Diversity of Microbial Traits in the Zebrafish-Gut Microbiota

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An estimated 10^{14} microbial cells are associated with the human body. Those microbial cells represent approximately 90% of the cells present[1] and most densely occupy the distal gut. In both presence and composition, the structure of the complex communities of microorganisms associated with the gut aid the development and health of the host.[2] In the model vertebrate zebrafish (*Danio rario),* the gut microbiota regulates aspects of gut-epithelium development,[3] primes the host immune system against the invasion of pathogens,[4,5] and regulates intestinal homeostasis.[6] Further, changes in the relative abundance of Bacteroidetes and Firmicutes, the two dominant bacterial divisions found in the distal gut,[7] occur in lean versus obese phenotypes in both humans and mice.[7,8] Moreover; several disease phenotypes (including inflammatory bowel disease) are associated with altered gut microbial compositions.[9] Despite the need to understand the ecological processes determining the structure of the vertebrate gut microbiota, elucidation of the processes structuring the gut microbiota is incomplete.

The vertebrate gut is a unique environment with a unique microbiota. Several interconnecting factors determine the gut microbiota; those

factors include diet,[8] environmental drivers,[10] host filtering,[10,11] and microbial community interactions.[2,12] Studies of co-occurring microbes and their interactions (e.g., niche competition, cooperation, facilitation, and inhibition) have focused on microbial communities associated with the human mouth. However, more recently, research has been gravitating towards studying the whole human microbiome as we realize its important role in human health (e.g., The Human Microbiome Project). I investigated the diversity of individual traits of the microbes in the gut microbiota because they may predict how well microbes establish and proliferate in the gut. To do this I used the *Danio rerio* model, which is ideal for this study because it can be reared germ-free and inoculated with known microbes.

We derived 66 individual taxa of microbes from conventionally-raised zebrafish and then isolated, identified, and characterized 19 taxa, representing 17 unique genera and 6 phyla of the known gut community. The primary methodology depended on measuring several common traits using well-established and efficient bacterial culture assays.[13] The measurements focused on motility (possession of a mechanism by which to move), speed, doubling time, hemolysis (production of a protein that opens blood cells), biofilm production (the ability of cells to attach to a surface and encase themselves within a protective structure made of polysaccharides and proteins), mono-associated colonization (assembly and growth of a population in the germ-free gut), and co-associated colonization (assembly and growth of a population in the germ-free gut along with another known microbe). The choice of these traits rested on the assumption that, if possessed by some organisms but not others of the community, there may be a possible advantage in assembly and persistence in the gut.

Materials and Methods

Bacterial strains and culture conditions. The bacterial strains in this study are listed in Table 1. All strains used in this experiment were maintained in glycerol stocks stored at −80°C. Each time an assay was conducted, these bacterial strains were grown in overnight cultures for the following day (except where indicated).

Isolation of bacterial strains. The bacterial strains listed in Table 1 that originated at the University of Oregon were derived by sampling dissected guts from zebrafish conventionally reared in the UO zebrafish facility. All bacterial strains (conditions in Table 1) were derived from fish

Strain ID	Phylum	Host Facility Origin	Culture Condition
ZOR0001	Gamma Proteobacteria	This study UO	Aerobic; 30°C; LB / TSA
ZOR0002	Gamma Proteobacteria	This study UO	Aerobic; 30°C; LB / TSA
ZOR0008	Gamma Proteobacteria	This study UO	Aerobic; 30°C; TSA
ZOR0011	Gamma Proteobacteria	This study UO	Aerobic; 30°C; BHI / TSA
ZOR0012	Gamma Proteobacteria	This study UO	Aerobic; 30°C; BHI / TSA
ZOR0014	Gamma Proteobacteria	This study UO	Aerobic; 30°C; NA / M17
ZOR0017	Beta Proteobacteria	This study UO	Anaerobic; 30°C; BHI; *
ZOR0019	Actinobacteria	This study UO	Aerobic; 30°C; BHI; *
ZOR0020	Actinobacteria	This study UO	Aerobic; 30°C; BHI; *
ZNC0006	Beta Proteobacteria Beta	U. North Carolina	Aerobic; 30°C; BHI
ZNC0007	Proteobacteria Beta	U. North Carolina	Aerobic; 30°C; BHI
ZNC0008	Proteobacteria Alpha	U. North Carolina	Aerobic; 30°C; BHI
ZNC0028	Proteobacteria Alpha	U. North Carolina	Aerobic; 30°C; BHI
ZNC0032	Proteobacteria Alpha	U. North Carolina	Aerobic; 37°C; stationary BHI broth
ZNC0037	Proteobacteria Gamma	U. North Carolina	Aerobic; 30°C; Columbia
ZWU0006	Proteobacteria	U. Washington	Aerobic; 30°C; BHI / LB /TSA
ZWU0009	Firmicutes	U. Washington	Aerobic; 30°C; BHI / LB
ZWU0011	Firmicutes	U. Washington	Aerobic; 30°C; BHI
ZWU0020	Gamma Proteobacteria	U. Washington	Aerobic; 30°C; BHI / TSA

LB = lysogeny broth TSA = tryptic soy agar BHI = brain heart infusion NA = nutrient agar

* sonicated gut for 4 minutes

Table 1: Strain ID, origin, and characteristics.

at various ages and by altering the growth conditions (aerobic, anaerobic, sonication, boiling) and the media (nutrient contents) in which they were incubated. Several of the strains listed in Table 1 originated from University of Washington and University of North Carolina.

Motility assays. Motility assays were performed on 2.5-2.7 mg ml^{-1} Bacto Agar plates consisting of 10 mg ml^{-1} tryptone and 5 mg ml^{-1} sodium chloride. Plates were stabbed with one fresh colony grown on Tryptic Soy Agar (Difco), incubated at 30°C for 16 hours, then placed stationary at 30°C for 4-6 hours. Controls for this assay consisted of a motile wild-type *E. coli* strain (positive control) and a non-motile mutant-type *E. coli* (ΔfliM) (negative control). (For more information on these strains, see Eberl & Boneca.)[5] The area of outward migration from the site of inoculation was recorded with an Apple digital camera. The standard size of *E. coli* growth area was compared to growth areas of the zebrafish isolate strains in Table 1 to determine binary results. Strains with growth areas most similar to wild-type *E. coli* were considered motile, and those with growth areas most similar to ΔFliM were considered non-motile.

Video speed assay. Brain Heart Infusion (BHI) broth was inoculated with one fresh colony plated on BHI agar and then grown at 30°C until the liquid culture reached an optical density at 600 nm (OD_{600}) of 0.05 ±.025, corresponding to mid-exponential phase. I used a protocol devised previously to record speed.[14] Samples of 2 µl for each culture were transferred into a 10-well microscope slide (MP Biomedicals), ten strains at a time. With a cover slip placed on top of the slide, videos were taken through a 203 objective lens at 15 frames per second using a Cohu CCD camera (model 4912) and USBVision Capture software. Videos were analyzed using custom particle-tracking programs written in MATLAB. Analysis was performed on inverted image frames (white bacterial particles on black background) of 352 x 240 pixels where 1 pixel

equaled 1.73 μm. Background and nonmotile particles for each frame were digitally removed by subtracting the average image intensity, integrated over the entire movie. Tracked particles were identified as local intensity maxima in high-pass filtered and thresholded images, with a filter size of 4 pixels and an intensity threshold of 99%. Each particle's position was assigned to be the centroid of the thresholded object. The bacterial length (approximately 3 mm) spans several pixels, and so centroid finding provides a measure of localization that is sufficiently precise relative to bacterial size. Particles were constructed into tracks by searching for the nearest particles in adjacent frames. Particles needed to be within 7 pixels of each other to be considered part of the same track. When edited, tracks longer than 1 sec or a frame-to-frame variance greater than 1 pixel were retained. Any segment of a bacterial track was included in the analysis if it lasted for at least 1 second (15 frames) without containing any of the following: a NaN (not a number; particle not tracked for that particular frame), a turn greater than 90 degrees, or movement less than 0.5 pixel for one frame. The average distance traveled per frame for all the included data is the mean speed (in pixels/frame).

Bacterial growth curve and doubling time calculations. Bacterial cultures were inoculated with BHI broth from a single colony that had grown overnight on BHI agar plate stationary at 30°C, then shaken at 200 RPM and 30°C. Culture samples of 1 ml were removed every 30 minutes for an average of 200 minutes and recorded. The natural log of the OD_{600} measurements for each time point were plotted versus time using R statistical software. A regression analysis of the data was used to calculate the doubling times at exponential growth.

Hemolysis assay. Hemolysis assays were performed using blood agar plates consisting of Fastidious Anaerobe Agar with 10% defibrillated sheep blood (Hemostat). Bacterial strains were inoculated on agar plates

from colonies grown overnight on BHI agar plates at 30°C. Bacterial cells were streaked on agar surface and stabbed into the agar to create oxygen labile pockets, incubated at 30°C, and observed frequently for three days for any clearing or discoloration of blood agar surrounding colonies. No control was used. Results were recorded with an Apple digital camera, and clearing and discoloration were compared across all plates.

Biofilm assay. Biofilm assays were performed using 18x150 mm Borosilicate glass culture tubes (VWR). Tubes containing 100 ml of BHI broth were inoculated with single colonies that were grown overnight at 30°C on BHI agar plates. The bacterial tube cultures were stationary and incubated at 30°C for 4 days and closely observed for visible biofilm formation at the air-fluid surface and glass-liquid interfaces. Observations were recorded with an Apple digital camera. After 4 days the cultures were diluted with 1 ml of sterile 1% potassium bisphosphate (PBS) solution. Next, 1 ml of 1% crystal violet was added to the cultures before they were set stationary for 15 minutes at room temperature. The culture was gently removed and the tubes gently rinsed with sterile PBS. Bacterial cultures that produced violet bands on the glass of tubes were considered to have formed a biofilm. The biofilms were then classified as biofilm, weak biofilm, and no biofilm, depending on the thickness and intensity of the violet bands.

Colonization assays. Zebrafish for this assay were derived by using a standard protocol for generating zebrafish embryos, deriving and rearing germ-free zebrafish, and colonizing zebrafish with microorganisms.[15] The sterilized eggs were transferred into flasks with 50 ml of growth medium (BHI or TSA broth depending on the medium of origin) and incubated until they reached 4 days post fertilization (dpf). Mono-associated colonization assays were performed by inoculating BHI and TSA broth (depending on which was broth of origin) from glycerol stocks of bacterial strains.

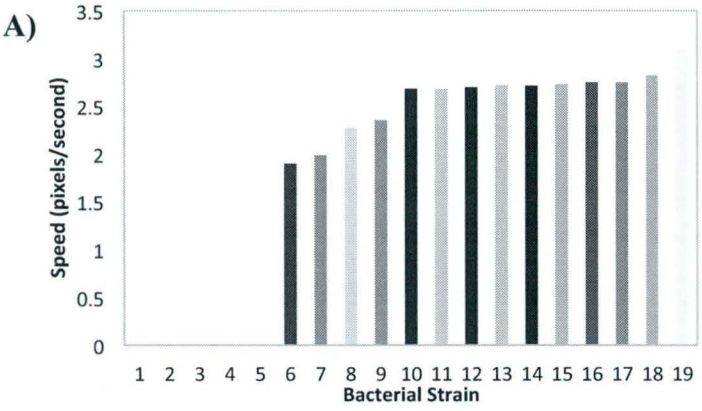
When the fish reached 6 dpf, their guts were dissected and manually homogenized before 10 µl samples were spot-inoculated onto BHI and TSA agar plates (depending on medium of origin). The plates were air dried, and then placed at 30°C overnight. Colony forming units (CFUs) were counted, and an industry-standard calculation was used to estimate the cellular concentration within the gut samples at the time of dissection. Co-association assays were conducted in the same way except that two strains at a time were introduced into the flasks instead of one. Seven strains that successfully colonized in mono-associations were co-inoculated with ZOR0012, which also successfully colonized in mono-associations.

DNA collection and extraction. DNA was extracted from isolated cultures using the Ultraclean microbial DNA extraction kit from MoBIO. Extracted 16S rDNA sequences from isolates were amplified by polymerase chain reaction (PCR) using 27F and 1492R primers. Sequences were purified using GeneJET PCR purification kit from ThermoScientific and sent to Sequetech (http://sequetech.com/) for sequencing. Strains were then assigned a taxonomic identification by matching sequences to a 16S sequence database (Green Database).

Results

Motility and speed traits across 19 taxa in zebrafish-gut microbial community. Motility and video speed assays revealed (Fig. 1) that 14 of the 19 strains measured exhibited motility. Among the motile strains, the motility speed ranged from 1.9 to 3.1 µm/second. I was unable to obtain speed data for one of the strains.

Hemolysis across 19 taxa in zebrafish-gut microbial community (Fig. 1). Oxygenic Beta hemolytic activity was observed in 9 of the 19 measured taxa. Aerobic Alpha hemolysis was observed in 1 of the strains, and 1 of the Beta hemolytic strains also exhibited anoxygenic (in oxygen labile pockets) Beta hemolytic activity.



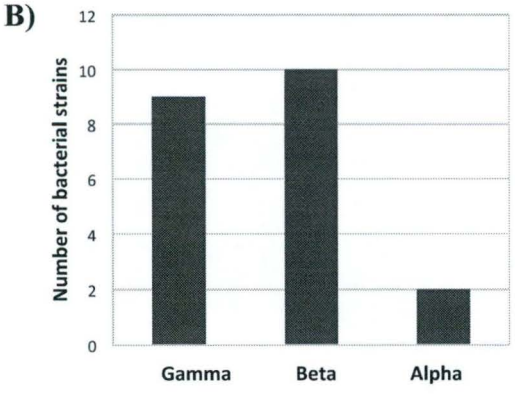
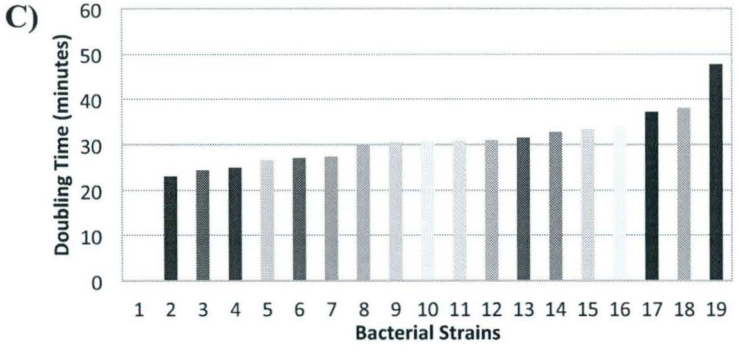


Figure 1 (A, B, C): Motility and speed observed. Strains are numbered arbitrarily in all charts. (A) Presence of a bar represents flagellar motility on the graph; speed is reflected by the magnitude of the bars. One strain (noted with an asterisk) exhibited twitch motility. (B) The magnitude of the bars represent the number of strains with enzymes for the type of hemolysis; Alpha = partial, Beta = complete, and Gamma = no hemolysis. (C) The magnitude of the bars represents the doubling time for strains during exponential growth phase.

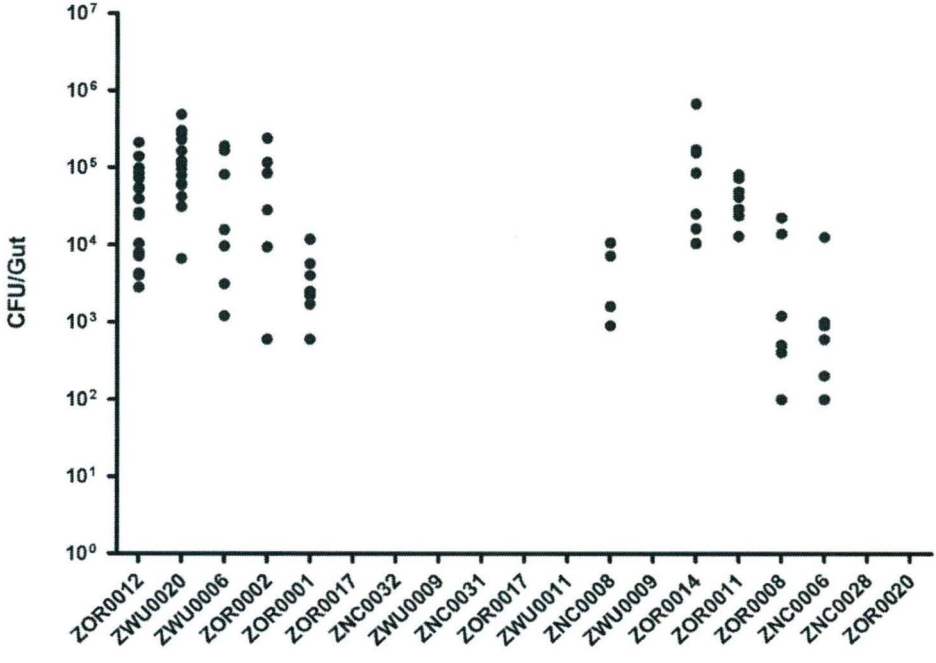


Doubling times across 19 taxa in zebrafish-gut micobial community (Fig. 1). Although the doubling times of these strains all appear to be relatively short (as expected), these results demonstrate there may be more diversity than expected for this trait.

Bofilm formation across 19 taxa in zebrafish-gut microbial community. As expected, not all 19 strains exhibited biofilm production; of the 14 that did, 4 exhibited nearly undetectable biofilm stain bands on the glass tubes in which they were grown.

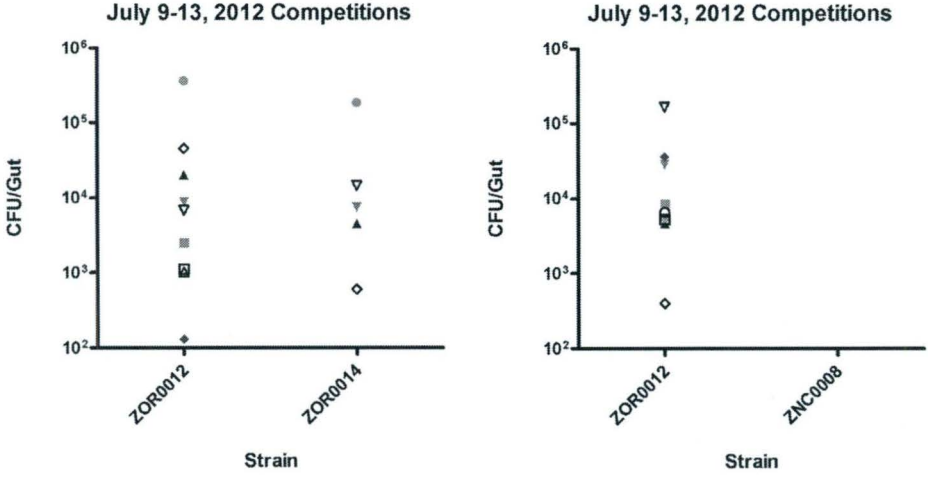
Colonization across 19 taxa in zebrafish-gut microbial community (Fig. 2). Of the 19 bacterial strains, 10 colonized successfully with

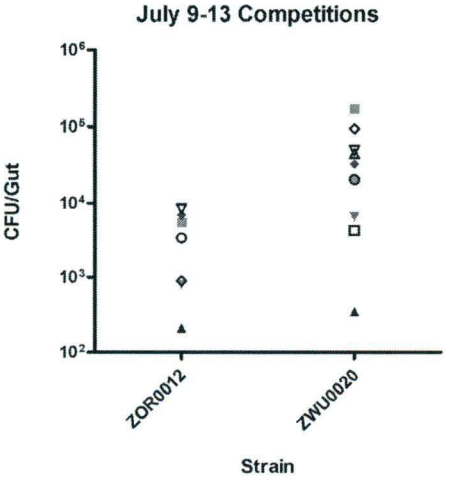
Figure 2: Co-association of isolates in zebrafish. Each point on the chart represents gut samples dissected, homogenized, inoculated on medium, and enumerated from one individual fish. CFUs varied within and across bacterial strains.

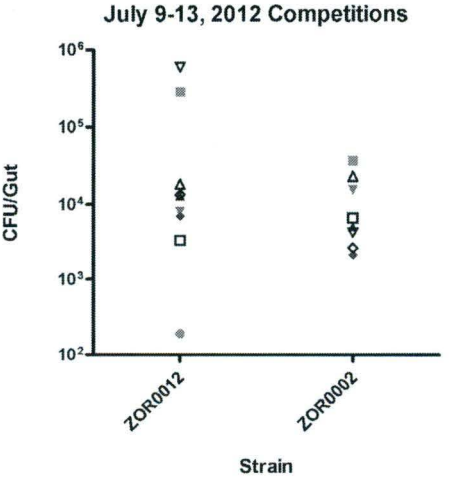


variability across gut samples, a result also demonstrated in previous work. The source of the variability across gut samples within strains is unknown. Possibly, the variability occurs because of the inconsistent size of gut dissections. Strain ZOR0012, which successfully colonized in mono-associations, was co-inoculated with 6 other strains that also successfully colonized in mono-associations. Co-inoculations demonstrated variability as well. In the co-inoculation of ZOR0012 and ZNC0008 (Fig. 3), ZOR0012 colonized and ZNC0008 never colonized successfully. In the co-inoculation of ZOR0012 and ZWU0006, again ZOR0012 colonized, yet ZWU0006 colonized in only 3 of the 12 fish sampled. In all other co-inoculations both strains were able to colonize. These results suggest the possibility that competition is occurring between some strains during colonization, and that the degree of competitive success may be affected by other variables (including host factors).

Figure 3: Co-association of isolates in the zebrafish gut. Each point on the charts represents one CFU enumeration and each set of points represents all the gut samples inoculated and enumerated from one group of fish inoculated with the same strain.




July 9-13 Competitions
CFU/Gut
10^6
10^5
10^4
10^3
10^2
ZOR0012
ZWU0020
Strain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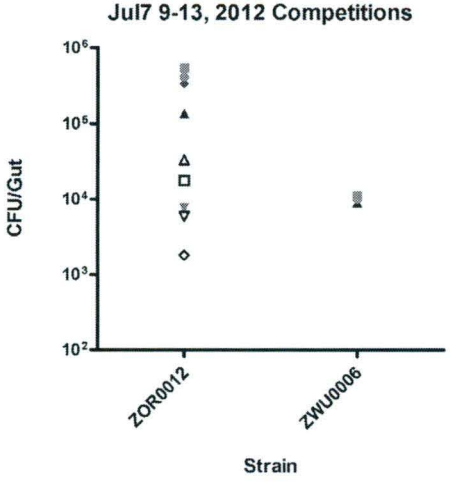
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CFU/Gut
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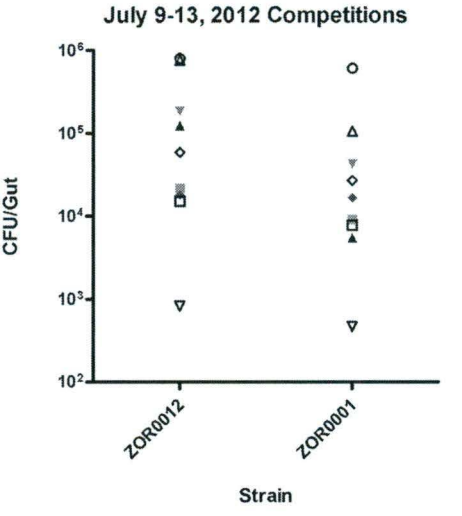
July 9-13, 2012 Competitions
CFU/Gut
10^6
10^5
10^4
10^3
10^2
ZOR0012
ZOR0001
Strain

Discussion

This study reveals that the 19 taxa of the zebrafish microbiota do not all share the same traits. Diversity of traits in a microbial community may mean that the gut habitat is not homogeneous (has a variety of niche, pH, and salinity levels) or that the gut does not strongly filter for specific types of microbes. Therefore, a more thorough examination of the whole gut community should be measured with more representatives of the gut community included. Future investigations should involve a power analysis to determine the number of representatives that would be most informative for a better understanding of the vertebrate-gut ecosystem.

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