**Diet Infection Study – Introduction, Results**

**Notes**

**Introduction:**

* Copy and pasted from MB 668 report
  + I have not added information about time or pathogen exposure

**Results/Discussion**:

* Section 1 is the most developed compared to the last two sections.
* I’m not super happy with the differential abundance analyses, or how I’ve thrown them in here.
  + I’ve been experimenting with ANCOM-BC, and want to compare the results of each. From what I’ve read, ancom results are more consistent and reliable compared to other DA methods
* Need to include beta-dispersion results for each section
* I need to add nitty-gritty statistical info for each result: F statistic, pvalue, etc.
* Figures need to be regenerated for article format
  + Sizing, significance bars/asterisks
* Too many potential figures, so will likely need to include several supplementary figures

**Introduction**

Zebrafish are an increasingly popular model organism in microbiome studies for a number of reasons, but notably they have a fully sequenced genome, their early embryonic development is fast and similar to humans, their husbandry is economic and their small size allows for generating large sample sizes, as well as the many well established methods across a number of disciplines. In particular for microbiome studies, the methods for deriving germ-free fish are relatively easy compared to other model systems, such as mice[(1)](https://www.zotero.org/google-docs/?4e2bCh). Together, these reasons make zebrafish an ideal organism to study.

Despite zebrafish’s increasing popularity and long history in research science, there is not a well established consensus on which diet to administer during experiments[(2–4)](https://www.zotero.org/google-docs/?x0OlqD). This is in stark contrast to other study systems which have long established protocols for nutritional requirements[(5)](https://www.zotero.org/google-docs/?2khwcm). This lack of consensus has led labs to use a variety of diets that can have varying compositions of macro- and micronutrients. Common zebrafish diets can vary in composition as much as 31% to 60% protein, 5% to 34% lipids, as well as varying amounts of minerals, vitamins and sources for ingredients[(6)](https://www.zotero.org/google-docs/?pao6rt). Additionally, optimal zebrafish nutritional requirements have yet to be determined[(2)](https://www.zotero.org/google-docs/?F5Lesn). While zebrafish's ability to live under diverse conditions, environments and diets is seen as a testament to their strengths as a model organism, this diversity in methodology is a weakness for reporting, reproducibility and translatability across studies[(3, 7, 8)](https://www.zotero.org/google-docs/?qehsap). Additionally, there have been few linking diet and the microbiome and none to our knowledge investigating how common laboratory zebrafish diets influence their microbiomes[(1, 9)](https://www.zotero.org/google-docs/?TkJhRy).

Diets can have a differential impact on zebrafish physiology[(2, 6)](https://www.zotero.org/google-docs/?tE3o4h). One study found significant differences in length, width and weight of zebrafish depending on the diet they were fed[(6)](https://www.zotero.org/google-docs/?ykPQmS). There have been some studies investigating the health outcomes of zebrafish fed certain diets, finding that diet and husbandry could influence tumor formulation[(10)](https://www.zotero.org/google-docs/?M2XK8a). The use of live feed (e.g. *Rotifer* and A*artemia*) has also been shown to indirectly impact zebrafish health as being a common source of pathogens[(11)](https://www.zotero.org/google-docs/?XX4mUH). The lack of dietary standards in zebrafish are concerning in their own right but are further compounded by our understanding of the microbiome’s influence on host health.

Diet is known to impact the gut microbiome composition of its host[(12)](https://www.zotero.org/google-docs/?Sa33DB). Consumption of high protein and high fat diets, like that of industrialized Western diets, is associated with disease (e.g. obesity and heart disease) and selects for certain compositions of microbiota[(13, 14)](https://www.zotero.org/google-docs/?jPyyKL). Whereas diets with plant based proteins and fats are associated with better health outcomes and a more diverse assortment of gut microbiota[(15, 16)](https://www.zotero.org/google-docs/?8UqP56). In addition to metabolism, the gut microbiome provides immune system support to the host, for example by fighting pathogens and inducing immune system responses[(17)](https://www.zotero.org/google-docs/?VVQyVl). It is clear that the immune system, the gut microbiome and diet are intimately linked to host health.

Here, we assessed the gut microbiome of four month old adult zebrafish fed one of three common laboratory diets (Gemma, Watts and ZIRC) in order to clarify how the gut microbiome varies in accordance with diet. At four months of age, we collected fecal samples and extracted their DNA for 16S rRNA sequencing. We found that microbiome diversity varied depending on diet. Variation was not uniform across diets. Each diet differed in the amount of inter- and intra-variation of microbial community compositions. Additionally, we collected physiological data and found that diet was associated with weight, but it did not associate with body condition. Sex of fish was a better predictor of weight and body condition score than diet. Together, our results clarify how diet influences the microbiome of zebrafish. While diet may not play a significant role in health outcomes of zebrafish, it did impact the composition of their gut microbiomes. This has important implications for zebrafish studies targeting the microbiome in that choice of diet will influence microbial composition of zebrafish guts and may challenge reproducibility of zebrafish studies.

**Results and Discussion**

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

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 and body condition, a general measure of physiology. In contrast to mice, zebrafish do not have a standard reference diet. Differences in husbandry choices involving diet induce variation in study outcomes and challenge efforts to compare results across studies. Zebrafish fed different commercial and laboratory diets resulted in different body condition outcomes. Moreover, fish fed a high versus low-fat diet manifested distinct gut microbiome communities. However, what is not known is if zebrafish gut microbiome communities differ between commonly used laboratory diets. Here, we assessed whether different common laboratory diets influenced zebrafish’s gut microbiomes and physiology.

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| A) | B) |
| C) | D) |
| Figure 1: Effects of diet on body condition score, gut microbiome diversity and community composition. A) Body condition score by sex; B) Body condition score by diet; C) gut microbiome diversity; and D) Capscale ordination based on the Bray-Curtis dissimilarity of gut microbiome composition. The analysis shows that Weight, body condition score, gut microbiome diversity and community composition significantly differs as a function of diet. | |

To assess the impact of diet on zebrafish physiology and gut microbiome, we fed zebrafish three different commonly used laboratory diets and compared their body condition scores, gut microbiome diversity and community compositions at 6 months post fertilization (mpf). Body condition score is a length normalized measure of weight and general measure of physiological health (citation). Female fish had higher body condition scores than males (statistical results; Figure #). ZIRC fed fish had the highest body condition scores compared to Gemma and Watts (statistical results; Figure #). The main effect of sex had a greater statistical effect on weight and body condition score than diet, but the interaction of sex and diet was not significant for either (statistical results; Fig S#). ZIRC fed fish had higher gut microbiome diversity at 6mpf, followed by Gemma and Watts fed fish, respectively (statistical results; Figure #). Moreover, a Bray-Curtis dissimilarity metric-based PERMANOVA analysis reveals that the interindividual variation in the composition of the gut microbiome associated with diet (statistical results; Figure #). <beta dispersion results>

We find diet differentially influences physiology and the gut microbiome. Fish fed ZIRC diet are heavier and have higher body condition scores compared to the Watts and the Gemma diets. Previous work found weight and condition score associated with diet 1–3. In contrast to our results, Fowler et al. found fish fed Gemma diet weighed more and had higher body condition scores compared to Zeigler (ZIRC) and Z12 (Watts) diets. These discrepancies between our studies can be explained by differences in body condition score calculations. We calculated BCS by dividing weight by length times width squared (see Methods), whereas Fowler divided weight by body length cubed. Also, we measured fish at 6 months old compared to 3 months old in Fowler’s study. In agreement with our study, Fowler found female fish were heavier than males. Together, our results show that diet manifests different weight and body condition scores at 6 months of age.

Next, we asked if diet associated with gut microbiome structure. Our results showed diet linked to higher microbiome diversity in ZIRC fed fish, and each diet stratified into distinct microbial community compositions. These results align with previous research in zebrafish, mice and humans 4–6. In these studies, nutrient profile (e.g. high-fat, high-protein) as well as source of ingredients (e.g. animal- or plant-based) were shown to influence host gut microbiome structure. In zebrafish, Wong *et al.* found that high dietary fat levels were linked to distinct gut microbiome compositions 7. Unlike prior work, our study compared microbiomes of fish fed commonly used laboratory diets, which have more consistent nutritional profiles to those in previous studies interrogating the microbiome and diet2,3,7,8. Thus, the strong associations we see between diet and the microbiome demonstrates that husbandry practices involving diet play a role in structuring the gut microbiome. Moreover, researchers using zebrafish as a model system should consider diet as a factor in their studies. We cannot, however, rule out possible confounding factor of a “feed microbiome” effect may play on shaping individuals microbiomes 9.

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

Zebrafish are developmentally considered adults by 3 months of age, but they continue to grow in weight and length (citation). Additionally, zebrafish microbiomes continue to develop as they age (citation). Prior to adulthood, zebrafish microbiome assembly is more susceptible to environmental influences of drift and dispersal, but with age these effects decline(citation). During adulthood, zebrafish microbiomes continue to diversify, but their community compositions stabilize (citation). To better understand the role of diet on the successional development of zebrafish, we compared body condition scores and gut microbiomes of 3 and 6 month old zebrafish.

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| A) | B)  Chart, box and whisker chart  Description automatically generated |
| C) | D)  Diagram  Description automatically generated |
| Figure 2:  A) Microbiome diversity increases from 3 to 6 mpf  B) ZIRC fed fish display a unique increase in gut microbiome diversity from 3 to 6 mpf  C) Bray-Curtis Capscale ordination of gut microbiome composition  D) Canberra Capscale ordination of gut microbiome composition | |

We find that development plays a role in gut microbiome diversity and composition. Regardless of diet, gut microbiome diversity increases with time. In particular, ZIRC fed fish displayed increased diversity at 3 months of age compared to the other diets, as well as an increase in diversity between 3 months and 6 months (statistical results; Figure #, Figure S). Watts fed fish diversity did not differ between time points (statistical results; Figure #, Figure S). The microbial community composition varies over time, but the temporal sensitivity of the abundant taxa in the microbiome is less than the sensitivity of these taxa to different in diet (statistical results; Figure #, Figure S). Rare microbiota, however, appear to vary more as a function of time than diet (statistical results; Figure #, Figure S). These patterns occur regardless of the specific diet being considered (statistical results; Figure #, Figure S#). <beta dispersion results>

31 genera significantly associated with diet in final control fish (statistical results; Figure #, Figure S#). In Gemma, Aeromonas and Cloacibacterium were significantly less abundant, while Plesiomonas, Cetobacterium and Chitinibacter were significantly more abundant compared to other diets. In Watts diet, Crenobacter and Shewanella were significantly less abundant, while Vibrio and ZOR0006 were significantly more abundant compared to other diets. In ZIRC, Acinetobacter was significantly more abundant compared to other diets.

Discussion:

* Main Observation:
  + We found the temporal stability of zebrafish gut microbiomes diversity differed by diet, and community compositions of rarer taxa to be more sensitive to the effects of time.
    - Stability, as in a lack of change in diversity/composition between time points
* Past studies support/refute observations:
  + Xiao 2021: Host development overwhelms environmental dispersal in governing the ecological succession of zebrafish gut microbiota
    - Found that certain keystone taxa, even those with low abundances, played important roles in affecting the microbial interactions and stability.
      * Do we see similar observations?
  + Xiao 2022: Stability of gut microbiota in zebrafish increase with host development
    - Diversity, interactions and stability increased with time
    - Caveat/Limitation:
      * These studies looked at <100 day old fish.
      * Changes could be caused by multitude of changes in diet from juvenile to maturity (paramecium at 5-8 dpf, boiled egg at 9-11 dpf, live brine shrimp at 12-19 dpf, and standard dry fish food >=20dpf). Stability of diet could be confounding stability of microbiome.
  + Stephens 2016: gut microbiome stability increases to a point, but decreases with senescence 10.
    - Stephens saw an overall decrease in diversity over time, while we saw an increase. They had more timepoints from 4 dpf to 380 dpf, and included parental samples. Comparing 75 to 380, they see an increase in diversity as we do we between 129 and 214. However, they note the decrease between 35 and 75 could be due to full development of zebrafish’s adaptive immune system as well as change to dry food.
* Novelty of present study’s methods/observations:
  + Fish in our study were fed the same diet from 0 to 1 mpf, and separate but consistent diets from >=1 mpf onward. Thus, eliminating the possibility of a diet change effect on the fish successional development after 1 mpf.
* Explanations for present studies observations compared to past studies
  + Because we fed each cohort a consistent diet from 1 mpf onward, we can be more confident that the differences in diversification across time is driven by type of diet.
* Caveats or limitations
  + Different diets, and sampling time points between our studies.
  + Ingredients in the diets could be influencing differences, but ingredients of diets is not known for commercial diets.
* Avenues for future research
  + Consistent diets used in microbiome studies
  + Sampling time point consensus
  + Transparency in source or ingredients of diets
  + More time points past 6 mpf to assess stability
* Major implications
  + Diet plays a role in the consistency of the gut microbiome across development.
    - Studies investigating the temporal effects of the microbiome may be confounded by inconsistencies in microbiome structure, such as those found in the ZIRC fed fish.

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| A) | B) |
| C) | D) |
| Figure 3: | |

ZIRC diet fed fish experienced a weakly significant decrease in body condition score over time (statistical results; Figure #, Figure S#), whereas Gemma and Watts diet fed fish do not (statistical results; Figure #, Figure S#). We analyzed the joint interaction effect between body condition score and diet on gut microbiome structure. Gut microbiome diversity uniquely increased as body condition score decreased in ZIRC fed fish, while microbiome diversity in Gemma and Watts diets remained stable across time (statistical results; Figure #, Figure S#). These observations suggest that there may be a physiological connection between ZIRC fed fish and the gut microbiome. Moreover, the composition of the gut microbiome of ZIRC fed fish were not distinct at 3 mpf, but at 6 mpf the gut microbiomes stratified by high and low body condition score (statistical results; Figure #, Figure S#). This pattern was not seen in Gemma and Watts fed fish (statistical results; Figure #, Figure S#). These observations suggest that over time, ZIRC fed fish uniquely select for distinct compositions of gut microbiome communities that correlate with body condition score.

33 genera were found to have a statistically significant relationship between body condition score in ZIRC fed fish. In particular, the genera Comamonadaceae, Bacteroides, Bosea and Paucibacter abundance decreased with increased body condition score, while Cetobacterium abundance increased with increased body condition score. Only Bacteroides was found to have an association with body condition score independent of diet. This observation suggests that the ZIRC diet is uniquely selecting for these taxa.

Time alone did not explain differences in body condition score, but ZIRC fed fish experienced an increase on body condition score (statistical results; Figure #, Figure S#). Interestingly, increased body condition score in ZIRC fed fish had lower gut microbiome diversity. Furthermore, at 3 months ZIRC fed fish microbiome’s did not differ compositionally, but at 6 months they displayed distinct communities that stratified by high and low body condition scores (statistical results; Figure #, Figure S#). Together, these results build upon our previous results, and demonstrate that diet plays a continued role across the development of zebrafish physiology and gut microbiome.

Discussion:

* Main Observation:
  + In ZIRC fed fish, there appears to be a physiological link to the microbiome structure
* Past studies support/refute observations:
  + a
* Novelty of present study’s methods/observations:
  + a
* Explanations for present studies observations compared to past studies
  + a
* Caveats or limitations
  + a
* Avenues for future research
  + A
* Implications
  + a

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

Zebrafish facilities are known to host many pathogens which can introduce non-protocol induced inconsistencies in study outcomes. One pathogen that is found in 40% of zebrafish facilities is *Mycobacterium chelonae*, and is hypothesized to be introduce through diet. *M. chelonae* causes gut inflammation in zebrafish. Previous work of ours has shown that pathogen exposure disrupted the gut microbiomes of zebrafish, 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 exposed zebrafish fed different diets to *M. chelonae* to and measured the effects on zebrafish physiology and microbiome across their development at 3 and 6 months.

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| A) | B) |
| Figure 4 | |

The gut microbiome diversity of exposed fish at 6 mpf were lower compared to unexposed fish at 6 mpf (statistical results; Figure #, Figure S#). Microbiome diversity of 6 mpf pre-exposed and 6 mpf exposed fish did not differ (statistical results; Figure #, Figure S#). When comparing between diets, the gut microbiome diversity of ZIRC fed fish are uniquely sensitive to pathogen exposure, while Gemma and Watts fed fish are resistant to the effects of pathogen exposure (statistical results; Figure #, Figure S#). 6 mpf unexposed ZIRC fed fish had significantly greater microbiome diversity compared to 3 mpf pre-exposed ZIRC fed fish, while 6 mpf exposed fish were significantly less diverse. Microbiome diversity of fish fed Gemma and Watts diets were not different between exposure groups. Together, these observations suggest that exposure to pathogens inhibits diversification of the gut microbiome, and fish fed the ZIRC diet are uniquely sensitive to pathogen exposure. Furthermore, pathogen exposure may be preventing diversification of gut microbiomes.

|  |  |
| --- | --- |
| A) | |
| B) | C) |
| Figure 5 | |

The gut microbiome community composition stratified by exposure group, regardless of diet (statistical results; Figure #, Figure S#). Dispersion did not differ between exposure groups at 6 mpf, but do differ when compared to 3 mpf pre-exposed fish (statistical results; Figure #). The effect of exposure group is secondary to diet (statistical results; Figure #). Suggesting that community composition is sensitive to pathogen exposure, but the primary driver of composition is diet. Dispersion differed between fish at 3 mpf and 6 mpf, but these differences were not significantly different between exposure groups (statistical results; Figure #). Together, these observations suggest that diet masks/overwhelms the effects of pathogen exposure on gut microbiome community composition.

Discussion:

* Main Observation:
  + a
* Past studies support/refute observations:
  + a
* Novelty of present study’s methods/observations:
  + a
* Explanations for present studies observations compared to past studies
  + a
* Caveats or limitations
  + a
* Avenues for future research
  + A

54 genera are differentially abundant across exposure groups at 6 mpf. In unexposed fish, Bacteroides and Vibrio were significantly more abundant, while Plesiomonas, Fluviicola, Flavobacterium and Shewanella were significantly less abundant in unexposed fish compared to pre-exposure and exposed fish. In exposed fish, Paucibacter, Cerasicoccus and Gemmobacter were significantly less abundant. These observations suggest that exposure has differential impacts on genera abundance.

Discussion:

* Main Observation:
  + a
* Past studies support/refute observations:
  + a
* Novelty of present study’s methods/observations:
  + a
* Explanations for present studies observations compared to past studies
  + a
* Caveats or limitations
  + a
* Avenues for future research
  + A

We found that the impact of *M. chelonae* exposure on the gut microbiome depended on diet. The gut microbiome diversity of ZIRC fed fish are uniquely sensitive to pathogen exposure, while Gemma and Watts fed fish are resistant to the effects of pathogen exposure. Post-exposure ZIRC fed fish had lower microbiome diversity, whereas microbiome diversity of fish fed Gemma and Watts diets were not different between exposure groups. However, the microbial community composition’s sensitivity to pathogen exposure is overwhelmed by the effects of diet. Microbiome compositions stratified into unique communities based on exposure type, these effects were dwarfed by the effects of diet. These results show that while pathogen exposure can impact the diversification of the gut microbiome across their development, diet plays a more influential role in the gut microbiome’s overall composition. Taken together, these results demonstrate that choice in husbandry practices involving diet can potentially mask or exacerbate the effects of pathogen exposure if not properly taken into consideration.

<Insert implication discussion here>

* Negative or positive health outcomes in other studies may be a function of diet, rather than treatment being tested.
* Choice in diet could minimize negative impacts of pathogen exposure

Discussion:

* Main Observation:
  + a
* Past studies support/refute observations:
  + a
* Novelty of present study’s methods/observations:
  + a
* Explanations for present studies observations compared to past studies
  + a
* Caveats or limitations
  + a
* Avenues for future research
  + A