**Common laboratory diets differentially influence zebrafish gut microbiome’s successional development and sensitivity to pathogen exposure**

Michael Sieler, Collen Al-Samarrie, Kristin Kasschau, Michael Kent, Thomas J. Sharpton

**Abstract**

Despite the long-established importance of zebrafish as a model organism and their increasing use in microbiome-targeted studies, relatively little is known about how husbandry practices involving diet impact the zebrafish gut microbiome. Given, the microbiome’s important role in mediating host physiology and the potential for diet to drive variation in microbiome composition, we sought to clarify how three different dietary formulations that are commonly used in zebrafish facilities impacts the gut microbiome. We reared 60 fish on each diet throughout their lifespan and compared the composition of their microbiomes at both 3- and 6-months post fertilization. Our analysis finds that diet has a substantial impact on the composition of the gut microbiome at both 3- and 6-months of age. Moreover, the developmental dynamics of the microbiome differ as a function of diet. We further evaluated whether the 6-month post fertilization microbiome compositions that result from dietary variation are differentially sensitive to infection by a common laboratory pathogen: *Mycobacterium chelonae*. Our analysis finds that the impact of *M. chelonae* infection on the gut microbiome differs as a function of diet, especially for moderate and low abundance taxa. Overall, our results indicate that diet drives the successional development of the gut microbiome as well as its sensitive to exogenous exposure. Consequently, investigators should carefully consider the role of diet in their microbiome zebrafish investigations, especially when integrate results across studies that vary by diet.

**Introduction**

Despite zebrafish’s long-established importance as a model organism and their increasing use in microbiome-targeted studies, key knowledge gaps remain about how diet influences their microbiome. In contrast to mice, zebrafish do not have a standard reference diet (Watts). Prior research has found husbandry choices involving diet can induce variation in study outcomes and challenge efforts to compare results across studies (Fowler, Watts). Moreover, experimental, commercial and laboratory diets result in different microbiome and health outcomes (Fowler, Leigh, Rawls, Others?). However, it remains unknown whether zebrafish gut microbiome communities differ between commonly used laboratory diets, and if these differences persist throughout development.

By 3 months of age Zebrafish are developmentally considered adults. Their immune systems have finished developing, they are sexually mature, and have reached full body size (citation). However, zebrafish microbiomes continue to develop as they age, becoming increasingly diverse and stable (Xiao). Prior to adulthood, zebrafish microbiome assembly is more susceptible to environmental influences of drift and dispersal, but with age these effects decline until senescence (Stephens2016). Additionally, the microbiome links to an array of health outcomes involving obesity, X, Y and Z across an array of organisms, including zebrafish (citations). Generally, microbiomes are stable once established. Therefore, early-life assembly of the gut microbiome could have long-term implications on host health, such as resistance to infection (citation).

Pathogen exposure is known to impact the gut microbiome of zebrafish (Gaulke), and the microbiome could mediate these effects, either protecting, exacerbating, or having a neutral influence (citation). Zebrafish facilities are known to host many pathogens, which can introduce non-protocol induced inconsistencies in study outcomes (Kent). One pathogen that is found in 40% of zebrafish facilities is *Mycobacterium chelonae*, and is hypothesized to be introduced through diet early in life (Stephens, Kent2012, Chang2019). *M. chelonae* forms granulomas in the gut intestine, which can cause gut inflammation, decreased fecundity and lifespan (Whipps2016, Varela). Previous work of ours has shown that pathogen exposure disrupted the gut microbiomes of zebrafish (Gaulke), but the joint effects of diet and pathogen exposure on zebrafish gut microbiomes and physiology remains unclear. Elucidating these relationships could offer microbiome-targeted treatments for preventing or minimizing the impacts of pathogen exposure on zebrafish health and study outcomes.

Here, we assessed whether different common laboratory diets influenced gut microbiomes and physiology of 3-month-old zebrafish (Fig. 1). Next, we investigated the role of diet on zebrafish’s development between 3 and 6-month-old zebrafish. Finally, we measured the diet-associated sensitivity of zebrafish to the pathogenic species *Mycobacterium chelonae*. Our study clarifies how common laboratory diets differentially impacts the successional development of zebrafish gut microbiome and sensitivity to pathogen exposure.

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| **Figure 1:** Experimental design showing treatments and husbandry events during the course of the study. An “X” indicates when an event occurred (e.g., fecal sampling took place when fish were age 129 and 214). |

**Results**

1. **Diet differentially influences physiology and gut microbiome**

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| **Figure 2:** Effects of fish fed one of three diets (Gemma, Watts, or ZIRC) on physiology and microbiomes of zebrafish. **(A)** Weight of ZIRC significantly differs from Watts and Gemma. Gemma and Watts do not differ from each other. **(B)** Body condition score is a length normalized measure of weight. ZIRC fed fish have significantly higher body condition scores from Gemma and Watts diets. **(C)** Simpson’s Index of diversity shows that gut microbiome diversity significantly differs between Gemma and Watts, ZIRC and Watts, but not between Gemma and ZIRC. **(D)** Capscale ordination based on the Bray-Curtis dissimilarity of gut microbiome composition. The analysis shows that physiology and gut microbiome composition significantly differs between the diets. “ns” indicates not significantly different, \*, \*\*, \*\*\* indicates significant differences below the 0.05, 0.01, and 0.001 levels, respectively. | |

To investigate how diet may impact the zebrafish gut microbiome diversity, composition, and relative abundance, we fed zebrafish one of three commonly used laboratory diets (Gemma, Watts, and ZIRC; see Table in supplementary material). At 3 months of age, we collected fecal samples and used 16S rRNA gene sequencing to identify microbial taxa. Additionally, we measured weight, length, and body condition score to assess how these diets may impact zebrafish physiology. Body condition score is a length normalized metric of weight (for equation, see Methods) and serves as a general indicator of health in zebrafish.

We first determined if physiology, represented here by weight and body condition score, differed between diets. Wilcoxon Signed-Rank Tests found that diet and sex significantly associated with weight and body condition. Female fish had higher weight and body condition scores compared to males (Z = 1,505, P < 0.001; Table S1.1). The ZIRC diet had the highest mean body condition score compared to the Gemma (Z = 301, P = 0.44) and Watts diets (Z = 225, P = 0.006, Table S1.1.1). Fish fed the Gemma and Watts diets did not significantly differ from one another in terms of weight and body condition scores. We did not observe a significant interaction between diet and sex on weight and body condition score. Collectively, results indicate that diet has an effect on physiology.  
  
Next, we asked if diet associated with gut microbiome diversity and composition. First, we built generalized linear models (GLM) to determine if diet associated with variation in one of three measures of microbiome alpha-diversity: richness, Simpson’s Index, and Shannon Entropy. An ANOVA test of these GLMs revealed that alpha diversity varies as a function of diet for all three measures of diversity (p < 0.05; Fig 1C; Table S1.2.2). A post hoc Tukey test clarified that ZIRC- and Watts-diet fed fish exhibited significant differences in diversity for all three metrics, whereas ZIRC- and Gemma-diet fed fish only differed when considering the Simpson’s index (p < 0.05; Table S1.2.3). Gemma and Watts only differed significantly in terms of richness, and ZIRC and Gemma only differed when considering the Shannon diversity index.

Next, we used the Bray-Curtis and Canberra dissimilarity metrics to evaluate how diet associates with microbiome composition. A PERMANOVA test revealed that gut microbiome communities fed different diets are significantly different from one another in their composition (p = NNN). Additionally, we assessed beta-dispersion, a measure of variation of microbiome communities, by calculating each gut microbiome community’s distance from their respective centroid. Beta-dispersion levels for Bray-Curtis differed significantly, where Watts had higher dispersion and differed from the other two diets, but ZIRC and Gemma did not differ from each other. For the Canberra measure, Gemma fed fish had the least dispersion and was significantly different from ZIRC and Watts, but ZIRC and Watts did not differ from each other.

Finally, to better understand the interactions between the diets and the gut microbiome, we quantified differential abundance using ANCOM-BC. We observed 24 taxa were significantly abundant in at least one of the three diets. Collectively, these results indicate that commonly used zebrafish laboratory diets have a differential effect on microbiome structure at 3 months of age.

1. **Diet impacts the successional development of the zebrafish gut microbiome**

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| **Figure 3:** Development is associated with altered microbiome composition. **(A)** Shannon Index for diversity of 3 and 6 month old fish, and **(B)** appears to be diet dependent. Capscale ordination of gut microbiome composition based on the **(C)** Bray-Curtis dissimilarity by time and **(D)** Canberra measure by time. **(E)** Body condition score did not differ between time points for either diet, but in ZIRC fed fish a **(F)** body condition score negatively associated gut microbiome diversity. The analysis shows that physiology and gut microbiome composition significantly differs between the diets across development, and there may be diet-dependent link with physiology. “ns” indicates not significantly different, \* indicates significant differences below the 0.05 level. | | |

Given the associations we observed above between diet, the gut microbiome and physiology at 3 months of age, we next asked how microbiome structure and physiology differs between the diets across development at 6 months of age. Based on linear regression, we observed a statistically significant main effect of diet, time and and interaction effect between diet and time on gut microbiome diversity across all diversity indices (p < 0.05; Fig A&B, Table S2.2.2.1). A post hoc Tukey test showed microbiome diversity was significantly different between 3 and 6 months in Gemma and ZIRC fed fish in Shannon and Simpson’s Indices (p < 0.05; Table S2.2.2.3), but Watts microbiome diversity was not significantly different between 3 and 6 months. We next sought to determine if diet influences microbiome composition across development. We find the microbiome community composition varies over time, but the temporal sensitivity of the abundant taxa in the microbiome is less than the sensitivity to diet. A PERMANOVA test using the Bray-Curtis dissimilarity metric revealed that community composition was best explained by diet (p < 0.05; Fig 2C, Table S2.4.3), but an analysis using the Canberra measure found that variation in microbiome composition was best explained by time (p < 0.05; Fig 2D, Table S2.4.3). Within each diet, beta-dispersion significantly differed between 3 and 6 months in Gemma and ZIRC diets (p < 0.05; Fig S2.5.3), while Watts remained consistent between 3 and 6 months. An ANOVA test revealed significant beta-dispersion in metrics that emphasize abundant taxa (e.g., Bray-Curtis) and metrics that emphasize rare taxa (e.g., Canberra) of ZIRC fed fish (p < 0.05; Fig S2.5.3), while Gemma had significant beta-dispersion among abundant taxa (p < 0.05; Fig S2.5.3). Finally, we used ANCOM-BC to determine if the abundance of taxa associated with development for each diet. We found 33 taxa that were significantly abundant at the genus levels in at least one diet between 3 and 6 months (p < 0.05; Table S2.6.1-2). Collectively, our results indicate that development differentially impacts fish gut microbiome structure depending on diet.

To determine if physiology differed between diets across development, we used Wilcoxon Signed-Ranks Tests to identify parameters that best explained the variation in body condition score. Body condition score did not significantly differ between time points across all diets (p < 0.05; Fig 2E, Table S2.1.1). We observed a significant interaction uniquely in ZIRC fed fish between gut microbiome diversity and body conditions score (p < 0.05; Fig 2F, Table S2.2.1). In ZIRC fed fish, body condition score negatively associates with an increase in microbiome diversity across development. A PERMANOVA test did not find a significant interaction effect of body condition score and diet (Table S2.2.2). Moreover, body condition score did not explain variation in the abundance of specific gut taxa. These results indicate that in the ZIRC diet there is a link between alpha diversity and body condition score across development.

1. **Diet influences gut microbiome’s sensitivity to pathogen exposure**

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| **Figure 4:** Exposure to *Mycobacterium chelonae* inhibits diversification of gut microbiome. **(A)** Shannon Index for diversity of pre-exposed 3-month-old fish, 6-month old exposed and unexposed fish, and **(B)** for exposure groups within each diet. Capscale ordination based on the Bray-Curtis dissimilarity of gut microbiome composition of 6-month-old fish colored by **(C)** exposure groups (exposed versus unexposed) and **(D)** diet. **(E)** Log observed abundances of Mycobacterium of pre-exposed, exposed and unexposed fish within each diet as calculated by ANCOM-BC. The analysis shows gut microbiome’s sensitivity to pathogen exposure is linked to diet, but Mycobacterium’s abundance is diet-dependent. “ns” indicates not significantly different, and \* indicates significant differences below the 0.05. | | |

<Results>

Lastly, we sought to elucidate the potential interactions between the intestinal pathogen *Mycobacterium chelonae*, common laboratory diets and the gut microbiome. Briefly, after collecting fecal samples at 3 months old, we injected *Mycobacterium chelonae* into the coelomic cavity of the fish in the exposed treatment group. Using linear regression, we find that microbiome diversity differs between exposure groups in Observed and Shannon indices (P < 0.05; Table S3.1.2.2), but we did not find a significant interaction effect between diet and exposure. The statistical effect of diet was far greatest across all diversity indices (Table S3.1.2.2). Furthermore, a post hoc Tukey test showed microbiome diversity was significantly different in unexposed ZIRC fed fish between pre-exposed and exposed groups across all diversity metrics; and unexposed Gemma fish were significantly different to pre-exposed fish in Shannon index, and Unexposed watts fish were significantly different to exposed fish in Observed index (P < 0.05; Fig 3B, Table S3.1.2.3). Moreover, we assessed how pathogen exposure influenced microbiome composition across the diets. For all beta-diversity metrics, we find significant main effects of diet (Fig 3C) and pathogen exposure (Fig 3D); and we find interaction effects of diet and exposure group in Canberra (P < 0.05; Table S3.2.3). In all beta metrics, diet’s statistical effect was greatest (Table S3.2.3). Finally, to determine if diet impacted *Mycobacterium* abundance we used ANCOM-BC. We find *Mycobacterium* taxa were significantly abundant in at least one group across the diet and exposure groups (W = 26.6, Q < 0.001; Fig 3 E, Table S3.4.1). Mycobacterium was present in pre-exposed groups at 3 months, and abundance increased in unexposed fish at 6 months (W = 19, Q = 0.003; Table S2.6). Relative to unexposed fish, we find that Mycobacterium had significantly decreased abundance in exposed fish in Gemma and ZIRC fed fish (P < 0.05; Table S3.4.2). We did not see a pathogen exposure effect on physiology. We also do not find a diet by exposure interaction with body condition score. Collectively, these results indicate that gut microbiomes of fish fed different diets vary in their sensitivity following pathogen exposure.

**Discussion**

Zebrafish are emerging as an important model organism in microbiome-targeted studies. Diet is an important contributor to gut microbiome structure in other animal systems. Zebrafish research does not employ a standard reference diet which could introduce variation across microbiome-targeted studies. Previous studies have found differences in microbiome outcomes of zebrafish fed different diets. However, diets used in these studies vary greatly in nutritional content. Moreover, the diets used in these studies differ in nutritional content to diets typically used in zebrafish husbandry and research experiments. Furthermore, the effects of diet on the zebrafish gut microbiome’s sensitivity to a common intestinal pathogen has not been investigated. Our study clarifies the effects of nutritionally similar and commonly used laboratory diets on the successional development of zebrafish gut microbiome and its sensitivity to pathogen exposure.

We find that commonly used laboratory diets with relatively similar nutritional compositions stratify the gut microbiome of adult zebrafish. Previous research has found that diets with varying compositions of key macronutrients: protein, lipids and fiber impacts zebrafish physiology and gut microbiome (Leigh, Wong, Fowler). However, the nutritional compositions used in these studies targeting the gut microbiome differ from what is typically used in zebrafish husbandry and research. Our results show that even minor differences in nutritional composition can have profound impacts on the gut microbiome assembly in 4-month-old zebrafish. The differences we observe in the gut microbiome assembly between these diets could be explained by the exact ingredient formulations, where the ingredients were sourced, and the methods of preparation. Studies in Atlantic salmon and the common carp found that not all protein sources in diet are equal. Soy bean meal is a common protein source for hatchery-reared fish, but studies show it is implicated in causing intestinal inflammation. Moreover, variation in ingredient sources caused by inconsistent commodity sources in the case of commercial diets may inadvertently introduce contaminants, such as phytoestrogens, or pathogenic microbes, such as M. cholonae, to zebrafish (Watts/D’ambro, Kent). Furthermore, studies using the same diets may not in fact be the same due to differences between batch production (Watts/D’ambro). Therefore, zebrafish researchers seeking to target the microbiome in their studies should consider using a standard refence diet. A standard reference diet would have the benefit of transparently disclosing ingredient sources, nutritional compositions, and methods of preparation. However, in some instances variability in diet may be beneficial to researchers seeking to model the variability of diets found in human populations.

We also observed variation in the successional dynamics of gut microbiomes of fish fed different diets across their development in adulthood. We find the composition of abundant taxa are driven primarily by diet, but rarer taxa are sensitive to the effects of time. We also saw interaction effect of diet and development on the gut microbiome. For instance, gut microbiome diversity and community variation increased across development in fish fed the Gemma and ZIRC diets, but remained stable in Watts-diet fed fish. One explanation for this stability is that the Watts diet is a laboratory designed and produced diet, which might offer more consistency in ingredient sources, nutritional composition and methods of preparation to the other two commercially-derived diets. Another important point of consideration is the transition of fish from juvenile to adult diet formulations at 4 months of age. Again, we find more consistency between the Watts juvenile amd adult diets compared to the Gemma and ZIRC juvenile and adults diets. In particular, the ZIRC adult diet formulation is a combination of four different diets that differ in nutritional composition and ingredient sources, whereas the Watts adult diet changes only in lipid content. The consistency in the Watts diet formulations could be linked to the consistency of the Watts-diet fed fish gut microbiome diversities across development. Previous research investigating the successional development of the gut microbiome in zebrafish finds zebrafish gut microbiome diversity is higher in juvenile fish but declines as they age. Our results contrast this overall trend, but when we compare similar time points between studies, we do find a similar increase in diversity between 4 and 7 months of age. A limitation of these past studies is inconsistency in diet and tank environment which could explain the variability of gut microbiomes of juvenile zebrafish. Future studies should seek methodological consistency across diet and microbiome sampling to aid efforts in cross-study comparisons. A unique strength of our study is the consistency in diet and tank environment. We clearly demonstrate the effects of diet, development, and their interaction on the successional development of zebrafish gut microbiome.

Differences in early-life assembly of the gut microbiome caused by diet could have long-term impacts on the health of the host. For instance, diets that select for gut microbiomes that more efficiently metabolize nutrients early in life could provide a fitness advantage to the host and improve their reproductive success, longevity, and ability to resist disease. Previous studies find diet-related impacts to physiology and reproduction (Fowler), as well as implicated certain taxa to physiological outcomes in zebrafish (Rawls, Leigh). Indeed, in the case of ZIRC-diet fed fish we find physiological effects linked to the gut microbiome, where body condition score and microbiome diversity are negatively associated. However, we did not find specific taxa that associated with physiological measurements of body condition score across any of the diets, even within ZIRC-diet fed fish. Therefore, the ZIRC diet may not be enriching for particular taxa that influence body condition score. Instead, the intestinal environment found within high body condition score ZIRC-diet fed fish may be inhospitable to cultivating a diverse microbiome. Compared to previous studies linking specific taxa to physiological outcomes in zebrafish fed different diets, the diets we used differed minimally which may explain why we do not find similar taxa-associated effects. Another explanation could be that body condition score is not the optimal metric for identifying physiologically important. For instance, previously mentioned studies measured more specific physiological measurements such as fat tissue, intestinal length, and gut enzymatic activity.

Finally, we find that exposure to the intestinal pathogen *Mycobacterium chelonae* inhibited diversification of gut microbiomes, and microbiome community composition was driven primarily by diet rather than pathogen exposure. Additionally, *Mycobacterium’s* abundance differed between the diets. Exposed Watts-diet fed fish had more *Mycobacterium*, but Exposed Gemma and ZIRC had fewer relative to controls. It’s important to note that nonpathogenic *Mycobacterium* species are a common member of the zebrafish gut microbiome community. Due to the limitations of 16S analysis it’s not possible to disentangle whether the Mycobacterium abundance we observed is the injected pathogenic strain or non-pathogenic species naturally present in the fish. Despite this limitation, we can see pathogen exposure effects across the diets. The gut microbiome diversity of ZIRC fed fish is uniquely sensitive to pathogen exposure, while Gemma- and Watts-diet fed fish were more stable. Higher gut microbiome diversity is linked to higher stability and greater ability to resist pathogens (Xiao, Gaulke?, Other?). Thus, it is possible *Mycobacterium* taxa might have been uniquely situated in Watts fed fish to take advantage of lower stability to gain habitat space. However, the effects of pathogen exposure on microbiome community composition were secondary to diet, and this might explain why our results differ from previous microbiome-pathogen studies that saw increased microbiome community variation following pathogen exposure (Gaulke). Specifically, Gaulke et al found microbiome diversity and community composition increased in variation within pathogen exposed fish, while we find the opposite effect of exposed fish microbiome communities becoming more similar to one another and decreased diversity compared to controls. These differences could be due to the differences in pathogens, where Gaulke et al exposed fish to an intestinal helminth and we used a bacterial pathogen. Therefore, the gut microbiome may respond differently to pathogensis. While we did not find an effect of infection on the gut microbiome, there could be other ways that pathogen exposure exerts influence on the gut microbiome. For instance, the presence of pathogenic bacteria may induce an immunological inflammatory response that affects the gut microbiome. Additionally, to ensure exposure to *M. chelonae* we injected fish with the pathogen, but this is not the natural route of transmission. Future studies could include immunological endpoints as well as expose zebrafish using a natural route of transmission to clarify the effect of *M. chelonae* on the gut microbiome.

Beyond zebrafish husbandry, our results have important implications to the field of conservation biology for wildlife management and rehabilitation, particularly for fish species such as salmonids. The differences in nutritional composition found across the diets we investigated here can be seen as analogous to the variability in nutritional or resource availability caused by habitat fragmentation driven by the expansion of human urbanization (e.g., damming of rivers preventing salmon migration and spawning). These challenges to wildlife’s ability to gather necessary resources to survive and reproduce, negatively impact their fitness. Moreover, previous research finds gut microbiomes of wildlife in their natural environments differ from those in captivity. Two proposed reasons for the variation in wild and captive animal microbiomes are the differences in diet and immune system development between their natural and captive environments. Furthermore, these differences are suspected as playing a role in the success or failure of wildlife reintroduction given the microbiomes role in digesting nutrients and supporting the immune system. However, more research is needed to clarify the microbiome’s impact on successful reintroduction of wildlife. Our characterization of zebrafish gut microbiome dynamics across their development provides a useful resource for researchers and wildlife managers seeking to integrate the microbiome in their conservation efforts.

In conclusion, this study represents, to our knowledge, the first assessment to date of common laboratory diets’ long-term impact on the successional development of the zebrafish gut microbiome and its sensitivity to pathogen exposure. In particular, we find diet is one of the most important factors driving variation in the zebrafish gut microbiome. Unlike prior studies, including the extensive research conducted in mammalian models, that have evaluated dietary effects on the gut microbiome using diets that fundamentally differ in macronutrient composition, our study reveals that even relatively consistent diets that are commonly selected as normal husbandry practices elicit these large impacts on microbiome composition. It may be worth establishing a standard reference diet for microbiome-targeted zebrafish studies to improve our understanding of zebrafish health and nutrition, advance knowledge of how the diet and microbiome interact, and support efforts towards reproducibility and interpretability of results across studies. Although, zebrafish diets may benefit from a variety of diets to model the variation in diets and microbiomes we see in human populations. One important challenge to establishing a standard reference diet is its ability to be made germ-free and nutritionally equivalent to conventional diets (Rawls). Significant progress is being made on this front, which supports efforts to better understand the connection between diet and the microbiome in zebrafish (Rawls, Watts). Collectively, our results indicate that researchers should carefully consider the role of diet in zebrafish microbiome studies, and the microbiome should be considered an important factor in wildlife management and rehabilitation efforts.

**Methods**

**Fish Husbandry**

A total of 270 1-month-old AB line zebrafish were randomly divided into eighteen 2.8 L tanks (15 fish/ tank). During the experiment, temperature was recorded daily and ranged from 25.5-28.3°C, with the exception of two isolated overnight temperature drops below that range due to two separate power loss events that affected the source water sump heater. All other water conditions were monitored weekly, pH ranged from 7.0-7.6, total ammonia ranged from 0-0.25 ppm (measured with pH and ammonia API test kits; Mars Fishcare North America Inc. Chalfont, PA), and conductivity ranged from 109 −166 microsiemens. Light in the vivarium was provided for 14 hours/day. One plastic aquatic plant piece approximately 6 inch in length was added to each tank for enrichment when fish were 4-months old. A stock of similarly aged Casper line fish were maintained for the duration of the experiment, with a third of the stock being maintained on each of the diet regimens matching the AB line zebrafish. These fish served as filler fish and were added to the tanks after each histological sampling time point to maintain the 15 fish/tank ratio required to maintain the prescribed diet volumes per feeding.

**Diets**

Fish were all fed the same nursery diet until 1-month old, a combination of paramecia, brine shrimp, and the ZIRC Nursery Mix: Zeigler AP Larval Diet (Ziegler Bros Inc., Gardners, PA) and freeze dried rotifers. Fish were then transferred to the OSU facility and assigned randomly to one of three juvenile diets: Gemma Micro 150/300 (Skretting, Fontaine­les-Vervins, France), Watts High-Fat Juvenile Mix, or ZIRC Juvenile Mix, twice daily (9 AM and 3 PM local time) until 2-months old. From 2-months of age onward, OSU fish were not fed on weekends and 1-day holidays as per the facility institutional animal care and use protocol. The total quantity fed daily was 3% fish body weight. This continued until fish were 4-months old and then they were transitioned to the adult version of their previously assigned juvenile diet: Gemma Micro 500 (Skretting, Fontaine­les-Vervins, France), Watts Low-Fat Adult Mix, or ZIRC Adult Mix, twice daily (9 AM and 3 PM local time), except weekends and 1-day holidays. The total quantity fed daily was 3% fish body weight. The prescribed amounts of each diet regiment, for both the juvenile and adult diets were delivered by 3D printed spoons specific to the diet and stage of life. These spoons were paired with conical tubes retrofitted with leveling wires to ensure consistent feeding volumes as prescribed. All fish were only fed once, in the afternoons, on sampling days.

**Diet and Pathogen Exposure**

Each of the eighteen tanks was assigned one of the three diet regimens: Gemma, Watts, or ZIRC. There were three tank replicates per diet regimens for a total of nine tanks that were exposed to M. chelonae via intraperitoneal injection. The remaining nine tanks were similarly assigned to diet regimens and were exposed to a sterile 1X-phosphate buffered saline (PBS) solution via intraperitoneal injection. Each fish was injected with 10 uL of either the M. chelonae inoculum or saline solution. The injections were completed over the course of two days and the M. chelonae inoculum was prepared as a 0.5 McFarland each day. Day 1 M. chelonae inoculum was afterwards determined by plating to be 3.1x10^3 dose per fish. Day 2 M. chelonae inoculum was determined by plating to be 1.0x10^5 dose per fish.

**Growth Parameters and Sex Determination**

Growth and sex parameters were collected at 3-months of age (101-102 dpf), 4-months of age (129-130 dpf), and 7-months of age (213-214 dpf) for interfacility comparison. Additionally these parameters were also collected at 164-165 dpf which was 5 weeks post exposure that were evaluated in comparison to the 7-months of age (213-214 dpf) measurements which were 15 weeks post exposure for evaluation of disease effects.

Sex was determined by gross differences in morphology and confirmed by histology for all samples collected for disease severity evaluation. Following overnight fecal collection, individual fish would be placed in a pre-anesthetic solution of 50 ppm MS-222 prepared with Tricaine-S (Western Chemical Inc., Ferndale, WA; a subsidiary of Aquatic Life Sciences Inc.) briefly before being transferred to a 150 ppm MS-222 anesthetic solution in a petri dish on centimeter grid paper to be photographed. Standard length and width were evaluated via photographs taken with an iPhone (Apple Inc., Cupertino, CA) and analyzed with ImageJ software (https://imagej.net).

Body condition score (BCS) was calculated using the following equation: BCS = Weight/Length^3

Weight was taken while the fish was still under the effects of anesthesia by transferring them from the photography petri dish to the petri dish on a scale with a volume of tared fish water. Excess water was removed

**Histopathology**

Fish were preserved in Dietrich’s solution, processed, and slides stained with Kinyoun’s acid-fast. Severity was scored by counting total numbers of granulomas containing acid fast bacteria in the coelomic cavity, ovaries, and kidney. Score of 1 was 1–2 granulomas, 2 = multiple granulomas observed, 3 = prominent infections with granulomatous lesions occupying a large amount of the coelom or gonad. In addition, an overall severity of infection score was assigned based on the average scores of the individual structures evaluated (cite previous Kent lab paper).

**Fecal Collection**

Fecal material was collected from individual fish at the same sample intervals as outlined for the growth parameters. Fecal collection was set up the day before growth parameter sampling. Fish were transferred to 1.4 L tanks (1 fish/tank) containing ~0.4 L of fish water at least 30 minutes after the last feeding of the day. Fish were left to defecate overnight and all feces present were collected from each tank the following morning. Fecal samples were immediately snap frozen on dry ice and stored at -80 ˚C until processing.

**16S Sequencing**

Microbial DNA was extracted from zebrafish fecal samples and 16S rRNA gene sequence libraries were produced and analyzed following established approaches (Kundu et al., 2021). Briefly, the DNeasy PowerSoil Pro DNA kits (Qiagen) were used to extract and purify DNA. The V4 region of the 16S rRNA gene was PCR amplified using the Earth Microbiome Project 16S index primers and protocols (Walters et al., 2016). PCR products were visualized on a 1.5% agarose gel and quantified on a Qubit 2.0 (Thermofisher Scientific) using the Qubit dsDNA HS Assay. One hundred ng of each PCR sample was pooled, cleaned using the QIAquick PCR Purification Kit (Qiagen), and quality was verified on the Agilent TapeStation 4200. The prepared library was submitted to the Oregon State University Center for Quantitative Life Sciences (CQLS) for 300 bp paired-end sequencing on an Illumina MiSeq System (RRID:SCR\_016379).

**Analysis**

All microbiome DNA sequence analyses and visualizations were conducted in R (v 4.2.1). Fastq files were processed in using the DADA2 R package (v 1.18.0). Briefly, forward and reverse reads were trimmed at 280 and 230 bp, respectively, subsequently merged into contigs, and subject to amplicon sequence variant (ASV) identification. ASVs unannotated at the Phylum level were removed to result in 292 remaining detected ASVs. We used Wilcoxon Signed-Ranks Tests to identify parameters that best explained the variation in weight and body condition scores. Alpha-diversity was calculated using the estimate\_richness function (Phyloseq v 1.38.0) and transformed using Tukey’s Ladder of Powers. After transformation, scores were normalized from 0 to 1 by dividing each score by the maximum value, which allowed us to compare results across alpha-diversity metrics using general linear models (GLMs). Two-way ANOVA assess these GLMs. Beta-diversity models were generated using methods described previously (Kundu et al., 2021). Briefly, we evaluated three beta-diversity metrics—Bray-Curtis, Canberra, and Sorensonand resolved the relationship between experimental parameters and beta-diversity by applying a step-wise model selection approach as implemented in the capscale function (vegan package v2.5). Optimal models were subsequently subject to PERMANOVA analysis to determine if the selected model parameters significantly explained the variation in microbiome composition across samples. Differential abundance was measured using ANCOM-BC (v ).

**Supplementary Tables and Figures**

**1) Diet**

**1.1) Physiology**

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**1.2) Alpha Diversity**

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**1.3) Beta Diversity**

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**1.4) Beta-Dispersion**

1.4.1) Diet

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| Bray-Curtis | Canberra |
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1.5) Differential Abundance

1.5.1)

1.5.2)

1.5.3)

**2) Development**

**2.1) Physiology**

2.1.1)Table

Description automatically generated

**2.2) Physiology ~ Microbiome**

2.2.1) Table

Description automatically generated

2.2.2) Table

Description automatically generated

**2.3) Alpha Diversity**

2.3.1) **Time**

2.3.1.1) Table

Description automatically generated

2.3.1.2) Table

Description automatically generated

2.2.1.3) Table

Description automatically generated

2.2.2) **Time:Diet**

2.2.2.1) Table

Description automatically generated

2.2.2.2) Table

Description automatically generated

2.2.2.3) Table

Description automatically generated

**2.4) Beta Diversity**

2.4.1)Table

Description automatically generated

2.4.2)Table

Description automatically generated

2.4.3)Table

Description automatically generated

**2.5) Beta-Dispersion**

**2.5.1) Diet**

|  |  |
| --- | --- |
| Bray-Curtis  Chart, box and whisker chart  Description automatically generated | Canberra  Chart, box and whisker chart  Description automatically generated |
| Table  Description automatically generated  Table  Description automatically generated | Table  Description automatically generated  Table  Description automatically generated |

**2.5.2) Time**

|  |  |
| --- | --- |
| Bray-Curtis  Chart, box and whisker chart  Description automatically generated | Canberra  Chart, box and whisker chart  Description automatically generated |
| Table  Description automatically generated  Table  Description automatically generated | Table  Description automatically generated  Table  Description automatically generated |

**2.5.3) Diet:Time**

|  |  |
| --- | --- |
| Bray-Curtis  Chart, box and whisker chart  Description automatically generated | Canberra  Chart, box and whisker chart  Description automatically generated |
|  |  |

**2.6 Differential Abundance**

2.6.1)

2.6.2)

**3) Exposure**

**3.1) Alpha Diversity**

**3.1.1) Exposure**

3.1.1.1) Table

Description automatically generated

3.1.1.2) Table

Description automatically generated

3.1.1.3) Table

Description automatically generated

3.1.2) Diet:Exposure

3.1.2.1) Table

Description automatically generated

3.1.2.2) Table

Description automatically generated

3.1.2.3) Table

Description automatically generated

**3.2) Beta Diversity**

3.2.1) Table

Description automatically generated

3.2.2) Table

Description automatically generated

3.2.3Table

Description automatically generated

**3.3) Beta-Dispersion**

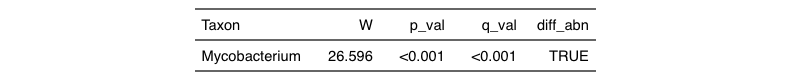
3.3.1) Exposure

|  |  |
| --- | --- |
| Bray-Curtis | Canberra |
|  |  |

3.3.2) Diet:Exposure

|  |  |
| --- | --- |
| Bray-Curtis | |
| Canberra | |
| Bray-Curtis | Canberra |

**3.4) Differential Abundance**

3.4.1)

3.4.2) Table

Description automatically generated

3.4.3) Chart, box and whisker chart

Description automatically generated