

Investigating the Impact of Gnotobiotic Diets on Gut Bacteria in Zebrafish

by

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A dissertation accepted and approved in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

in Physics

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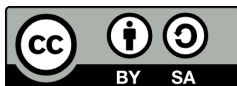
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Fall 2025

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DISSERTATION ABSTRACT

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The gut microbiota is a complex ecosystem within the gastrointestinal tract of humans and other animals, whose interactions with the host influence health, development, and nutrition. Studying the gut microbiota in humans is challenging due to limited accessibility and the difficulty of experimental manipulation. Zebrafish offer a valuable alternative: they are easy to maintain and manipulate, and their early-stage optical transparency enables direct observation of bacterial traits such as aggregation and motility. Food is a key factor modulating the microbiota, affecting not only its composition but also bacterial behavior. However, studies in zebrafish that aim to understand how food ingestion influences bacterial communities are limited by the difficulty of maintaining gnotobiotic conditions where it is known the bacterial strains present. In this dissertation, we describe a method to reduce the native bacterial load associated with rotifers — a common food source for zebrafish larvae — by irradiating them with UV light. Using these treated rotifers, we examine bacterial persistence during feeding periods, finding that some genus, such as *Enterobacter*, decline within days, while others, like *Aeromonas*, persist longer. We also use this system to observe how food alters bacterial behavior *in vivo*. In monoassociation

experiments with *Enterobacter* and *Vibrio*, we find that feeding larvae with UV-treated rotifers induces a transition of *Enterobacter* from an aggregated, non-motile state to a planktonic, motile state, whereas *Vibrio* exhibits a decrease in pathogenic activity. Overall, the use of UV-treated rotifers extends the utility of zebrafish as a model organism to study host-microbe interactions by incorporating the influence of diet. This approach opens new opportunities to investigate how food affects both the microbiota and host health.

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Eduardo Pulgar, Cornelia Schwyer, Néstor Guerrero, Loreto López, Susana Marquez Rosales, Steffen Härtel, Rodrigo Soto, Carl-Philipp Heisenberg, & Miguel L. Concha (2021). Apical contacts stemming from incomplete delamination guide progenitor cell allocation through a dragging mechanism. *eLife* 10:e66483.

Susana Márquez Rosales, German Reig, Miguel Concha, & Rodrigo Soto (2019). Cell migration driven by substrate deformation gradients. *Physical Biology* 16 066001.

ACKNOWLEDGMENTS

I want to thank everyone who has accompanied me all these years. Raghu for being my PI, Stephanie for listening me in the hardest times, Karen for her advices and people in her lab, and the rest of the committee. Thanks to all members of the Parthasarathy Lab with whom I had the pleasure of sharing during these years. Thank you Jonah for make things work. Thanks to Rose for her support with fish and rotifers. Thank you Peter, Emily, Cydney and Ryan for your help and support in the lab. Sharing successful and failures with you has been a big learn.

I want to thank and agradecer all people out of the lab with whom I shared a talk, a dinner, a walk. You gave to my life in Eugene the best moments. I want to specially thank Havisha just for being there in the right moments.

María y Luchito muchas muchas gracias por aguantarme, por su paciencia, por las conversaciones, discusiones, cosas compartidas y sufridas. Llegar a la casa y que ‘haya alguien’ me hizo no perder la cabeza.

I want to especially thank Piyush. Only you and Havi understand how important, significant, valuable it has been to share this adventure with you. Of course, I wouldn't be writing this if it weren't for your support.

Muchas gracias queridxs todxs en Chile! tías, tío, primas, amigxs, que pese a la distancia me han acompañado todos estos años. A Andrea por su apoyo especialmente en los peores momentos. Abuela, sus chombas y frazada me abrazaron y ayudaron a sobrevivir

cada días de invierno. A mi familia por su compañía y por ser bacanes. Gabi, todas tus visitas con almuerzo incluido fueron un regalo. Tote, estuviste todos los días presente con tus recomendaciones musicales. Mami y Papi gracias por arreglarselas para estar todos los días presente de alguna u otra manera, gracias por las aventuras y su generocidad.

DEDICATION

Para Berta y su hija Isabel, mi abuela. Pensar en ustedes me ayudo a lograrlo.

Para Tato y Ester, y Antígona por siempre estar presente.

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CHAPTER I

INTRODUCTION

Our entire body houses on the order of 100 trillion microorganisms distributed across the skin, mouth, respiratory system, and gastrointestinal tract—an especially dense environment [1]. In recent years, interest in the bacterial communities of the gastrointestinal tract has increased due to their correlation with health and disease [2]. What we understand by “good microbiota” is still debated; however, the literature generally agrees that higher diversity tends to be beneficial. In contrast, the overgrowth of certain bacterial species and the loss of others leads to an imbalance known as dysbiosis [3]. Studies have observed that people suffering from cardiovascular, respiratory, and neurological disorders, as well as inflammatory bowel disease, often exhibit dysbiosis.

Studying gut microbiota is challenging due to its complex nature. In terms of bacterial diversity, most human studies rely on fecal samples or biopsies combined with 16S rRNA sequencing, which provides an overview of community composition across major bacterial groups [4]. Despite the valuable insights obtained with these methods, they do not capture bacterial functional activity or behavior, and results can be affected by contamination or incomplete detection of species [5]. Moreover, diversity is only one aspect of microbiota research; questions related to bacterial stability and spatial organization remain difficult to address.

Longitudinal studies, which analyze multiple time points from the same individual, have helped to characterize microbial stability and the factors influencing temporal variation. Strong evidence indicates that each person harbors an individualized microbiota, with the core bacterial community composition remaining relatively stable over years despite daily compositional fluctuations ([6], [7]). Changes in diet, social interactions, or lifestyle can modulate microbiota composition to varying degrees, while factors such as antibiotic consumption can produce long-lasting disruptions ([6], [8]).

These limitations have motivated the use of animal models for studying microbiota dynamics and the impacts on the host. Among them, zebrafish has emerged as one of the main systems for microbiota research, for reasons detailed below.

1.1 Experimental setup

1.1.1 Zebrafish

Native to southern Asia, zebrafish (*Danio rerio*) have become a valuable and versatile model in diverse research areas, including developmental biology, cancer research, neurological disease, and host–bacterial interactions. Their small size and capacity for co-housing — compared with other animal models like mice — make them cost-effective. They also have high fecundity, rapid development, and share approximately 70% of their genes with humans. The complete sequencing of their genome has enabled the generation of transgenic lines, including those expressing fluorescent immune or neural cells, which have

facilitated a wide range of in vivo studies. Another important characteristic is that eggs and larvae are fairly transparent, at least during their first weeks, allowing direct visualization of biological processes ([9],[10],[11]).

This transparency is also an advantage for studying bacteria–host interactions. In Parthasarathy’s lab, we take advantage of partial larval transparency to visualize these interactions. The protocol consists of adding the bacteria of interest to the water. At approximately 4 days post-fertilization, the opening of the mouth allows water—and the bacteria suspended in it—to enter the digestive tract, leading to initial gut colonization. Experiments are usually conducted between days 5 and 7 post-fertilization and involve quantifying the bacterial load—either from whole larvae or dissected guts—and imaging the larva’s gut ([12],[13],[14],[15],[16],[17]).

1.1.2 Light sheet fluorescent microscopy

The observation of bacteria in the larva’s gut involves multiple cellular scales and requires a platform capable of capturing highly dynamic processes. Imaging these interactions therefore imposes stringent technical demands: methods must provide sufficient three-dimensional resolution to resolve individual bacteria, rapid acquisition to capture movements and host responses without motion blur, and low phototoxicity to allow continuous observation of fluorescent protein reporters over the duration of the interaction.

We use a home-built light sheet fluorescence microscope (LSFM) with a configuration close to Keller et al. [18]. In brief, the laser beams—488 nm for GFP-tagged

bacteria and 561 nm for dTomato-expressing bacteria—are rapidly scanned with a galvanometer mirror, then demagnified to form a thin sheet of excitation light that sweeps across the specimen. The sheet illuminates only a single optical plane at a time, and fluorescence from that plane is collected perpendicularly by the imaging objective. This arrangement reduces phototoxicity and enables fast, high-resolution three-dimensional imaging of living samples, making it ideal for live imaging of the zebrafish gut ([17],[19]).

To capture the whole gut, imaging is performed in four overlapping segments covering the anterior–posterior axis (around 1000 μm), the dorsal–ventral axis (around 300 μm), and the left–right axis (around 150 μm). Larvae are anesthetized using 120 $\mu\text{g}/\text{ml}$ MS-222 (tricaine methanesulfonate), and held in glass capillaries using 0.7% agar, submerged in embryo media at 28°C as the protocol states.

1.1.3 Bacteria

To study zebrafish gut bacterial organization and dynamics, we use fluorescently labeled symbiotic strains previously isolated from this host [20]. The combination of larval transparency, fluorescent bacteria [10], and LSM provides a powerful platform to investigate spatial organization and bacterial behavior *in vivo* [17]. For example, in monoassociation, *Enterobacter* sp. ZOR0014 and *Aeromonas* sp. ZOR0001 tend to form aggregates in the midgut, with a smaller fraction of planktonic, non-motile cells, while *Vibrio cholerae* ZWU0020 remains highly motile and distributed throughout the gut [12]. Experiments that combine two or more species have revealed interspecies interactions, such

as *Aeromonas* displacement in the presence of *Vibrio* due to increased peristaltic amplitude [21], and *Enterobacter* disaggregation upon the invasion of *Aeromonas* MB4, a mutant strain obtained through repeated passaging of non-aggregated fractions in GlcNAc-containing medium [14]. In addition to the presence of other species, sub-inhibitory antibiotic doses may also impact bacterial load and behavior [16].

1.2 The challenge of gnotobiotic conditions

The term gnotobiotic comes from the Greek words *gnotos* (known) and *bios* (life). In the context of microbiota, gnotobiotic conditions imply knowledge of the bacteria present, in this case in the zebrafish gut. The relevance of working under gnotobiotic conditions lies in the ability to isolate and study the effects and behaviors of specific bacterial strains or communities in the absence of other microbial species.

In zebrafish larvae, established methods to work under gnotobiotic conditions begin with the derivation of germ-free eggs. The standard protocol, detailed in [22], involves incubating eggs in an antibiotic cocktail for approximately five hours, followed by brief treatments with dilute polyvinylpyrrolidone (PVP) and chlorine solutions, rinsing with sterile embryo medium, and maintaining them under sterile conditions. Once the chorion breaks, larvae remain germ-free as no environmental bacteria are present. Subsequently, by inoculating the larvae with specific bacterial strains to be studied, gnotobiotic conditions are maintained. This procedure works until day 7 post-fertilization; beyond this point, the yolk

is depleted and larvae require external feeding. Maintaining fed larvae under gnotobiotic conditions beyond this stage has remained a major challenge.

We have discussed the relevance of microbiota and factors that influence it, such as diet, and highlighted zebrafish as a platform to study host–microbe interactions. Despite this, this model has been underutilized in exploring the dynamic interplay among diet, microbiota, and host ([23],[24],[22]). Larval zebrafish rely heavily on vision to hunt, so motile prey are essential for triggering capture behavior and encouraging feeding ([25],[26]). Thus, zebrafish facilities commonly feed larvae with rotifers, *Artemia* (brine shrimp), or *Paramecia*—all of which, however, carry their own bacterial load [27]. Among the efforts to feed larvae and keep them under gnotobiotic conditions, Melancon et al. used germ-free, ciliated *Tetrahymena thermophila*. Although this protozoan is highly motile and supplies adequate nutrition for larvae, initializing and maintaining it required many steps and was time-consuming [22]. Other attempts to find sterile alternatives have shown less satisfactory results: for instance, chicken yolk is a high-fat diet that affects normal larval development [28], and dry diets do not supply enough nutrition, producing less healthy larvae in terms of standard length [29]. Their non-motile nature also restricts hunting behavior. Thus, the key challenge lies in developing feeding methods that minimize the bacterial load on the micro-animals used as food while preserving normal zebrafish growth.

Developing gnotobiotic food provides a platform to explore key questions related to bacteria–host interactions during the feeding regime. For example, it could enable

investigations of how food ingestion impacts bacterial behavior when bacteria are mono-associated or in communities.

Similarly, studying how food affects bacterial interactions in communities with two or more species can reveal changes in behaviors such as aggregation, motility, or competitive interactions. Additionally, it could facilitate long-term studies of bacterial stability, keeping larvae alive beyond 7 dpf. Beyond the microbiota itself, a gnotobiotic feeding system provides a means to explore how food ingestion affects host physiology, including gut motility, immune-cell activity, or epithelial responses. Therefore, developing gnotobiotic food does more than solve a technical problem; it offers a practical framework for better understanding and exploring host–bacteria–food interactions.

The following chapters explore whether reducing the bacterial load in larval food can help maintain gnotobiotic conditions, allowing quantification of bacterial persistence and direct observation of behavior in the zebrafish gut.

The first paper (Chapter II) presents a method to reduce bacterial load in rotifers by UV irradiation, enabling longer maintenance of gnotobiotic zebrafish. This approach allows the study of bacterial stability under feeding conditions, revealing species-specific differences in persistence. The second paper (Chapter III) studies how rotifer ingestion affects bacterial behavior within the zebrafish gut. We observed that for *Enterobacter*, food intake changes spatial organization and motility, whereas in *Vibrio*, pathogenic activity is reduced.

CHAPTER II

UV-IRRADIATED ROTIFERS FOR THE MAINTENANCE OF GNOTOBIOTIC
ZEBRAFISH LARVAE

This work is adapted from previously published co-authored work by S. Marquez, P. I. Bouchard, E. M. Olmstead, and R. Parthasarathy, UV-irradiated rotifers for the maintenance of gnotobiotic zebrafish larvae, *mSphere* 10:e00698-24 (2025). I was involved in the experiments, analysis and writing of this work.

2.1 Abstract

Host-associated microbial communities profoundly impact the health of humans and other animals. Zebrafish have proven to be a useful model for uncovering mechanisms of host-microbe interactions, but the difficulty of maintaining germ-free or gnotobiotic zebrafish beyond one week post-fertilization has limited their utility. To address this, we have developed a simple protocol using ultraviolet (UV) irradiation of rotifers, a common and nutrient-rich prey species for larval zebrafish, to reduce the bacterial load associated with the rotifers by several orders of magnitude while maintaining their motility and viability. We find that though feeding with UV-treated rotifers does not preserve the sterility of germ-free fish, it enables the maintenance of pre-existing bacterial communities. Normal feeding, in striking contrast, leads to the near total depletion of these prior populations. We measure the abundance of single- and three-species consortia of zebrafish-commensal

bacteria inoculated into initially germ-free larvae in a series of experiments extending to 8 days of feeding, or 13 days post-fertilization. We find, in fish fed UV-treated rotifers, persistence of bacterial populations on timescales of days, together with strong species-specific variation. In addition, re-inoculation of differently labeled strains of the same zebrafish-commensal species alongside feeding leads to colonization by the new bacteria without displacement of earlier microbes. Our method will facilitate the use of gnotobiotic zebrafish for investigations of phenomena that emerge later in animal development and for studies that probe microbiome composition fluctuations and stability over extended timescales.

2.2 Importance

All animals, including humans, are host to vast microbial communities that contribute to health and disease through mechanisms that remain largely mysterious. These microbiomes are challenging to study, spurring the use of various model organisms, including zebrafish. Zebrafish, however, are difficult to raise beyond one week post-fertilization under gnotobiotic conditions, in other words germ-free or with known microbial constituents, a consequence of normally feeding on live prey that bring their own, generally unknown, microbes. We therefore developed a simple protocol in which UV irradiation of rotifers, a widely used small-animal food for larval zebrafish, facilitates the maintenance of gnotobiotic larvae. We show that pre-existing bacterial communities in larvae are minimally affected by feeding on UV-treated rotifers, in strong contrast to feeding

on untreated rotifers. We demonstrate that this feeding method allows investigations of zebrafish-associated bacterial community stability over several days, allowing investigation of previously intractable questions about microbiome stability.

2.3 Introduction

The human body sustains nearly 100 trillion microorganisms, most of which form dense communities in the intestinal tract, communicating with host organs and influencing the course of health and disease [2]. Manipulating and controlling microbial variables to uncover mechanisms of host-microbe interactions is challenging in humans, which has long motivated the use of animal models and the development of strategies to raise them germ-free, i.e. devoid of microorganisms, or under gnotobiotic conditions, i.e. with known microbial strains or communities. Reports of germ-free guinea pigs date from 1896 [30], and since then, researchers have successfully derived various germ-free animals, including mammals, birds [31], amphibians [32], and fish. In 1942, Baker et al. reported the first germ-free fish (platyfish, *Xiphophorus maculatus* [33]), which was followed by other aquatic species including zebrafish (*Danio rerio* [23],[22]), a species whose attributes such as rapid development, genetic tractability, and transparency at young ages have made it a widely used animal model ([9],[10],[11]). Zebrafish-based studies of host-microbe interactions have illuminated the varied roles of commensal microbes in early development [10] including effects on epithelium maturation, secretory cell proliferation, enzymatic activity ([23],[34],[35],[36]), immune cell numbers ([37],[38],[39]), the abundance of insulin-producing *b η*

cells ([40],[41]), and neural anatomy influencing behavior [42]. Cell-resolved live imaging of gut microbes in larval zebrafish [19], so far impossible in other vertebrates, together with a library of fluorescently-labeled zebrafish-native bacterial species [20], has revealed diverse aggregation states among gut microbes ([12],[14],[43]), influences of bacterial motility on host immune response [15], effects of antibiotics on bacterial motility and persistence [16], and non-pairwise interactions among gut bacterial species [44].

One major aspect of microbiome research to which zebrafish have contributed far less as a model is the investigation of long timescales, necessary for studying bacterial community stability and changes over developmental periods extending to the whole lifespan. This is important because studies in humans show significant variations of timescales bacterial of persistence throughout a host's life, ranging from mere days or weeks, particularly during early developmental stages [45], to extended periods stretching up to months or even years in adults [6],[46],[47]. Perturbations like changes in diet or antibiotic introduction can also alter the microbiota on timescales of days or weeks, though a full return to the original state can take years ([48],[49],[50],[8]). For all these temporal changes, the underlying mechanisms are poorly understood [51]. Zebrafish could help illuminate these issues for the reasons noted above, but they have suffered from a key limitation: the difficulty of maintaining gnotobiotic animals beyond approximately 7 days post-fertilization (dpf), at which point, having exhausted their yolk, they require food. While it is possible to provide sterilized, powdered chow [23], zebrafish larvae raised on dry food show diminished growth and development compared to larvae fed live food [52]. Larval

zebrafish are primarily visual hunters, and the motion of prey is crucial to stimulating capture and food intake ([25],[26]).

Several research groups have therefore pursued the derivation of germ-free prey species. These include *Artemia* (brine shrimp), which researchers since the 1950s have made germ-free through egg sterilization ([53],[54],[55],[56]). Recently, Jia *et al.* [57] showed that a different fish species (medaka, *Oryzias melastigma*) could be raised germ-free to adulthood solely on *Artemia*. Zebrafish are capable of eating *Artemia* by 9 or 10 dpf, a few days after yolk depletion, calling for another, intermediate, prey species. For earlier feeding of zebrafish, groups have reported antibiotic treatment of the unconventional prey species *Tetrahymena thermophila* ([55],[54]). While successful for producing gnotobiotic fish, such methods involving these prey species, especially *Artemia*, are highly labor intensive [22] and are not widely used. Moreover, antibiotics, even at low concentrations, can have large impacts on the gut microbiome ([8],[16]) and can alter animal metabolism and growth [58], further motivating our efforts to investigate alternative methods. We consider rotifers (*Brachionus plicatilis*), an easily grown and widely used live food for zebrafish larvae [59], [60], and ultraviolet (UV) irradiation. Rotifers have been the target of axenic method development for over half a century at least [61], for example through sterilization of amictic eggs [62].

Though the aim of such efforts has usually been to facilitate studies of rotifers themselves rather than their use as prey, we note that because of their role as filter feeders, rotifers are capable of harboring a wide variety of environmental bacteria, including fish

pathogens [63]. Regardless of the motivation for sterilization, ultraviolet sterilization of rotifers has rarely been investigated. In 1999, Munro et al. reported an ultraviolet irradiation protocol that reduced the bacterial load of rotifers by 99% and showed that these rotifers could be successfully fed to turbot [64]. No investigations have been made, however, on the gnotobiotic status of fish fed UV-irradiated food. We therefore aimed to establish a simple method based on UV-exposed rotifers that can maintain gnotobiotic zebrafish for extended durations. We developed and compared two separate procedures to minimize native bacterial populations in rotifers: antibiotic treatment and a simple UV irradiation protocol. We assessed the effect of consumption of treated rotifers on pre-existing bacterial populations in larval zebrafish. While both prey treatments reduced the rotifers' bacterial load, consumption of antibiotic-treated rotifers led to dramatic drops in commensal bacterial abundance. In contrast, consumption of UV-treated rotifers left larval bacterial populations largely intact. Then, we examined bacterial populations in fed larvae to 13 dpf, demonstrating greater stability in fish fed UV-treated rotifers than in untreated rotifers. The methods described here will facilitate studies of gut microbiome dynamics and host-microbe interactions in gnotobiotic animals.

2.4 Results

We aimed to establish an easily implemented method for feeding rotifers to zebrafish larvae that maintains gnotobiotic conditions. The key step is to reduce as much as possible the bacteria that naturally accompany the rotifers while preserving rotifer survival and

motility. We investigated two methods: one using ultraviolet light exposure and the other using antibiotics.

The ultraviolet irradiation method uses a 270-280 nm UV-C LED attached to the cap of a flask containing a rotifer suspension and a stir bar to ensure mixing (Figure Figure 2.1 A; see Materials and Methods). To find an exposure protocol that leads to a significant reduction in bacterial numbers while maintaining a viable rotifer population, we assessed cycles of UV light consisting of alternating 30-minute exposure and rest periods. By three exposure cycles, plating the rotifer suspension showed a drop in bacterial abundance of nearly four orders of magnitude (Fig. Figure 2.1 B).

UV irradiation causes rotifers to become slow or non-motile. By five UV cycles, the fraction of motile organisms decreased to less than 50%, with the non-motile animals lacking apparent internal organ activity and therefore likely being dead, and the average speed of motile rotifers was less than half that of unexposed rotifers (Fig. Figure 2.1 C, Supplemental Fig. Figure 2.5, see Materials and Methods).

We therefore chose four UV exposure cycles to reduce the rotifers' bacterial load while keeping the majority of animals motile (Fig. Figure 2.1 C) and therefore likely to stimulate the prey capture response of hunting zebrafish larvae.

While a large variety of antibiotics exist, we focused for simplicity on the same cocktail of antibiotics typically used for germ-free zebrafish derivation [22] (see Materials and Methods). After 3.5 hours of incubation, the antibiotics reduced the bacterial load of rotifer suspensions by three orders of magnitude (Fig. Figure 2.1 D), similar to the results

from UV irradiation. Following antibiotic treatment, we observed no impact on rotifer swimming speed or the proportion of motile rotifers.

We evaluated the efficacy of the UV and antibiotic treatments for maintaining germ-free fish after feeding. Beginning with germ-free larvae, we administered feeding regimes spanning one, two, and three days starting at 5 dpf (Figure Figure 2.1 E), followed by bacterial abundance quantification via larval homogenization and plating (see Materials and Methods). Notably, we measured bacterial load from entire larvae, which will be dominated by gut microbes but can include skin-resident microbes. Figure Figure 2.1 F presents the results detailing bacterial loads from larvae fed with UV-treated rotifers, antibiotics-treated rotifers (AB), and control (untreated) rotifers, alongside unfed germ-free fish and unfed conventionalized (CVZ) larvae, the latter initially germ-free and then raised in standard, non-sterile aquaculture water. As expected, unfed conventionalized larvae and larvae fed control rotifers showed $\{10^4\}$ - $\{10^5\}$ bacteria (colony forming units (CFU)) per fish, while unfed germ-free fish typically had undetectable numbers of bacteria (Figure Figure 2.1 F). Fish fed with AB-treated rotifers showed low bacterial abundance after one day of feeding (10-100 CFU), though bacterial numbers increased to several thousand by two or three days of feeding. Fish fed with UV-treated rotifers lost their germ-free status after just one day of feeding, with bacterial abundance around $\{10^4\}$ indicating that the small amount of bacteria remaining in the rotifers after UV treatment are sufficient to colonize the larvae, compromising the gnotobiotic conditions. We also measured larval length after two or three days of feeding as an indicator of fish health. Larvae in all fed groups were, on average,

longer than unfed larvae (Supplemental Figure Figure 2.6). The length of fish fed with UV-treated rotifers showed no significant difference compared to those fed with control rotifers, implying that the decrease in motility and speed of rotifers due to UV treatment did not negatively affect their capture by hunting fish.

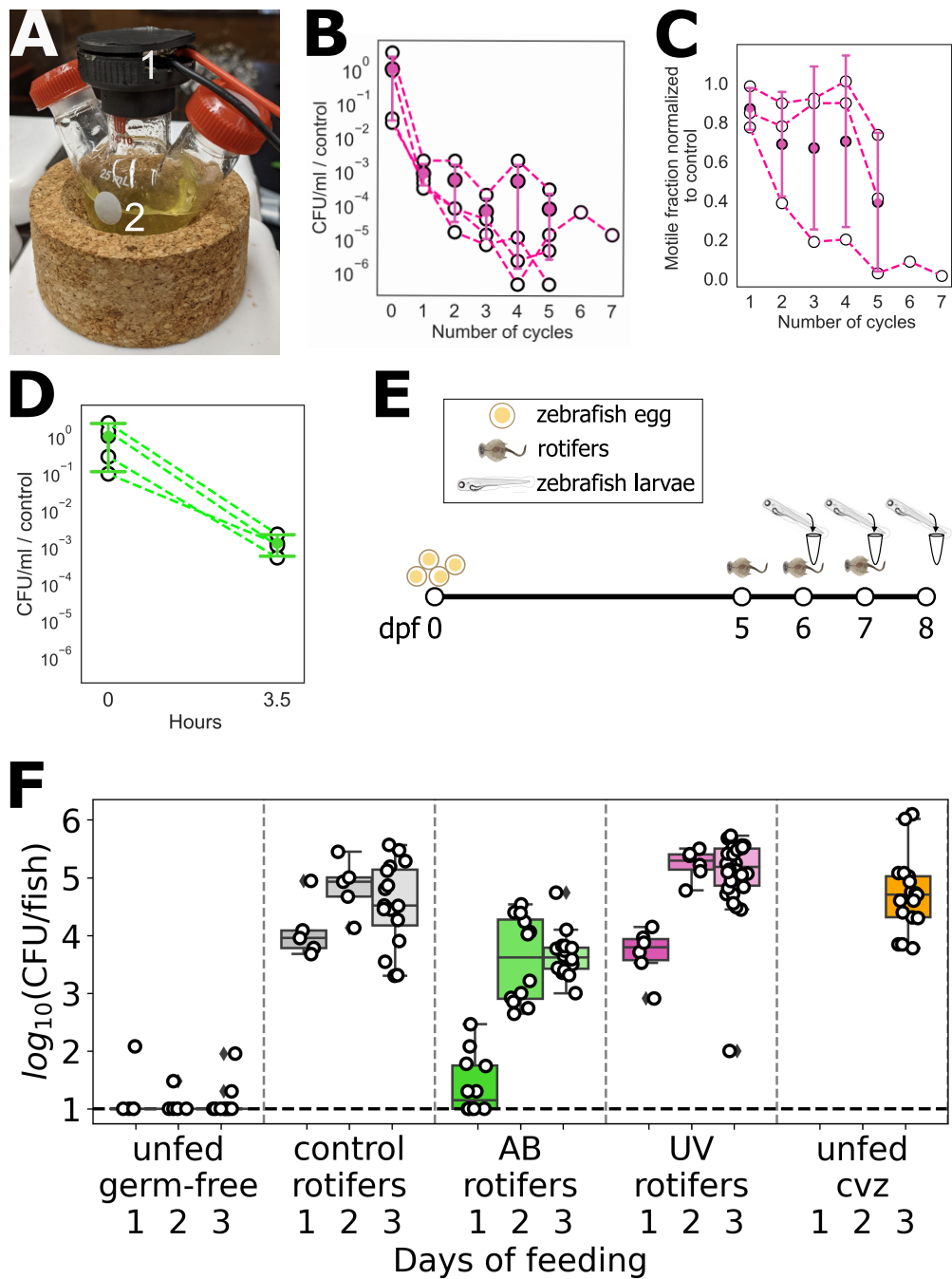


Figure 2.1. A Experimental setup for UV irradiation of rotifers, including (1) a UV-C LED mounted in a cap of a round bottom flask and (2) rotifers in a 4ppt NaCl solution. **B** Bacterial concentration in the rotifer suspension as a function of the number of UV exposure cycles, each 30 minutes on / 30 minutes off. **C** Fraction of motile rotifers as a function of the number of UV exposure cycles. In B and C, open symbols represent different replicates, and solid symbols and error bars indicate the mean and standard deviation. **D** Bacterial concentration in the rotifer suspension before and after incubation with antibiotics for 3.5 hours. **E** Schematic diagram of the protocol for assessing bacterial load in initially germ-free zebrafish subject to different feeding methods. **F** Bacterial abundance per larval zebrafish after 1, 2, and 3 days of feeding with rotifers under different treatments, or unfed at the same days. Each symbol indicates a measurement from an individual fish; boxes indicate the median (line within the box), first and third quartiles (top and bottom of the box), and 95% confidence intervals (bars). The dashed line indicates the approximate limit of detection, 10 bacteria per fish.

Though the methods being examined cannot maintain zebrafish germ-free, they nonetheless may be capable of keeping larvae under gnotobiotic conditions, sustaining intentionally inoculated bacterial communities with minimal perturbation. We hypothesized that symbiotic strains may out-compete the bacteria that accompany rotifers. To assess this, as indicated schematically in Figure Figure 2.2 A, we started by establishing a bacterial community at 4 dpf by inoculating germ-free larval fish with previously studied zebrafish-native strains expressing fluorescent proteins (see Materials and Methods) ([20],[44]) *Pseudomonas mendocina* ZWU0006 (PS), *Aeromonas veronii* ZOR0001 (AE), and *Enterobacter cloacae* ZOR0014 (EN). On each of the next three days (5 - 7 dpf), we fed the fish and also re-introduced the fluorescent strains to the aqueous medium. At 8 dpf, we homogenized and plated the whole fish as above (see Materials and Methods), noting the

abundance of fluorescent colonies, representing the intentionally inoculated species, and non-fluorescent colonies, representing the species introduced by feeding.

We found that for fish fed AB-treated and UV-treated rotifers, the abundance of fluorescent bacteria was over 90% of the total bacterial load (0.98 ± 0.016 , mean $\pm$ standard deviation, and 0.90 ± 0.087 , respectively) (Figure Figure 2.2 B). In contrast, the abundance of fluorescent bacteria in fish fed control rotifers was considerably smaller, 0.7 ± 0.1 of the total. Strikingly, the overall number of intentionally inoculated (fluorescent) bacteria was approximately an order of magnitude lower in fish fed AB-treated rotifers than in fish fed UV-treated or control rotifers (Figure Figure 2.2 C). Plating on chromogenic agar, on which colonies of the different intentionally inoculated strains show different colors [44] (see Materials and Methods) showed that the depletion of bacterial numbers following feeding with AB-treated rotifers was strongly species dependent (Figure Figure 2.2 D), with the EN population being particularly affected and completely absent in most larvae (Figure Figure 2.2 D). The AE population was two orders of magnitude smaller compared to other fed groups, while PS was similar (Figure Figure 2.2 D). To gain insights into intestinal abundance in particular, we imaged the whole intestines of live larvae using Light Sheet Fluorescence Microscopy ([12],[43]). We could not detect fluorescent bacteria in the gut of larvae fed with AB-treated rotifers, while fluorescent bacteria were clearly evident in larvae fed with UV-treated or control rotifers (Figure Figure 2.2 E).

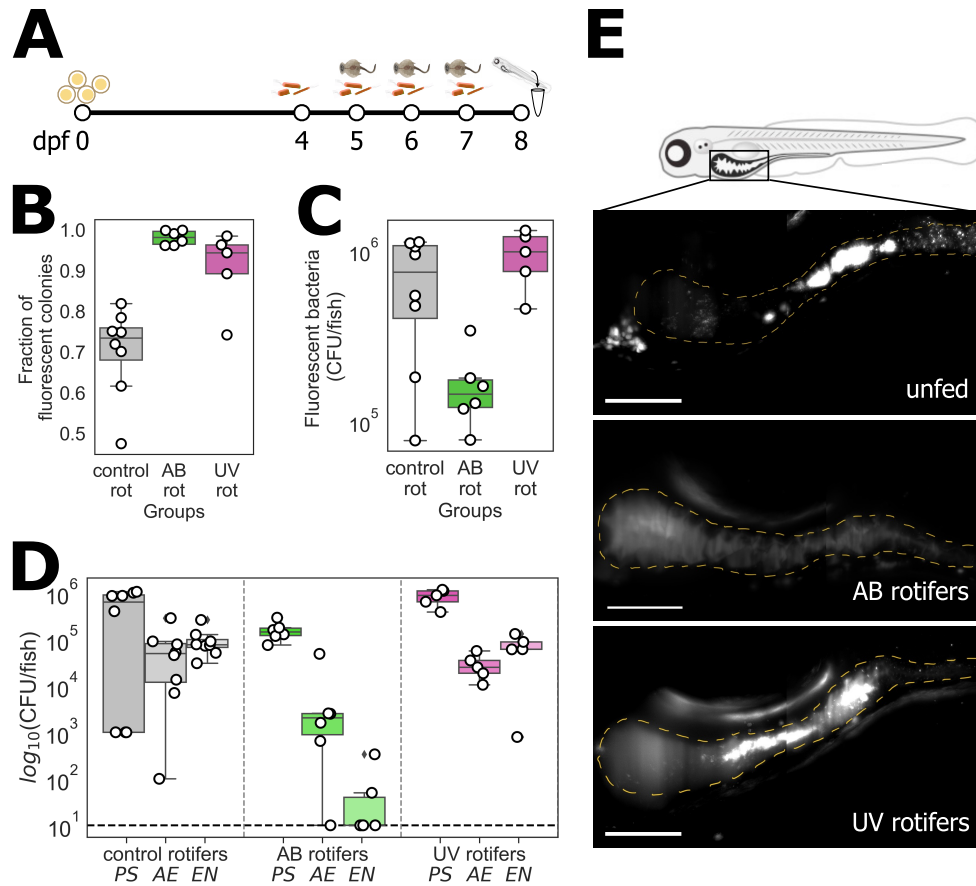


Figure 2.2. **A** Schematic diagram of the protocol to evaluate the permanence of gnotobiotic larvae after three days of feeding, in which initially germ-free zebrafish are inoculated with three fluorescently labeled bacterial species. **B** Fraction of fluorescent colonies from plating whole larvae, indicating the fraction of intentionally inoculated bacteria, after three days of feeding and re-inoculation. **C** Total abundance of fluorescent bacteria per larva after three days of feeding and re-inoculation. **D** Abundance of each intentionally inoculated strain. PS: *Pseudomonas mendocina* ZWU0006, AE: *Aeromona veronii* ZOR0001, EN: *Enterobacter cloacae* ZOR0014. In B-D, each symbol indicates a measurement from an individual fish; boxes indicate the median and quartiles. **E** Maximum intensity projections of representative 3D image stacks from light sheet fluorescence microscopy of the guts of live larval fish, taken after one day of feeding with antibiotic- or UV-treated rotifers, or on the equivalent day for unfed fish. Dashed lines trace approximate intestinal boundaries. Bar = 200 μm .

Having established a method using UV irradiated rotifers to feed zebrafish larvae, we investigated inoculated *Enterobacter cloacae* (EN) populations over several days, noting that of the three species examined this strain was the most sensitive to food-induced perturbation (Fig. Figure 2.2 D). To distinguish bacteria descended from those inoculated on the first day, colonizing germ-free fish, from bacteria introduced on subsequent days, we used differently tagged EN strains [20], expressing green fluorescent protein (GFP) and dTomato, respectively. As above, non-fluorescent bacteria represented unintended colonizers from rotifers or other sources. Fish were fed from 5 to 7 dpf or from 7 to 9 dpf. The first set allowed us to include unfed fish as a control group. The second set tested bacterial dynamics into the regime that requires feeding. For both sets, we included fish fed with control (untreated) rotifers to determine the efficacy of the UV treatment.

The study comprised four distinct fish groups for each set of days, as illustrated in Figure Figure 2.3 A. All fish were inoculated on the initial day (4 or 6 dpf) with GFP-labeled EN, fed with UV-treated or control rotifers on each of the next three days unless in the unfed set, and then plated to determine bacterial load on various days including the final day (Fig. Figure 2.3 A). Groups 2, 3, and 4 were, in addition, inoculated with dTomato-labeled EN on the first, second, and third day of feeding, respectively (Fig. Figure 2.3 A; see Materials and Methods). Representative larvae were homogenized and plated as above to quantify bacterial load, with all fish assessed by the final day, at 8 or 10 dpf. Together, these groups provided insight into the persistence of EN introduced before feeding and of stability following re-inoculation.

We present the results in Figure Figure 2.3, indicating the feeding types by different colors and the sets of days by different symbols. Considering fish only inoculated on the first day, by EN-GFP, the initial bacterial load assessed from unfed fish at one day post inoculation (dpi) was roughly $\{10^5\}$ - $\{10^6\}$ per fish, as expected (Fig. Figure 2.3 B). At 4 dpi, almost no EN were found in fish fed with untreated rotifers, in contrast to roughly $\{10^3\}$ - $\{10^5\}$ in fish fed UV-treated rotifers. Interestingly, unfed fish at 8dpf showed fewer EN (Fig. Figure 2.3 B), suggesting that the depletion of yolk has consequences for resident bacteria. We next considered the total EN population in Groups 2, 3, and 4, as a function of days following inoculation of the second (EN-dTomato) bacterial strain (Fig. Figure 2.3 C). Again, almost no EN were found in fish fed with untreated rotifers at 2 and 3 dpi, while unfed fish and fish fed UV-treated rotifers showed sizable bacterial numbers ($\{10^4\}$ - $\{10^5\}$ per fish, Fig. Figure 2.3 C). Considering separately the EN-GFP population, descended from the initial colonizers, and the EN-dTomato population, descended from later entrants, we found similar orders-of-magnitude for the abundance of each group in the unfed fish and fish fed UV-treated rotifers (Fig. Figure 2.3 D, E). The relative abundance of the two groups, quantified as the GFP-labeled fraction of the total EN abundance, showed considerable variation (Fig. Figure 2.3 F), indicating that neither the initial nor later colonizers consistently dominates the population.

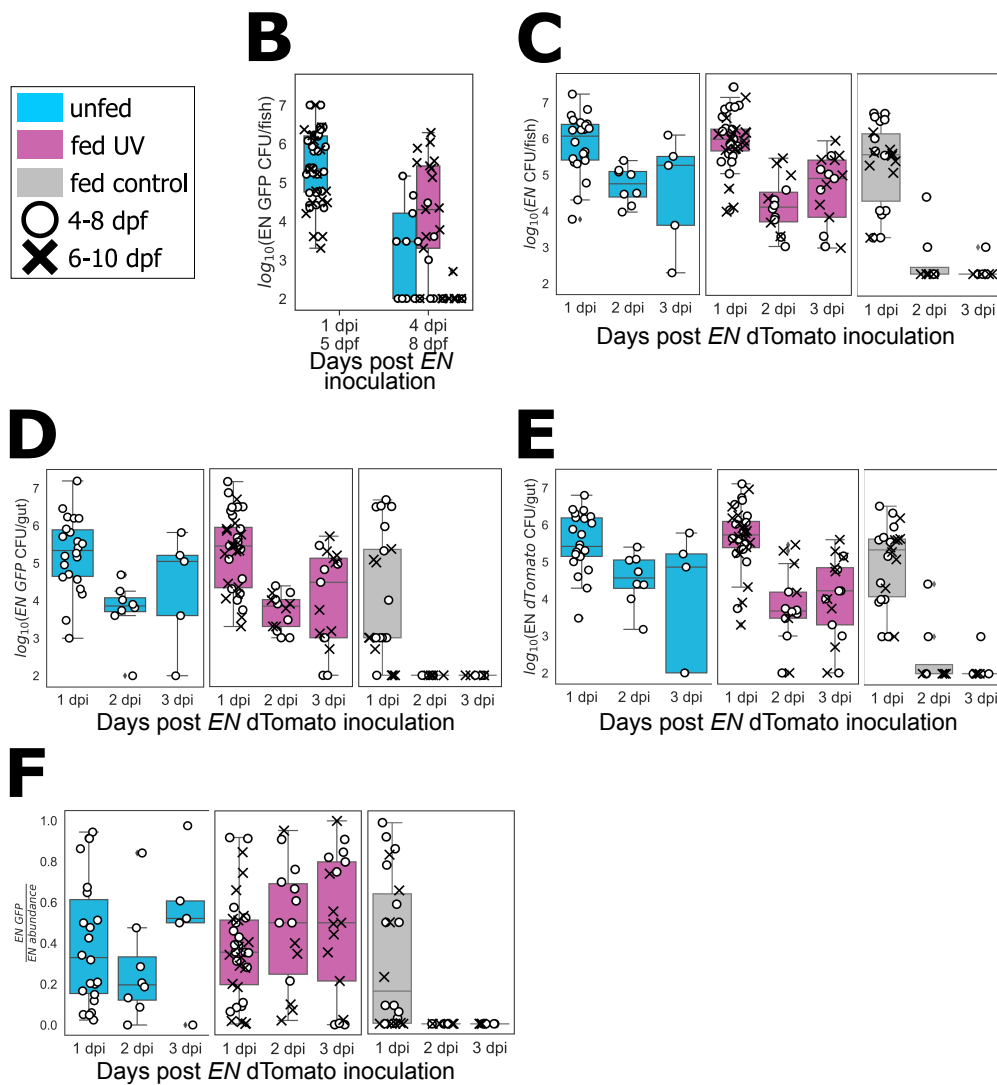
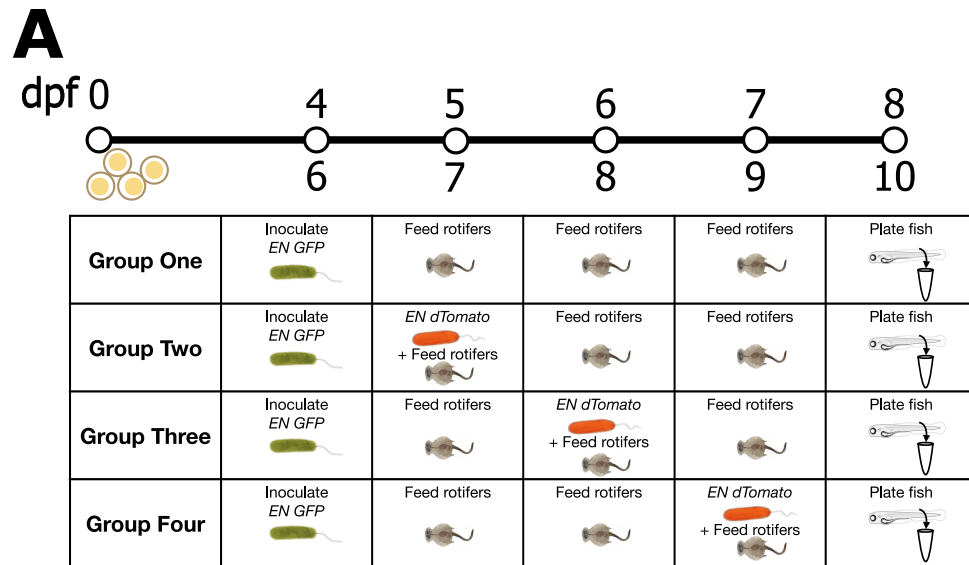


Figure 2.3. A Schematic diagram of the protocol to evaluate *Enterobacter* stability after feeding and re-inoculation. As described in the main text, initially germ-free fish were inoculated with GFP-labeled EN and fed UV-treated or control rotifers or left unfed for three days, starting at 4 or 6 dpf. Groups 2-4 were re-inoculated with dTomato-labeled EN on different days. **B-F** Final EN abundance in unfed larvae (cyan), larvae fed with UV treated rotifers (pink), and larvae fed with untreated rotifers (gray). Circles represent data from larvae fed from 5 to 7 dpf, and crosses from 7 to 9 dpf. **B** Fluorescent EN-GFP abundance in Group 1 larvae assessed one day post-inoculation (before the start of feeding) and four days post-inoculation (after three days of feeding). **B-E** From groups 2, 3, and 4, total fluorescent EN abundance (GFP and dTomato) (C), EN-GFP abundance (D), and EN-dTomato abundance (E) one, two, or three days post EN dTomato inoculation. **F** Fraction of EN-GFP relative to the total EN abundance one, two or three days post EN-dTomato inoculation.

Finally, we examined the stability of a multi-species bacterial consortium in zebrafish up to 13 dpf, several days longer than is possible without feeding. We again considered PS, AE, and EN (as in Fig. Figure 2.2) and again considered differently inoculated groups. We inoculated all larvae with GFP-labeled PS, AE, and EN at 4 dpf and Group 2 larvae additionally with dTomato-labeled PS, AE, and EN at 8 dpf, the fourth day of feeding (Fig. Figure 2.4 A; see Materials and Methods). Both groups were fed for 7 or 8 days after which individuals were homogenized and plated to assess bacterial load. Both groups included fish fed UV-treated rotifers and control (untreated) rotifers.

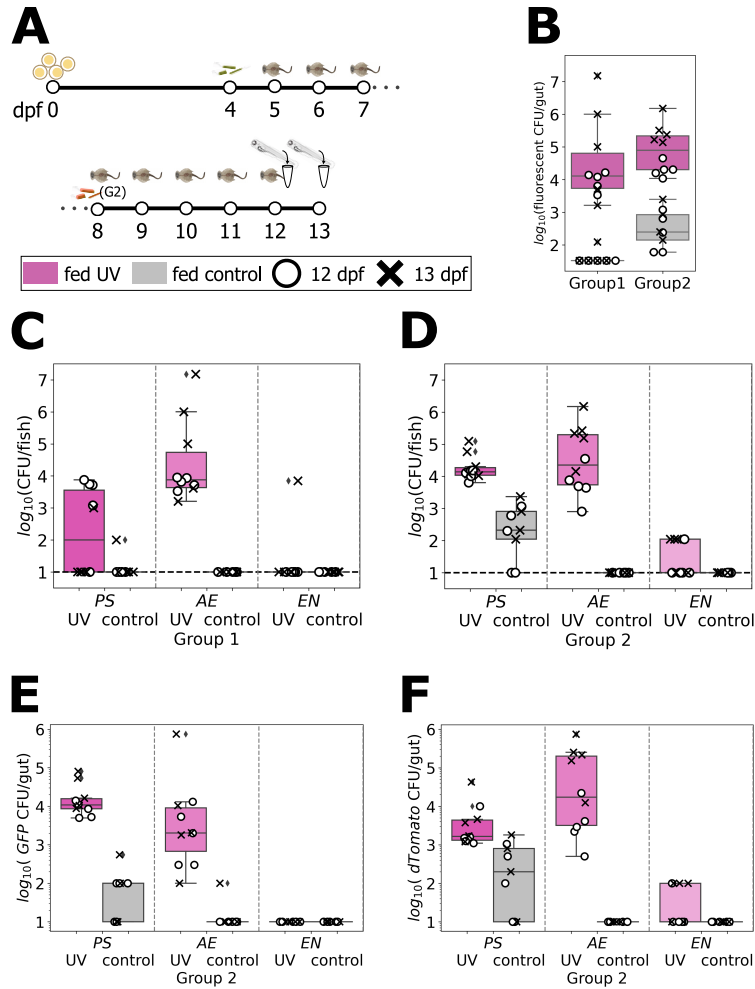


Figure 2.4. **A** Schematic diagram of the protocol to evaluate the stability of multiple bacterial species in larvae fed until 13 dpf. Initially germ-free larvae were inoculated with GFP-labeled PS, AE, and EN at 4 dpf and split into two groups. Zebrafish in Group 2 were re-inoculated with dTomato-labeled PS, AE, and EN at 8 dpf. Both groups were fed from 5 to 11 or 12 dpf and homogenized and plated at 12 or 13 dpf. **B** Fluorescent bacterial abundance per larva after 7 or 8 days of feeding. Pink corresponds to larvae fed with UV treated rotifers, and gray to larvae fed with untreated rotifers. **C** and **D** Bacterial abundance of each inoculated per strain for fish in Group 1 (**C**) and Group 2 (**D**). **E** Total abundance of GFP-labeled bacteria, descended from initial colonizers, of each strain in larvae from Group 2. **F** Total abundance of dTomato-labeled bacteria, descended from later colonizers, in larvae from Group 2.

Considering the total abundance of fluorescent (intentionally inoculated) bacteria at the experimental endpoint, fish in Group 1 and Group 2 that were fed UV-treated rotifers showed sizeable populations, roughly 10^4 - 10^5 CFU/fish, while fish fed with control rotifers showed bacterial numbers that were on average two orders of magnitude lower (Fig. Figure 2.4 B). Quantifying each inoculated bacterial species separately showed considerable differences (Fig. Figure 2.4 C, D). AE was the most stable species, with abundances similar to those measured one day after bacterial inoculation in earlier experiments (Fig Figure 2.2 D), and EN was the least stable species. For all species, abundances in control-rotifer-fed fish were low or undetectable (Fig. Figure 2.4 C, D). Considering separately, in Group 2 fish, the GFP-labeled population, descended from the initial colonizers, and the dTomato-labeled population, descended from later entrants, we found similar orders-of-magnitude for the abundance of each in the fish fed UV-treated rotifers (Fig. Figure 2.4 D, E). Intriguingly, PS and AE showed opposite behaviors for the abundance of dTomato- relative to GFP-labeled bacteria, with PS dominated by the descendants of initial colonizers and AE the later colonizers (Fig. Figure 2.4 E, F). Variation was large, however, prohibiting strong conclusions.

2.5 Discussion

To improve the utility of zebrafish as a model for studying animal-associated microbiomes, we have developed a method for feeding zebrafish larvae that allows live, motile food while minimizing the disruption of existing bacterial communities. Our method

uses UV-C LEDs that are inexpensive, easy to deploy, and sufficient to reduce within a few hours the bacterial density that naturally accompanies rotifers by four orders of magnitude. Although maintaining truly germ-free zebrafish larvae was not achievable with this method of preparing prey (Fig. Figure 2.1 F), likely due to the ability of remaining microbes to proliferate in the sterile larval environment, we found that feeding with UV-irradiated rotifers allows zebrafish-native bacteria associated with the fish prior to feeding, or reintroduced together with feeding, to persist at high abundance (Fig. Figure 2.2, Figure 2.3). Importantly, the similarity between the abundances of intentionally inoculated bacteria in unfed fish and those fed with UV-treated rotifers, and the marked contrast with fish fed untreated rotifers, validates the UV treatment protocol for studying zebrafish-associated bacterial stability. Normal feeding on untreated rotifers, in contrast, diminishes fish native bacteria by orders of magnitude.

Treatment of rotifers with an antibiotic mixture commonly used for deriving germ-free fish, though effective for reducing the bacterial concentration in the rotifer suspension (Fig. Figure 2.1 D) leads after ingestion to large and species-specific drops in fish-associated bacterial abundance (Fig. Figure 2.2 C, D). This is perhaps unsurprising, as the antibiotics are diluted only by a factor of 15 from the concentration used for deriving germ-free fish (see Materials and Methods). It may be possible, for example through filtration or centrifugation, to separate the rotifers from their antibiotic treated suspension prior to feeding, but this adds extra procedural steps, and moreover it is possible that the antibiotics are preferentially concentrated in the rotifers. Furthermore, studies of humans [65] and zebrafish [16] have

shown that even low doses of antibiotics can have large consequences on gut microbiomes, in part due to antibiotic-induced changes in bacterial morphology and motility [16], providing further cautions for antibiotic-based procedures for gnotobiotic prey. We believe, therefore, that our use of UV-irradiation highlights a gnotobiotic strategy that is less likely to lead to strong perturbations of animal-associated microbiomes, and we focus on further characterization of this method.

Extending the timescale over which zebrafish can be maintained under gnotobiotic conditions opens up new research opportunities. For example, week-old gnotobiotic zebrafish larvae have enabled insights into connections between gut bacterial species and innate immune responses (e.g. [66]), but studying the adaptive immune system requires older fish, as the maturation of the adaptive immune system in zebrafish starts at three weeks post-fertilization with the appearance of CD4+/CD8+ lymphocytes [67]. Given increasingly appreciated connections between host-associated microbes and autoimmune and chronic inflammatory disorders [68], older gnotobiotic zebrafish could serve as valuable experimental tools.

Most generally, improved gnotobiotic methods enable the extension of timescales of experimental investigation in zebrafish. Of particular interest are timescales of gut microbiome stability and instability, whose underlying determinants in humans and other animals remain mysterious [51]. Given the presence of a bacterial strain, what is the likelihood of its persistence some time later, and is this altered by immigration of members of the same or different strains?

We have demonstrated that studying these questions is tractable, for example examining a three-species consortium in larvae fed for up to eight days, to 13 dpf. We find strong species-dependence of stability, with a zebrafish-native *Enterobacter* nearly disappearing while other species persist (Fig. Figure 2.4). Notably, this *Enterobacter* species also shows the greatest decline when fish are fed with antibiotic-treated rotifers (Fig. Figure 2.2 D). These observations are consistent with previous results from short-duration studies that show that *Enterobacter* is disfavored in competition with *Aeromonas* ([44],[14]) and is highly sensitive to weak antibiotics [16]. *Enterobacter* is also highly aggregated [12], and we suspect that underlying all of these phenomena is a connection between aggregation and intestinal transport. With the ingestion of food, a frequent perturbation of obvious importance to humans and other animals, the physical space occupied by food particles and the mechanics of its digestion may induce species-specific displacement, particularly impacting more aggregated strains. Further experiments, including studies using live imaging (as in Fig. Figure 2.2 E), can directly probe and quantify these processes in a useful model animal.

2.6 Materials and Methods

Animal care. All experiments with zebrafish were done in accordance with protocols approved by the University of Oregon Institutional Animal Care and Use Committee and by following standard protocols [69].

Zebrafish gnotobiology. Wild-type ABC X TU zebrafish (*Danio rerio*) were derived germ-free as described previously [22] with slight modifications. In brief, fertilized eggs were collected and placed in sterile antibiotic embryo medium (EM) containing 100 µg/ml ampicillin, 250 ng/ml amphotericin B, 10 µg/ml gentamicin, 1 µg/ml tetracycline, and 1 µg/ml chloramphenicol for approximately 5 hours. The eggs were then washed in sterile EM containing 0.003% sodium hypochlorite and then in sterile EM containing 0.1% polyvinylpyrrolidone-iodine. Washed embryos were distributed into tissue culture flasks containing 50 ml of sterile embryo medium at a density of one embryo per ml. Flasks were inspected for sterility before being used in experiments.

Bacterial strains. We used the following strains, each originally isolated from the zebrafish intestine: *Aeromonas* sp. strain ZOR0001, *Pseudomonas mendocina* (ZWU0006), and *Enterobacter* sp. strain ZOR0014 (EN). From each, engineered strains constitutively expressing green fluorescent protein (GFP) or dTomato were previously generated [20]. Stocks of bacteria were maintained in 25% glycerol at ≥80 °C.

Inoculation of larval zebrafish. One day prior to fish inoculation, bacteria from frozen glycerol stocks were grown overnight in lysogeny broth (LB medium; 10 g/l NaCl, 5 g/l yeast extract, 12 g/l tryptone, 1 g/l glucose) at 30 °C with shaking. For inoculation of germ-free fish with a single species (*Enterobacter* sp. strain ZOR0014), 1 ml of overnight culture was washed once by centrifuging for 2 minutes at 7000 × g, removing the supernatant, and adding 1 ml of fresh sterile embryo medium. Then 100 µl of the bacterial mix was added to a 50 ml tissue culture flask containing approximately 50 germ-free 4 dpf

zebrafish larvae, giving a bacterial concentration of approximately 10^6 CFU/ml. For experiments involving multiple bacterial species, we combined 1 ml of each overnight culture, diluting each as needed in lysogeny broth to achieve similar optical densities ($OD_{600} \approx 5.0$) and therefore similar concentrations. The bacterial mixture was further prepared and added to the flasks containing larvae as above, at a bacterial concentration of approximately 10^6 CFU/ml. For re-introduction of bacteria to previously colonized fish, we prepared a 1000-fold dilution of the *Enterobacter* overnight culture in LB for single-species experiments and a 100-fold dilution of the bacterial mixture for multi-species experiments. Then, 100 μ l of the bacterial suspension was combined with 900 μ l of the rotifer suspension and added to the flasks containing zebrafish larvae as above, giving bacterial concentrations of approximately 10^4 CFU/ml and 10^5 CFU/ml (about 10^4 CFU/ml per species) for the single- and multi-species experiments, respectively.

Rotifers. Rotifers (*Brachionis plicatilis*) were provided by the University of Oregon Zebrafish Facility. Rotifer cultures were raised in the facility in 5-gallon containers, fed with the “Rotigrow Plus” algae mixture (Reed Mariculture), and maintained at 4 ppt salinity in “Instant Ocean” commercial sea salt mix (Instant Ocean). Rotifers at a density of roughly 2000/ml were obtained as needed from the facility and used the same day.

Ultraviolet irradiation of rotifers. Ultraviolet light, 270–280 nm, was provided by LEDs (E275-3-S UVC LED Chip on Board, International Light Technologies; UVC LED Driver ILT-PWRTYLED.3W, International Light Technologies). An LED was glued to a 3D-printed cap designed to fit a three-neck round bottom flask (Synthware Round Bottom

Flasks, Three Neck, Threaded, 25 ml; Fisher Scientific Catalog No. 31-502-121); CAD files for the cap are provided in the Thingiverse repository, item no. 6737329. The remaining necks of the flask, which allow easy extraction of the rotifer suspension, were kept loosely capped to allow oxygenation. For rotifer treatment, rotifers were diluted 5× in 4 ppt NaCl, reducing the opacity of the suspension, to a concentration of roughly 400/ml and 15 ml of this suspension was placed in each round bottom flask. The maximum depth of the suspension was 2.2 cm. A 2 mm stir bar was used for continuous stirring. The UV intensity was measured to be 0.48 mW/cm² at the location of the water surface, 5.4 cm away from the LED.

Antibiotic treatment of rotifers. We used the same antibiotic solution and concentrations noted in the Zebrafish Gnotobiology section, treating 1 ml of rotifer suspension for 3.5 hours.

Rotifer motility assessment. Rotifer motility was assessed by tracking individual rotifers in brightfield movies captured at 10 frames per second with a scale of 6.5 μm/px. Using custom software, images were inverted and regions of interest containing rotifers were identified as local intensity maxima. Rotifers were localized in each frame using a symmetry-based algorithm [70] and connected across frames using nearest-neighbor linkage. A rotifer was considered motile if the mean speed of its trajectory exceeded 50 μm/s, which clearly distinguished moving and stationary rotifers.

Feeding. Before feeding, larvae were transferred to a new tissue culture flask with fresh sterile embryo medium. To 15 ml of the flask medium, we added 1 ml of rotifer

suspension, corresponding to roughly 400 rotifers, or roughly 30 per fish. Fish were allowed to feed *ad libitum*.

Light sheet fluorescence microscopy. Imaging was performed using a home-built light sheet fluorescence microscope based on the design of Keller et al. [18]. The protocols to mount the fish and the details of the microscope can be found in [17], [13], [21], and [15]. In brief, we anesthetize larvae with MS-222 (tricaine, 20 μ l/ml) and mount the fish in glass capillaries containing 0.7% agarose gel suspended vertically, head up, in a custom imaging chamber containing EM and MS-222. Lasers with excitation wavelengths of 488 and 568 nm were used to excite green fluorescent protein.

2.7 Acknowledgments

We thank Rose Sockol, Tim Mason, and the University of Oregon Zebrafish Facility staff for fish husbandry, providing rotifers, and general advice. We also thank Jonah Sokoloff for experimental assistance and Brendan Bohannon, Judith Eisen, Karen Guillemin, and John Rawls for advice and feedback.

This work was supported by the New Tools for Advancing Model Systems in Aquatic Symbiosis program of the Gordon and Betty Moore Foundation (doi:10.37807/GBMF9320), awarded to R.P. Additional support was provided by the National Science Foundation through the Research Traineeship (NRT) Award DGE 2022168 to S. M. R. and the National Institutes of Health through Award 1R01GM149827 to R. P. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

2.8 Supplemental Figures

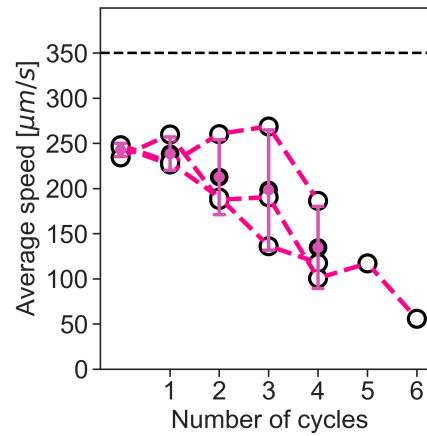


Figure 2.5. Average speed of motile rotifers as a function of the number of UV exposure cycles, each 30 minutes on / 30 minutes off. Dotted line at 350 $\mu\text{m/s}$ represents the average speed of rotifers before UV irradiation. Open symbols represent different replicates, and solid symbols and error bars indicate the mean and standard deviation.

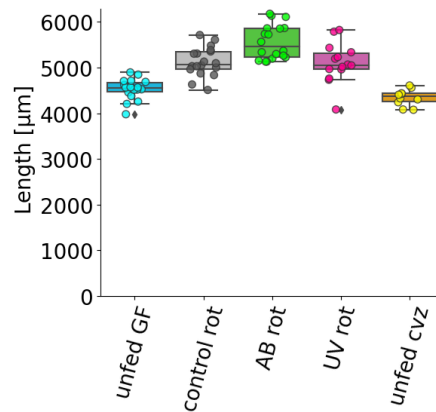


Figure 2.6. Fish length on 7 or 8 dpf after 2 or 3 days of feeding with rotifers under different treatments, or unfed at the same days. Each symbol indicates a measurement from an individual fish; boxes indicate the median (line within the box), first and third quartiles (top and bottom of the box), and 95% confidence intervals.

CHAPTER III

IMAGING THE IMPACT OF FOOD ON BACTERIAL BEHAVIORS IN THE ZEBRAFISH GUT

3.1 Abstract

The gut microbiota forms a diverse and complex ecosystem that influences the host's health, development, and nutrition. The ways in which food can alter bacterial behavior remain largely unknown, due primarily to the difficulty of assessing behavior *in situ*. In this project, we use *in vivo* imaging of zebrafish larvae to study how the ingestion of a common food (rotifers) affects the behavior of two commensal bacterial strains that, in the absence of food, show markedly different aggregation behavior. The optical transparency of zebrafish at early developmental stages and their ability to be prepared germ-free and then associated with specific bacterial species, combined with the use of light sheet fluorescence microscopy to visualize bacteria and rotifers inside the guts of live larvae, enables us to characterize physical properties of bacterial communities, such as their aggregation state and motility. We focus on a zebrafish-commensal *Enterobacter* species that forms large aggregates in unfed larvae. Surprisingly, we find that it disperses and becomes motile in the presence of rotifers. We also examine a pathobiont *Vibrio* species, motile and planktonic in unfed as well as fed larvae, feeding induces a marked decrease in the activity of the bacterial Type VI Secretion System (T6SS), leading to a decrease host tissue damage. Our results reveal that

feeding strongly shapes bacterial behavior and should be considered in models of gut interactions.

3.2 Introduction

Diet is one of the major factors that shapes and modulates the gut microbiota. Different nutrients influence the microbiota directly by altering substrate availability in the gut, and indirectly, by affecting the host, for instance increasing inflammation or changing intestinal permeability. Many studies have quantified how diet alters bacterial composition and diversity [71]. For example, fiber-rich diets increase the availability of microbiota-accessible carbohydrates (MACs), which promote greater microbial diversity and support the growth of beneficial species such as *Bifidobacterium* [72]. In contrast, high-fat and high-sugar diets shift the microbiota toward dysbiosis, characterized by overgrowth of pro-inflammatory Firmicutes and Proteobacteria and a reduction in Bacteroidetes ([73],[74]). Beyond composition, diet can also influence microbiota function by altering bacterial gene expression. For instance, *H. pylori* changes its gene expression under high-salt conditions [75], and food additives like carboxymethylcellulose (CMC) increase expression of bioactive flagellin, enhancing pro-inflammatory potential [76]. Such changes in gene expression may translate into altered bacterial behaviour within the gut. These behavioral changes can include differences in motility, spatial distribution, activation of self-defense mechanisms, or morphology. In vitro, advances in genomics, biochemistry, and imaging have uncovered

some of these changes. However, *in vivo* and in the gut environment, bacterial behavior remains challenging to study, especially in mammals.

Other animal models, such as zebrafish, offer a platform for studying bacterial behaviour *in vivo*. Zebrafish have extensive use in research due to genetic similarities with humans, rapid generation time, and cost-effectiveness. Additionally, their transparency during the larval stage makes it possible to observe bacterial dynamics in real time [10]. A library of zebrafish symbiotic bacteria tagged with fluorescent proteins [20], together with light sheet fluorescent microscopy (LSFM) — which provides rapid, three - dimensional, cell-resolved imaging—enables detailed observation of bacterial behavior and spatial organization within the gut.

Within the zebrafish symbiotic bacteria library, two strains stand out for their contrasting behaviours in the larval gut: *Enterobacter* ZOR0014 and *Vibrio cholerae* ZWU0020. In monoassociation, the first forms dense aggregates in the midgut [12] and has been shown to be less persistent under feeding regimes [77]. In contrast, *Vibrio cholerae* is mainly planktonic, highly motile, and usually fills the entire gut. This strain also drives strong gut contractions and carries a type VI secretion system (T6SS) that damages the posterior opening of the gut (vent). Using both the bacterial library and LSFM, we have also been able to observe other perturbation-driven behavior changes: for example, antibiotics induce *Vibrio* filamentation, shifting it from a planktonic to an aggregated state that makes it more vulnerable to peristalsis and expulsion [16], while *Aeromonas* MB4 invades and disrupts *Enterobacter* aggregates [14].

So far, however, these studies have used unfed larvae because feeding while maintaining gnotobiotic conditions remains a major challenge. Therefore, the question of how food affects symbiotic bacteria in the zebrafish gut has not been addressed. In previous work we developed a protocol to reduce the bacterial load in rotifers—a microanimal standardly used as a nutrient source in fish facilities—by irradiating them with UV light. This procedure enables us to maintain larvae under near-gnotobiotic conditions for at least one day after feeding and inoculation, while keeping them healthy [77].

In this study, we use UV-treated rotifers to investigate how food ingestion influences bacterial communities within the zebrafish gut. We first visualize food in fed larvae using LSM, and then examine how rotifers change the bacterial behaviour of *Enterobacter cloacae* (EN) and *Vibrio cholerae*. We show that food ingestion modifies bacterial behavior in distinct ways: EN shifts from non-motile aggregates to planktonic motile cells, and *Vibrio* reduces T6SS activity, leading to less vent damage.

3.3 Results

As noted above, we used recently developed methods to obtain nearly sterile rotifers using ultraviolet irradiation (see Materials and Methods). Because larval zebrafish hunt live rotifers, the time and amount of their consumption is variable. We first asked whether we could detect through imaging rotifers in the intestines of individual live larvae, to establish that feeding had occurred. Zebrafish larvae were derived germ-free using established methods [22] and then allowed to hunt UV-irradiated rotifers for around 20 hours at day 5

post-fertilization (dpf). Larvae were imaged the next day (6 dpf) using brightfield and light sheet fluorescence microscopy (LSFM) (method). Rotifers range from 100 μm to 300 μm — approximately the same order of magnitude as the larval gut—so we anticipated they would be visible within the gut. Bright-field images confirmed our expectation: several fed larvae showed a noticeably larger and darker anterior region compared with unfed larvae (Fig. Figure 3.7 B–C).

We also used LSFM to record fluorescence in two channels: one with excitation at 488 nm and emission at 510-550 nm (green), and another with excitation at 561 nm and emission at S $\text{Irange}\{590\}\{650\}\{\text{nm}\}$ (red). Because neither rotifers nor larvae expressed fluorescent proteins, signals in green and red channels only reflected autofluorescence. We observed the autofluorescence intensity in red and green channels was higher in fed larvae than in the unfed ones, with folded morphology that likely came from rotifers (Fig. Figure 3.7 C). To confirm this difference, we measured the maximum autofluorescence intensity in the green channel and the total autofluorescence intensity in the red channel (Materials and Methods). Both measurements consistently showed higher values in fed larvae than in unfed larvae demonstrating that the feeding state of larval zebrafish is clearly resolvable through imaging (Fig. Figure 3.7 D).

To gain a sense of the timescale of rotifer transit from the anterior to the posterior gut, we tracked them by imaging the larval gut every 15 minutes for 8 hours (Materials and Methods). We observed the rotifers remained in the anterior region of the gut for extended periods (hours) before descending to the vent, a process that lasted less than 1 hour. In the

example shown in Fig. Figure 3.7 E, a rotifer stayed in the anterior gut for 2 hours; however in other larvae, rotifers remained in the anterior region for more than 5 hours before descending. Overall, the timescale for the presence of rotifers in the gut is on the order of hours.

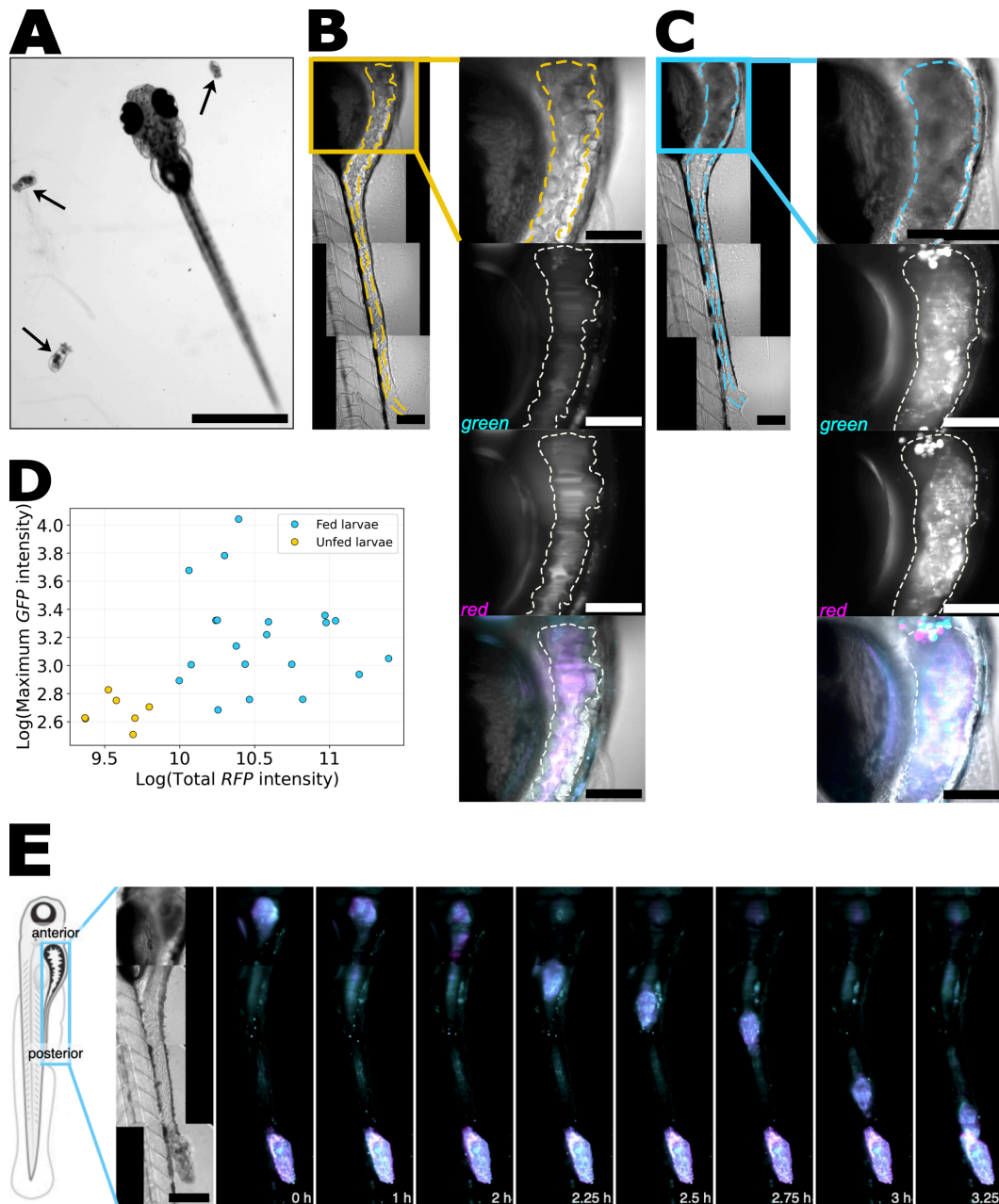


Figure 3.7. A A Zebrafish larva at 5 days post-fertilization (dpf) alongside rotifers (arrows). Scale bar: 1 mm. **B** The gut and surrounding regions of a germ-free, unfed larva at 6 dpf. The dashed line indicates the approximate intestinal boundary. The yellow box includes the anterior gut region, shown in the zoomed-in panels. From top to bottom: bright field, autofluorescence imaged with light-sheet fluorescence microscopy (LSFM) using blue/green excitation/emission wavelengths (excitation 488 nm, emission 510-550 nm, denoted ‘green’), autofluorescence using green/red excitation/emission wavelengths (excitation 561 nm, emission 590-650 nm, denoted ‘red’), and a merged composite image (gray: bright field, cyan: green autofluorescence, magenta: red autofluorescence). **C** Gut of a germ-free 6 dpf larva fed with rotifers, with a rotifer in the anterior gut. Zoomed-in panels include bright field, green and red autofluorescence, and a composite image as in (B). Scale bar: 100 μm . **D** Comparison of gut autofluorescence intensity in unfed (yellow dots) and rotifer-fed (light blue dots) larvae. Each dot represents one larval gut. Autofluorescence was measured in the green and red channels as described in the Methods. **E** From left to right: schematic of a larval zebrafish with the same orientation as in the following panels. Bright field image of a rotifer in the anterior gut at the initial imaging time. Composite overlays of images (cyan : green autofluorescence, magenta: red autofluorescence) from a series of time points showing rotifer transit. Time intervals are not uniform. Scale bar: 200 μm .

To qualitatively compare the organizational states, we segmented objects in images of unfed and fed larvae and compared their sizes (Materials and Methods, Fig. Figure 3.8 C). Large segmented objects corresponded to bacterial aggregates, whereas small objects corresponded to planktonic bacteria. On average, the segmented objects in unfed larvae measured two order of magnitude larger than the fed larvae ($277.59 \pm 70.4 \mu\text{m}^2$ mean $\pm$ SEM, and $2.68 \pm 0.1 \mu\text{m}^2$, respectively; Fig. Figure 3.8 D–F). These results show that EN forms aggregates in unfed larvae but shifts to a planktonic state in fed larvae.

In addition to being dispersed rather than aggregated, we also observed that EN became motile in fed larvae (video). We recorded videos in single optical planes at 20 ms intervals and tracked EN over 80 frames (Fig. Figure 3.8 G) (see Materials and Methods). With this data, we quantified EN mean speed of $25.12 \pm 7.36 \mu\text{m/s}$ (Fig. Figure 3.8 H), consistent with the run-speed observed in other EN strains [78]. Together, these findings show that rotifer ingestion changes EN behavior *in vivo*, shifting the bacteria from aggregated and non-motile to planktonic and motile.

To investigate whether this behavioral change was persistent after rotifers transit, we imaged an EN-inoculated and fed larval zebrafish every 15 minutes for 6 hours. After the rotifer completed its transit, EN re-forms dense aggregates (Fig. Figure 3.8 I).

To determine whether rotifers alone are sufficient to induce the shift in EN aggregation state, we dissected the guts of unfed larvae containing EN-GFP aggregates and incubated them in petri dishes (see Materials and Methods) with rotifers (concentration 2000 per ml) for 3 hours. We found that the aggregates stayed intact (Supplemental Fig. Figure 3.11), providing evidence that the presence of rotifers being digested within the gut environment is the driver of the bacterial transition, though it should be noted that the density of rotifers in the dish is considerably lower than the density of rotifers in the intestinal space.

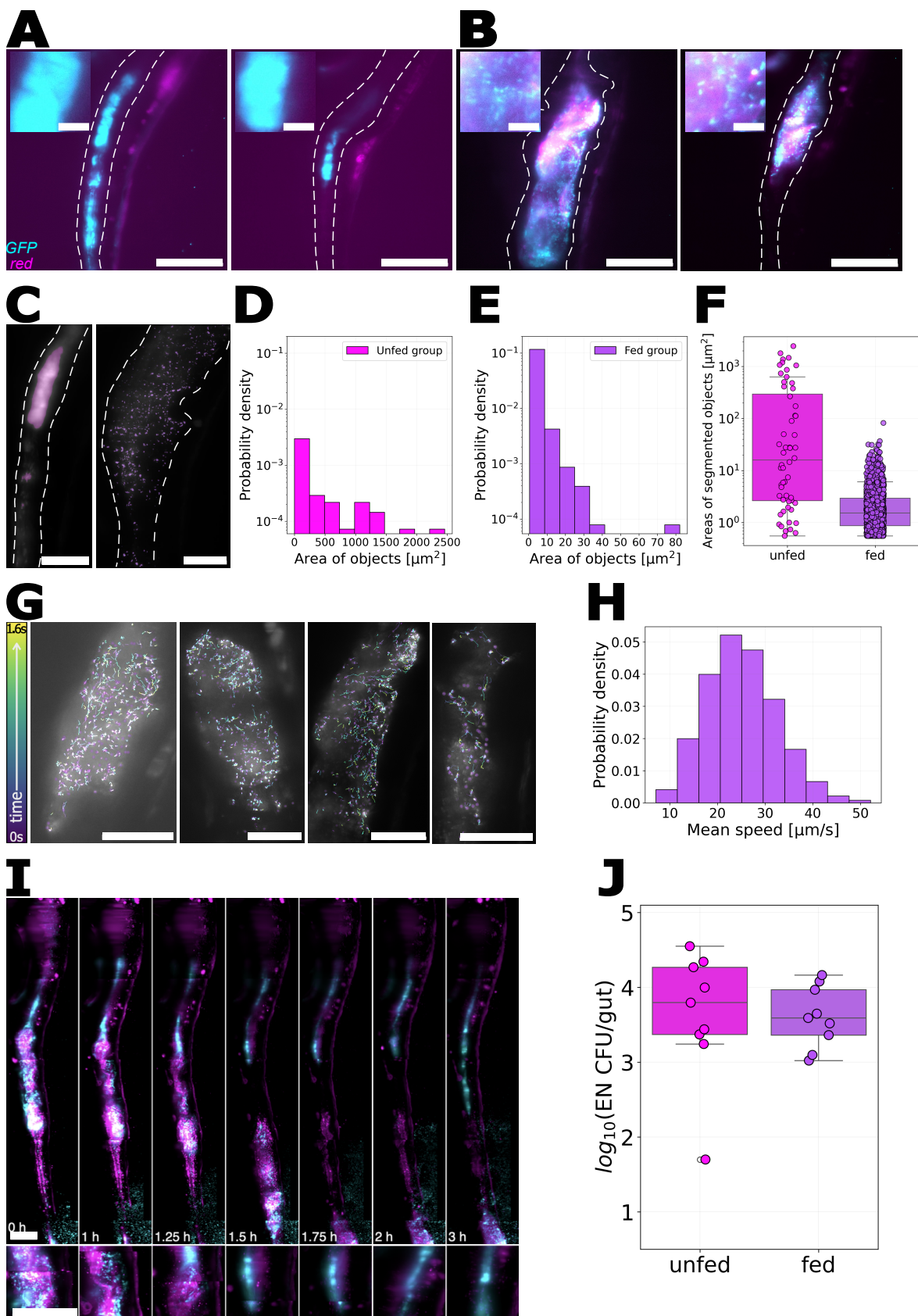


Figure 3.8. A-B Composite images of zebrafish intestines showing GFP-labeled *Enterobacter* (EN) (cyan) and red autofluorescence from the gut and rotifers (magenta). Dashed lines indicate approximate intestinal boundaries. Scale bar: 100 μ m. **A** EN aggregates in the midgut of unfed larvae. **B** Planktonic EN and rotifers in the guts of fed larvae. **C** Overlay of EN images and their corresponding segmentation (purple) in the gut of an unfed larva (left) and a fed larva (right). Scale bar: 50 μ m. **D-F** Histogram of sizes of fluorescent objects in images of **D** unfed larvae and **E** fed larvae, and **F** a summary comparison. $P < 0.001$. **G** Examples of EN tracking within rotifers in the gut of fed larvae. Scale bar: 50 μ m. **H** Probability distribution of EN speed within rotifers in guts of fed larvae. **I** Time-lapse of rotifer transit from anterior to the posterior gut of a larva inoculated with EN. After the rotifer reaches the posterior region, EN forms visible aggregates again. Images are composite overlays of the GFP signal (cyan) and autofluorescence in the red channel (magenta). Below each gut image is a zoomed-in view of the midgut, initially showing a rotifer and planktonic EN, followed by the appearance of bacterial aggregates. Time intervals are not uniform. Scale bar: 100 μ m. **J** EN abundance in dissected guts from unfed and fed larvae. $P = 0.37$.

Next, we describe the impact of rotifer feeding on a bacterial species that behaves very differently in unfed animals: *Vibrio cholerae* (ZWU0020), which is planktonic and motile in unfed larvae (Fig. Figure 3.9 A). We followed a protocol similar to the EN experiments: we began with germ-free larvae, inoculated them with *Vibrio*-GFP at either 5 or 6 dpf, fed them UV-treated rotifers three hours later, and imaged them the following day (6 or 7 dpf). In parallel, we maintained a control group of unfed larvae. We dissected and plated guts from both groups (see Materials and Methods) and found that *Vibrio* abundance appeared slightly higher in fed larvae ($\text{Log}_{10}(\text{CFU}) 4.36 \pm 0.39$, mean $\pm$ SD) than in unfed larvae (3.71 ± 0.66), though both values remained within the same overall range. LSFM imaging showed

that in both groups *Vibrio* preserved its planktonic organization and motility in the gut (Fig. Figure 3.9 B). To quantify this, we applied the same segmentation and tracking analysis used for EN. Segmented objects from both unfed and fed larvae had mean sizes of the same order of magnitude (unfed group: $3.54 \pm 0.14 \mu\text{m}^2$, mean $\pm$ SEM; fed group: $8.04 \pm 0.76 \mu\text{m}^2$; Fig. Figure 3.9 D–F), supporting that *Vibrio* remained planktonic regardless of feeding status. For motility, we tracked *Vibrio* in LSM videos recorded at 20 ms intervals over 80 frames (see Materials and Methods; Fig. Figure 3.9 G–H). The mean speed was 43.94 ± 12.4 mms (mean $\pm$ SD) in unfed larvae and 42.02 ± 13.39 in fed larvae (Fig. Figure 3.9 I), confirming that rotifer ingestion does not affect *Vibrio* motility. As noted earlier, a key feature of *Vibrio*'s intestinal behavior is the activity of its Type VI Secretion System (T6SS), a signature of which is damages to intestinal tissue, particularly resulting in widening of the gut vent (the posterior opening of the gut) ([79], [21]). We confirmed these results finding the unfed larvae inoculated with *Vibrio* had nearly double the vent width (103.53 ± 31.2 micro m, mean $\pm$ SD) compared with germ-free larvae (51.08 ± 12.87 micro m) and fed, non-inoculated larvae (50.02 ± 16.98 micro m). Strikingly, vent width was reduced in fed larvae inoculated with *Vibrio* to a value closer to that of germ-free or non-inoculated fish (63.34 ± 21.09 ; Fig. Figure 3.10 A-B). This result led us to ask whether rotifer ingestion might alter *Vibrio* behavior not by changing its organizational state or motility, but by influencing T6SS activity.

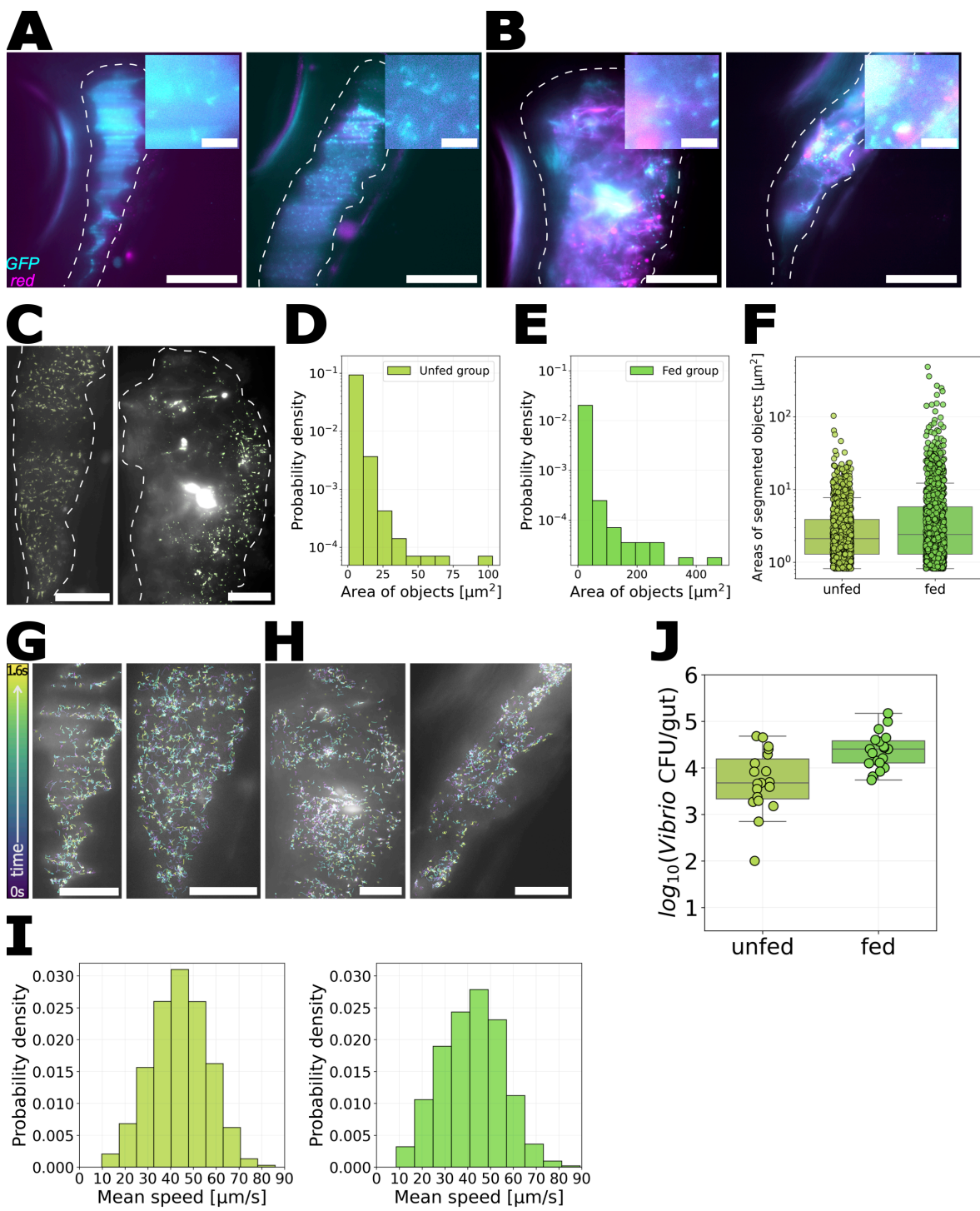


Figure 3.9. **A-B** Composite LSMF images of zebrafish intestines showing GFP-labeled *Vibrio* (cyan) and red autofluorescence from the gut and rotifers (magenta). Dashed lines indicate approximate intestinal boundaries. Scale bar: 100 micro m. **A** *Vibrio* in the anterior gut region of unfed larvae. **B** *Vibrio* and rotifers in the guts of fed larvae. **C** Overlay of *Vibrio* (gray) and their corresponding segmentation (green) in the guts of unfed larva (left) and fed larvae (right). Scale bar: 50 μ m. **D-F** Histogram of sizes of fluorescent objects in images of **D** unfed larvae and **E** fed larvae, and **F** a summary comparison. ($P < 0.001$). **G, H** Examples of tracking *Vibrio* in **G** unfed and **H** fed larvae. Trajectories are color-coded by time. Scale bar: 50 micro m. **I** Probability distribution of *Vibrio* speeds in guts of unfed larvae (left) and fed larvae (right). **J** *Vibrio* abundance in dissected guts from unfed and fed larvae. ($P = 0.014$).

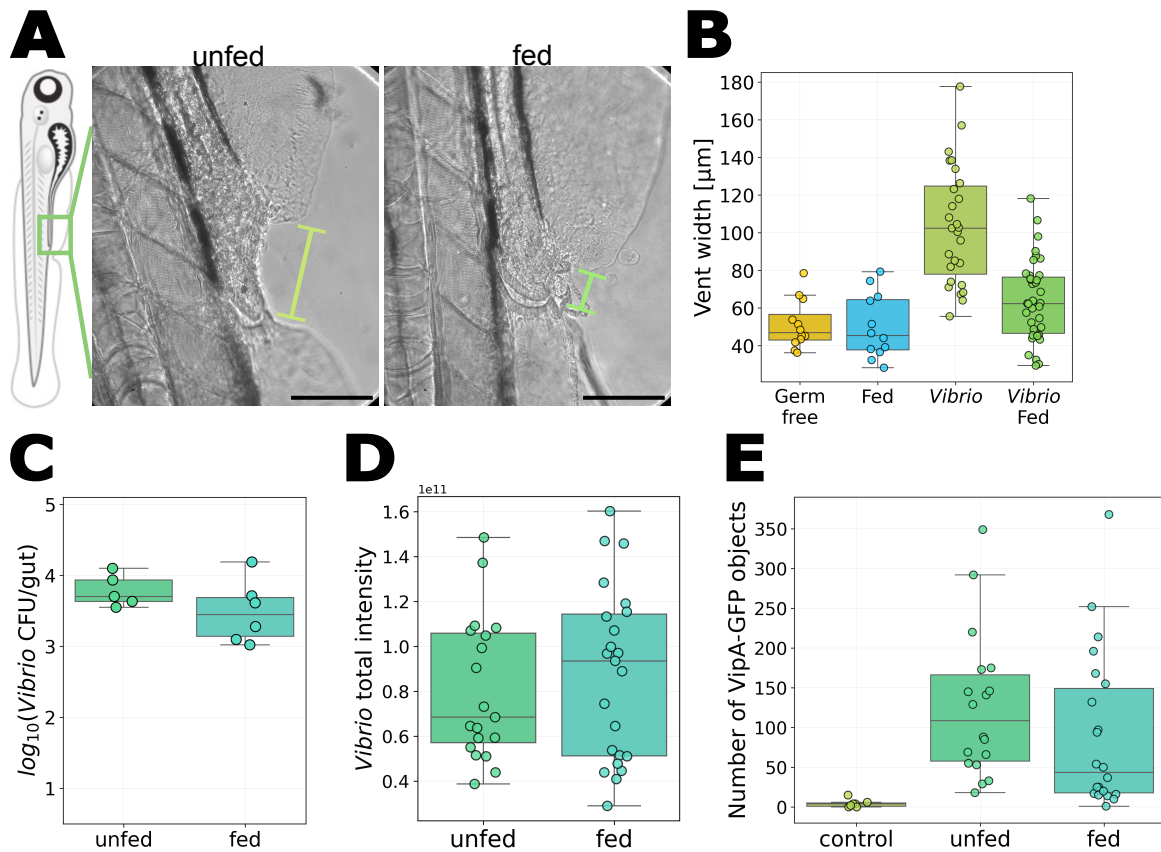


Figure 3.10. A Bright field images of the vent region in fed and unfed larvae mono-associated with *Vibrio*. Green lines indicate vent width. Scale bar: 100 μm . **B** Vent width of unfed and fed larvae, when they were germ-free or mono-associated with *Vibrio*; comparing *Vibrio* unfed and fed gives $P < 0.001$. **C** *Vibrio* abundance in dissected guts from unfed and fed larvae. $P = 0.708$ **D** Total fluorescence intensity of constitutively expressed dTomato, proportional to overall bacterial abundance, in the vicinity of the posterior vent in unfed and fed larvae. **E** The number of VipA-GFP puncta in the same fish and imaging regions as (C). The control group is unfed larvae mono-associated with *Vibrio*-dTom. $P = 0.075$ }

We repeated the experiment described for *Vibrio*, but this time inoculated larvae with the strain *Vibrio*-dTom;VipA-GFP, which constitutively expresses dTomato and carries a VipA-GFP fusion. In this strain, VipA-GFP appears as fluorescent puncta when the T6SS sheath assembles, and as diffuse fluorescence throughout the cell when it does not [80]. Although *in vivo* larval imaging cannot fully resolve subcellular puncta, differences in VipA-GFP fluorescence intensity allow us to compare relative T6SS activity. Dissected guts showed that rotifer ingestion did not alter bacterial load (Log10(CFU): 3.78 ± 0.23 in unfed larvae, mean $\pm$ SD, and 3.49 ± 0.44 in fed larvae; $P = 0.329$; Fig. Figure 3.10 C). We then studied fluorescence intensity, focusing the analysis on the posterior quarter of the gut, where intestinal damage is concentrated. We considered overall fluorescence from constitutive dTomato expression in the manually outlined intestinal volume, and the number of GFP puncta as an indicator of T6SS activity. The total fluorescence intensity in the constitutive bacterial channel was comparable between unfed ($8.75 \pm 3.84\text{e-}10$, mean $\pm$ SD) and fed larvae ($8.07 \pm 3.19\text{e-}10$; $P = 0.707$; Fig. Figure 3.10 D), indicating that the vent damage

difference was not explained by bacterial load or redistribution. Finally, we segmented and counted GFP puncta in the vent region (see Materials and Methods; Supplemental Fig. Figure 3.12) and found that fed larvae had fewer puncta (mean: 91.32) than unfed larvae (mean: 125.88; $P = 0.075$; Fig. Figure 3.10 E). Together, these findings suggest that rotifer ingestion alters *Vibrio* behavior in the larval gut, lowering T6SS activity and thereby reducing vent damage.

3.4 Discussion

In this work, we used UV-treated rotifers to observe their impact on symbiotic bacteria in the zebrafish gut after ingestion. We first demonstrated that LSFM provides a reliable approach to detect rotifers in the larval gut based on their morphology and autofluorescence. We then evaluated changes in bacterial behavior in two species: EN and *Vibrio*. With these results, we showed that one of the most used food sources for zebrafish larvae — rotifers — can influence bacterial behavior in very different ways depending on the species. Additionally, the UV treatment for rotifers ensures the observed behavioral changes are due to the food ingestion and no additional bacteria.

For EN, we found a shift from non-motile aggregates in unfed larvae to motile planktonic cells in fed ones. While for *Vibrio*, rotifer ingestion affected its behavior in a different way, decreasing the activity of the T6SS and the resulting tissue damage in the vent. This suggests that diet can directly modulate bacterial virulence and pathogenicity, thereby influencing the host's susceptibility to infection and immune response.

The molecular mechanisms that trigger the behavioral changes we observed were not resolved in this paper. Therefore, an interesting future direction is comparing the gene expression of EN and *Vibrio* in unfed and fed larvae to evaluate the regulatory pathways responsible for the observed shifts in motility, aggregation, and T6SS activity. Alternatively, specific genetic reporters could be used to visualize how key genes are activated in real time within the gut environment. These approaches would help connect the behavioral changes we observe under the microscope to the underlying molecular responses driving them.

Here, we focused on mono-associations to isolate the effects of food ingestion on each bacterial species. Since in general bacteria are forming communities in the gut, future work could examine how food ingestion influences these communities and whether the presence of other strains alters the behavioral responses of EN and *Vibrio*. Understanding whether EN's planktonic state and *Vibrio*'s reduced virulence persist in multi-species contexts will reveal new insights into bacteria–bacteria interactions within the larval gut.

Zebrafish have proven to be a powerful model for studying host–microbe interactions. With this work, we propose to extend these studies to also include the relationship with diet, given the relevance of the diet–microbiota–host triad in health and disease. Because rotifers are often used for nursing in fish facilities, we can use this nutritional source as a control to study how other diets, such as high-fat (chicken yolk), affect bacterial behavior and its impact on the host.

Additional studies could also explore how host responses contribute to the bacterial behavioral changes we observed. Because EN dispersion occurs only *in vivo*, it is likely

influenced by host-derived factors such as immune cell activity or signaling from intestinal epithelial cells. These questions could be addressed using transgenic zebrafish that express fluorescent labeled immune or gut cell types, allowing us to visualize host–microbe interactions directly. For *Vibrio*, future work could determine whether rotifer ingestion also reduces inflammation in other gut regions, such as the intestinal bulb. This could be examined using zebrafish lines that carry fluorescent inflammation reporters—for example, lines expressing GFP under the control of the *tnfal* promoters—to gain deeper insight into how food intake modulates host responses and bacterial behavior.

3.5 Materials and Methods

Animal care All experiments with zebrafish were done in accordance with protocols approved by the University of Oregon Institutional Animal Care and Use Committee and by following standard protocols [69].

Zebrafish gnotobiology Wild-type ABC X TU zebrafish (*Danio rerio*) were derived germ-free as described previously [22] with slight modifications. In brief, fertilized eggs were collected and placed in sterile antibiotic embryo medium (EM) containing 100 mg/ml ampicillin, 250 ng/ml amphotericin B, 15 mg/ml gentamicin, 1 mg/ml tetracycline, and 1 mg/ml chloramphenicol for approximately 5 hours. The eggs were then washed in sterile EM containing 0.003% sodium hypochlorite and then in sterile EM containing 0.1% polyvinylpyrrolidone-iodine. Washed embryos were distributed into tissue culture flasks

containing 15 ml of sterile embryo medium at a density of one embryo per. Flasks were inspected for sterility before being used in experiments.

Bacterial strains We used the bacterial strains isolated from the zebrafish intestine *Enterobacter* sp. strain ZOR0014 (EN) and *Vibrio* Cholerae ZWU0020 (*Vibrio*). From each, engineered strains constitutively expressing GFP were previously generated. Stocks of bacteria were maintained in 25% glycerol at $\geq 80^{\circ}\text{C}$.

Inoculation of larval zebrafish One day prior to fish inoculation, bacteria from frozen glycerol stocks were grown overnight in lysogeny broth (LB medium; 10 gram/liter NaCl, 5 gram/liter yeast extract, 12 gram/liter tryptone, and 1 gram/liter glucose) at 30°C with shaking. For inoculation, 1 ml of overnight culture was washed once by centrifuging for 2 minutes at $7,000 \times g$, removing the supernatant, and adding 1 ml of fresh sterile embryo medium. Then 30 ml of the bacterial mix was added to a 15 ml tissue culture flask containing approximately 15 germ-free 5 dpf zebrafish larvae, giving a bacterial concentration of approximately 10^6 CFU/mL.

Rotifers Rotifers (*Brachionis plicatilis*) were provided by the University of Oregon Zebrafish Facility. Rotifer cultures were raised in the facility in 5 gallon containers, fed with the “Rotigrow Plus” algae mixture (Reed Mariculture), and maintained at 4 ppt salinity in “Instant Ocean” commercial sea salt mix (Instant Ocean). Rotifers at a density of roughly 2000/liter were obtained as needed from the facility and used the same day.

UV irradiation of rotifers We follow the same protocol described in [77]. In short, we diluted rotifers 1:4 (volume/volume) with 4 ppt NaCl, and placed 15 ml of this

suspension in a three-neck round bottom flask, with a stir bar, for continuous stirring. The center cap has LED which provides UV light and the remaining necks of the flask were kept loosely capped to allow oxygenation. The rotifers are exposed to 4 cycles of UV light consisting of alternating 30-minute exposure and rest periods.

Feeding To 15 ml of the flask medium, we added 1 ml of UV-treated rotifer suspension, corresponding to roughly 400 rotifers, or roughly 30 per fish. Fish were allowed to feed ad libitum.

Light sheet fluorescence microscopy Imaging was performed using a home-built light sheet fluorescence microscope based on the design of Keller et al. [18]. The protocols to mount the fish and the details of the microscope can be found in references [17],[13],[21], [15]). In brief, we anesthetize larvae with MS-222 (tricaine, 20 microliter/mililiter) and mount the fish in glass capillaries containing 0.7% agarose gel suspended vertically, head up, in a custom imaging chamber containing EM and MS-222. Lasers with excitation wavelengths of 488 and 568 nanometer were used to excite green fluorescent protein and dTomato labeled bacteria, respectively. To image the entire extent of the intestine covering the gut (approximately $1,200 \times 300 \times 150$ micrometer) we move the specimen in z-steps of 1 micrometer and sequentially image four or five sub-regions computationally registering the images after acquisition. Registration is performed using custom software, publicly available in a GitHub repository (https://github.com/pamitabh/batch-processing/tree/main/use_friendly_downsampling_mip_stitch_batchprocess_code; function run2), which uses stage positions saved during image acquisition to align and stitch image stacks with the correct

offsets. For time series imaging, scans occurred at 15 or 20 minutes intervals for 8 hours durations.

Plating and bacterial abundance measurement To determine the intestinal abundance of bacterial species, dissections of larval zebrafish were performed at 6 dpf or 7 dpf in parallel with the imaging. Zebrafish were euthanized by hypothermal shock. Intestines were removed by dissection, placed in 1000 ml of sterile embryo medium, and homogenized with zirconium oxide beads using a bullet blender. The homogenized gut solution was diluted to 10^{-1} and 10^{-2} , and 100 ml volumes of these dilutions were spread onto agar plates. All plating abundance data are provided in Table 1.

Image Analysis All image processing and data analysis were performed with code written in Python using the NumPy [50], SciPy [51], scikit-image [52], and pandas [53] libraries.

Comparison of gut autofluorescence intensity in unfed and rotifer-fed larvae (no inoculation) We consider the first and second regions of the posterior part gut (bulb and mid-gut) because rotifers remain longer in that area than in the posterior side. We use the maximum intensity projection of red channel fluorescent images to create a coarse mask that allows us to exclude the yolk - highly autofluorescent - from the analysis. We compute the maximum fluorescence intensity in the green channel as the maximum pixel value among all the z-stacks images (within the mask limits). For the total intensity in the red channel, we sum all the intensities in all the z stacks within the mask.

Compute sizes of fluorescent objects in images of unfed and fed larvae In fed larvae, we consider the specific region of each fish where the rotifer is visible (usually first or second region), and in the unfed larvae, the region of the gut where the aggregates are visible (usually second region). The analysis per larvae is on the green channel maximum intensity projection of the 20 planes around the mid sagittal plane of the fish. First of all, as the gut has a lot of autofluorescence we use a hand drawn mask to segment out the lumen as that is the region in which bacteria will be found. Then we do a background subtraction followed by a high-pass filter. High-pass filter sharpens an image by highlighting its high-frequency details, which represent the edges and fine textures. This improves the detection of aggregates or bacterial puncta we fine tune it and verify the results by eye. Finally, we binarize the image with an Otsu threshold. We use the Python functions `label'` and `regionprops'` from `skimage` package to find the objects and their respective areas.

Tracking bacteria EN in fed larvae For the bacteria tracking we use 2D videos with a frame rate of 20 fps. The first step is to find a global Otsu threshold for all the frames. Since the number of bacteria on focus changes in each frame, the Otsu threshold also varies being very small on those with lower number of bacteria on focus. If that number is too small, the binarization does not exclude some regions without bacteria. Therefore, we start by computing the Otsu filter for each frame and use the average of them as global Otsu. We follow the next steps. In each frame we subtract the media of all the frames to discard the objects that are not moving, most of the time parts of rotifers. We use a high-pass filter to enhance the bacteria's positions and a mask to eliminate any brightness out of the gut. With

these images we compute the global threshold value. After that we binarize the images to use the `label` and `regionprops` functions from the *skimage* package of Python to identify a list of (x,y) positions and the `link_df` function from the *Trackpy* package to find trajectories. Finally we filter the trajectories whose maximum displacement is lower than 6 pixels, which probably does not correspond to motile bacteria, and those with length smaller than 5 frames. These parameters were set by direct observations. Finally for each trajectory we compute the average distance and with the information of the frame rate we conclude a mean speed value per bacteria. We used 8 fish and plotted the histogram of all the mean speed of the bacteria among all the larvae.

Tracking bacteria *Vibrio* in unfed and fed larvae The procedure is similar to the one used for EN, except for the value of the Gaussian parameter for the High filter (20) and the parameters to filter the final trajectories (maximum displacement 5 pixels and trajectories with length smaller than 4 frames). Compared with EN, *Vibrio* has a bigger size and higher speed. For the analysis we used 8 unfed and fed larvae.

Segmentation of VipA objects We only worked with the 4th position and manually made masks. For each z stack we first subtract the background and multiply the image with the mask. We use a High pass filter with a Gaussian sigma of 10. We binarized the 3D image and use the `label` and `regionprops` function from *skimage* package to detect the objects in the binarized images.

3.6 Acknowledgments

We thank Rose Sockol, Tim Mason, and the University of Oregon Zebrafish Facility staff for fish husbandry, providing rotifers.

This work was supported by the New Tools for Advancing Model Systems in Aquatic Symbiosis program of the Gordon and Betty Moore Foundation (doi:10.37807/GBMF9320). Additional support was provided by the National Science Foundation under the Research Traineeship (NRT) Award DGE 2022168. The funders had no role in study design, data collection and analysis, decision to publish, or preparation.

3.7 Supplemental Figures

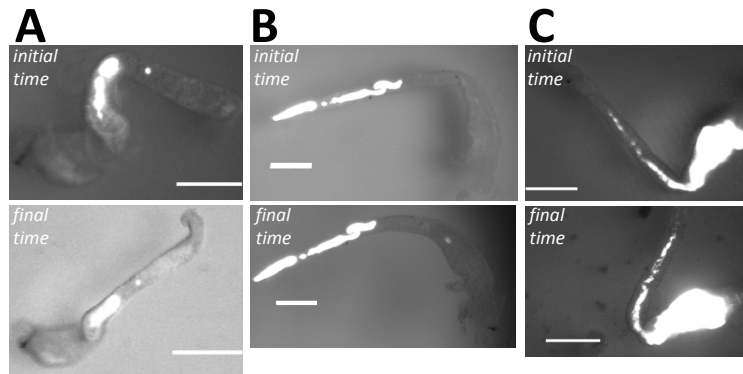


Figure 3.11. Dissected guts of zebrafish larvae containing fluorescent EN aggregates. Panels A–C correspond to different larvae. The upper panel shows the gut image at the initial time point in the green channel; the lower panel shows the final time point. Scale bar: 50 μm .

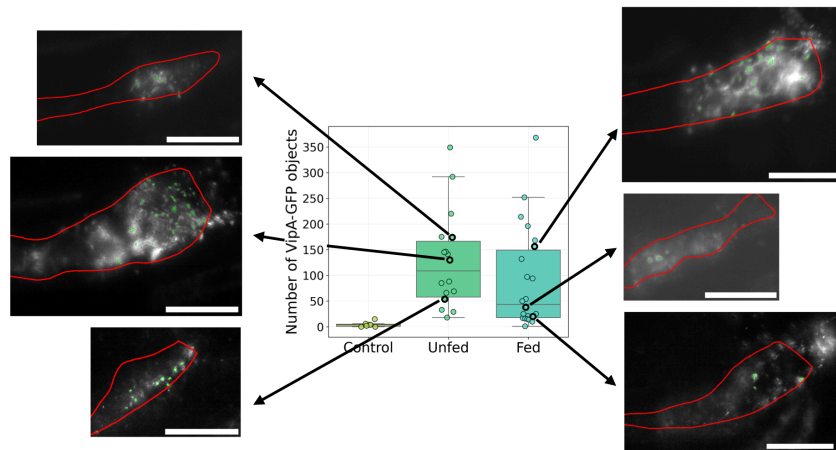


Figure 3.12. Examples of punctually segmented VipA in unfed and fed larvae, taken from the results shown in Figure Figure 3.10 E. Scale bar: 100 μm .

CHAPTER IV

CONCLUSIONS AND FUTURE WORK

In this thesis the bacterial-work interaction work with zebrafish was extended to investigate how food — specifically rotifers — affects bacterial communities. We first developed a method to decrease the bacteria load in the rotifers that uses UV-light. We used this method, together with other techniques, such as plating (entire larvae or dissected guts), and imaging using a light sheet fluorescent microscope, to address two fundamentally different inquiries: one related to bacterial stability, and the other to bacterial behavioral changes in the guts of fed larvae.

UV treatment was demonstrated to be efficient in maintaining specific bacterial strains or communities *in vivo* for longer periods than would be possible without feeding, enabling the study of the bacterial persistence and the effects of reinoculation. For more extended periods of feeding, we observed that some bacterial species persisted better than others when UV-treated rotifers were used, while untreated rotifers tended to reduce or even eliminate bacterial communities.

To study behavior changes we considered two bacterial species in monoassociation, revealing the rotifers modified them in very distinct manners: *Enterobacter* moved from aggregated non-motile to planktonic and motile, whereas *Vibrio*, decreased its T6SS activity and the resulting tissue damage in the vent. Using UV-treated rotifers ensured that the

observed shifts were due to the effect of food in the larvae and not attributable to other bacteria. This dissimilarity in behavioral changes motivates further observations of other bacterial monoassociations when larvae are fed.

The proposed methods opened the door to explore new aspects in the relation among food, microbiota and host, understudied so far in this animal model, offering new opportunities to investigate bacterial persistence, interspecies relation, and host-microbe interactions. For example, since it is already known that other symbiotic species such as *Aeromonas* and *Plesiomonas* also form aggregates in unfed larvae, it would be of interest to determine whether those aggregates disperse when the larvae ingest rotifers or whether other behavioral shifts occur as a result.

Because this work is mainly focused on bacterial effects, one of the pending aspects to further studies is to examine how the food ingestion affects the host. Today, there are multiple resources to elucidate effects of microbiota on the host. Transgenic lines are one of those, and combined with LSM techniques, could provide insights of variations in gut cell activity, immune cells, inflammation, and even in the effects on the neural system. For example, larvae with fluorescent neutrophils (*Tg(mpx:eGFP)*) and macrophages (*Tg(mpeg1:mCherry)*) combine with image analysis pipeline to characterize its motility could demonstrate whether food ingestion impact on immune cells. We could also evaluate larvae inflammation changes by measuring $\text{TNF}\alpha$ (*Tg(tnfa:GFP)*) and if rotifers ingestion increase or decreases this signal. This experiment could have relevance specially in fed larvae mono-associated with *Vibrio*, where we observe a decrease in the tissue damage. Other

experiments to explore host changes are related to quantifying the strength of gut peristaltic movements, which in the past have shown to have an important role on bacterial aggregate expulsion.

The mechanism by which *Enterobacter* and *Vibrio* alter their behavior is not resolved here, and additional experiments are required. We propose studying bacterial gene expression to determine which genes or pathways are differentially regulated under the conditions that trigger aggregation or motility. Using RT-qPCR, we could examine the expression of genes associated with biofilm formation and surface adhesion (such as *csgA*, *bssS*, or *fliC*) as well as motility regulation (such as *flhD*, *motA*, or *cheA*). This approach would allow us to determine whether the presence of rotifers directly or indirectly induces the activation of these genetic programs. Additionally, further studies on the host, such as those mentioned above, would also contribute to a better understanding of the phenomenon.

As we mentioned at the beginning, food is not the only factor with strong effects on the microbiota. Antibiotics, circadian cycle, social interactions are all elements which are currently being studied separately, and eventually can be combined with food consumption, using UV-treated rotifers. An example is how an appropriate diet, or in this case, rotifers consumption may benefit bacterial communities from the antibiotics effects when ingesting them.

Taking advantage of the different zebrafish symbiotic bacteria available, another interesting direction is to explore how food affects bacterial consortia. From previous

studies, it is known that the presence of other species can modify bacterial abundance and behavior. In the presence of rotifers, will the planktonic motile behavior of *Enterobacter* persist if it is inoculated into a community of five bacterial species, when some of the drastic bacterial effects are attenuated? What will occur under di-association?

Finally, even though the UV treatment effectively reduced bacterial load in rotifers and enabled the described experiments, feeding larvae and maintaining them germ-free were challenges that were not achieved in this study, and they definitely represent a direction for future researches.

REFERENCES CITED

- [1] K. Hou et al., Microbiota in health and diseases, *Signal Transduction and Targeted Therapy* **7**, 135 (2022).
- [2] D. Haller, *The Gut Microbiome in Health and Disease* (Springer, 2018).
- [3] S. Carding, K. Verbeke, D. T. Vipond, B. M. Corfe, and L. J. Owen, Dysbiosis of the gut microbiota in disease, *Microbial Ecology in Health and Disease* **26**, 26191 (2015).
- [4] D. Peterson, K. S. Bonham, S. Rowland, C. W. Pattanayak, R. Consortium, and V. Klepac-Ceraj, Comparative analysis of 16S rRNA gene and metagenome sequencing in pediatric gut microbiomes, *Frontiers in Microbiology* **12**, 670336 (2021).
- [5] Q. Tang, G. Jin, G. Wang, T. Liu, X. Liu, B. Wang, and H. Cao, Current sampling methods for gut microbiota: a call for more precise devices, *Frontiers in Cellular and Infection Microbiology* **10**, 151 (2020).
- [6] J. J. Faith et al., The long-term stability of the human gut microbiota, *Science* **341**, 1237439 (2013).
- [7] X. Zhou et al., Longitudinal profiling of the microbiome at four body sites reveals core stability and individualized dynamics during health and disease, *Cell Host & Microbe* **32**, 506 (2024).
- [8] L. Dethlefsen and D. A. Relman, Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation, *Proceedings of the National Academy of Sciences* **108**, 4554 (2011).
- [9] D. J. Grunwald and J. S. Eisen, Headwaters of the zebrafish—emergence of a new model vertebrate, *Nature Reviews Genetics* **3**, 717 (2002).
- [10] K. Stagaman, T. J. Sharpton, and K. Guillemin, Zebrafish microbiome studies make waves, *Lab Animal* **49**, 201 (2020).

- [11] K. Milligan-Myhre, J. R. Charette, R. T. Phennicie, W. Z. Stephens, J. F. Rawls, K. Guillemin, and C. H. Kim, Study of host–microbe interactions in zebrafish, *Methods in Cell Biology* **105**, 87 (2011).
- [12] B. H. Schlomann, T. J. Wiles, E. S. Wall, K. Guillemin, and R. Parthasarathy, Bacterial Cohesion Predicts Spatial Distribution in the Larval Zebrafish Intestine, *Biophysical Journal* **115**, 2271 (2018).
- [13] T. J. Wiles, M. Jemielita, R. P. Baker, B. H. Schlomann, S. L. Logan, J. Ganz, E. Melancon, J. S. Eisen, K. Guillemin, and R. Parthasarathy, Host gut motility promotes competitive exclusion within a model intestinal microbiota, *Plos Biology* **14**, e1002517 (2016).
- [14] D. Sundarraman, T. J. Smith, J. V. Kast, K. Guillemin, and R. Parthasarathy, Disaggregation as an interaction mechanism among intestinal bacteria, *Biophysical Journal* **121**, 3458 (2022).
- [15] T. J. Wiles, B. H. Schlomann, E. S. Wall, R. Betancourt, R. Parthasarathy, and K. Guillemin, Swimming motility of a gut bacterial symbiont promotes resistance to intestinal expulsion and enhances inflammation, *Plos Biology* **18**, e3000661 (2020).
- [16] B. H. Schlomann, T. J. Wiles, E. S. Wall, K. Guillemin, and R. Parthasarathy, Sublethal antibiotics collapse gut bacterial populations by enhancing aggregation and expulsion, *Proceedings of the National Academy of Sciences* **116**, 21392 (2019).
- [17] M. Jemielita, M. J. Taormina, A. R. Burns, J. S. Hampton, A. S. Rolig, K. Guillemin, and R. Parthasarathy, Spatial and temporal features of the growth of a bacterial species colonizing the zebrafish gut, *Mbio* **5**, 10 (2014).
- [18] P. J. Keller, A. D. Schmidt, J. Wittbrodt, and E. H. Stelzer, Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy, *Science* **322**, 1065 (2008).
- [19] M. J. Taormina, M. Jemielita, W. Z. Stephens, A. R. Burns, J. V. Troll, R. Parthasarathy, and K. Guillemin, Investigating bacterial-animal symbioses with light sheet microscopy, *The Biological Bulletin* **223**, 7 (2012).

- [20] T. J. Wiles, E. S. Wall, B. H. Schlomann, E. A. Hay, R. Parthasarathy, and K. Guillemin, Modernized Tools for Streamlined Genetic Manipulation and Comparative Study of Wild and Diverse Proteobacterial Lineages., *Mbio* **9**, e1877 (2018).
- [21] S. L. Logan, J. Thomas, J. Yan, R. P. Baker, D. S. Shields, J. B. Xavier, B. K. Hammer, and R. Parthasarathy, The *Vibrio cholerae* type VI secretion system can modulate host intestinal mechanics to displace gut bacterial symbionts, *Proceedings of the National Academy of Sciences* **115**, E3779 (2018).
- [22] E. Melancon, S. G. D. L. T. Canny, S. Sichel, M. Kelly, T. Wiles, J. Rawls, J. Eisen, and K. Guillemin, Chapter 3 - Best practices for germ-free derivation and gnotobiotic zebrafish husbandry, *Methods in Cell Biology* **138**, 61 (2017).
- [23] J. F. Rawls, B. S. Samuel, and J. I. Gordon, Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota, *Proceedings of the National Academy of Sciences* **101**, 4596 (2004).
- [24] L. N. Pham, M. Kanther, I. Semova, and J. F. Rawls, Methods for generating and colonizing gnotobiotic zebrafish, *Nature Protocols* **3**, 1862 (2008).
- [25] M. B. McElligott and D. M. O'malley, Prey tracking by larval zebrafish: axial kinematics and visual control, *Brain Behavior and Evolution* **66**, 177 (2005).
- [26] D. S. Mearns, J. C. Donovan, A. M. Fernandes, J. L. Semmelhack, and H. Baier, Deconstructing hunting behavior reveals a tightly coupled stimulus-response loop, *Current Biology* **30**, 54 (2020).
- [27] P. Coutteau, P. Lavens, and P. Sorgeloos, Manual on the production and use of live food for aquaculture, *FAO Fisheries Technical Paper* **361**, 7 (1996).
- [28] L. Ye, O. Mueller, J. Bagwell, M. Bagnat, R. A. Liddle, and J. F. Rawls, High fat diet induces microbiota-dependent silencing of enteroendocrine cells, *Elife* **8**, e48479 (2019).

- [29] A. P. Carvalho, L. Araújo, and M. M. Santos, Rearing zebrafish (*Danio rerio*) larvae without live food: evaluation of a commercial, a practical and a purified starter diet on larval performance, *Aquaculture Research* **37**, 1107 (2006).
- [30] G. H. F. Nuttall and H. Thierfelder., Thierisches Leben ohne Bakterien im Verdauungskanal., *Zeitschrift Für Physiologische Chemie* **23**, 231 (1897).
- [31] J. Reyniers, P. C. Trexler, R. Ervin, M. Wagner, T. Luckey, and H. Gordon, A Complete Life-Cycle in the " Germ-Free' Bantam Chicken, *Nature* **163**, 67 (1949).
- [32] H. A. Gordon, The germ-free animal: its use in the study of "physiologic" effects of the normal microbial flora on the animal host, *The American Journal of Digestive Diseases* **5**, 841 (1960).
- [33] J. A. Baker, M. Ferguson, and C. TenBroeck, Growth of platyfish (*Platyopocilus maculatus*) free from bacteria and other micro'organisms, *Proceedings of the Society for Experimental Biology and Medicine* **51**, 116 (1942).
- [34] J. M. Bates, E. Mittge, J. Kuhlman, K. N. Baden, S. E. Cheesman, and K. Guillemin, Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation, *Developmental Biology* **297**, 374 (2006).
- [35] J. V. Troll et al., Microbiota promote secretory cell determination in the intestinal epithelium by modulating host Notch signaling, *Development* **145**, dev155317 (2018).
- [36] I. Semova, J. D. Carten, J. Stombaugh, L. C. Mackey, R. Knight, S. A. Farber, and J. F. Rawls, Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish, *Cell Host & Microbe* **12**, 277 (2012).
- [37] C. C. Murdoch and J. F. Rawls, Commensal microbiota regulate vertebrate innate immunity-insights from the zebrafish, *Frontiers in Immunology* **10**, 2100 (2019).
- [38] M. Kanther, S. Tomkovich, S. Xiaolun, M. R. Grosser, J. Koo, E. J. Flynn III, C. Jobin, and J. F. Rawls, Commensal microbiota stimulate systemic neutrophil migration through induction of serum amyloid A, *Cellular Microbiology* **16**, 1053 (2014).

- [39] A. S. Rolig, R. Parthasarathy, A. R. Burns, B. J. Bohannon, and K. Guillemin, Individual members of the microbiota disproportionately modulate host innate immune responses, *Cell Host & Microbe* **18**, 613 (2015).
- [40] J. H. Hill, E. A. Franzosa, C. Huttenhower, and K. Guillemin, A conserved bacterial protein induces pancreatic beta cell expansion during zebrafish development, *Elife* **5**, e20145 (2016).
- [41] J. H. Hill et al., BifA, a microbiota-secreted membrane disrupter, disseminates to the pancreas and increases *bif* cell mass, *Cell Metabolism* **34**, 1779 (2022).
- [42] J. J. Bruckner, S. J. Stednitz, M. Z. Grice, D. Zaidan, M. S. Massaquoi, J. Larsch, A. Tallafuss, K. Guillemin, P. Washbourne, and J. S. Eisen, The microbiota promotes social behavior by modulating microglial remodeling of forebrain neurons, *Plos Biology* **20**, e3001838 (2022).
- [43] B. H. Schlomann and R. Parthasarathy, Gut bacterial aggregates as living gels, *Elife* **10**, e71105 (2021).
- [44] D. Sundarraman, E. A. Hay, D. M. Martins, D. S. Shields, N. L. Pettinari, and R. Parthasarathy, Higher-order interactions dampen pairwise competition in the zebrafish gut microbiome, *Mbio* **11**, 10 (2020).
- [45] C. J. Stewart et al., Temporal development of the gut microbiome in early childhood from the TEDDY study, *Nature* **562**, 583 (2018).
- [46] R. S. Mehta et al., Stability of the human faecal microbiome in a cohort of adult men, *Nature Microbiology* **3**, 347 (2018).
- [47] J. Lloyd-Price et al., Strains, functions and dynamics in the expanded Human Microbiome Project, *Nature* **550**, 61 (2017).
- [48] M. Rajilić-Stojanović, H. G. Heilig, S. Tims, E. G. Zoetendal, and W. M. de Vos, Long-term monitoring of the human intestinal microbiota composition, *Environmental Microbiology* **15**, 1146 (2013).

- [49] H. E. Jakobsson, C. Jernberg, A. F. Andersson, M. Sjölund-Karlsson, J. K. Jansson, and L. Engstrand, Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome, *Plos One* **5**, e9836 (2010).
- [50] L. A. David, A. C. Materna, J. Friedman, M. I. Campos-Baptista, M. C. Blackburn, A. Perrotta, S. E. Erdman, and E. J. Alm, Host lifestyle affects human microbiota on daily timescales, *Genome Biology* **15**, 1 (2014).
- [51] B. H. Schlomann and R. Parthasarathy, Timescales of gut microbiome dynamics, *Current Opinion in Microbiology* **50**, 56 (2019).
- [52] T. Forberg and K. Milligan-Myhre, Gnotobiotic fish as models to study host–microbe interactions, *Gnotobiotics* 369 (2017).
- [53] L. Provasoli and K. Shiraishi, Axenic cultivation of the brine shrimp *Artemia salina*, *The Biological Bulletin* **117**, 347 (1959).
- [54] S. G. de la Torre Canny, O. Mueller, C. V. Craciunescu, B. Blumberg, and J. F. Rawls, Tributyltin exposure leads to increased adiposity and reduced abundance of leptogenic bacteria in the zebrafish intestine, *Biorxiv* 2021 (2021).
- [55] O. Rendueles, L. Ferrières, M. Frétaud, E. Bégaud, P. Herbomel, J.-P. Levraud, and J.-M. Ghigo, A new zebrafish model of oro-intestinal pathogen colonization reveals a key role for adhesion in protection by probiotic bacteria, *Plos Pathogens* **8**, e1002815 (2012).
- [56] H. M. Lenhoff, Hatching brine shrimp larvae axenically and/or in a range of quantities, *Hydra: Research Methods* 39 (1983).
- [57] P.-P. Jia, Y.-F. Yang, W.-G. Li, J.-J. Duan, Y. Wang, and D.-S. Pei, Breaking the mold: The first report on germ-free adult marine medaka (*Oryzias melastigma*) models, *Biorxiv* 2023 (2023).
- [58] S. M. Limbu, L.-Q. Chen, M.-L. Zhang, and Z.-Y. Du, A global analysis on the systemic effects of antibiotics in cultured fish and their potential human health risk: a review, *Reviews in Aquaculture* **13**, 1015 (2021).

- [59] P. Dhert, G. Rombaut, G. Suantika, and P. Sorgeloos, Advancement of rotifer culture and manipulation techniques in Europe, *Aquaculture* **200**, 129 (2001).
- [60] C. Harper and C. Lawrence, *The Laboratory Zebrafish* (CRC Press, 2016).
- [61] E. C. Dougherty, B. Solberg, and L. G. Harris, Synxenic and Attempted Axenic Cultivation of Rotifers, *Science* **132**, 416 (1960).
- [62] N. T. N. Tinh, Nguyen Ngoc Phuoc, K. Dierckens, P. Sorgeloos, and P. Bossier, Gnotobiotically grown rotifer *Brachionus plicatilis sensu strictu* as a tool for evaluation of microbial functions and nutritional value of different food types, *Aquaculture* **253**, 421 (2006).
- [63] C. T. Chang, S. Benedict, and C. M. Whipps, Transmission of *Mycobacterium chelonae* and *Mycobacterium marinum* in laboratory zebrafish through live feeds, *Journal of Fish Diseases* **42**, 1425 (2019).
- [64] P. Munro, R. Henderson, A. Barbour, and T. Birkbeck, Partial decontamination of rotifers with ultraviolet radiation: the effect of changes in the bacterial load and flora of rotifers on mortalities in start-feeding larval turbot, *Aquaculture* **170**, 229 (1999).
- [65] I. Cho et al., Antibiotics in early life alter the murine colonic microbiome and adiposity, *Nature* **488**, 621 (2012).
- [66] A. S. Rolig, E. G. Sweeney, L. E. Kaye, M. D. DeSantis, A. Perkins, A. V. Banse, M. K. Hamilton, and K. Guillemin, A bacterial immunomodulatory protein with lipocalin-like domains facilitates host–bacteria mutualism in larval zebrafish, *Elife* **7**, e37172 (2018).
- [67] S. Lam, H. Chua, Z. Gong, T. Lam, and Y. Sin, Development and maturation of the immune system in zebrafish, *Danio rerio*: a gene expression profiling, in situ hybridization and immunological study, *Developmental & Comparative Immunology* **28**, 9 (2004).
- [68] W. E. Ruff, T. M. Greiling, and M. A. Kriegel, Host–microbiota interactions in immune-mediated diseases, *Nature Reviews Microbiology* **18**, 521 (2020).

- [69] M. Westerfield, *The Zebrafish Book; A Guide for the Laboratory Use of Zebrafish (Danio Rerio)* (University of Oregon Press, 2007).
- [70] R. Parthasarathy, Rapid, accurate particle tracking by calculation of radial symmetry centers, *Nature Methods* (2012).
- [71] P. J. Turnbaugh, V. K. Ridaura, J. J. Faith, F. E. Rey, R. Knight, and J. I. Gordon, The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice, *Science Translational Medicine* **1**, 6ra14 (2009).
- [72] N. K. Leeuwendaal, C. Stanton, P. W. O'toole, and T. P. Beresford, Fermented foods, health and the gut microbiome, *Nutrients* **14**, 1527 (2022).
- [73] A. Agus et al., Western diet induces a shift in microbiota composition enhancing susceptibility to Adherent-Invasive E. coli infection and intestinal inflammation., *Scientific Reports* **6**, 19032 (2016).
- [74] M. A. Hildebrandt, C. Hoffmann, S. A. Sherrill–Mix, S. A. Keilbaugh, M. Hamady, Y. Chen, R. Knight, R. S. Ahima, F. Bushman, and G. D. Wu, High-fat diet determines the composition of the murine gut microbiome independently of obesity, *Gastroenterology* **137**, 1716 (2009).
- [75] J. T. Loh, A. C. Beckett, M. B. Scholz, and T. L. Cover, High-salt conditions alter transcription of *Helicobacter pylori* genes encoding outer membrane proteins, *Infection and Immunity* **86**, 10 (2018).
- [76] B. Chassaing, T. Van de Wiele, J. De Bodt, M. Marzorati, and A. T. Gewirtz, Dietary emulsifiers directly alter human microbiota composition and gene expression ex vivo potentiating intestinal inflammation, *Gut* **66**, 1414 (2017).
- [77] S. Márquez Rosales, P. I. Bouchard, E. M. Olmstead, and R. Parthasarathy, UV-irradiated rotifers for the maintenance of gnotobiotic zebrafish larvae, *Msphere* **10**, e698 (2025).

- [78] S. Lim, X. Guo, and J. Q. Boedicker, Connecting single-cell properties to collective behavior in multiple wild isolates of the *Enterobacter cloacae* complex, *Plos One* **14**, e214719 (2019).
- [79] J. S. Ngo, P. Amitabh, J. G. Sokoloff, C. Trinh, T. J. Wiles, K. Guillemin, and R. Parthasarathy, The *Vibrio* type VI secretion system induces intestinal macrophage redistribution and enhanced intestinal motility, *Mbio* **16**, e2419 (2025).
- [80] M. Kudryashev, R. Y.-R. Wang, M. Brackmann, S. Scherer, T. Maier, D. Baker, F. DiMaio, H. Stahlberg, E. H. Egelman, and M. Basler, Structure of the type VI secretion system contractile sheath, *Cell* **160**, 952 (2015).