Video Article

Examination of Host Phenotypes in *Gambusia affinis* Following Antibiotic Treatment

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Abstract

The commonality of antibiotic usage in medicine means that understanding the resulting consequences to the host is vital. Antibiotics often decrease host microbiome community diversity and alter the microbial community composition. Many diseases such as antibiotic-associated enterocolitis, inflammatory bowel disease, and metabolic disorders have been linked to a disrupted microbiota. The complex interplay between host, microbiome, and antibiotics needs a tractable model for studying host-microbiome interactions. Our freshwater vertebrate fish serves as a useful model for investigating the universal aspects of mucosal microbiome structure and function as well as analyzing consequential host effects from altering the microbial community. Methods include host challenges such as infection by a known fish pathogen, exposure to fecal or soil microbes, osmotic stress, nitrate toxicity, growth analysis, and measurement of gut motility. These techniques demonstrate a flexible and useful model system for rapid determination of host phenotypes.

Video Link

The video component of this article can be found at https://www.jove.com/video/55170/

Introduction

It has been established that antibiotics can disrupt the human microbiome leading to dysbiosis, meaning a microbial community imbalance. The microbiota's compositional alteration after antibiotic treatments has been shown to lower the community's diversity, reduce key members, and alter community metabolism, especially in the gut[1,2]. Antibiotic disturbance of the gut microbiome can reduce colonization resistance to *Clostridium difficile*[3,4] and *Salmonella*[5].

Additionally, the disruption of the microbiota has been linked to the development of many syndromes and diseases in humans (*e.g.,* antibiotic-associated enterocolitis, inflammatory bowel disease, metabolic disorders, *etc.*). Antibiotics are also widely implemented in agriculture as growth promoters in livestock and poultry production[6]. The usage of these powerful tools is not without collateral effects, which is evident in the rapid rise of antibiotic resistance, as well as the effects of a disrupted microbiome has with its inhabited host. Many studies have shown that broad-spectrum antibiotic usage has long lasting consequences to the structure and function of the microbiota, yet the side effects from an antibiotic-disrupted microbiome impacting host physiology are only speculations which have yet to be supported.

The interplay between host, microbiota, and antibiotics is far from being understood in a concise manner. Therefore, a simple and more tractable model is advantageous to shedding light on the highly complex mammalian system. Mucosal surfaces in humans, including the gut, harbor the highest density and diversity of microbes, and also the most intimate microbe-host interactions. The mucosal skin microbiome of fish offers several advantages as a model system. The *Teleostei* (bony fish) is one of the earliest lineages to diverge within the *Vertebrata* meaning that teleosts have both innate and acquired immune systems that have co-evolved a relationship with commensal bacterial communities[7]. Fish skin shares many characteristics with type 1 mucosal surfaces of mammals, such as physiological functions, immunity components, and arrangement of mucus-producing cells[8]. The external location of the fish skin mucosal surface offers a microbiome easy to experimentally manipulate and sample.

The Western mosquitofish, *Gambusia affinis (G. affinis)*, is a model fish that has been used in the past for studying mating and toxicology[9,10,11]. Given the small size, population abundance in the wild as an invasive species, minimal care cost, and hardy nature, we have developed *G. affinis* as a mucosal microbiome model. Further, *Gambusia* share the physiology of giving birth to live young with viviparous mammals, which is uncommon in fish species. We completed the most extensive study at the time of fish skin normal microbiota using 16S profiling with *Gambusia*[12]. Further work demonstrated three negative effects on the host following disruption of the skin and gut microbiota using a broad-spectrum antibiotic[13].

Five different effects were examined in the fish following antibiotic exposure. The most well established host benefit of the microbiome is competitive exclusion of pathogens. The fish pathogen *Edwardsiella ictaluri* is known to cause outbreaks of enteric septicemia in commercial

catfish farms[14]. *E. ictaluri* has also been shown to lethally infect zebrafish[15,16] and *Gambusia*[17]. A challenge with this pathogen from the water column can serve as a measure of exclusion. As a comparison to susceptibility to an individual pathogen, survival during exposure to a high density of mixed organisms was also carried out. Feces and organic-rich soil were used as commonly-encountered sources of microbial communities.

Another established role the bacterial gut community performs is nutrient processing and energy harvest, thus affecting the overall nutritional uptake for the host. As a gross measurement of nutrition, fish body weight was compared before and after one month of being fed a standard diet. Antibiotic-treated fish as an average lost weight while control fish on average gained weight over the month. The mechanism for this lack of weight gain is unclear. One possible contributing factor is transit time of food in the gut. A GI motility method was adapted from zebrafish (Adam Rich, SUNY Brockport, personal communication) to determine transit time. It has not yet been determined if antibiotic-treated fish have an altered transit time.

A common challenge experienced in the natural environment by all organisms, especially fish, is osmotic stress. *Gambusia* have been shown to quickly adapt when acutely stressed in high concentrations of salinity[18]. Surprisingly, fish with an antibiotic-altered microbiome exhibited lowered survival to a high salt stress. The mechanism for this novel phenotype is under investigation. Another common stress on aquatic animals, especially in aquaria, is toxic forms of nitrogen (ammonia, nitrate, and nitrite). Survival against nitrate was not significantly different between antibiotic-treated and control fish. The methods presented in this manuscript can be used with *Gambusia* or similar fish model organisms, such as zebrafish and medaka, to measure phenotypes in the fish following experimental manipulation.

Protocol

All animal experiments were conducted under approval of IACUC protocols, numbered 14-05-05-1018-3-01, 13-04-29-1018-3-01, and 14-04-17-1018-3-01.

1. Animal Collection, Handling, and Ethical Care

1. Collect *Gambusia affinis* from the field site (identification guide at http://www.sms.si.edu/irlspec/Gambusia_affinis.htm) using a small dip net and place into 19 L buckets. Use visual inspection to identify species.
2. Rest fish for 1 - 2 d in a bucket with pond water. Afterwards, transfer fish into 76 or 189 L aquarium tanks filled with half pond water/half tap water at 25 °C, aerated with a bubbling airstone. Use a small dip net when handling fish as to minimally disturb their skin microbiome.
   NOTE: Fish density should be no higher than 20 fish/38 L of aquarium water. Fish are acclimated to the aquarium for at least 5 d before experimentation.
3. Feed fish on a daily regimen with 5 mg of flake food per fish. Hold fish with a 12 h light/ 12 h dark cycle.

2. Initial Antibiotic Exposure for All Experiments

1. Drawing all fish from each experiment from the same aquarium, place into two separate groups (treated: exposed to antibiotic & control: unexposed) in 19 L buckets. Previous data collected shows that fish in the same aquarium have homogenized skin microbiomes that have little variation in community composition.[12]
2. During experiments, keep fish in 2 L of artificial pond water (APW; 0.33 g/L $CaCl_2$, 0.33 g/L $MgSO_4$, 0.19 g/L $NaHCO_3$) that is sterilized by autoclaving before usage.
3. Prepare the rifampicin antibiotic stock solution by adding 50 mg/mL into dimethyl sulfoxide (DMSO). Rifampicin is a representative broad-spectrum antibiotic that is non-toxic to the fish. In humans and mice, it distributes well in the tissues.
4. From the rifampicin stock administer a final concentration of 25 µg/mL in 2 L of APW for antibiotic exposure of the treated fish group for 3 days. Note that after 3 d, culturable skin bacterial numbers return similar to that of pretreated fish skin.
5. In parallel, keep a group of untreated fish in APW and give an equivalent amount of DMSO (0.5 mL per L of APW) into the water column to act as a control group.
6. As experiments are intended to measure effects of an antibiotic-altered microbiome on fish phenotypes, in order to avoid effects directly from the antibiotic itself, following the three-day antibiotic exposure (or control) period, transfer fish into a bucket with 2 L of fresh APW.
   1. Use a 10 h rest period so the antibiotic is removed by the bodies of the fish. Base this elimination time on experiments where fish were exposed to 25 µg/mL of antibiotic for three days. Euthanize the fish by snipping the spinal cord followed by pithing, then homogenize the entire body using a tissue grinder.
   2. Determine antibiotic presence by placing 50 µL of this suspension after 0.2 µm filtering into the center of an agar petri dish. Make a hole for this suspension by pressing an upside down sterile 200 µL pipette tip into the agar, which removes a small plug.
   3. Use agar plates with 20 mL measured volume of nutrient agar, and spread evenly using a cotton swab with an indicator organism, *Bacillus subtilis*, which is sensitive to rifampicin. Obtain the *B. subtilis* from a nutrient agar incubated at 25 °C O/N and using the cotton swab to obtain a colony. Observing a zone of inhibition after overnight incubation at 25 °C indicates presence of antibiotic in the fish tissues.

3. Microbiome Extraction

1. Extract a **skin microbiome** sample from the outer mucosal epidermis by placing the fish into a sterile 15 mL conical tube filled with 2 mL sterile PBST (137 mM NaCl, 10 mM phosphate, 0.1% Tween-20, pH 7.4) solution and vortexing at a setting of 10 for 1 min with 10 s pausing increments. Detergent and salt in the buffer promotes even dispersion of bacteria in solution. Comparable results were found with 0.85% saline but lowered bacterial counts with pure water.

2. Transfer the fish from the conical tube to a recovery bucket. Lethality of skin extraction is typically lower than 10%. Either, analyze the bacterial suspension in the tube immediately upon extraction or pellet the bacteria by a 2 min spin in a centrifuge at room temperature at 15,000 x g and store at -80 °C for future analysis.
   NOTE: All culture and biochemical analyses are immediate; DNA or protein extraction can be from frozen samples.
3. To obtain a **gut microbiome** sample, first place the fish in a sterile petri dish. Euthanize the fish by severing the cervical spinal cord directly behind the head with surgical scissors, followed by pithing, and then externally sanitize the body with 70% ethanol wipes.
4. After dissection, remove the entire gut by cutting after the esophagus and before the anus.
5. Cut the gut into small sections 1 - 2 mm in length and place all sections into two mL PBST solution in a conical tube. After vortexing for 1 min, remove supernatant into another clean tube (take caution to not draw up the gut sections).
   NOTE: The supernatant contains extracted gut bacteria that can either be used for direct analyses or pelleted by the previous method mentioned for skin samples.

4. Infection Model Preparation and Bath of a Specific Pathogen

1. Obtain an infectious strain of *Edwardsiella ictaluri* (*E. ictaluri*) and keep the culture stored at -80 °C. Strain 93 - 146 used in this study, isolated from an infected catfish, was obtained from Mark Lawrence, College of Veterinary Medicine, Mississippi State University.
2. Streak *E. ictaluri* stock onto a selective & differential media called *E. ictaluri* media (EIM) agar[19] to culture the bacteria and incubate for 2 d at 27 °C.
3. Transfer a pure isolated colony from the culture and inoculate a 150 mL flask containing 40 mL of nutrient broth (NB; 5 g/L peptone, 4 g/L beef extract). Place flask in a shaker set at 150 rpm for 2 d at 27 °C.
4. After incubation, dilute a sample of the culture in PBST to a spectrometer reading of 0.2 OD at 650 nm to calibrate *E. ictaluri* culture for desired infectious dose volumes.
5. Next transfer both treated (after 3 d of antibiotic exposure) and untreated fish groups into individual plastic cups containing 130 mL of fresh artificial pond water, or APW for approximately 10 h for drug clearance from fish tissues.
6. Then from both groups, transfer again each fish into 8-ounce plastic cups containing 130 mL of clean APW.
7. Give each fish a lethal dose containing 2.8 x 10^6 CFU/mL of *E. ictaluri* culture into the APW (bath infection model) and keep in a 27 °C incubator for 24 h.
8. After the *E. ictaluri* bath, transfer fish individually into new cups with 130 mL of APW.
9. Following water replacement, record fish mortality over the duration of a week. Fish are not fed over this period. In contrast to catfish, *Gambusia* show no external signs of infection, therefore it is essential to use lethality as an experimental endpoint.

5. Polymicrobial Challenge with Feces & Soil

1. **Feces Treatment**
   1. Obtain fresh human feces from a healthy volunteer subject who has not received antibiotics in the previous two weeks, using sterile gloves and a sterile 50 mL conical tube.
   2. Upon collection, gather fecal matter in a sterile plastic bag and then transfer into a sterile 15 mL conical tube. Create a stock concentration by suspending feces in PBST to give a stock solution of 500 - 800 mg/mL. This thick mixture is best to transfer using a 1 mL or larger plastic pipette tip that has been cut at the bottom with sterile scissors so that the opening is larger.
   3. After initial antibiotic exposure of treated and control untreated fish groups, separate into individual plastic cups containing fecal suspension at final concentrations of either 15 mg/mL or 10 mg/mL into APW with a total volume of 130 mL. Hold cups in an incubator at 25 °C.
   4. Record fish mortality during fecal exposure by close observation over 2 d.

2. **Soil Treatment**
   1. Collect 1 - 2 kg of rich organic topsoil (should contain the highest density and diversity of microbes) approximately 7 - 15 cm down in depth and place into a clean plastic container. Note that the soil should be used within two days following collection.
   2. Directly after initial exposure period, separate both groups of antibiotic treated and untreated fish into individual plastic cups containing 18.2 g of soil in 130 mL of APW. Mix the soil in the water well by hand before adding the fish. Most of the particulates do not suspend and stay at the bottom of the cup.
   3. Expose fish for a duration of 3 d at 25 °C and record any mortality.

6. Osmotic Stress Challenge

1. After treatment followed by a 10 h rest period as described above, individualize fish into separate cups containing NaCl at 17.5 mg/mL (300 mM) in 150 mL of APW. This is half of the typical salinity of seawater, which is not fully lethal (<50%) to control fish but is strongly stressful, requiring effective osmoregulation.
2. Observe for fish mortality over a duration of 36 h.

7. Nitrate Toxicity Challenge

1. Rest fish for a 10 h period after antibiotic exposure, and then separate fish into individual cups containing a sodium nitrate concentration of either 10 mg/mL (118 mM) or 17.5 mg/mL (206 mM) in 130 mL of APW.
2. Record for mortality over 4 d for low dose and 1 d for high dose.

8. Growth Analysis of Individualized or Grouped Fish

1. Complete 3 d antibiotic exposure or control period as groups in buckets.
2. For *Individual*, fill 8-oz Styrofoam cups with 150 mL APW each, transfer an individual fish into the cup and record total body weight for initial assessment.
    1. Repeat for all fish in both treated and untreated groups. Use white Styrofoam cups instead of clear plastic cups because fish will not eat when in the clear cups.
3. Feed fish daily with the standard diet of two pellets per fish. Pellets were used rather than flakes because pellets have low variance in individual mass (3.53 ±0.42 mg each, or 8% variation), resulting in fish receiving similar amounts of food. Monitor consumption by visually recording uneaten pellets. Consumption rates were typically above 80%.
4. At the end of each week over 4 weeks, determine fish weight. Prepare a new cup with fresh APW, place on a balance, and then record the weight. Then, pour a fish into a dip net from the original cup and transfer into the new cup, which is then weighed again. Fish are not handled to avoid stress.
5. For *Groups*, place 2 L of APW into a 19 L bucket and tare the scale. Keep fish together in both groups when transferring into new APW buckets then record total group weight. Record fish group weight each week over a month time span by transferring to a new bucket.

9. Gut Transit Time

1. Use fluorescein-labeled 70 kDa anionic dextran. Dextran is not significantly digested, and is too large to be absorbed across the gut layer, thus passage will measure transit time through the gut. Labeled dextran was incorporated into fish food.
2. Into a sterile 1.7 mL tube, add 360 µL of sterile deionized water and 52 mg of gelatin (13% solution), and place the tube on a 60 °C heat block until the gelatin melts and is fully dissolved.
    1. Grind goldfish food flakes to a fine powder using a mortar and pestle, and add 40 mg of this powder to the tube, along with 20 µL of fish oil. After mixing, add 1.6 mg of FITC-dextran.
3. Dispense the hot liquid mixture into 20 µL drops onto Parafilm on the lab bench, and let them quickly cool and solidify. Drops can be stored for up to 4 d before they become too hard for fish to eat. Store drops in the refrigerator by placing the Parafilm onto half of a petri dish, which is then floated on sterile water inside a sealed plastic container, which keeps the drops humidified.
4. Just before feeding, quarter drops into sections using a razor blade. Acclimate the fish to the Styrofoam cups individually for 2 days without feeding (Note: Only unexposed control fish were used). Place four quarters of the food into the cup with the fish, and observe the fish for 30 minutes for how many pieces of the food they each eat.
5. To begin the monitoring period, transfer fish individually into cups with 80 mL of APW using a dip net. Every 2 h, gently swirl the APW by hand, and then take a 1 mL sample and store in a labeled 1.7 mL tube at 4 °C. Take samples for 36 h.
6. Record fluorescence, with a fluorescence spectrophotometer, of all samples at the same time after completion of the assay. Place the entire 1 mL sample into a cuvette, and record the fluorescence measured using excitation at 495 nm and emission at 520 nm.

Representative Results

An overall schematic diagram of the experimental system used to study fish host effects from antibiotic exposure[13] is represented in **Figure 1A** and includes the technique for extracting the skin (**Figure 1B**) and gut (**Figure 1C**) microbiomes from the fish. Three days was selected as the antibiotic period of exposure because previous data reveals that while the total skin culturable number drops early in treatment, it has returned to pre-treatment levels after 3 d. Meanwhile, the community composition, as determined by 16S profiling, has been strongly altered. Therefore, the 3-day period should be optimal for analyzing the effects from a changed community of approximately the same density. Note that culture analysis, using colony numbers on agar plates, may leave out a number of species. Efficiency of the skin microbiome dispersion method (**Figure 1B**) was analyzed by comparing colony number on plates from suspension buffer (typically in range of 10^4 - 10^5 CFU/g fish weight) to colony number from several fish subjected to the same suspension procedure a second time (to quantify any remaining bacteria). Counts from this second suspension were lower than 100 CFU/g, suggesting the suspension method is effective (unpublished).

Fish with an antibiotic-altered microbiome appeared to be more susceptible to an *E. ictaluri* infection than control fish (**Figure 2**). The difference in mean time to death of 56.1 ±15 h for the treated fish and 98.5 ±48 h for control fish was not statistically significant (two-tailed Student's t-test, p = 0.12). This is likely due to the small group size (treated n=6, control n=5). Group sizes of at least a dozen are therefore recommended. An advantage of this infection model is the bath protocol, which does not require needles for infection or tracking of food intake. *E. ictaluri* naturally invades catfish from the water column. Safety is high because *E. ictaluri* is temperature sensitive, and thus poorly infectious to humans. Other studies have revealed that the time to death in *G. affinis* correlates with the initial dose of bacteria. The incubation temperature of 27 °C was selected as optimal for both bacteria and fish.

Treated or control fish did not have significant differences in survival in water contaminated with high counts of mixed microbes. No mortality was observed over 4 days for control (n=8) or treated (n=9) fish when soil was used as the microbial source. While some mortality did occur with human feces as the source of microbes (**Table 1**), antibiotic treatment made no difference. When the concentration of feces in APW was at 20 mg/mL, 40 - 50% of the fish died. However, the dissolved oxygen concentration was only 10% (compared to >80% in buckets or aquaria), thus the hypoxic conditions confounded the interpretation. When lower levels of 16 or 10 mg/mL were used, the survival rates were matched (two-sided Fisher's exact test, p=0.95 or greater for a difference between groups). Dissolved oxygen levels in these two trials were 65% or above. *Gambusia* are a very hardy invasive species that can live in low-quality water. It would be interesting to use these assays of natural microbial exposure to measure survival of other species against a challenge of contaminated water. Samples of water from specific environmental sites, especially those subjected to eutrophication, could be used in a similar assay, although water quality (dissolved O_2, nitrate, salinity, *etc.*) would need to be determined, as a potentially confounding factor.

Fish exposed to rifampicin were more susceptible to osmotic stress than control fish (**Figure 3**). The log-rank test observed a significant difference ($p = 0.049$) in survival rate (43% death in control group and 88% death in treated group, $n = 9$ for both groups) when challenged with elevated salinity. Results from this assay agreed with a determination of 18.1 mg/mL as the LC_{50} for NaCl with *G. affinis* at 24 h in freshwater[20]. Signs of saline stress that fish exhibit include reddening around the gills and decreased swimming movement. With this assay, salinity levels above 18 mg/mL resulted in rapid fish death.

When exposed to the toxin nitrate in the water column, pre-exposure to antibiotic did not affect survival (**Table 2**). For each trial, death was recorded at both a short and long time point, representing acute and more chronic effects. The concentration of 10 mg/mL was selected based on it being the LD_{50} for *G. affinis* in freshwater at 48 h[21]. Results from this assay were consistent with this LD_{50} value, with a 50% lethality in both treated and control groups after 90 h. A higher challenge of nitrate (17.5 mg/mL) was also examined, leading to more rapid death. Again there was not difference in treatment groups. Nitrite is dramatically more toxic than nitrate to *G. affinis*, with an LD_{50} of 0.0015 mg/mL at 48 h[22]. Community biochemical analysis using the analytical profile index system (unpublished) shows that the fish skin microbiota has the potential to reduce nitrate. However, nitrite levels in the APW during both trials remained below the detection limit (0.001 mg/mL). This challenge method could be used with any small soluble chemicals.

When individualized in cups and fed the same amount to each fish for a month, a trend was observed for the antibiotic-treated fish to not gain weight as well as control fish, without a statistically significant difference (data not shown). To avoid the stress of individualization, fish were housed together as a group for treated and untreated fish in buckets for one month and given matched amounts of food. The limitation of this setup is that fish may not be receiving the same food on an individual basis. However, in the group model, treated fish on average lost weight and control fish on average gained weight (**Table 3**). The amount of food the fish were consuming did not seem to be affected by prior antibiotic exposure, so appetite suppression is an unlikely candidate explanation for lack of weight gain. Numerous other factors could contribute, including changes in inflammation in the gut, levels of mucus production, gut permeability, and/or gut motility. A major advantage of this assay to measure nutritional effects is simplicity. It is inexpensive, and only requires a laboratory balance as instrumentation. It is suitable to screen for an effect, leading to other involved experimentation to determine mechanism.

One example factor to examine related to fish weight gain is food transit time, which is related to gut motility. FITC-labeled dextran can be incorporated into gelatinized food and is nonlethal to the fish. Measuring fluorescence in the surrounding water over time gives a measurement of how fast the FITC-dextran is passing through the gut. Fluorescence above background can be detected as soon as 2 hours, with a maximum reached after 16 hours post-feeding (**Figure 4**). This result is with control fish from an aquarium. Reliable results from antibiotic-treated fish have not yet been obtained. One limitation of this procedure is that a 2-d starvation period is required for fish to eat the food. An advantage of the protocol is high sensitivity (low background fluorescence), as fish eating only one food section can give results, although data is more consistent when two sections are eaten. This protocol is less complicated than a similar and lethal method for mice[23].

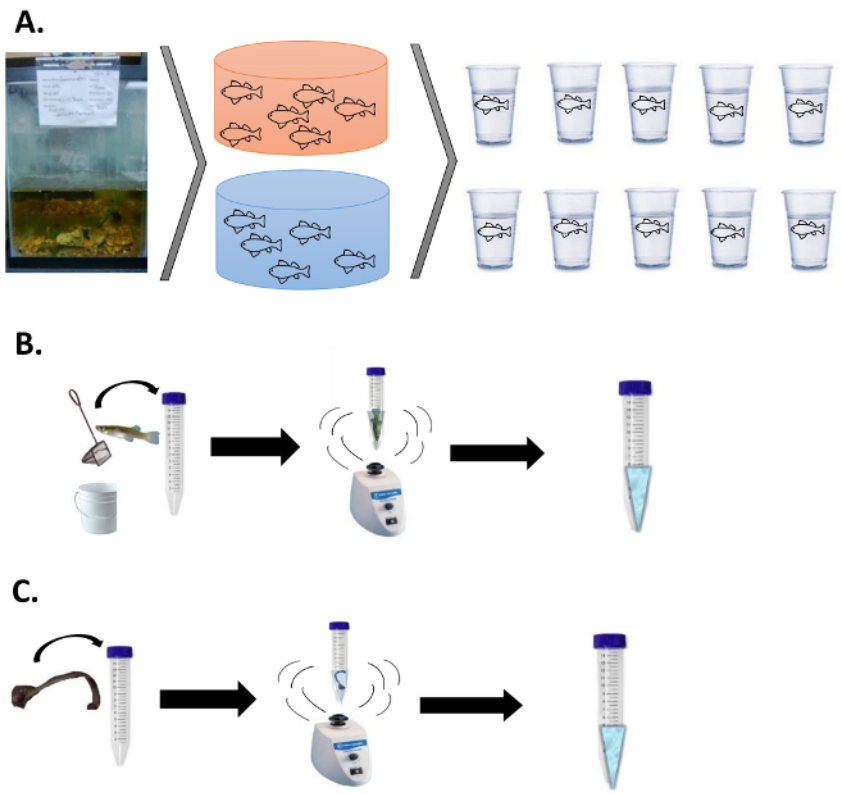

Figure 1: Schematic Overview of the Common Experimental Protocol. A is a flow chart illustrating, left to right, fish transferred from aquarium tank, separated into antibiotic-treated (represented by red water) or control (blue) groups, and then placed into individual cups to track phenotypes. **B** and **C** depict the process of extracting the fish skin and gut microbiomes. After vortexing, bacteria are suspended into the solution for analysis of the microbial community. Please click here to view a larger version of this figure.

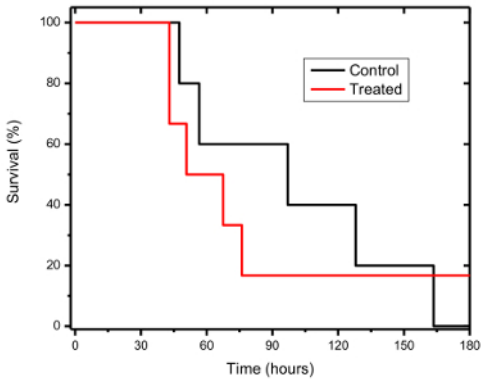

Figure 2: Pathogen Susceptibility. A survival curve during exposure to *E. ictaluri* for fish pre-treated with rifampicin (red line) or an untreated control group (black line) fish. Please click here to view a larger version of this figure.

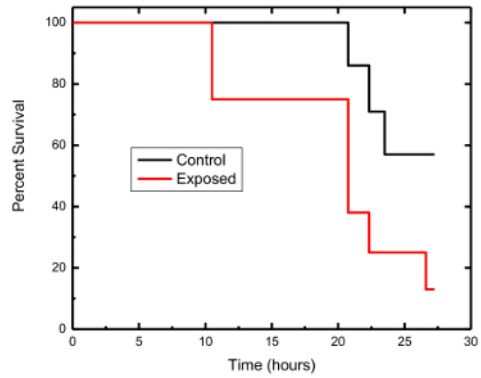

Figure 3: Susceptibility to Osmotic Stress. A survival curve during exposure to high salinity for fish in both antibiotic-treated and untreated groups. Please click here to view a larger version of this figure.

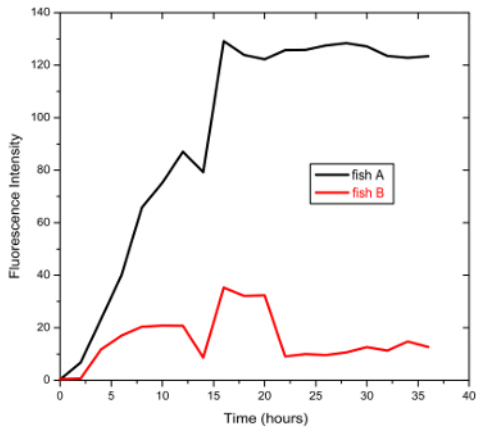

Figure 4: Food Transit Time. Fluorescence over time in the water from two fish fed FITC-dextran. Fish A ate two gelatin food sections and fish B ate one. Please click here to view a larger version of this figure.

		Death Ratio	
Trial	Concentration of feces	Control	Antibiotic
1st	20 mg/mL	2:4	2:5
2nd	16 mg/mL	5:7	5:7
3rd	10 mg/mL	2:5	1:6

Table 1: Susceptibility to High Microbial Environments. Exposure to human fecal matter was assessed at 3 different concentrations. Death ratio of x:y shows the total number of fish dead at the indicated time point x compared to the total number of fish in the experimental group y.

Trial	Nitrate concentration	Time of death	Death ratio	
			Control	Antibiotic
1st	10 mg/mL	45 hr	0:8	0:6
		90 hr	4:8	3:6
2nd	17.5 mg/mL	6.5 hr	6:8	4:8
		24 hr	8:8	7:8

Table 2: Nitrate Toxicity Challenge. Recorded time of fish mortality in treated and control groups throughout exposure to a toxic nitrate concentration. Death ratio of x:y shows the total number of fish dead at the indicated time point x compared to the total number of fish in the experimental group y.

	Trial 1			Trial 2		
Group	Δweight	N	weight/fish	Δweight	N	weight/fish
Control	23%	7	0.43 g	41%	6	0.48 g
Antibiotic	-20%	5	0.49 g	-11%	6	0.32 g

Table 3: Growth Analysis. Changes in total body weight after one month following antibiotic or control treatment. The percent difference in initial mean body weight (for the fish in that trial group) compared to final mean body weight is the column Δweight. N is the number of fish in that group. The mean weight per fish at the end of the trial is underweight/fish.

Discussion

Some challenges require a rest period in clean APW after antibiotic treatment for the drug to be depleted in fish tissues. If the rest period is skipped then antibiotic presence can confound the results, especially when the assay involves exposure to bacteria. In order to examine effects from an altered microbiome composition without large changes in the total number of microbes on the host, preliminary experiments monitoring microbiome composition (16S profiling or whole genome sequencing) and population density (16S quantitation via qPCR) during antibiotic exposure would be required. While 3 d is optimal in this system, changing the host and/or antibiotic would require recalibration.

Typical of biomedical research, the mouse model is commonly utilized for microbiome studies. The most common fish model is zebrafish. In order to get a better understanding of the universal properties of the structure and function of mucosal microbiomes and host interactions, new and atypical models are a necessity. Our WT fish model serves as an authentic source for studying host-microbiome interactions by including natural host genetic variability that other models have lost due to generations of lab-raised animal inbreeding[24]. A major experimental advantage of *Gambusia* is their hardy nature, tolerating a wide range of conditions. High survival rates have been observed in water that varies in temperature (4 - 38 °C), salinity (0 - 17 mg/mL), dissolved oxygen (110 - 25%), and pH (4 - 8). This allows not only examining many environmental conditions, but also for multi-step experimental procedures that are often too stressful for other fish species.

The procedures presented here are suited for rapid screening to discover host effects from a particular treatment. Follow-up studies are required to determine direct causality and mechanism. Example extension studies for the fish microbiome host effects include: measuring intestinal inflammation, examination of fish health by quantifying fat stores, and measurement of mucus levels on the skin and in the gut. A gnotobiotic system is being developed to study in detail links of specific microbes to particular host phenotypes.

Disclosures

The authors have nothing to disclose.

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