The Identification and Characterization of Zebrafish Housed at Goucher College

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Abstract

To gain a better understanding of the *Denio rario*, zebrafish, gut microbiota composition and function and how it contributes to the health of the zebrafish, three zebrafish guts were cultured. The average density of the zebrafish gut microbiota was 2.55 CFU/mL ± 1.03 CFU/mL with a Simpson's diversity between 1.12 – 1.39. Seven species were identified at the genera level though Sanger sequencing of the 16S rRNA sequencing and comparison to the NCBI database. Species isolated included an *Enterococcus* species, *Micrococcus* species, *Shewanella* species, two *Aeromonas* species, and two *Vibrio* species. All gram-stains matched the literature, excluding the *Aeromonas* species 1, which had inconclusive results. Biochemical tests indicated that the isolated strains were capable of glucose fermentation, gas production, acetoin production, phenylalanine deamination, urea hydrolysis, citrate utilization. *Micrococcus* species, *Aeromonas* species 1, and both *Vibrios* species were capable of producing antibiotics, and each species demonstrated antibiotic resistance. Additionally, isolated species were tested for growth at 37°C to determine if they could survive the highest temperatures of the zebrafish's range, and every isolate except *Aeromonas* species 2 experienced a drastic reduction in growth. Understanding the composition and functions of the gut microbiota, as well as the temperature range of the microbes, will help us better understand the health of the zebrafish and predict how the microbiota may change as the temperature of their habitat increases.

Importance

A species health relies on its microbiota, and zebrafish are not an exception. Many microbes call an organism's gut home; however, knowing that microbes are in the gut does not tell us anything about what they are doing to contribute to the health of the organism. By isolating the zebrafish gut microbes, we are able to see if the laboratory zebrafish at Goucher College have similar microbes to the zebrafish gut microbes that others researchers have studied. Discovering the microbial capabilities gives us a picture of how the gut community works together to help the zebrafish function optimally. Knowing what antibiotics can be produced or resisted will help caretakers provide more holistic treatment in the event of infection. Additionally, understanding temperature range of the microbiota will help predict how zebrafish health may change through global warming. Together this study aids in our understanding of maintaining the health of the zebrafish.

Introduction

It is now accepted that the health and function of an individual relies on more than just one's DNA. The microbes associated with an individual can be considered a part of that individual's functional metagenome. The microbiota plays a role in many aspects of health ranging from digestion, to immunity, to neurological development (1). Therefore, when studying a species, one is actually handling an entire community of organisms, and must take into account the affect those microbes are having. These microbial communities are found at various places on and within their bodies (1). The species, *Denio rario*, or zebrafish, is no exception to this (1). In the zebrafish, the largest concentration microbial organisms is contained in the gut (1). Typically, these microbes are more than squatters in the zebrafish gut; these microbes usually produce necessary molecules, help with immunity, and facilitate development (1). A study by Rawls et al. determined that the zebrafish gut microbiota are responsible for the regulation of 212 genes (1).

While previous studies have determined some of the microbes present, as well as their roles, it is important to note that there are often variabilities in the gut microbiotas of zebrafish housed in

different facilities (2). When working with zebrafish, the composition of the gut microbiota must be determined to understand what microbes are present, and what functional roles are they playing in the overall health of the zebrafish. If the core functional roles of the gut microbiota are determined, then researchers may know what roles the microbes are playing in the health of their zebrafish, even when housed in a different laboratory.

To lend insight into possible core microbial functions in zebrafish, our objective is to characterize the gut microbiota of the zebrafish housed at Goucher College. The gut microbes will first be identified through culturing. The density of the gut microbiota will be calculated using the colony forming units. The species diversity will be determined using PCR analysis. Then the capabilities and functions will be determined using biochemical analyses. Isolates will also be tested for antibiotic production, antibiotic resistance, and viability at different temperatures.

Previous studies looking at the metagenomics and metatranscriptomics only give scientists a small picture of what microbes are present due to the reliance on a database. In order to determine the functional capabilities of the microbes, they must be cultured. By culturing the gut microbiota of the zebrafish, we will gain a better understanding of the intricate relationships necessary for the health of the zebrafish. Since the microbiota are responsible for maintaining the health of the zebrafish, dysbiosis may contribute to zebrafish illness. As the zebrafish can withstand a gut temperature between 6°C - 38°C and a pH level above 7.5, we must determine if the microbiota can survive throughout that range (3, 4). Determining which factors, including temperature and antibiotics, result in dysbiosis, the findings will ensure that the zebrafish at Goucher are handled in a way that maintains the microbial community.

We hypothesize that the Goucher College zebrafish will have a set of core microbes and core microbial functions. We expect to find species from the phylum of Proteobacteria, Firmicutes, Fusobacteria, Actinobacteria, and Bacteroidetes (2). Based off of Romero, Ringo, and Merrifield, these species will probably produce enzymes, vitamins, and short chain fatty acids that the zebrafish needs to survive (2). If the zebrafish housed at Goucher College do have gut microbes similar to other zebrafish, we may be able to predict how temperature and antibiotics effect the microbes of zebrafish housed elsewhere.

Materials and Methods

Fish

Three zebrafish, *Danio rerio*, were obtained from Dr. Lenkowski's research laboratory at Goucher College. The fish were healthy and had an average weight of 0.37g ± 0.06g.

Sterile Media

Tryptic Soy Agar (TSA) media was created using 15.0g TSB powder, 7.5g agar, and 500mL dH_2O. de Man, Rogosa and Sharpe (MRS) media was created using 34.1g MRS media and 500mL dH_2O. Tyrptic Soy Broth (TSB) media was created using 15g TSB powder and 500mL dH_2O. The chemicals used to make the media were purchased from Merck & Co Inc.

Gut Dissections and serial dilutions

The three zebrafish were euthanized by submersion in Tricaine-S (MS-222) for 10 minutes, and then soaked in 95% ethanol. The gut was dissected, and placed in a microcentrifuge tube with 1000 µl of phosphate buffered saline (PBS) and a glass bead. Each sample was vortexed for 5 minutes. Serial

dilutions of the zebrafish gut in PBS were performed in 1:10 µl dilution increments to obtain a final dilution factor of 1:100,000 µl, and a PBS control. Using spread plate method, 0.1mL of each sample was plated on both TSA and MRS plates. Anaerobic MRS plates kept at ~24°C were created using dilutions 1:1 – 1:1000. The rest of the plates were incubated at 26°C, and moved to 4°C after sufficient growth occurred.

Serial Dilution Analysis

Colonies were counted for each individual plate. CFUs/ml and Simpson's Diversity Index was determined. Colonies were described using a Modified Smibert and Krieg (5) proposed protocol. The average densities were calculated. The following descriptions were made for each colony: colony number, media type, diameter, pigmentation, form, elevation, margin, texture appearance, opacity, texture with a needle. The representative colonies were then streak plated. The streak plates were repeated to obtain pure colonies.

Subculturing

Representative colonies were subcultured as liquid cultures in 5mL of TSB, and then incubated in a shaker. The anaerobic colony 1 was kept in kept at room temperature under anaerobic conditions. The representative colonies were also subcultured on TSB plates, and the anaerobic colony was subcultured on a MRS plate. However, colonies 3, 5, 7, and 12 did not grow, and were removed from further analysis.

Glycerol Stock Preservation

In cryogenic tubes, a working stock and a permanent stock was prepared for every representative culture. For the isolates that grew in liquid culture, 1mL was placed in each cryogenic tube with 1 mL of 80% glycerol. For isolates that only grew on solid media, 3mL of TSB was added to the plate, the plate was gently scraped, then 1mL was collected and combined with 1mL of 80% glycerol. Glycerol stocks were vortexed, and stored at -80°C.

Gram Staining

Each representative colony was smeared and heat fixed to a glass slide along with a gram negative control strain, *Escherichia coli*, and a gram positive control strain, *Staphylococcus epidermidis*. Slides were gram-stained according to Smith and Hussey (6).

Biochemical Analysis

Using the wire tip of an EnteroPluri-*Test* tube, a large amount of each colony was picked up and used to inoculate each sector of the system. After inoculating every compartment, the notch of the needle was broken and used to punch the plastic film of the sectors with Ardonitol, Lactose, Sorbitol, VP, Dulcitol/PA, Urea, and Cirtrate while the other sectors remained anaerobic. These tests were incubated at 26°C for 24 hours. Color changes were noted. For the H_2S/Indole compartment, 3 drops of Kovac's Indole reagent was added, and color changes were noted after 15 seconds. In the VP compartment, a hole was punched in the plastic film, 3 drops of α-naphtol and 2 drops of potassium hydroxide were added. After 20 minutes, color changes were noted.

Simple boil lysis of Gram-positive bacteria

Fresh liquid cultures were grown up using a pure culture for each isolate. Five hundred µL of the fresh liquid cultures were vortexed and then centrifuged at 10,000 rpm for 2 minutes. The supernatant was discarded, and the cells were vortexed for minutes with a glass bead and 200 µL of sterilized dH_2O. After placing the samples in boiling water for 10 minutes, the samples were placed in a -80°C freezer for 10 minutes, then placed on ice for 10 minutes. Samples were then centrifuged at 10,000 rpm for 10 minutes, and 150 µL of supernatant was collected.

PCR of 16S rDNA

A thermal cycler was used for PCR to amplify the 16s rDNA gene. For each isolate, the reaction was run using 1 µL of each primer, 2 µL of a DNA template, 25 µL of 2X Apex red Master Mix, and 21 µL of water. The DNA template was the isolated using the simple boil lysis described above. In addition to the isolates, a negative and positive control was also run. In the positive control reaction, DNA known to amplify with 16S_1492R and 16S_27F primers was used for the DNA template. In the negative control reaction, no DNA template was added. After an initial denaturation at 95°C of 10 minutes, 35 cycles of the following conditions were applied: 1 minute of denaturation at 95°C, 30 seconds of annealing at 50°C, and 2 minutes of extension at 72°C. A final extension was applied at 72°C for 10 minutes. Upon completion of the program, samples were stored at -20°C.

Table 1. Primers used to amplify the 16s rDNA gene

Primer Name	**5'-3' Sequence**	**Position**
16S_1492R	GGTTACCTTGTTACGACTT	1492-1510
16S_27F	AGAGTTTGATCMTGGCTCAG	8-27

Gel Electrophoresis

The PCR products were examined using electrophoresis in a 0.8% (w/v) agarose gel with 1 drop of ethidium bromide. The PCR samples were run with a positive and negative control at 120V for 25 minutes.

PCR purification of amplicons

The PCR products were purified using a modified version of Wizard SV gel and PCR Cleanup system. In a minicolumn assembly, 45 µL of PCR product were combined with 45 µL of binding buffer. After a minute this was centrifuged at 10,000 rpm for 1 minute. The flow-through was discarded. Then 700 µL of membrane wash solution were added and centrifuged at 10,000 rpm for 1 minute. Five hundred µL of membrane wash solution were added and centrifuged at 10,000 rpm for 5 minutes. Flow-through was discarded, and was re-centrifuged at 10,000 rpm for 1 minute. The minicolumn was transferred to a 1.5 ml microcentrifuge tube. Then 30 µL of elution buffer were added. After a 1 minute incubation, this was centrifuged at 10,000 rpm for 1 minute, and stored at -20°C.

Sequence Analysis

The purified PCR products were sequenced via Sanger Sequencing using primers 16S_1492R and 16S_27F. The sequencing data was opened in Chromas. Sequences were manually cropped to obtain only clear peaks, exported to OneNote, and converted to FASTA format. FASTA sequences were entered into NCBI BLASTn. The top three hits were recorded for both the forward primer and reverse primer. For

each hit the accession number, description, max-score, query coverage, E-value, Max-identity, and organism information was recorded.

Antibiotic Resistance

Liquid cultures and streak plates of TSB were prepared, and each isolate was spread on Mueller-Hinton agar plates. The following antibiotic disks were placed on the Mueller-Hinton plates with five to seven disks per plate: chloramphenicol (30 mcg), erythromycin (15 mcg), kanamycin (30 mcg), neomycin (30 mcg), novobiocin (30 mcg), penicillin G (10 mcg), streptomycin (10 mcg), tetracycline (30 mcg), vancomycin (30 mcg), Sulfamethoxazole/Trimethoprim (23.75 mcg/1.25 mcg). A replicate was performed for each isolate. The plates were then incubated at 26° C. They were moved to 4°C when a lawn of growth was observed. The zones of clearing were recorded for each antibiotic tested, and the relative antibiotic resistance was calculated by taking the reciprocal of the average diameter of antibiotic clearing. The standard error was also calculated, and these values were graphed for each isolate.

Antibiotic Production

Using liquid cultures of susceptible indicator strains, gram-positive *Micrococcus luteus*, gram negative *Escherichia coli*, and *Escherichia coli* $smpA^{\Delta}$ $surA^{\Delta}$ (a gram negative double mutant with increased susceptibility to vancomycin), 150µL of each culture was spread onto LA plates. All seven isolates were patched onto the LA indicator lawn plates. Three replicates were created for each susceptible indicator strain. The *E. coli* plates were incubated at 37°C and the *M. luteus* plates were incubated at 26°C for until a lawn of growth was observed. This procedure was repeated for the *Enterococcus* species, but these were incubated in anaerobic conditions. Any zones of clearing were recorded.

Temperature Tolerance

The growth of each isolate was tested at both 26°C and 37° C. Each isolate was plated on TSA, and a colony was picked and grown in 5 mL of TSB at 26°C with 3 replicates per isolate. After a 24 hour incubation, 3 replicates of the isolates were grown from the liquid cultures in 5mL of TSB. One was used as a control, one was incubated at 26°C, and the other was incubated at 37° C. After 24 hours, serial dilutions were performed in 1:10 µl dilution increments to obtain a final dilution factor of 1:10,000 µl, using TSB. Using spread plate method, 0.05mL of each sample was plated on TSA and incubated at 26°C. When counting CFUs, plates from the 10,000 µl with more than 300 colonies were given the value of 1000 CFU in order to calculate CFU/mL. For each replicate, CFU/mL was calculated, and the (CFU/mL at 26°C)/(control CFU/mL) was compared graphically to (CFU/mL at 37°C)/(control CFU/mL). Standard deviation was calculated for the replicates.

Results

Colony Counts

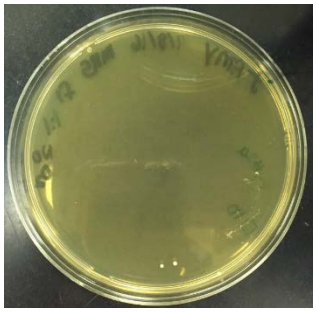
Due to sufficient growth, or the presence of a lawn of cells, after one day, TSA plates with the gut microbiota from the following fish and dilutions were moved from the incubator to 5°C: Fish 1: dilutions 1:1-1-1:1000, Fish 2: dilutions 1:1 and 1:1000, and fish 3: dilutions 1:1 and 1:10. After 4 days,

TSA plates with the gut microbiota from the following fish and dilutions were moved from the incubator to 5°C: Fish 1 dilution of 1:10,000, and Fish 2 dilution of 1:10. The rest of the plates remained in the incubator for 5 days. The number of colonies on each plate were counted as shown in Table 2. This table also shows that there was no growth on the MRS plates kept in aerobic conditions. However, there were colonies on the MRS plate with a 1:1 dilution kept in aerobic conditions. Since the TSA control plate for fish 2 was contaminated, the contamination colony was subtracted from the total colony count if present on other plates.

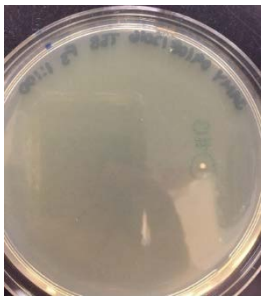
Table 2. Number of colonies observed from all serial dilutions with the zebrafish guts and PBS plated on TSA, MRS, with MRS plates kept in anaerobic conditions.

Colony Counts							
MRS							
Dilution	1:1	1:10	1:100	1:1k	1:10K	1:100k	Control
F1	0	0	0	0	0	0	0
F2	0	0	0	0	0	0	0
F3	0	0	0	0	0	0	0
TSA							
Dilution	1:1	1:10	1:100	1:1k	1:10K	1:100k	Control
F1	TMTC	TMTC	TMTC	154	6	0	0
F2	36	5	4	Contamination	0	0	Contamination
F3	TMTC	25	2	0	0	0	0
Anaerobic MRS							
Dilution	1:1	1:10	1:100	1:1k	1:10K	1:100k	Control
F1	5	0	0	0	0	0	0
F2	0	0	0	0	0	0	0
F3	0	0	0	0	0	0	0

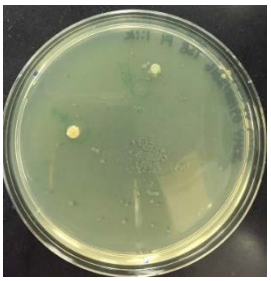
Fish 3, 1:1 TSA



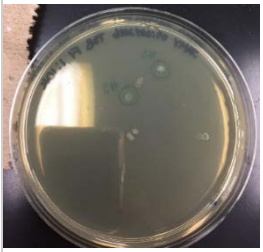
Fish 1, 1:1 MRS



Fish 3, 1:100 TSA



Fish 1, 1:1000 TSA



Fish 1, 1:10,000 TSA

Density of gut microbiota

The density of the gut microbiota was calculated for each fish using the plate that contained 30-300 colony forming units from table 1. For fish 1 the dilution plate of 1:1000 was used. For fish 2 the dilution plate of 1:1 was used. For fish 3, the dilution plate of 1:10 was used as it was the result nearest 30 colonies. Using these plates, the density of the gut microbiota was determined for each fish to obtain an average of 2.55 CFU/mL with a standard variance of 1.03 CFU/mL among the three fish samples as shown in table 3.

Table 3. The density of the gut microbiota for each fish calculated using the standard formula (CFUs/(Dilution factor x Volume plated)), and the average between the three fish.

Density of Gut Microbiota	
F1	1.54 CFU/mL +06 CFU/mL
F2	3.60 CFU/mL +02 CFU/mL
F3	2.50 CFU/mL +03 CFU/mL
Average Density: 2.55 CFU/mL ± 1.03 CFU/mL	

Simpson's Diversity Index

Using the representative plate for each fish, Simpson's Diversity Index was calculated. The reciprocal of Simpson's Diversity Index was calculated as shown in table 4. The diversity reciprocals range from 1.12 – 1.39 indicating that the diversity of the three fish were consistent when taking into account species richness and species evenness.

Table 4. The reciprocal of Simpson's Diversity Index calculated for the gut microbiota's of three zebrafish.

Simpson's Diversity Index	
Fish number	1/D

F1	1.12
F2	1.12
F3	1.39

Colony Morphology

Seven representative colonies were selected among the different plates, and the colony morphology was determined as shown in table 5. The colonies are certainly distinct because table 5 indicated that no representative colonies had the same morphology.

Table 5. Morphology of 7 representative colonies obtained from serial dilutions with the zebrafish gut and PBS. MRS was kept in anaerobic conditions and TSA was kept in aerobic.

		Colony Morphology							
Species	Media	Diameter (mm)	Pigmentation	Form	Elevation	Margin	Texture Appearance	Opacity	Texture with needle
Enterococcus Species	MRS	1	White	Circular	Convex	Entire	Smooth	Shiny	Moist
Vibrio Species 1	TSA	3	Cream	Circular	Flat	Entire	Smooth	Translucent	Butyrous
Vibrio Species 2	TSA	2	Clear	Circular	Convex	Entire	Smooth	Transparent	Mucoid
Micrococcus Species	TSA	1	Cream	Circular	Convex	Entire	Smooth	Translucent	Viscous
Aeromonas Species 1	TSA	2	Cream	Circular	Flat	Entire	Mucoid	Translucent	Mucoid
Aeromonas Species 2	TSA	4	Cream	Circular	Umbonate	Entire	Mucoid	Opaque	Mucoid
Shewanella Species	TSA	4	Orange	Circular	Umbonate	Entire	Smooth	Opaque	Butyrous

Subculturing

All isolates exhibited growth in liquid culture of TSB.

Biochemical Assays

Using EnteroPluri-*Tests,* the biochemical tests shown in table 6 were performed to determine the different functional capabilities of the isolates. As shown in Table 6, the *Enterococcus* Species, both *Vibrio* Species, and both *Aeromonas* Species could ferment glucose. Other than *Vibrio* Species 2, the same isolates could produce gas. Table 6 indicates that the *Aeromonas* Species 2 was the only isolate capable of producing acetoin and deaminating phenylalanine. The *Shewanella* Species was the only isolate capable of hydrolyzing urea and utilizing citrate as shown in Table 6.

Table 6. Biochemical assay results obtained using EnteroPluri-*Tests.* Positive and negative results were determined by the color changed that occurred.

Biochemical Assays							
	Isolate #						
Capabilities	*Enterococcus* Species	*Vibrio* Species 1	*Vibrio* Species 2	*Micrococcus* species	*Aeromonas* Species 1	*Aeromonas* Species 2	*Shewanella* Species
Glucose Fermentation	+	+	+	-	+	+	-
Gas Production	+	+	-	-	+	+	-
Lysine Decarboxylation	-	-	-	-	-	-	-
Ornithine Decarboxylation	-	-	-	-	-	-	-
Hydrogen Sulphide Production	-	-	-	-	-	-	-
Indole Test	-	-	-	-	-	-	-
Adonitol Fermentation	-	-	-	-	-	-	-
Lactose Fermentation	-	-	-	-	-	-	-
Arabinose Fermentation	-	-	-	-	-	-	-
Sorbital Fermentation	-	-	-	-	-	-	-
Acetoin Production	-	-	-	-	-	+	-
Dulcitol Fermentation	-	-	-	-	-	-	-
Phenylalanine Deamination	-	-	-	-	-	+	-
Urea Hydrolysis	-	-	-	-	-	-	+
Citrate Utilisation	-	-	-	-	-	-	+

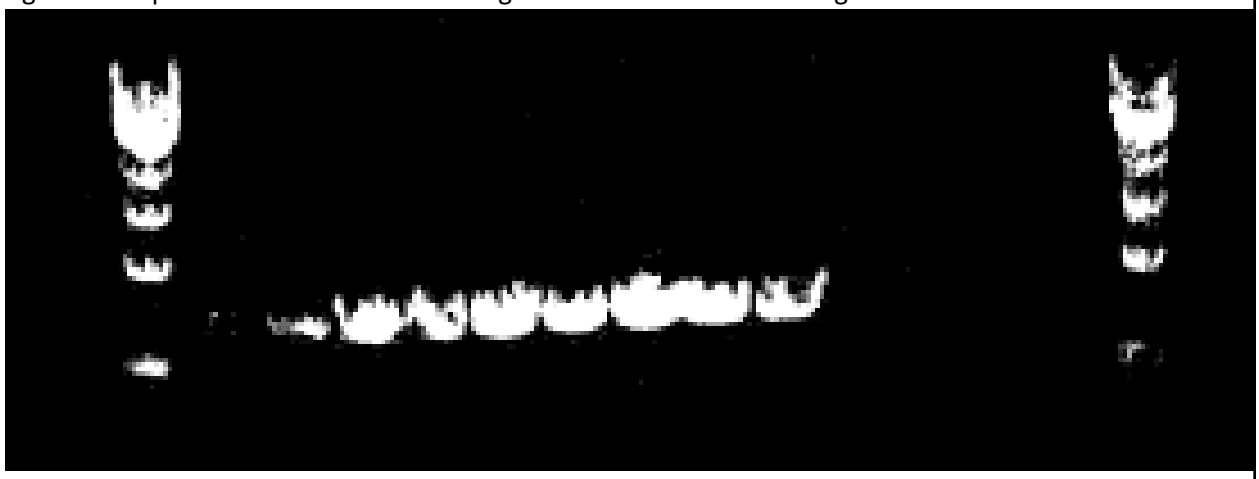
Gram Staining

After Gram staining each isolate, the slides were viewed under a light microscope. The stains were compared to the positive and negative controls and, as shown in Table 7, *Vibrio* Species 1, *Vibrio* Species 2, *Aeromonas* Species 2, and *Shewanella* Species were classified as gram-negative. While *Enterococcus* Species and *Micrococcus* species were classified as gram-positive. The stain of *Aeromonas* Species 1 did not match the gram-positive nor gram-negative controls making our results inconclusive.

Gel electrophoresis of PCR 16S rDNA products

As shown in figure 1., each isolate had one amplicon. The positive control had one amplicon, and there were no bands present in the negative control. Due to the fact that the simple boil lysis procedure was sufficient to isolate DNA for PCR, the PCR products were purified in order to be sequenced.

Figure 1. Amplicons of the 16s rRNA for eight isolates of the zebrafish gut.



1 kb Ladder #1 #2 #4 #6 #8 #9 #10 #11 Positive Negative 1kb Ladder

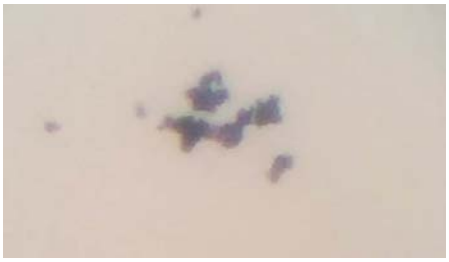
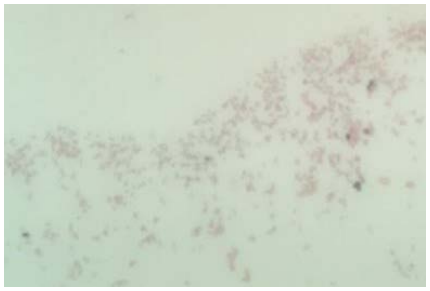
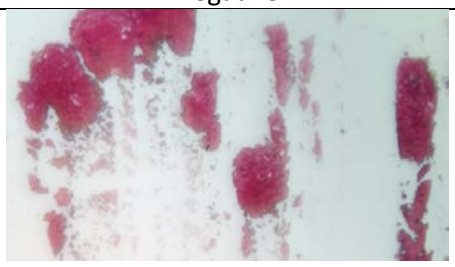
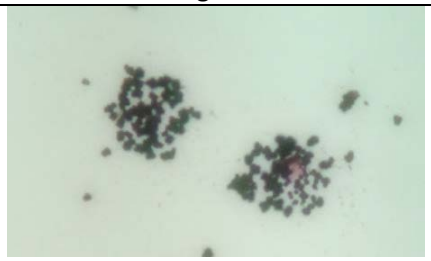
#1: *Enterococcus* Species
#2: *Vibrio* Species 1
#4: *Vibrio* Species 2
#6: *Micrococcus* species
#8: *Vibrio* Species 2
#9: *Aeromonas* Species 1
#10: *Aeromonas* Species 2
#11: *Shewanella* Species

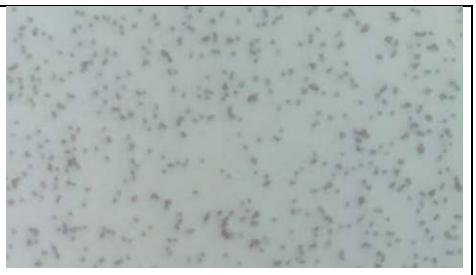
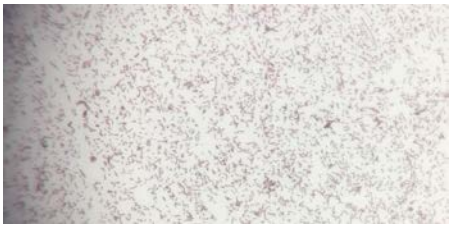
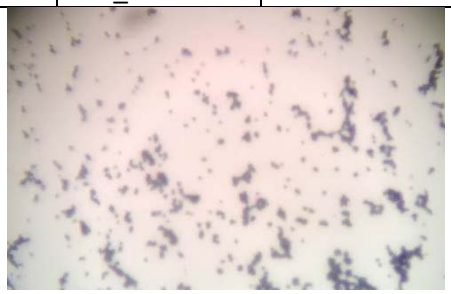
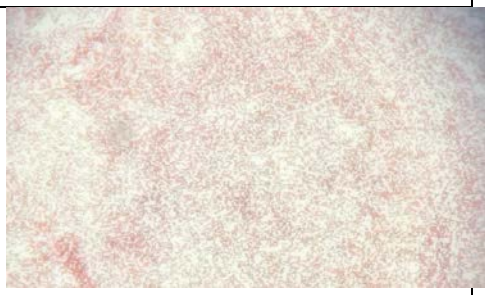
Sequence Analysis

Upon comparison of the sequencing data to the NCBI database, we identified isolate 1 as an *Enterococcus* species (Table 7). Most isolates provided equally close alignments with different species, so we were only able to identify isolates at the genus level. Table 7 indicates that isolate 2 and 4 were identified as *Vibrio* species; however, they have different biochemical abilities, so we identified them as *Vibrio* species 1 and 2, respectively. Isolate number 6 was identified as a *Micrococcus* species as shown in table 7. The three closest alignments for the forward and reverse primer were the same three species (table 7). Isolate numbers 9 and 10 were identified as *Aeromonas* species, yet they had different gram stains and different biochemical capabilities (table 7). As shown in table 7, Isolate 11 was identified as a *Shewanella* strain. The three closest alignments for the forward and reverse primer were the same three species. All alignments used had a query coverage and max identity of at least 97%, and an E-Value of 0.

Table 7. A culture isolate summary table displaying the characterization of seven Zebrafish gut microbial isolates.

16S rRNA Identification	Isolate #	Accession #	Biochemical Analysis Results	Gram stain results

Enterococcus Species	1	Forward: NR_113907.1 NR_117976.1 NR_115762.1 Reverse: NR_133741.1 NR_113933.1 NR_113932.1	Glucose Fermentation, Gas Production	 Positive
Vibrio Species 1	2	Forward: NR_135208.1 NR_118570.1 NR_118569.1 Reverse: NR_025476.1 NR_029259.1 NR_112229.1	Glucose Fermentation, Gas Production	 Negative
Vibrio Species 2	4	Forward: NR_118569.1 NR_135208.1 NR_113604.1 Reverse: NR_029259.1 NR_112229.1 NR_117894.1	Glucose Fermentation	 Negative
Micrococcus species	6	Forward: NR_134088.1 NR_075062.2 NR_116578.1 Reverse: NR_116578.1 NR_075062.2 NR_134088.1	-	 Positive
Aeromonas Species 1	9	Forward: NR_037013.2 NR_119040.1 NR_112838.1 Reverse: NR_136829.1 NR_074841.1 NR_113342.1	Glucose Fermentation, Gas Production	 Inconclusive

Aeromonas Species 2	10	Forward: NR_112838.1 NR_118947.1 NR_119045.1 Reverse: NR_136829.1 NR_074841.1 NR_113342.1	Glucose Fermentation, Gas Production, Acetoin Production, Phenylalanine Deamination	 Negative
Shewanella Species	11	Forward: NR_116732.1 NR_113582.1 NR_044863.1 Reverse: NR_116732.1 NR_044863.1 NR_113581.1	Urea Hydrolysis, Citrate Utilization	 Negative
Gram Stain Controls	 Positive: *Staphylococcus epidermidis*			 Negative: *Escherichia coli*

Antibiotic Resistance

By determining the zone of inhibition for each antibiotic, we were able to identify the relative antibiotic resistance for each isolate. As shown in figure 2, *Enterococcus* species expressed total resistance to Streptomycin and moderate resistance to kanamycin, neomycin, and sulfamethoxazole/trimethoprim. As shown in figure 2, *Vibrio* species 1 demonstrated total resistance to erythromycin and moderate resistance to streptomycin and sulfamethoxazole/ trimethoprim. Figure 2 shows that *Vibrio* species 2 showed strong resistance to streptomycin, penicillin G, and vancomycin (Figure 2). The *Micrococcus* species showed moderate resistance to neomycin, vancomycin, and sulfamethoxazole/trimethoprim as shown in figure 2. Both *Aeromonas* species expressed total resistance to penicillin G, while expressing strong resistance to novobiocin and vancomycin (Figure 2). The *Shewanella* species expressed total resistance novobiocin. It also expressed resistance to tetracycline and vancomycin (Figure 2).

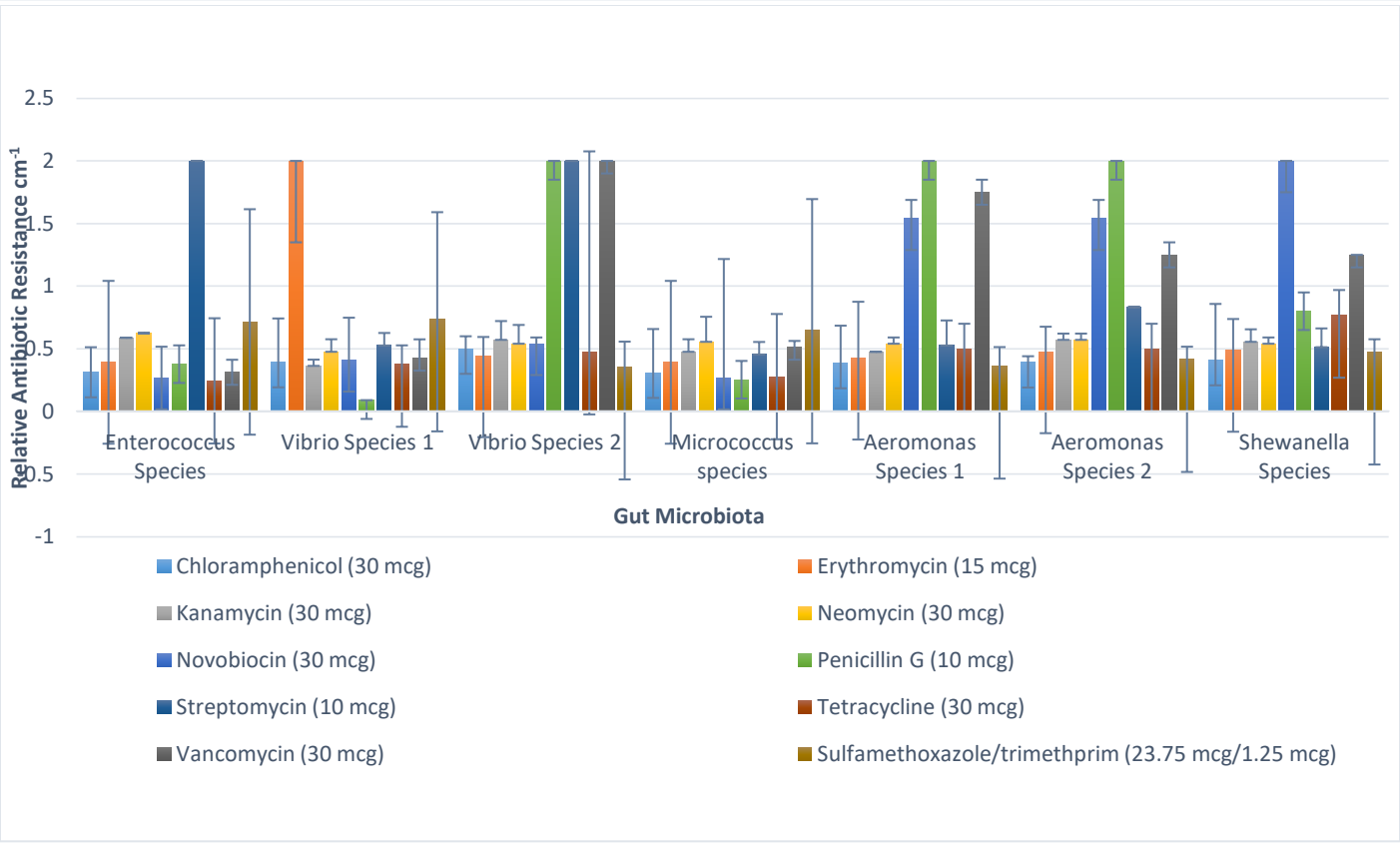


Figure 2. Level of antibiotic resistance among seven species isolated from the zebrafish gut. Each isolate was tested for antibiotic resistance against the 10 antibiotics listed. Standard error bars represent one standard error of the mean.

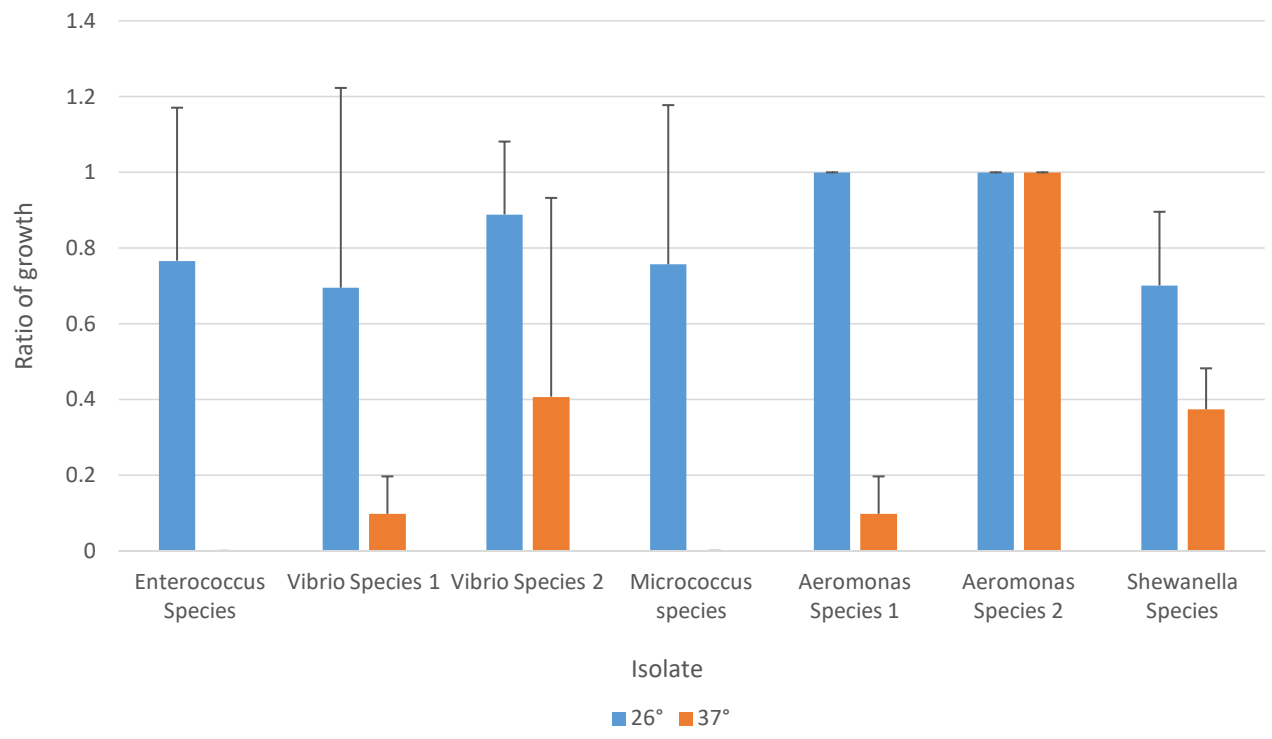
Antibiotic production

By determining the zone of inhibition against susceptible indicator strains, we were able to identify antibiotic production capabilities for each isolate. *Vibrio* species 1*, Micrococcus* species, and *Aeromonas* species 1 were capable of producing antibiotic against *Micrococcus luteus*. *Vibrio* species 2 was capable of producing antibiotic against wild-type *E. coli.* No other isolates demonstrated antibiotic production.

Temperature tolerance

By comparing the CFU/mL for each isolate at 26°C and 37° C, we were able to compare the isolates viability at the two different temperatures. As shown in figure 3, all isolates except *Aeromonas* species 2 demonstrated a drastic decrease in growth. The *Enterococcus* species and *Micrococcus* species were not viable at 37° C (figure 3). Figure 3 shows that the *Aeromonas* species 2 experienced no difference in growth between 26°C and 37° C.

Figure 3. Growth of isolates at 26°C and 37°C in relation to the control. Error bars represent the standard deviation between the mean three replicates.



Discussion

To gain insight into the health of the zebrafish at Goucher College, we needed to understand what communities were residing within the zebrafish. We wanted to know what microbes make up the gut microbiota, as well as what they were doing. We expected to find the core microbiota present among other zebrafish which includes: Proteobacteria, Firmicutes, Fusobacteria, Actinobacteria, and Bacteroidates (2). Starting with the zebrafish gut, we worked to isolate pure cultures. During this process, it was discovered that colonies 3, 5, 7, and 12 were not able to grow in pure cultures, as they only grew with other species present, so they were removed from further analysis. We hypothesize that these were reliant on another species, but further studies are needed to test this. When aligning sequences, *Vibrio* species 1 and isolate 8 were determined to be the same species as they had the 16S rRNA sequence, gram stain results, and biochemical capabilities.

Overall, seven different strains were isolated from five different genera: *Enterococcus*, *Vibrios*, *Micrococcus*, *Aeromonas*, and *Shewanella*. Certainly, the zebrafish gut possessed rich diversity. Research by Roeselers et al., indicated the presence of a core microbiota from the following phyla: Proteobacteria, Firmicutes, Fusobacteria, Actinobacteria, and Bacteroidates (2). The *Enterococcus* species falls within the phylum of Firmucutes. The two *Vibrios*, *Aeromonas,* and *Shewenella* species are types of Proteobacteria. The *Micrococcus* strain belongs to the phylum of Actinobacteria. We did not isolate any core microbiota from the phyla Fusobacteria or Bacteroidates; however, we used different

methods. Roeselers et al., started their research by isolating DNA directly from the gut, but we only isolated genetic material from species isolated in pure culture (2). Most microbes have not been cultured to this day, and we cultured the microbes under a narrow range of conditions. In the future, we could try to culture under conditions that support Fusobacteria and Bacteroidates, including a completely anaerobic environment, to isolate the entire zebrafish core microbiota (7). The average density of the zebrafish gut microbiota was 2.55 CFU/mL ± 1.03 CFU/mL. The Simpson's diversity ranged between 1.12 – 1.39 when taking into account species richness and species evenness, we were not able to culture a great amount of diversity. As indicated by researchers, Roeselers et al., we found common zebrafish genera which include *Aeromonas* species, *Vibrio* species, and *Shewanella* species. We certainly were able to isolate many of the common species.

The *Enterococcus* species that we cultured was characterized as a gram-positive species capable of glucose fermentation and gas production. This matches Fisher and Phillips characterization of *Enterococcus* species as being gram-positive, however, they are considered lactose fermenters. Further research is required to investigate this discrepancy (8). The *Vibrios* species 1 and *Vibrios* species 2 that we cultured were characterized as a gram-negative species capable gas production, and species 1 was also capable of fermenting glucose. In accordance with literature, the top hits were all gram-negative species associated with eukaryotes in aquatic environments (9). The *Micrococcus* species that we cultured was characterized as a gram-positive species. One hit was a free-living microbe, but the other two, were gram-positive plant microbes, *Micrococcus yunnanensis* and *Micrococcus aloeverae* (10). Further investigation is necessary to identify the specific species isolated.

The *Aeromonas* species 1 that we cultured was characterized as capable of Glucose Fermentation and Gas Production, but with inconclusive gram-stain results. As *Aeromonas* are gram-negative species, further research is necessary (11). Our hypothesis is that *Aeromonas* species 1 may be a capsule forming species. The *Aeromonas* species 2 that we cultured was characterized as a gram-negative species capable of Glucose Fermentation, Gas Production, Acetoin Production, Phenylalanine Deamination. Two of the hits were comprised of *Aeromonas hydrophila* strains which are gram-negative mesophiles, making them the species most likely isolated (11). The *Shewanella* species that we cultured was capable of hydrolyzing urea and producing citrate. The first hit for the forward and reverse primer, NR_116732.1, was isolated from costal sea sediments. This *Shewanella xiamenensis* strain is the most probable match based on location. It is also a gram-negative species capable of producing citrate (12). While we were not able to identify the isolates at the species level, we know that we isolates many microbes common to the zebrafish based on the genera isolated.

The species that we isolated were able to perform a diverse range of biochemical capabilities including, glucose fermentation, gas production, acetoin production, phenylalanine deamination, urea hydrolysis, citrate utilization. Most of the species were able to ferment glucose and produce gas. This is probably the main energy source in the gut where little oxygen is available. Acetoin production and phenylalanine deamination may indicate that *Aeromonas* Species 2 is capable of breaking down proteins ingested by the zebrafish. The *Shewanella* species was capable of urea hydrolysis and citrate utilization which probably indicate that this species can utilize urea and citrate for carbon and energy sources. This species may breakdown molecules that the zebrafish and other species are not able to utilize.

By understanding which species demonstrate antibiotic resistance and production, we are able to choose antibiotics that cause the least harm to the gut microbiota when the zebrafish requires

treatment. Species isolated from the gut microbiome expressed the most resistance to Penicillin G (10 mcg), Streptomycin (10 mcg), Vancomycin (30 mcg), and Novobiocin (30 mcg). Thereby, treating zebrafish with these antibiotics whenever possible will cause minimal harm to their gut microbiotas. The use of antibiotics can certainly be detrimental to the microbiota, and therefore, the health of the zebrafish. Our results show that our isolates can resist some of these effects. What appears to be a more detrimental factor to the viability of the microbiota, is the temperature. As global warming continues, the temperature of the of the zebrafish habitat rises. While the zebrafish can live at 37°C, most of our isolates had a drastic reduction in viability (3). The *Enterococcus* species and *micrococcus* species could not grow at that temperature, while the other species demonstrated minimal growth in comparison to the growth at 26°C. The only species whose growth did not differ at different temperatures was *Aeromonas* Species 2. The loss and drastic reduction in the core microbiota could severely impact the health of the zebrafish. The microbial community would be disrupted, necessary nutrients may no longer be synthesized for the zebrafish, and pathogens may be able to invade.

Conclusion

We were able to culture an *Enterococcus* Species, two *Vibrio* Species, a *Micrococcus* species, two *Aeromonas* Species, and a *Shewanella* Species. These isolates represent the phyla of the Firmucutes, Proteobacteria, and Actinobacteria. These isolates matched the literature for their characterization, and presented a broad range of biochemical capabilities. Each isolate demonstrated antibiotic resistance, while the *Micrococcus* species, *Aeromonas* Species 1, and both *Vibrio* Species demonstrated antibiotic production. Every species isolated, except *Aeromonas* Species 2, revealed a drastic decrease in viability at 37°C. While zebrafish can survive this temperature, our results indicate that much of the microbiota cannot; this suggests that global warming may cause detrimental harm to the microbiota, and thereby, the health of the zebrafish. As we were able to isolate much of the core microbiota, we believe that our results may be applicable to zebrafish housed in other places or in the wild. Future studies should work to isolate the gut microbiota under more diverse conditions to culture the rest of the core, which includes Bacteroidetes and Fusobacteria. Future studies are also needed to confirm that the data is consistent with other populations in order to extrapolate our findings to zebrafish everywhere.

Acknowledgments

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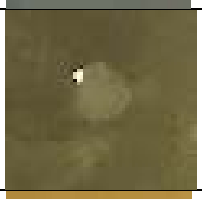
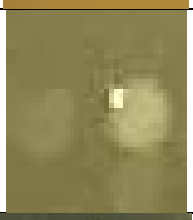
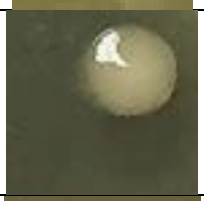
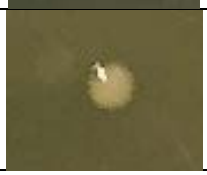
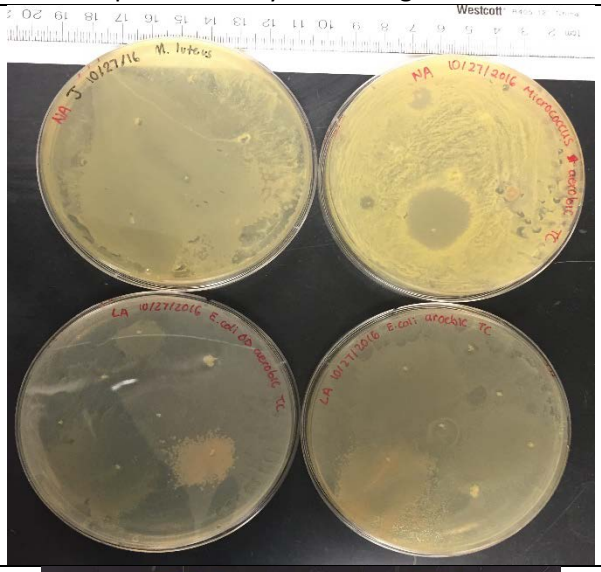
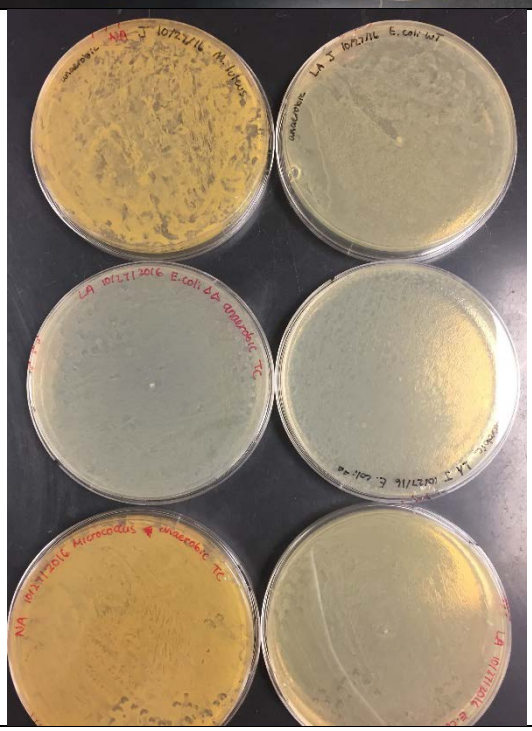
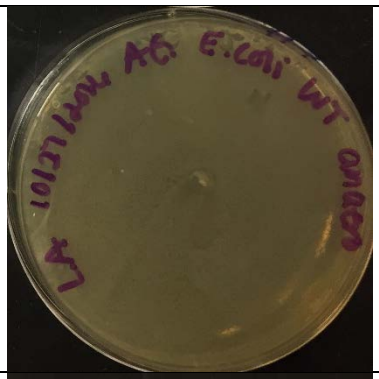
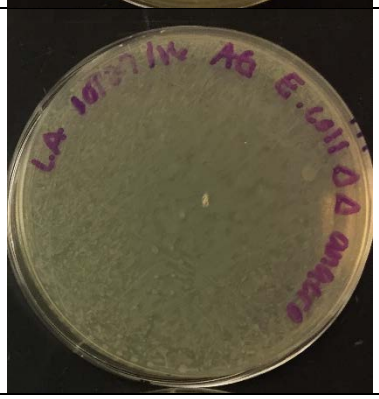
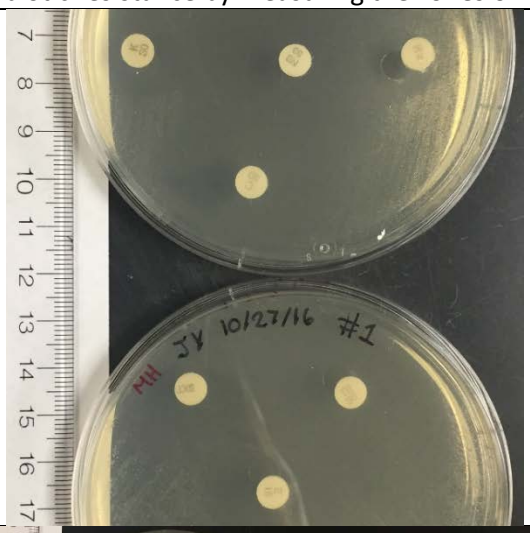
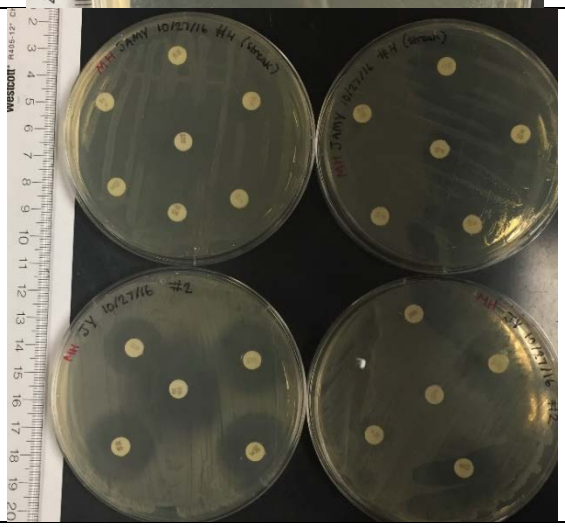
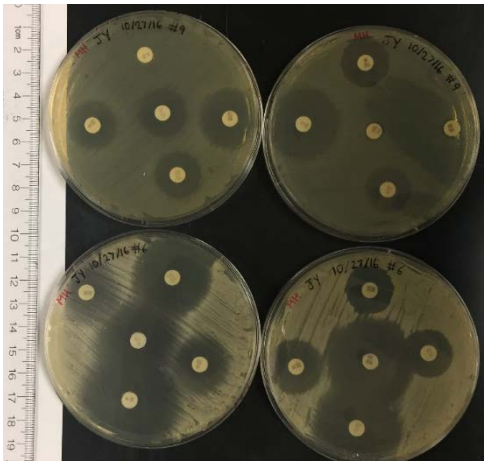
We thank Dr. Lenkowski for her zebrafish and Dr. Jozwick for her technical support.

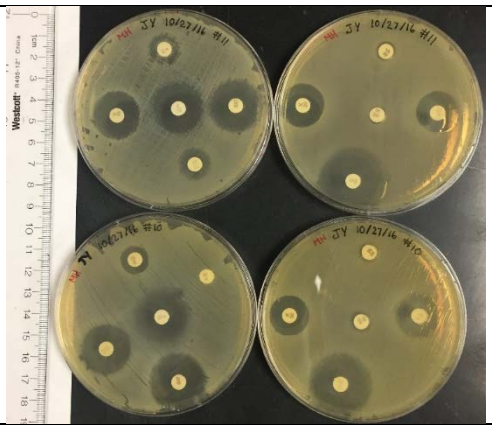
Supplemental

Supplemental 1. Colony morphology of each isolate.	
Enterococcus Species	
Vibrio Species 1	
Vibrio Species 2	
Micrococcus species	
Aeromonas Species 1	
Aeromonas Species 2	
Shewanella Species	

Supplemental 2. Testing isolates for antibiotic production by measuring the zones of inhibition.	
Aerobic: Top Right: NA *M. luteus* Top Left: NA *M. luteus* Bottom right: LA *E. Coli ΔΔ* Bottom left: LA *E. Coli*	
Anaerobic: Top Right: NA *M. luteus* Top Left: LA *E. coli* Middle Right: LA *E. coli ΔΔ* Middle Left: LA E. coli ΔΔ Bottom right: LA *M. luteus* Bottom left: LA *E. coli*	

Anaerobic LA *E. coli*			
Anaerobic LA *E. coli* ΔΔ			

Supplemental 3. Testing isolates for antibiotic resistance by measuring the zones of inhibition.	
Enterococcus species	
Top: *Vibrio* species 2 Bottom: *Vibrio* speces 1	
Top: *Aeromonas* species 1 Bottom: *Micrococcus* species	

Top: *Shewanella* species Bottom: *Aeromonas* species 2			

Works Cited

1. **Romero, J., E. Ringø, and D. L. Merrifield.** 2014. The Gut Microbiota of Fish. Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics. .

2. **Roeselers, G., E. K. Mittge, Z. Stephens, D. M. Parichy, C. M. Cavanaugh, K. Guillemin, and J. F. Rawls.** 2011. Evidence for a core gut microbiota in the zebrafish. The International Society for Microbial Ecology. **5:**1595-1608.

3. **Watts, S. A., M. Powell, and L. R. D'Abramo.** 2014. **Fundamental Approaches to the Study of Zebrafish Nutrition**. NIH Public Access. . doi: 10.1093/ilar.53.2.144.

4. **Verri, T., G. Kottra, A. Romano, N. Tiso, M. Peric, M. Maffia, M. Boll, F. Argenton, H. Daniel, and C. Storelli.** 2003. **Molecular and functional characterisation of the zebrafish (*Danio rerio*) PEPT1-type peptide transporter** . FEBS Letters. **549:**115-122. doi: 10.1016/S0014-5793(03)00759-2.

5. **Smibert, R. M. and N. R. Krieg**. 1994. Phenotypic characterization, p. 615. *In* P. Gerhardt, R. Murray, W. Wood, and N. Krieg (ed.), Methods for general molecular bacteriology. ASM Press, Washington, DC.

6. **Smith, A. C., and M. A. Hussey.** 2005. Gram Stain Protocols. American Society for Microbiology. 1-9.

7. Finegold SM. Anaerobic Gram-Negative Bacilli. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; 1996. Chapter 20.

8. **Fisher, K., and C. Phillips.** 2009. The ecology, epidemiology and virulence of Enterococcus. Microbiology. **155:**1749-1757. doi: 10.1099/mic.0.026385-0.

9. **Thompson, F. L., G. Gevers, C. C. Thompson, P. Dawyndt, S. Naser, B. Hoste, C. B. Munn, and J. Swings.** 2005. **Phylogeny and Molecular Identification of Vibrios on the Basis of Multilocus Sequence Analysis**. Applied and Environmental Microbiology. **71:**5107-5115. doi: 10.1128/AEM.71.9.5107-5115.2005.

10. **Zhao, G., J. Li, S. Qin, Y. Zhang, W. Zhu, C. Jiang, L. Xu, and W. Li.** 2009. Micrococcus yunnanensis sp. nov., a novel actinobacterium isolated from surface-sterilized Polyspora axillaris roots. Int. J. Syst. Evol. Microbiol. **59:**2383-2387. doi: 10.1099/ijs.0.010256-0.

11. **Martinez-Murcia, A. J., S. Benlloch, and M. D. Collins.** 1992. Phylogenetic Interrelationships of Members of the Genera Aeromonas and Plesiomonas as Determined by 16s Ribosomal DNA Sequencing: Lack of Congruence with Results of DNA-DNA Hybridizations. International Journal of Systematic Bacteriology. **42:**412-421.

12. **Huang, J., B. Sun, and X. Zhang.** 2010. Shewanella xiamenensis sp. nov., isolated from coastal sea sediment. Int. J. Syst. Evol. Microbiol. **60:**1585-1589. doi: 10.1099/ijs.0.013300-0.