically compare timepoints to one another. In Figure 3.3 panel B, pink colored branches indicate a significant clustering defined at an alpha value of < 0.05. Yellow branches indicate a non-significant clustering (p-value > 0.05). Green branches were not tested for significance based on the threshold for minimum number of observations (n=9). Finally, blue branches were not tested for significance due to its parent branch determined to not be statistically significant to reduce change of type-one error. Hierarchical clustering analysis revealed significant grouping within the 2-8 dph larvae group, and the 13-76 dph larvae group (p-value = 0.00266) (Fig. 3.3B).

We next sought to determine if ASVs were consistently found across larval microbiome samples from the entire study period. Here, we defined these core ASVs as those occurring in at least 75% of larval samples from across all 8 sampling timepoints at a minimum detection

threshold of 0.05%. Four ASVs were core by these criteria, including three from the gammaproteobacterial genus *Vibrio* (ASVs 1, 17, and 19) and one from the alphaproteobacterial family Rhodobacteraceae.

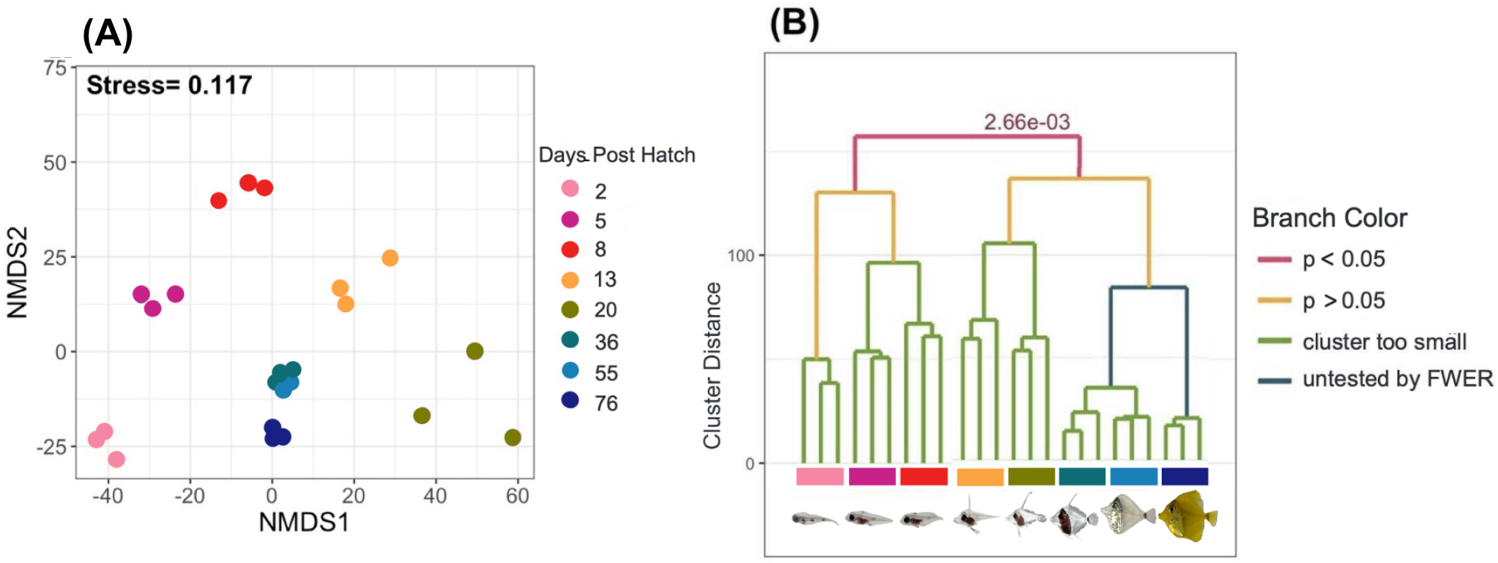


Figure 3.3: Ordination and hierarchical clustering of larval yellow tang samples from 2-76 days post-hatch. **(A):** Non-metric multidimensional scaling (NMDS) ordination generated using Aitchison's distances among larval microbiomes. **(B)** Dendrogram of larval microbiomes from hierarchical clustering with Aitchison's distances. Color bars indicate days post-hatch as in panel A. Significance of clustering indicated by the red and yellow color of the node. Family-wise error rate (FWER) controlling procedure only tests daughter nodes if FWER- corrected significance was achieved at the parent node. Green nodes were not tested for significant based on the minimum observation threshold of nine.

Next, changes in the larvae associated microbiota were investigated from 2 dph to 76 dph at the phylum (Fig. 3.4) and genus (Fig. 3.5) level. Proteobacteria, found ubiquitously in larvae samples across the study period, was the most abundant phylum in 7/8 timepoints (12.32-99.68%). Other taxa proved to be more stage specific. Though below detection levels from 2-55 dph, Fusobacteriota encompassed 52.72 ± 15.15% of the relative abundance at 76 dph. Similarly, Firmicutes encompasses < 1% of the relative abundance from 2 to 55 dph but accounted for 5.57% of the relative abundance at 76 dph. The gammaproteobacterial genus, *Vibrio* was

represented at all developmental stages and ranged from 3.92-97.10%. On average across all developmental stages, *Vibrio* was the single most abundant genera within the phylum Proteobacteria.

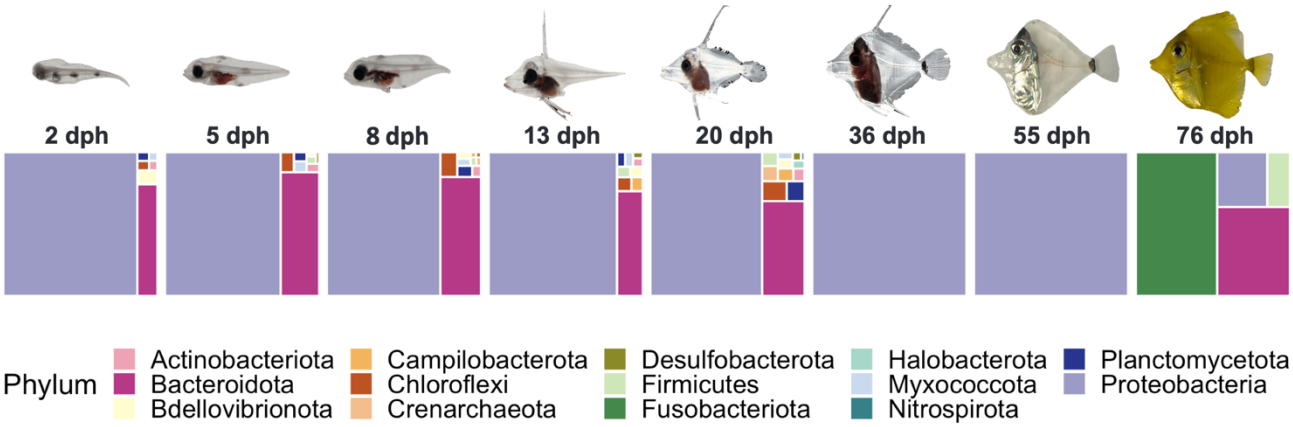


Figure 3.4: Treemap of the phylum-level mean relative abundance within microbiomes from eight timepoints spanning 2 to 76 days post-hatch for yellow tang larvae. The size of each tile is the mean relative abundance across replicates (n=3) at each developmental stage.

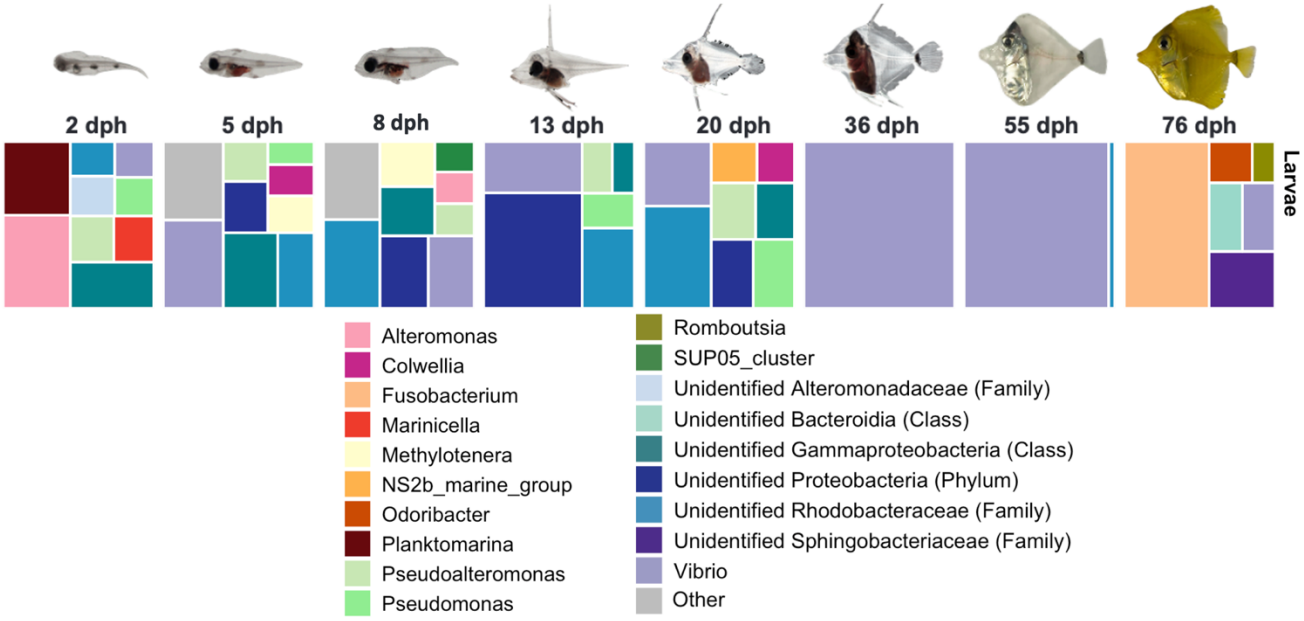


Figure 3.5: Treemap of the genus-level mean relative abundance within microbiomes from eight timepoints spanning 2 to 76 days post-hatch for yellow tang larvae. If taxonomy was not able to be resolved at the genus level, the next most specific taxonomic classification provided. The size of each tile is the mean relative abundance across replicates (n=3) at each developmental stage.

Across the dataset, 20 *Vibrio* ASVs were identified, with eight encompassing more than 1.5% of the relative abundance across the sampling events (Fig. 3.6). The ASVs themselves as well as their abundance changed from 2 to 76 dph. In larvae samples, ASVs 4 and 17 dominated in abundance in early timepoints 2, 3 and 4. At timepoints 6 and 7 a spike in *Vibrio* relative abundance (97.17% and 94.17% respectively) occurred almost entirely made up of ASV 17 and 19. The final timepoint had a low abundance of *Vibrio*, comparatively to other timepoints, encompassed primarily by ASV 2, 8, and 19.

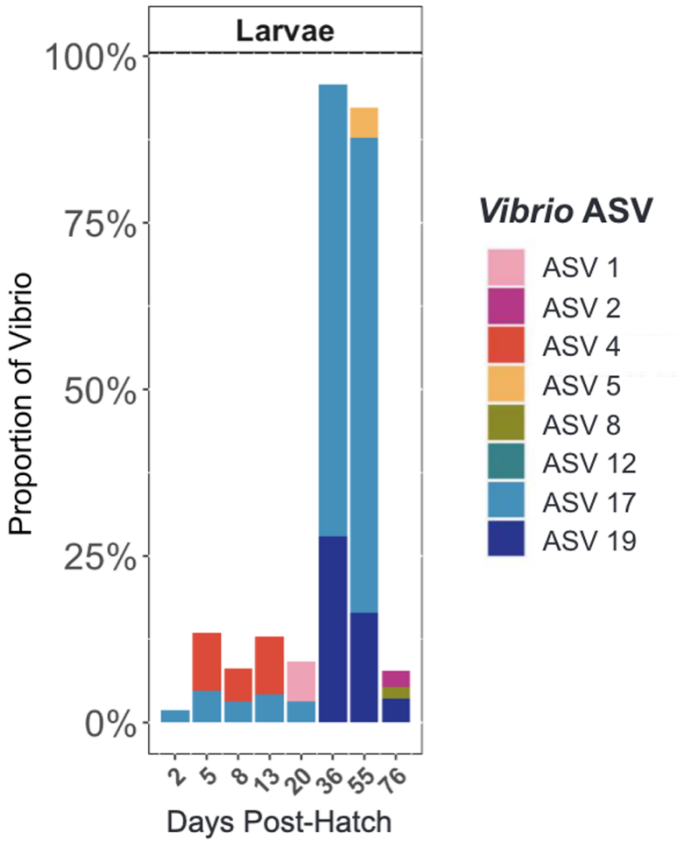


Figure 3.6: Relative abundance of *Vibrio* amplicon sequence variants (ASV) > 1.5% of the relative abundance across larvae replicates (n=3) for each sampling event. Each bar represents the average proportion of *Vibrio* at each timepoint. Color distinguishes *Vibrio* ASVs.

Phylogenetic analysis showed ASVs identified in this study are variable in their evolutionary relationships. Several of the *Vibrio* ASVs grouped closely with marine *Vibrio* spp. not associated with vibriosis. For example, ASV 7 has an identical sequence match within the V4 region to several *Salinivibrio* species. However, the most abundant *Vibrio* ASV identified in this study, ASV 17, has an identical V4 region sequence with well-known pathogens including *Vibrio fluvialis*, *V. tubiashii*, *V. neptunis, V. furnissii*, and *V. ostreae, V. intestinalis, and V. ichthyoenteri* (Fig. 3.7). Similarly, ASV 19, is closely related with several *Vibrio* spp. within the pathogenic Harveyi clade (Lin et al., 2012; Goudenège et al., 2012) including *Vibrio alginolyticus*, *V. azureus, V. harveyi*, *V. mytili*, and *V. natriegens* (Fig. 3.7).

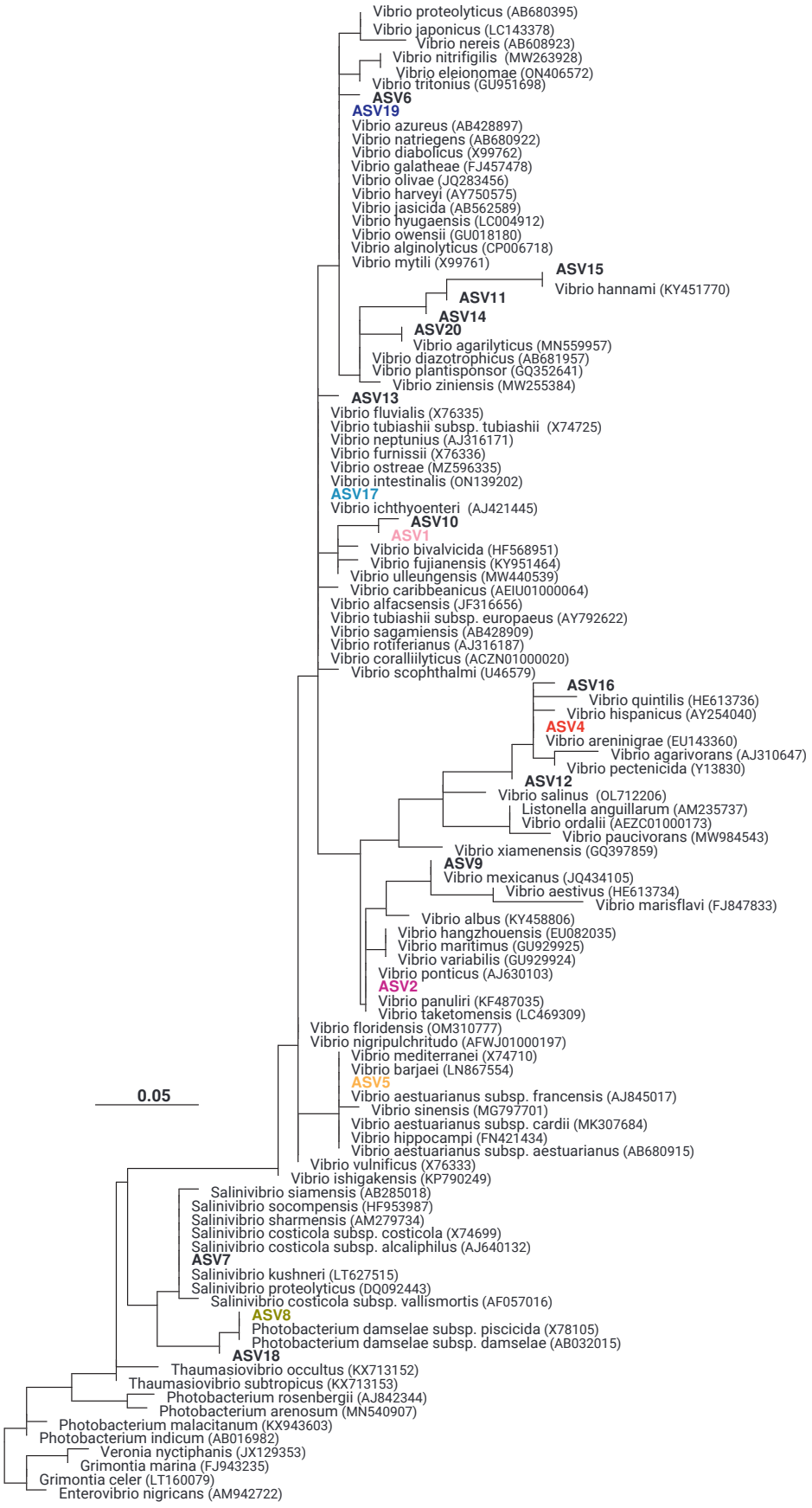

Vibrio proteolyticus (AB680395)
Vibrio japonicus (LC143378)
Vibrio nereis (AB608923)
Vibrio nitrifigilis (MW263928)
Vibrio eleionomae (ON406572)
Vibrio tritonius (GU951698)
ASV6
ASV19
Vibrio azureus (AB428897)
Vibrio natriegens (AB680922)
Vibrio diabolicus (X99762)
Vibrio galatheae (FJ457478)
Vibrio olivae (JQ283456)
Vibrio harveyi (AY750575)
Vibrio jasicida (AB562589)
Vibrio hyugaensis (LC004912)
Vibrio owensii (GU018180)
Vibrio alginolyticus (CP006718)
Vibrio mytili (X99761)
ASV15
Vibrio hannami (KY451770)
ASV11
ASV14
ASV20
Vibrio agarilyticus (MN559957)
Vibrio diazotrophicus (AB681957)
Vibrio plantisponsor (GQ352641)
Vibrio ziniensis (MW255384)
ASV13
Vibrio fluvialis (X76335)
Vibrio tubiashii subsp. tubiashii (X74725)
Vibrio neptunius (AJ316171)
Vibrio furnissii (X76336)
Vibrio ostreae (MZ596335)
Vibrio intestinalis (ON139202)
ASV17
Vibrio ichthyoenteri (AJ421445)
ASV10
ASV1
Vibrio bivalvicida (HF568951)
Vibrio fujianensis (KY951464)
Vibrio ulleungensis (MW440539)
Vibrio caribbeanicus (AEIU01000064)
Vibrio alfacsensis (JF316656)
Vibrio tubiashii subsp. europaeus (AY792622)
Vibrio sagamiensis (AB428909)
Vibrio rotiferianus (AJ316187)
Vibrio coralliilyticus (ACZN01000020)
Vibrio scophthalmi (U46579)
ASV16
Vibrio quintilis (HE613736)
Vibrio hispanicus (AY254040)
ASV4
Vibrio areninigrae (EU143360)
Vibrio agarivorans (AJ310647)
Vibrio pectenicida (Y13830)
ASV12
Vibrio salinus (OL712206)
Listonella anguillarum (AM235737)
Vibrio ordalii (AEZC01000173)
Vibrio paucivorans (MW984543)
Vibrio xiamenensis (GQ397859)
ASV9
Vibrio mexicanus (JQ434105)
Vibrio aestivus (HE613734)
Vibrio marisflavi (FJ847833)
Vibrio albus (KY458806)
Vibrio hangzhouensis (EU082035)
Vibrio maritimus (GU929925)
Vibrio variabilis (GU929924)
Vibrio ponticus (AJ630103)
ASV2
Vibrio panuliri (KF487035)
Vibrio taketomensis (LC469309)
Vibrio floridensis (OM310777)
Vibrio nigripulchritudo (AFWJ01000197)
Vibrio mediterranei (X74710)
Vibrio barjaei (LN867554)
ASV5
Vibrio aestuarianus subsp. francensis (AJ845017)
Vibrio sinensis (MG797701)
Vibrio aestuarianus subsp. cardii (MK307684)
Vibrio hippocampi (FN421434)
Vibrio aestuarianus subsp. aestuarianus (AB680915)
Vibrio vulnificus (X76333)
Vibrio ishigakensis (KP790249)
0.05
Salinivibrio siamensis (AB285018)
Salinivibrio socompensis (HF953987)
Salinivibrio sharmensis (AM279734)
Salinivibrio costicola subsp. costicola (X74699)
Salinivibrio costicola subsp. alcaliphilus (AJ640132)
ASV7
Salinivibrio kushneri (LT627515)
Salinivibrio proteolyticus (DQ092443)
Salinivibrio costicola subsp. vallismortis (AF057016)
ASV8
Photobacterium damselae subsp. piscicida (X78105)
Photobacterium damselae subsp. damselae (AB032015)
ASV18
Thaumasiovibrio occultus (KX713152)
Thaumasiovibrio subtropicus (KX713153)
Photobacterium rosenbergii (AJ842344)
Photobacterium arenosum (MN540907)
Photobacterium malacitanum (KX943603)
Photobacterium indicum (AB016982)
Veronia nyctiphanis (JX129353)
Grimontia marina (FJ943235)
Grimontia celer (LT160079)
Enterovibrio nigricans (AM942722)

Figure 3.7: Phylogenetic analysis illustrating relationships among the hypervariable region V4 of the16S rRNA gene from taxonomically described *Vibrio* and ASVs recovered in this study. The scale bar corresponds to 0.05 substitutions per nucleotide position. ASVs > 1.5% of the total relative abundance color coded.

3.4.3 Evaluation of Sample Type Dissimilarity

Multivariate analysis of all sample types (larvae, diet, and rearing water) regardless of time of sampling revealed microbiomes were more similar within the same sample type than those from different sample types (p-value= 0.01). Pairwise comparisons revealed that larval microbiomes were significantly different from rearing water (p-value= 0.021) and from food stock (p-value= 0.043), but were not significantly different from tank food (Table 3.2). Ordination and clustering echoed the results of the multivariate analysis showing close grouping between larval replicates and tank food samples. However, at 76dph a greater distance between larvae and both food and water samples were noted (Fig. 3.7).

Table 3.2: Results of pairwise multilevel comparisons using adonis for Larvae, Tank Food, Food Stock, and Water samples.

Variable	R^2	P-value*
Larvae vs Water	0.06	**0.021**
Larvae vs Tank Food	0.034	0.357
Larvae vs Food Stock	0.063	**0.043**
Water vs Tank Food	0.064	0.503
Water vs Food Stock	0.138	**0.004**
Tank Food vs Food Stock	0.124	**0.026**

**P-values were calculated from a distribution of 999 permutations post Bonferroni correction.*

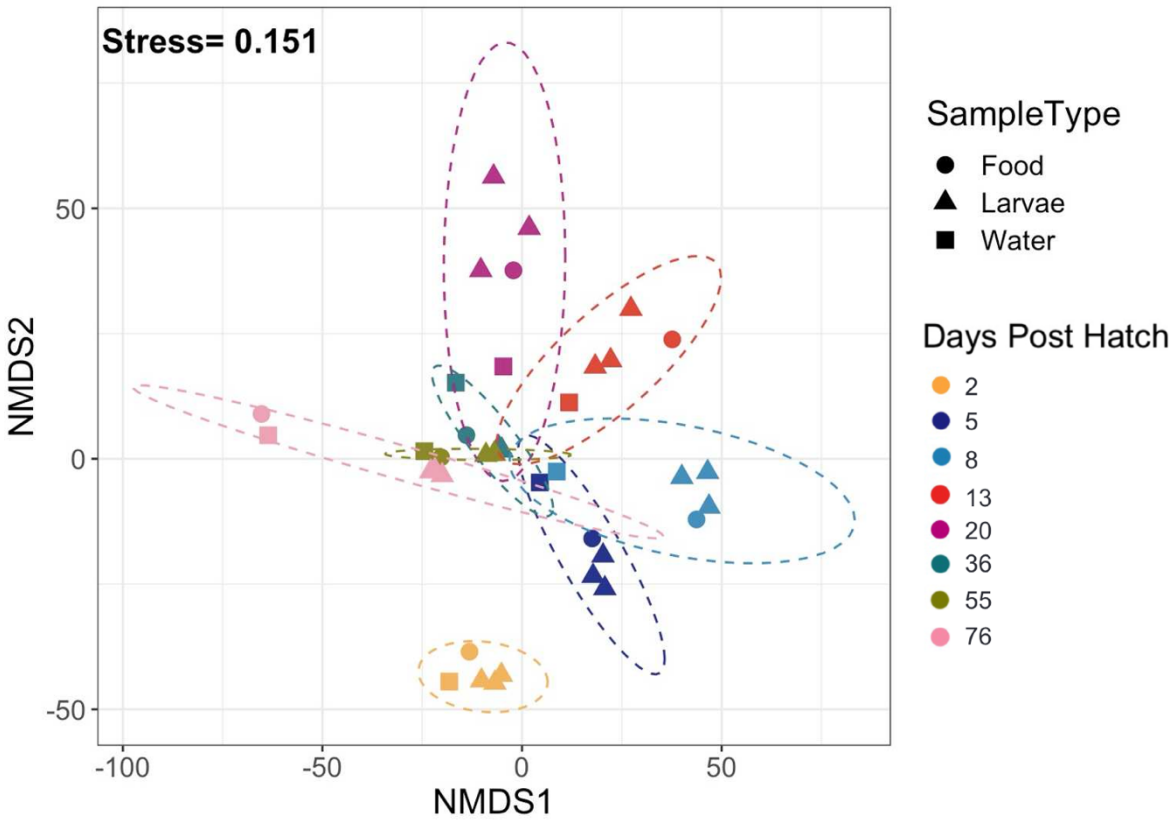


Figure 3.8: Non-metric multidimensional scaling (NMDS) ordination among tank food, water, and larvae samples from 2-76 dph. Generated with Aitchison's distances. Days post-hatch is differentiated by color and ellipses while sample type is indicated by shape.

We next looked at the Vibrio ASVs present in the food and water and found *Vibrio* to encompass a much greater proportion of the relative abundance in food samples compared to rearing water. Following a similar pattern to larvae samples, ASVs 4 and 17 dominated in abundance in early timepoints 2, 3 and 4 for diet samples. At timepoints 6 and 7 a spike in *Vibrio* relative abundance occurred almost entirely made up of ASV 17 and 19. In food stock, ASV 19 remained elevated at 76 dph.

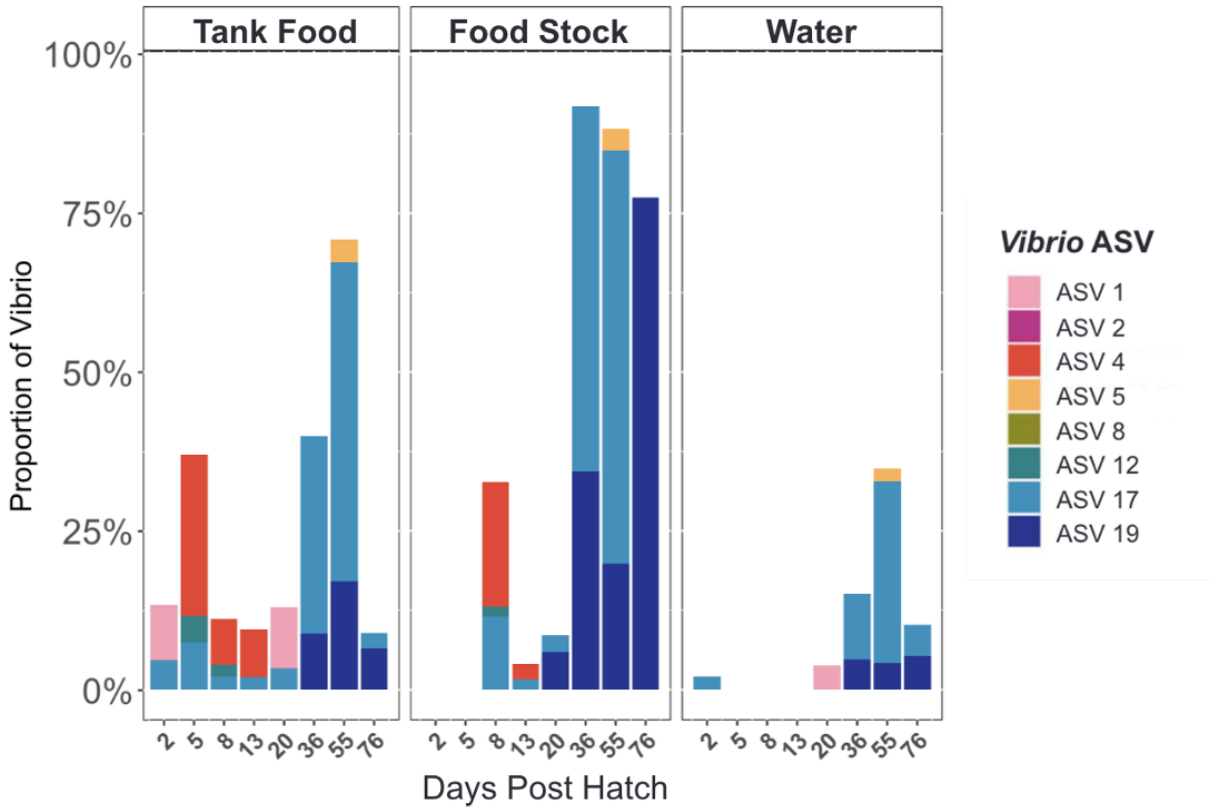


Figure 3.9: Relative abundance of *Vibrio* amplicon sequence variants (ASV) > 1.5% of the relative abundance in tank food, food stock, and water samples from 2 to 76 dph. Samples not collected for food stock at 2 and 5 dph. Color distinguishes *Vibrio* ASVs.

3.5 Discussion

3.5.1 Introduction

Producing high-quality juveniles is a bottleneck in marine aquaculture, especially for pelagic spawning ornamental species like the yellow tang (Moorhead & Zeng 2010; Tlusty 2002; Wabnitz et al., 2003). It has become increasingly evident that unfavorable microbial interactions are a major driver in larval mass mortalities (Villamil et al., 2003; Ransangan & Manin, 2010; Vadstein et al., 2013; Yang et al., 2022). Intensive aquaculture that fosters higher concentrations

of organic material and densities of organisms than is typically observed in the wild is particularly vulnerable to episodic outbreaks of opportunistic pathogens (Sanches-Fernandes et al., 2022). Newly hatched larvae that lack an established gut microbiota and immune system are less equipped to ward pathogens from invasion than their juvenile and adult counterparts (Auclert et al., 2024). Thus, the initial recruitment of favorable gut microbiota appears paramount to larvae survivorship and development (Auclert et al., 2024).

Despite the importance of the gut microbiome to host survival, its initial assembly is poorly defined (Luan et al., 2023). Here, using high throughput sequencing of the hypervariable V4 region of the 16S rRNA gene we documented the gut microbiome assembly of a captive-reared yellow tang cohort from hatch to settlement, and assessed how the respective microbial communities of their diet and rearing water may influence the initiation and development of the early gut microbiome. In sum, we provide the first insights into the initial assembly of the gut microbiome within this difficult to rear fish.

3.5.2 Larvae Gut Microbiota Community Structure

Previous studies characterizing marine teleost gut microbiomes have largely been conducted on the model zebrafish species *Danio rerio* (Rawls et al., 2004; Roeselers et al., 2011; Stephens et al., 2015). In an interesting parallel, we found that bacteria of the phylum Fusobacteriota (previously called Fusobacteria) only appeared in the developing microbiome of the yellow tang at 76 dph, when the yellow tang reached settlement and have transitioned to juveniles. This echoes previous zebrafish microbiome studies that found this phylum to be scarce in larvae, but abundant in adult intestines (Stephens et al., 2015). However, Stephens et al. reports 90% of reads within Fusobacteriota belonged to the genus *Cetobacterium* (Stephens et al., 2015), an obligate gut associate well-known for the synthesis of vitamin B12 and improving

host resistance to pathogens (Qi et al., 2023). Here, *Fusobacterium* was the primary genus within Fusobacteriota and instead is associated in high quantities with intestinal inflammation and gastrointestinal diseases (Engevik et al., 2021).

Other phyla commonly reported as major components within the gut microbiota of healthy marine fish include Firmicutes and Bacteroides (Huang et al., 2020; Talwar et a., 2018). These phyla are known to contain species that provide an array of essential health benefits to the host including short-chain fatty acid (SCFA) synthesis (Biddle et al., 2013) and immunomodulation (Fu & Jiang et al., 2018). On average across the larval stage, Bacteroides and Firmicutes encompassed only 14% and 1% of the relative abundance respectively for gut samples. In contrast, we found Proteobacteria to dominate the gut microbiota (77%) with *Vibrio* being the largest component of this phylum at 34.16%. Proteobacteria, despite being another common phylum in fish gut microbiomes (Huang et al., 2020), when present in large abundances can be an indication of dysbiosis due to the pathogenic species associated with this phylum (Xavier et al., 2023; Mekasha & Linke, 2021). Taken together, the relatively small abundance of known gut symbionts and the increased prevalence of potentially pathogenic taxa provide evidence supporting a general dysbiosis of the larval yellow tang gut microbiota (Xavier et al., 2023).

3.5.3 Gut Microbiota Displays Significant Temporal Variability & Small Core Community

We next sought to investigate if the gut microbiota exhibits change from hatch to the juvenile stage and if so, identify what taxa may be driving these changes. Hierarchical clustering, a strategy designed for high dimensional and low replicate datasets, supported the grouping of the larval phase into two distinct periods. Given the number of replicates able to be gathered at each sampling timepoint, pairwise comparisons of timepoints lacked strong statistical power.

However, visualization of gut samples via ordination and clustering provide support the enteric microbiota associated with the same developmental stage are more similar than those from different stages. These data collectively assert the gut microbiome of the yellow tang during the larval stage is a dynamic assemblage, which displays unique signatures over the timescale of just days to weeks.

Changes across larval development were particularly noticeable by the relative abundance patterns of *Vibrio*. Despite being ubiquitous within the gut across larval development, and on average being the most relatively abundant genus, the proportion of *Vibrio* varied considerably across developmental stages. Rapid restructuring of the gut microbiota during early life has been previously documented in other marine teleosts (Stephens et al., 2018) and in humans (Stewart et a., 2018). Thus, we conclude larval yellow tang follow this previously observed pattern of a variable gut microbiota in the early stages of life. In adult vertebrates, a more stable gut microbiome is typically observed, though factors like diet and disease can still be influential in its overall structure and composition (Sieler Jr. et al., 2023). Continuing to document the succession of the yellow tang gut microbiome through the juvenile stage and into adulthood in a future study would be beneficial to identify if the same pattern is observed for this species.

In alignment with results illustrating a rapidly restructuring enteric microbiota, a very small number of "core" microbiota were able to be detected among gut samples. Of the four core ASVs identified, three of which belonged to the genus *Vibrio*. Core microbiota generally refers to taxa that are consistently present across most individuals or samples across populations (Sharon et al., 2022). Previous studies, in vertebrates such as mice (Wang et a., 2019) and zebrafish (Roeselers et al., 2011), have sought to identify core microbial taxa characteristic of

these hosts under the assumption that the consistent presence of these taxa indicates an important symbiosis. For example, Roeselers et al. in 2011 identified 21 shared operational taxonomic units (OTUs) present among the guts of zebrafish raised within different aquaculture facilities as well as those that were recently captured from the wild. These OTUs were comprised of 12 genera within the γ-Proteobacteria, β-Proteobacteria, Fusobacteriota, Bacilli, Flavobacteria and Actinobacteria classes, with *Aeromonas* and *Shewanella* being the most common genera. Here, the lack of persistent taxa across the larval stage suggests that host selection and maintenance of microbiota is likely limited during the early larval stage.

3.5.4 Diet as a Key Vector of Microbiota

The next question we sought to answer was, if the gut microbiota is exhibiting change across the larval period, what is driving these changes and how may this be impacting larval survival. Diet and rearing water are known vectors of both favorable and pathogenic microbiota to larvae (Sanches-Fernanes et al., 2022). In this study, pairwise testing using the non-parametric PERMANOVA strategy indicated homogeneity between tank food and the larvae, providing support for a close association between the microbiota of these environments. Ordination of the tank food, gut, and water sample types provided concurring evidence of this pattern. In agreement with previous studies (Wang et al., 2022; Stephens et al., 2016; De Angelis et al., 2020; Sieler et al., 2023), we conclude diet is playing an influential role in early gut microbiota seeding and is likely a mechanism driving the observed changes in gut microbiota structure across the study period. Rearing water, despite likely playing a role in gut microbiota seeding in addition to diet (Giatsis et al., 2015), appeared more distinct from the larval gut microbiota.

The close association between the larvae microbiota and microbiota associated with their diet promoted a closer investigation into microbial community structure. The diet provided to

captive reared larval yellow tang is dominated by live feed. Live feed, including copepods, Rotifers (*Branchionus* spp.), and brine shrimp (*Artemia*) are essential initial prey items in larviculture due to their small size and nutritional potential (Sanches-Fernanes et al., 2022). However, due to the high load of organic material present in first feed cultures, proliferation of opportunistic bacteria is often observed (Sanches-Fernanes et al., 2022). Rotifers, introduced to yellow tang tanks at ~10dph, are known to associate with an array of widely distributed marine microbes including the genera *Marinomonas*, *Pseudoalteromonas*, *Aeromonas*, and *Photobacterium* (Ishino et a., 2012; Dhont et al., 2013; Rombaut et al., 2001). However, Rotifers can also host a variety of pathogenic *Vibrio* spp. in large abundances. For example, well-known aquaculture pathogens *Vibrio alginolyticus* (Gómez-León et al., 2005), *V. anguillarum* (Frans et al., 2011), *V. parahaemolyticus* (Yang et al., 2022), *V. harveyi* (Zhang et al., 2020), and *V. rotiferianus* (Zhang et al., 2014) have been previously isolated from Rotifer cultures (Sanches-Fernanes et al., 2022). *Artemia* are also believed to associate with similar pathogens including *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. anguillarum*, *V. harveyi* and *V. hispanicus* (Sanches-Fernanes et al., 2022).

Phylogenetic analysis showed ASV 19 to have an identical sequence match within the V4 region to *Vibrio alginolyticus* and *Vibrio harveyi*, both established pathogenic species (Gómez-León et al., 2005; Zhang et al., 2020) known to be associated with Rotifers and *Artemia* cultures (Sanches-Fernanes et al., 2022). Though we cannot definitively resolve if ASV 19 is one of those exact species, most of the other species it shares the exact same V4 sequence with are also known pathogens. The close relationship of abundant *Vibrio* ASVs observed in this study with pathogenic species provides evidence that the high relative abundance of Vibrio may be contributing to larval yellow tang mortality. Moreover, the phylogenic analysis encourages

further investigation into the role diet may be playing in exposing the larvae to these pathogenic species.

We interestingly noted an increase in the dissimilarity between tank food and gut samples particularly at 76dph, despite their close association from 3-55dph. A similar pattern has been documented previously in the model species, zebrafish. For example, Stephens et al. found the microbiota of the surrounding environment to be more similar to the gut microbiota of larval individuals than adults (Stephens et al., 2016). Additionally, Stephens et al. found increasing interindividual variation among a cohort of zebrafish through the larval stage and into adulthood. These findings suggest an increasing ability of teleosts to curate favorable microbiota based on physiological requirements and exclude unfavorable taxa introduced through environmental factors. Furthermore, we hypothesize upon settlement, host selective pressures become a more deterministic factor in gut microbiota structure for yellow tang. This results in the gut microbiota becoming more distinct from environmental factors like their diet and may contribute to the increased survival rate that is characteristic of settlement.

3.6 Conclusions

Understanding and managing host-microbiota interactions is critical to achieving a more sustainable aquaculture industry (Perry et al., 2020; Auclert et al., 2024). Here, for the first time we characterize the gut microbiota assembly for the difficult to culture ornamental species, yellow tang. Data presented in this study indicate the larval microbiome to be largely dominated by *Vibrio*, with diet likely a key vector of this genus. We hypothesize the prevalence of potentially pathogenic *Vibrio* spp. in combination with an unestablished gut microbiome may be contributing to the high mortality rates characteristic of the larval stage. Moreover, earlier

establishment of a favorable gut microbiota may increase larval resistance to pathogens they are episodically exposed to throughout the larval stage. Improving our understanding of interactions between captive-raised yellow tang with the microbial landscape in an aquaculture environment is a vital step in improving juvenile output. Increasing production of juveniles is key to generating a sustainable supply of yellow tang for aquarium trade demands and preserving wild populations of this ecologically and economically important species.

3.7 Future Directions

The data presented here provide the groundwork for further investigation of microbial dynamics found within yellow tang larviculture. Moreover, this work points to several interesting avenues worthy of further investigation. Here we found several lines of evidence supporting a dysbiosis of gut microbiota throughout the larval stage. We hypothesize that in adult and juvenile individuals where survivorship increases to > 90%, a more favorable and established microbiome is present. Exposing yellow tang larvae to juvenile and/or adult yellow tang fecal matter would be an interesting exploration to determine if this exposure would promote the assimilation of symbiotic gut microbiota present in mature gut microbiome earlier in the larval stage and if this phenomenon impacts larval survival. In this study, interindividual variation among larvae were not explored, however, this could also be an interesting area of study as previous work has indicated marine fish larvae become increasingly distinct from one another and their environment through the larval stage (Stephens et al., 2018). Another important avenue of future study would be to resolve which *Vibrio* spp. yellow tang larvae are exposed to in the aquaculture setting. Based on the results presented here, we recommend specific investigation into the live feed cultures. For more robust species identification, alternative methods such as qPCR assays or

metagenomics may be explored. Reliable identification of *Vibrio* spp. the larvae routinely encounter through their diet is of prime importance for clarifying the role this genus may be playing in larvae mortality and better inform strategies to mitigate exposure.

3.8 Citations

Apprill, A., McNally, S., Parsons, R., & Weber, L. (2015). Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic Microbial Ecology*, 75(2), 129–137. https://doi.org/10.3354/ame01753

Auclert, L. Z., Chhanda, M. S., & Derome, N. (2024). Interwoven processes in fish development: Microbial community succession and immune maturation. *PeerJ*, 12, e17051. https://doi.org/10.7717/peerj.17051

Biddle, A., Stewart, L., Blanchard, J., & Leschine, S. (2013). Untangling the Genetic Basis of Fibrolytic Specialization by Lachnospiraceae and Ruminococcaceae in Diverse Gut Communities. *Diversity*, 5(3), Article 3. https://doi.org/10.3390/d5030627

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, 37(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, 13(7), 581–583. https://doi.org/10.1038/nmeth.3869

Callan, K, C., Burgess, A., Rothe, C., & Touse, R. (2018). Development of Improved Feeding Methods in the Culture of Yellow Tang, *Zebrasoma flavescens*. *Journal of the World Aquaculture Society*. 49. https://doi.org/10.1111/jwas.12496

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., & Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America*, 108 Suppl 1(Suppl 1), 4516–4522. https://doi.org/10.1073/pnas.1000080107

Cruaud, P., Vigneron, A., Fradette, M.-S., Charette, S. J., Rodriguez, M. J., Dorea, C. C., & Culley, A. I. (2017). Open the SterivexTM casing: An easy and effective way to improve DNA extraction yields. *Limnology and Oceanography: Methods*, 15(12), 1015–1020. https://doi.org/10.1002/lom3.10221

De Angelis, M., Ferrocino, I., Calabrese, F. M., De Filippis, F., Cavallo, N., Siragusa, S., Rampelli, S., Di Cagno, R., Rantsiou, K., Vannini, L., Pellegrini, N., Lazzi, C., Turroni, S., Lorusso, N., Ventura, M., Chieppa, M., Neviani, E., Brigidi, P., O'Toole, P. W., … Cocolin, L. (2020). Diet influences the functions of the human intestinal microbiome. *Scientific Reports*, 10(1), 4247. https://doi.org/10.1038/s41598-020-61192-y

DiMaggio, M. A., Cassiano, E. J., Barden, K. P., Ramee, S. W., Ohs, C. L., & Watson, C. A. (2017). First Record of Captive Larval Culture and Metamorphosis of the Pacific Blue Tang, *Paracanthurus hepatus*. *Journal of the World Aquaculture Society*, 48(3), 393–401. https://doi.org/10.1111/jwas.12426

DLNR (Department of Land and Natural Resources). 2015 Report on the findings and recommendations of effectiviness of the West Hawai‘i regional fishery management area. Report of the Thirtieth Legislature. Accessed January 20, 2024 at chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://dlnr.hawaii.gov/dar/files/2015/01/ar_hrs188_2015.pdf

Egerton, S., Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). The Gut Microbiota of Marine Fish. *Frontiers in Microbiology*, 9. https://www.frontiersin.org/article/10.3389/fmicb.2018.00873

El-Saadony, M. T., Alagawany, M., Patra, A. K., Kar, I., Tiwari, R., Dawood, M. A. O., Dhama, K., & Abdel-Latif, H. M. R. (2021). The functionality of probiotics in aquaculture: An overview. *Fish & Shellfish Immunology*, *117*, 36–52. https://doi.org/10.1016/j.fsi.2021.07.007

Engevik, M. A., Danhof, H. A., Ruan, W., Engevik, A. C., Chang-Graham, A. L., Engevik, K. A., Shi, Z., Zhao, Y., Brand, C. K., Krystofiak, E. S., Venable, S., Liu, X., Hirschi, K. D., Hyser, J. M., Spinler, J. K., Britton, R. A., & Versalovic, J. (2021). Fusobacterium nucleatum Secretes Outer Membrane Vesicles and Promotes Intestinal Inflammation. *mBio*, 12(2), e02706-20. https://doi.org/10.1128/mBio.02706-20

Frans, I., Michiels, C. W., Bossier, P., Willems, K. A., Lievens, B., & Rediers, H. (2011). Vibrio anguillarum as a fish pathogen: Virulence factors, diagnosis and prevention. *Journal of Fish Diseases*, 34(9), 643–661. https://doi.org/10.1111/j.1365-2761.2011.01279.x

Fu, C., & Jiang, A. (2018). Dendritic Cells and CD8 T Cell Immunity in Tumor Microenvironment. *Frontiers in Immunology*, 9, 3059. https://doi.org/10.3389/fimmu.2018.03059

Giatsis, C., Sipkema, D., Smidt, H., Heilig, H., Benvenuti, G., Verreth, J., & Verdegem, M. (2015). The impact of rearing environment on the development of gut microbiota in tilapia larvae. *Scientific Reports*, 5, 18206. https://doi.org/10.1038/srep18206

Gómez-León, J., Villamil, L., Lemos, M. L., Novoa, B., & Figueras, A. (2005). Isolation of Vibrio alginolyticus and Vibrio splendidus from Aquacultured Carpet Shell Clam (Ruditapes decussatus) Larvae Associated with Mass Mortalities. *Applied and Environmental Microbiology*, 71(1), 98–104. https://doi.org/10.1128/AEM.71.1.98-104.2005

Goodman, A. L., Kallstrom, G., Faith, J. J., Reyes, A., Moore, A., Dantas, G., & Gordon, J. I. (2011). Extensive personal human gut microbiota culture collections characterized and

manipulated in gnotobiotic mice. *Proceedings of the National Academy of Sciences*, 108(15), 6252–6257. https://doi.org/10.1073/pnas.1102938108

Goudenège, D., Boursicot, V., Versigny, T., Bonnetot, S., Ratiskol, J., Sinquin, C., LaPointe, G., Roux, F. L., & Delbarre-Ladrat, C. (2014). Genome sequence of Vibrio diabolicus and identification of the exopolysaccharide HE800 biosynthesis locus. *Applied Microbiology and Biotechnology*, 98(24), 10165–10176. https://doi.org/10.1007/s00253-014-6086-8

Holt, G. J., Leu, M.-Y., Callan, C. K., & Erisman, B. (2017). Large Angelfish and Other Pelagic Spawners. In Marine Ornamental Species Aquaculture (pp. 251–278). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119169147.ch15

Huang, Q., Sham, R. C., Deng, Y., Mao, Y., Wang, C., Zhang, T., & Leung, K. M. Y. (2020). Diversity of gut microbiomes in marine fishes is shaped by host-related factors. *Molecular Ecology*, 29(24), 5019–5034. https://doi.org/10.1111/mec.15699

Kimes, P. K., Liu, Y., Neil Hayes, D., & Marron, J. S. (2017). Statistical significance for hierarchical clustering. *Biometrics*, 73(3), 811–821. https://doi.org/10.1111/biom.12647

Lin, B., Wang, Z., Malanoski, A. P., O'Grady, E. A., Wimpee, C. F., Vuddhakul, V., Alves Jr, N., Thompson, F. L., Gomez-Gil, B., & Vora, G. J. (2010). Comparative genomic analyses identify the Vibrio harveyi genome sequenced strains BAA-1116 and HY01 as Vibrio campbellii. *Environmental Microbiology Reports*, 2(1), 81–89. https://doi.org/10.1111/j.1758-2229.2009.00100.x

Llewellyn, M. S., Boutin, S., Hoseinifar, S. H., & Derome, N. (2014). Teleost microbiomes: The state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in Microbiology*, 5, 207. https://doi.org/10.3389/fmicb.2014.00207

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, null, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., … Schleifer, K.-H. (2004). ARB: A software environment for sequence data. *Nucleic Acids Research*, 32(4), 1363–1371. https://doi.org/10.1093/nar/gkh293

Ludwig, W., Viver, T., Westram, R., Francisco Gago, J., Bustos-Caparros, E., Knittel, K., Amann, R., & Rossello-Mora, R. (2021). Release LTP_12_2020, featuring a new ARB alignment and improved 16S rRNA tree for prokaryotic type strains. *Systematic and Applied Microbiology*, 44(4), 126218. https://doi.org/10.1016/j.syapm.2021.126218

Mekasha, S., & Linke, D. (2021). Secretion Systems in Gram-Negative Bacterial Fish Pathogens. *Frontiers in Microbiology*, 12. https://doi.org/10.3389/fmicb.2021.782673

Moorhead, J. A., & Zeng, C. (2010). Development of Captive Breeding Techniques for Marine Ornamental Fish: A Review. *Reviews in Fisheries Science*, 18(4), 315–343. https://doi.org/10.1080/10641262.2010.516035

Mougin, J., Flahaut, C., Roquigny, R., Bonnin-Jusserand, M., Grard, T., & Le Bris, C. (2020). Rapid Identification of Vibrio Species of the Harveyi Clade Using MALDI-TOF MS Profiling With Main Spectral Profile Database Implemented With an In-House Database: Luvibase. *Frontiers in Microbiology*, 11, 586536. https://doi.org/10.3389/fmicb.2020.586536

Neu, A. T., Allen, E. E., & Roy, K. (2021). Defining and quantifying the core microbiome: Challenges and prospects. *Proceedings of the National Academy of Sciences*, 118(51), e2104429118. https://doi.org/10.1073/pnas.2104429118

Olivotto, I., Zenobi, A., Rollo, A., Migliarini, B., Avella, M., & Carnevali, O. (2005). Breeding, rearing and feeding studies in the cleaner goby *Gobiosoma evelynae*. *Aquaculture*, 250(1), 175–182. https://doi.org/10.1016/j.aquaculture.2005.02.057

Parada, A. E., Needham, D. M., & Fuhrman, J. A. (2016). Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental Microbiology*, 18(5), 1403–1414. https://doi.org/10.1111/1462-2920.13023

Perry, W. B., Lindsay, E., Payne, C. J., Brodie, C., & Kazlauskaite, R. (2020). The role of the gut microbiome in sustainable teleost aquaculture. *Proceedings of the Royal Society B: Biological Sciences*, 287(1926), 20200184. https://doi.org/10.1098/rspb.2020.0184

Pruesse, E., Peplies, J., & Glöckner, F. O. (2012). SINA: Accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics*, 28(14), 1823–1829. https://doi.org/10.1093/bioinformatics/bts252

Qi, X., Zhang, Y., Zhang, Y., Luo, F., Song, K., Wang, G., & Ling, F. (2023). Vitamin B12 produced by Cetobacterium somerae improves host resistance against pathogen infection through strengthening the interactions within gut microbiota. *Microbiome*, 11(1), 135. https://doi.org/10.1186/s40168-023-01574-2

Ransangan, J., & Manin, B. O. (2010). Mass mortality of hatchery-produced larvae of Asian seabass, Lates calcarifer (Bloch), associated with viral nervous necrosis in Sabah, Malaysia. *Veterinary Microbiology*, 145(1–2), 153–157. https://doi.org/10.1016/j.vetmic.2010.03.016

Rawls, J. F., Samuel, B. S., & Gordon, J. I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America*, 101(13), 4596–4601. https://doi.org/10.1073/pnas.0400706101

Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Cavanaugh, C. M., Guillemin, K., & Rawls, J. F. (2011). Evidence for a core gut microbiota in the zebrafish. *The ISME Journal*, 5(10), 1595–1608. https://doi.org/10.1038/ismej.2011.38

Sanches-Fernandes, G. M. M., Sá-Correia, I., & Costa, R. (2022). Vibriosis Outbreaks in Aquaculture: Addressing Environmental and Public Health Concerns and Preventive Therapies Using Gilthead Seabream Farming as a Model System. *Frontiers in Microbiology*, 13. https://doi.org/10.3389/fmicb.2022.904815

Sieler, M. J., Al-Samarrie, C. E., Kasschau, K. D., Varga, Z. M., Kent, M. L., & Sharpton, T. J. (2023). Disentangling the link between zebrafish diet, gut microbiome succession, and Mycobacterium chelonae infection. *Animal Microbiome*, 5(1), 38. https://doi.org/10.1186/s42523-023-00254-8

Stamatakis, A. (2006). RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, 22(21), 2688–2690. https://doi.org/10.1093/bioinformatics/btl446

Stephens, W. Z., Burns, A. R., Stagaman, K., Wong, S., Rawls, J. F., Guillemin, K., & Bohannan, B. J. M. (2016). The composition of the zebrafish intestinal microbial community varies across development. *The ISME Journal*, 10(3), 644–654. https://doi.org/10.1038/ismej.2015.140

Talwar, C., Nagar, S., Lal, R., & Negi, R. K. (2018). Fish Gut Microbiome: Current Approaches and Future Perspectives. *Indian Journal of Microbiology*, 58(4), 397–414. https://doi.org/10.1007/s12088-018-0760-y

Tlusty, M. (2002). The benefits and risks of aquacultural production for the aquarium trade. *Aquaculture*, 205(3), Article 3. https://doi.org/10.1016/S0044-8486(01)00683-4

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I. (2007). The Human Microbiome Project. *Nature*, 449(7164), Article 7164. https://doi.org/10.1038/nature06244

Vadstein, O., Bergh, Ø., Gatesoupe, F.-J., Galindo-Villegas, J., Mulero, V., Picchietti, S., Scapigliati, G., Makridis, P., Olsen, Y., Dierckens, K., Defoirdt, T., Boon, N., De Schryver, P., & Bossier, P. (2013). *Microbiology and immunology of fish larvae. Reviews in Aquaculture*, 5(s1), S1–S25. https://doi.org/10.1111/j.1753-5131.2012.01082.x

Villamil, L., Figueras, A., Toranzo, A. E., Planas, M., & Novoa, B. (2003). Isolation of a highly pathogenic Vibrio pelagius strain associated with mass mortalities of turbot, Scophthalmus maximus (L.), larvae. *Journal of Fish Diseases*, 26(5), 293–303. https://doi.org/10.1046/j.1365-2761.2003.00461.x

Wabnitz, C., Taylor, M., Green, E. P., Razak, T. (2003). From Ocean to Aquarium: The global trade in marine ornamental species. UNEP-WCMC: Cambridge (UK).

Wang, M., Fan, Z., Zhang, Z., Yi, M., Liu, Z., Ke, X., Gao, F., Cao, J., & Lu, M. (2022). Effects of Diet on the Gut Microbial Communities of Nile Tilapia (*Oreochromis niloticus*) Across

Their Different Life Stages. *Frontiers in Marine Science*, 9. https://www.frontiersin.org/articles/10.3389/fmars.2022.926132

Wilkes Walburn, J., Wemheuer, B., Thomas, T., Copeland, E., O'Connor, W., Booth, M., Fielder, S., & Egan, S. (2019). Diet and diet-associated bacteria shape early microbiome development in Yellowtail Kingfish (Seriola lalandi). *Microbial Biotechnology*, 12(2), 275–288. https://doi.org/10.1111/1751-7915.13323

Williams, I. D., Walsh, W. J., Claisse, J. T., Tissot, B. N., & Stamoulis, K. A. (2009). Impacts of a Hawaiian marine protected area network on the abundance and fishery sustainability of the yellow tang, Zebrasoma flavescens. *Biological Conservation*, 142(5), 1066–1073. https://doi.org/10.1016/j.biocon.2008.12.029

Xavier, R., Severino, R., & Silva, S. M. (2024). Signatures of dysbiosis in fish microbiomes in the context of aquaculture. *Reviews in Aquaculture*, 16(2), 706–731. https://doi.org/10.1111/raq.12862

Yang, F., Xu, L., Huang, W., & Li, F. (2022). Highly lethal Vibrio parahaemolyticus strains cause acute mortality in Penaeus vannamei post-larvae. *Aquaculture, 548*, 737605. https://doi.org/10.1016/j.aquaculture.2021.737605

Zhang, X.-H., He, X., & Austin, B. (2020). Vibrio harveyi: A serious pathogen of fish and invertebrates in mariculture. *Marine Life Science & Technology,* 2(3), 231–245. https://doi.org/10.1007/s42995-020-00037-z

Zhang, X.-J., Yan, B.-L., Bai, X.-S., Bi, K.-R., Gao, H., & Qin, G.-M. (2014). Isolation and Characterization of Vibrio parahaemolyticus and Vibrio rotiferianus Associated with Mass Mortality of Chinese Shrimp (Fenneropenaeus chinensis). *Journal of Shellfish Research,* 33(1), 61–68. https://doi.org/10.2983/035.033.0108

Appendix

For Appendix Table 1 & 2 visit

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