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

**Abstract**

Despite zebrafish’s long-established importance as a model organism and their increasing use in microbiome-targeted studies, there is a lack of consensus regarding husbandry practices involving type of diet. Diet is known to influence the structure of the gut microbiome and physiology of zebrafish. Given, the microbiome’s important role in maintaining host health through digestion of nutrients and fighting pathogens, diet-associated differences in the microbiome could impact host health and study outcomes. However, key knowledge gaps remain about whether commonly used laboratory diets influence zebrafish microbiomes, and if these diets differentially affect the gut microbiome’s successional development and sensitivity to pathogen exposure. Here we show that diet drives gut microbiome succession and sensitivities to pathogen exposure. We found that at 3 months fish gut microbiomes stratified by diet, and these effects accumulated across development that resulted in diet-dependent differences in the microbiome and physiology at 6 months of age. Furthermore, we found that sensitivity to pathogen exposure depended on diet. Our results demonstrate that variation in husbandry practices around diet impacts the composition of the gut microbiome. Collectively, our results indicate that researchers should carefully consider the role of diet in their zebrafish microbiome studies and that diet should be controlled for when integrating microbiome data across studies.

**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. Prior research has found husbandry choices involving diet can induce variation in study outcomes and challenge efforts to compare results across studies (Watts). Moreover, experimental, commercial and laboratory diets result in different microbiome and health outcomes (Fowler, Leigh, Rawls, Others?). However, what is not known is if 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, but they continue to grow in weight and length. Their immune systems have finished developing, they are sexually mature, and reach 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 has been linked to an array of health outcomes involving obesity, X, Y and Z across an array of organisms, including zebrafish (citations). This is important because microbiomes are relatively stable once established. Therefore, early life assembly of the gut microbiome could have long-term implications on host health and their ability to resist infection (citation).

Pathogen exposure is known to impact the gut microbiome of zebrafish, and the microbiome could mediate these effects, either protecting, exacerbating, or having a neutral influence. 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 introduce through diet early in life (Stephens, Kent). *M. chelonae* causes gut inflammation in zebrafish (Kent). 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. 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.

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

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| **Figure 1:** 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)** Shannon 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. | |

<Results>

To investigate how the zebrafish gut microbiome diversity, composition, and relative abundance may be influenced by diet, 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 in fecal samples. 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 a general indicator of health in zebrafish. Here, we are assessing how commonly used laboratory zebrafish diets may impact the zebrafish physiology and gut microbiome structure.

Briefly, to determine if physiology differed between diets, we used Wilcoxon tests to identify parameters that best explained the variation in weight and body condition score. We find that diet and sex significantly associated with weight and body condition. Female fish had higher weight and body condition scores compared to males. ZIRC had the highest mean body condition score compared to Gemma and Watts. Gemma and Watts fed fish weight and body condition scores did not significantly differ. We did not observe a significant interaction between diet and sex on weight and body condition score.   
  
Next, we asked if diet associated with gut microbiome structure. To assess microbiome structure, we used Wilcoxon tests to identify if diet associated with variation in Simpson’s and Shannon Indices of diversity. We find that alpha diversity associated with diet. ZIRC fed fish had highest alpha diversity, followed by Gemma and Watts diets. Both indices found that ZIRC and Watts fish diversities differed significantly, but only in Simpson’s did ZIRC and Gemma differ significantly. Shannon diversity index was weakly significant. Watts and Gemma differed significantly in Shannon, but not Simpson’s indices.

Moreover, we used the Bray-Curtis and Canberra dissimilarity metrics to compare pairs of microbial community composition. A PERMANOVA test using both metrics revealed that gut microbial communities between the diets were significantly different in their composition. Beta-dispersion levels for Bray-Curtis metric differed significantly between the diets, with Watts fed fish displaying the most pairwise variation compared to ZIRC and Gemma. Using Canberra measure, beta-dispersion was weakly significant. Beta-dispersion between ZIRC and Gemma did not significantly differ in either measure.

Finally, to better understand the interactions between the diets and the gut microbiome, we quantified how relative abundance of taxa varied using ANCOM-BC. We found 24 families were significantly differentially abundant in at least one group across the three diets. Among fish fed Gemma diet, the families of Chitinibacteraceae were significantly abundant relative to Watts and ZIRC diets, and Aeromonadaceae were decreased. In Watts diet, the families of Xanthomonadaceae, Cellvibrionaceae, Microscillaceae, Erysipelotrichaceae, and Flabovacteriaceae were significantly abundant. In the ZIRC fed fish, families of Pseudomonadaceae and Weeksellaceae were significantly abundant.

<Discussion>

Here, we compared microbiomes of fish fed commonly used laboratory diets, which have more consistent nutritional profiles to those in previous studies interrogating the physiology, microbiome and diet. We found that diet differentially influences physiology and the gut microbiome of 3 month old zebrafish. Fish fed ZIRC diet are heavier and have higher body condition scores compared to the Watts and the Gemma diets. These results align with previous research investigating the effects of diet on zebrafish physiology (Watts, Fowler). Previous studies have found that different laboratory, commercial and experimental diets manifest inconsistent gut physiology, growth, health and reproductive outcomes (Leigh 2018, Fowler 2019). Leigh et al. found that in addition to nutritional composition, digestive enzyme activity played a role in shaping the physiological structure of the gut and could impact the gut microbiome of fish. We found that gut microbiome diversity differed by diet, and fish microbial communities were more similar to fish fed similar diets. López Nadal describe how zebrafish microbiomes are influenced by diet. However, in a recent study by Karlsen et al. has drawn attention to a “feed microbiome” effect potentially impacting fish microbiome studies (Karlsen). We cannot rule out the possibility that variance in gut microbial diversity seen here in fish fed different diets could be an artifact of microbial DNA present in their digesta collected during sampling, and may not necessarily be representative of the gut mucosa-associated microbes (Karlsen 2022). Therefore, future zebrafish microbiome-targeted research should include gut intestinal samples and feed samples alongside fecal samples to account for a potential feed microbiome effect. It’s important to note that while each of these diets have slightly different nutrient profiles to each other, they are far more consistent in composition to one another than the diets used in the previously mentioned analyses conducted around physiology, diet and the microbiome. Where previous studies tested more extreme ranges (e.g., high-fat diets), our study differs in that the three diets used are more consistent to one another. Together, we our results demonstrate that the gut microbiomes of 3-month-old zebrafish differ by diet, and highlights the importance of minor nutritional differences ability to affect the microbiome and physiology of zebrafish.

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

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| **Figure 2:** 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 diet 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 microbial 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, 0.01, and 0.001 levels, respectively. | | |

<Results>

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. Briefly, we measured gut microbiome diversity, composition and abundance, as well as body condition score at 6 months of age and compared these measures to the 3-month-old fish.

We find gut microbiome diversity increases over time from 3 to 6 mpf, but diet has a stronger statistical effect. A Wilcoxon test found a significant interaction effect between time and diet of ZIRC and Gemma fed fish in the Simpson’s Index, but Watts fed fish diversity did not differ between these time points. Furthermore, we find the microbial 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 Bray-Curtis dissimilarity metric revealed that community composition was best explained by diet, but Canberra measure found variation was best explained by time. Within each diet, beta-dispersion increased over time in Gemma and ZIRC diets, while Watts remained consistent between 3 and 6 months. We observed dispersion effects in Gemma and ZIRC. Beta-dispersion was significantly in abundant and rare taxa of ZIRC fed fish, while Gemma had significant beta-dispersion among abundant taxa.

To see which taxa may be contributing to the variation we see in gut microbial diversity, we ran ANCOM-BC to measure relative abundance. We found 3 and 23 taxa that were significantly abundant at the phylum and genus levels in at least one diet between 3 and 6 months. All three diets saw an increase in the phylum Actinobacteriota, Gemma and ZIRC fed fish saw increases in Bacteroidota, and ZIRC fed fish saw decreases in Protoebacteria. At the genus level, each diet saw increases in unnamed genera in Barnesiellaceae; Gemma and ZIRC saw increases in Macellibacteroides, Aurantisolimonas, Mycobacterium, unnamed genera in Rhizobiales and Sutterellaceae, and decreases in Flavobacterium; Watts and ZIRC saw increase in Fluviicola, and decrease in Shewanella; and Gemma and ZIRC saw increase in Glutamibacter, and decrease in Flavobacterium. Individually, Gemma saw an increases in Gordonia, Coxiella and an unnamed genus in Chitinibacteraceae, and a decrease in Chintinibacter; Watts saw an increase in Cloabacterium, and decrease in an unnamed genus in Comamonadaceae; and ZIRC saw decreases Exiguobacterium, Phreatobacter, Cetobacterium, Aeromonas, Plesiomonas and Acinetobacter. Together, we see that diet differentially impacts the abundance of certain phyla and genera over the course of development.

While body condition score did not significantly differ between time points across all diets, we observed a joint interaction uniquely in ZIRC fed fish between gut microbiome diversity on body conditions core. In ZIRC fed fish, body condition score negatively associates with an increase in microbiome diversity across development. Moreover, a PERMANOVA test showed a body condition score interaction with diet in Canberra index, but not Bray-Curtis. Body condition score did not explain variation in abundant taxa.

<Discussion>

Prior work has shown that zebrafish gut microbiome succession is increasingly governed by host development stages and stochastic processes (e.g., drift and dispersal from environment) as fish age. Additionally, prior research has shown that high-fat and high-protein diets impact gut microbiome structure, little is known how different commonly used zebrafish diets influence the successional development throughout adulthood. Here, we measured gut microbiome diversity, composition, and abundance at 3 and 6 months of age of fish fed one of three diets, and measured body condition score to assess impact to physiology.

We found the gut microbiome diversity increases and composition varies over time. Notably, in ZIRC and Gemma fed fish rare taxa appear more temporarily sensitive, and abundant taxa are sensitive to diet. Watts fed fish experienced less variation over time. At four months of age, all fish were switched from juvenile to adult diets. ZIRC adult feed incorporates several new feeds and minor changes to nutritional composition. Gemma adult feed changes in size but not nutrition. Watts feed decreases in lipid content but the ingredients remain the same. Changes in nutritional composition can alter the microbiome (Rawls). Xiao and Stephens observed similar increases in gut microbial diversity that they note may be due to dietary changes. Moreover, previously identified core, keystone taxa had diet dependent changes in abundance over time. Keystone taxa are believed to play important roles in maintaining gut microbiome homeostasis by digesting nutrients, fighting pathogens and communicating with the immune system. Thus, if certain diets are disproportionately enriching for or against keystone taxa early in zebrafish development it could have long-term implications on microbiome succession, and possibly physiological outcomes. Indeed, we observed a link between physiology and microbial diversity, where smaller fish fed the ZIRC diet had higher gut diversity, and vice versa. One explanation is that higher diversity could drive more competition for resources and habitat space, which may prevent taxa from gaining a foothold and efficiently metabolizing nutrients. Additionally, we can't exclude the possibility that sex plays a role in defining these differences in body condition score and microbiome structure (Ma 2018). Taken together, these results demonstrate that diet’s influence on early-life gut microbiome assembly can accumulate overtime to impact gut microbiome succession over the course of development, which may manifest differential physiological outcomes. <Insert a good take home message>

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

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| **Figure 3:** 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 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 zebrafish gut microbiome. Briefly, after collecting fecal samples at 3 months old, we interperitoneally exposed a cohort of zebrafish within each diet to *Mycobacterium chelonae*. Then at 6 months old, we measured gut microbiome diversity, composition, and abundance, as well as body condition score.

We find that the gut microbiome’s sensitivity to pathogen exposure is linked to diet.

Variation in gut microbiome diversity and composition was not significantly explained by presence of infection, nor was the interaction significant between diet and infection. We observed a diet-dependent effect of pathogen exposure in ZIRC fed fish, but not Gemma or Watts. Exposed ZIRC fed fish decrease in diversity, while unexposed fish increase in diversity. Unexposed Gemma fed fish increased in diversity compared to pre-exposed fish, but the results did not meet our significance threshold. Community composition differed by exposure group, but the effect is less significant than diet. Mycobacterium was present in all diets at 3 months prior to exposure, and increased between time points. Variance of Mycobacterium was best explained by diet. Watts fed fish had higher levels of Mycobacterium in exposed group compared to the unexposed controls, and Gemma and ZIRC exposed groups having lower levels of Mycobacterium compared to unexposed controls. We did not see a pathogen exposure effect on physiology. We also do not find a diet by exposure interaction with body condition score. However, a pairwise Wilcoxon test found that the mean body condition scores between exposure groups in Watts fed fish differed, but it did not reach our threshold for significance.

<Discussion>

We find that pathogen exposure inhibited diversification of gut microbiomes, and microbial community composition was driven primarily by diet rather than pathogen exposure. ZIRC fed fish are uniquely sensitive to pathogen exposure, Gemma and Watts diet were more resistant. Interestingly, Mycobacterium’s abundance differed between the diets. In exposed Gemma and ZIRC fed fish, Mycobacterium’s abundance was less than controls, but in Watts fed fish Mycobacterium was more abundant in exposed fish compared to controls. Higher gut microbial 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 to take advantage of lower stability to gain habitat space in Watts fed fish. However, the effects of pathogen exposure were secondary to diet, and might explain why our results differ from previous microbiome-pathogen studies that saw increased microbial community variation following pathogen exposure (Gaulke, others?). Here, we see the oppositive effect of microbiome communities becoming more similar after exposure to Mycobacterium. Three important limitations to this study are 1) fish were injected with mycobacterium, which is not the natural route of transmission; 2) prior to injection all fish had Mycobacterium species present; and 3) we do not know the strain abundance of the Mycobacterium present in our samples. Thus, the presence of pre-existing Mycobacterium species could have hindered our treatment of *M. chelonae’s* ability to successfully colonize exposed fish. Future research should attempt to expose zebrafish free of Mycobacterium using a natural route of transmission. Taken together, these data suggest that the microbiome might contribute to *M. chelonae* success depending on diet. It is unclear whether the microbiome exacerbates or protects zebrafish from pathogen exposure, but it illuminates the need for researchers to consider diet as a confounding factor that could alter the outcomes and interpretations of their study outcomes.

**Conclusion**

This study represents, to our knowledge, the first assessment to date of common laboratory diets long-term impact on host-pathogen-microbiome dynamics. We find that at 3 months old, fish fed different diets experience a difference in physiology and gut microbiome structure. These diet-associated differences accumulate through development at 6 months of age. We also find diet-dependent sensitivities of the gut microbiome to pathogen exposure. Together, these results demonstrate that diet and host health are intertwined with their microbiome’s development and sensitivity to pathogen exposure.

It may be worth establishing a standard reference diet for microbiome-targed 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. However, we do not suggest that one diet here is preferred for microbiome-targeted studies. Rather, 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 and 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 their zebrafish microbiome studies and that diet should be controlled for when integrating microbiome data across studies.