

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

Michael Sieler, Colleen 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

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(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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## Results

### 1. Experimental design and data collection

Treatments	Pathogen Exposure	Pre-exposure		Unexposed	
		Exposed	Unexposed	Exposed	Unexposed
Diets	Nursery diet	Juvenile diet	Adult diet (Gemma)	Adult diet (Watts)	Adult diet (ZIRC)
		Juvenile diet	Adult diet	(Watts)	(ZIRC)
Fish age (days)	0	30	129		214
# Fish sampled			89		87
Fecal sampling		X		X	
<i>M. Chelonae</i> injection		X			
Histopathology check				X	

**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).

In this study, our main goals were to reveal the influence different commonly used laboratory diets have on the zebrafish physiology, the successional development of zebrafish gut microbiome and its sensitivity to pathogen exposure. To that end, we reared 176 zebrafish that were assigned one of three diets from 30 to 214 days old, and at 129 days old exposed a cohort of fish to *Mycobacterium chelonae* (Figure 1). The diets include a commercial diet (Gemma), a defined laboratory diet (Watts), and a mixture of commercial diets (ZIRC). From 30 to 129 days old, fish were fed the juvenile versions of their respective diets. At 129 days old, they were switched to adult formulations of their respective diets. Fish were housed 15 fish per tank according to their assigned diet (e.g. Gemma, Watts and ZIRC) and exposure group (e.g. exposed and unexposed). We collected 89 fecal samples at 129 days, and then injected half the fish (e.g. exposed) to *Mycobacterium chelonae* of fish from each diet while the other half remained unexposed. Approximately 3 months later when fish were 214 days old, we collected 87 fecal samples from each diet and exposure group, and performed a histopathology check to assess infection burden of exposed fish. Our questions focused on the effect of diet on physiology, microbial gut diversity, community composition and bacterial abundance, as well as the gut microbiome's sensitivity to pathogen exposure.

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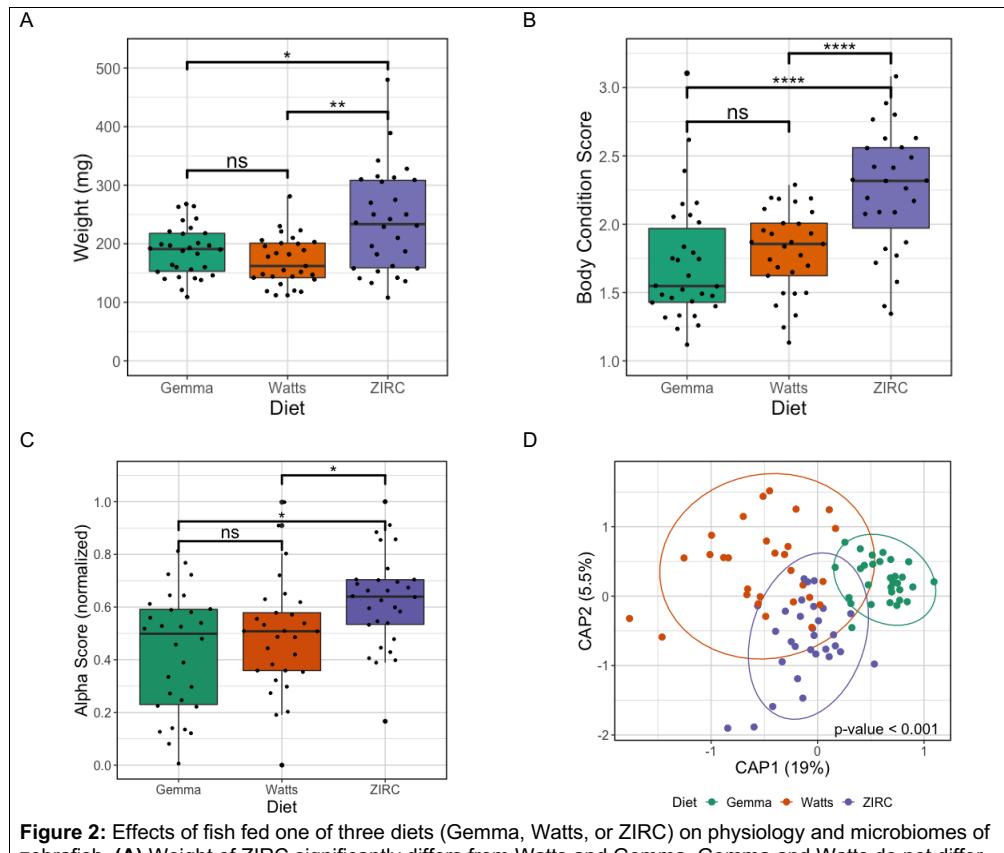
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## 2. Diet differentially influences physiology and gut microbiome



**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.

Zebrafish physiology and gut microbiome has been previously associated with diet, but these studies considered diets that differed greatly in nutritional content (e.g., high fat and protein vs. low fat and protein; citations). Moreover, our study is unique in that fish were fed the same diets from 30 to 214 days old. Here, we investigate how commonly used laboratory diets with relatively similar nutritional composition may impact the zebrafish physiology and gut microbiome diversity, composition, and relative abundance. These diets will be referred to as Gemma, Watts and ZIRC from hereafter.

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We first determined if physiology, represented here by weight and body condition score, differed between the three 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). Fish fed the ZIRC diet had the highest mean body condition score compared to fish fed 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. These results indicate that fish fed the ZIRC diet experience changes to their physiology that differ from fish fed the Gemma and Watts diets.

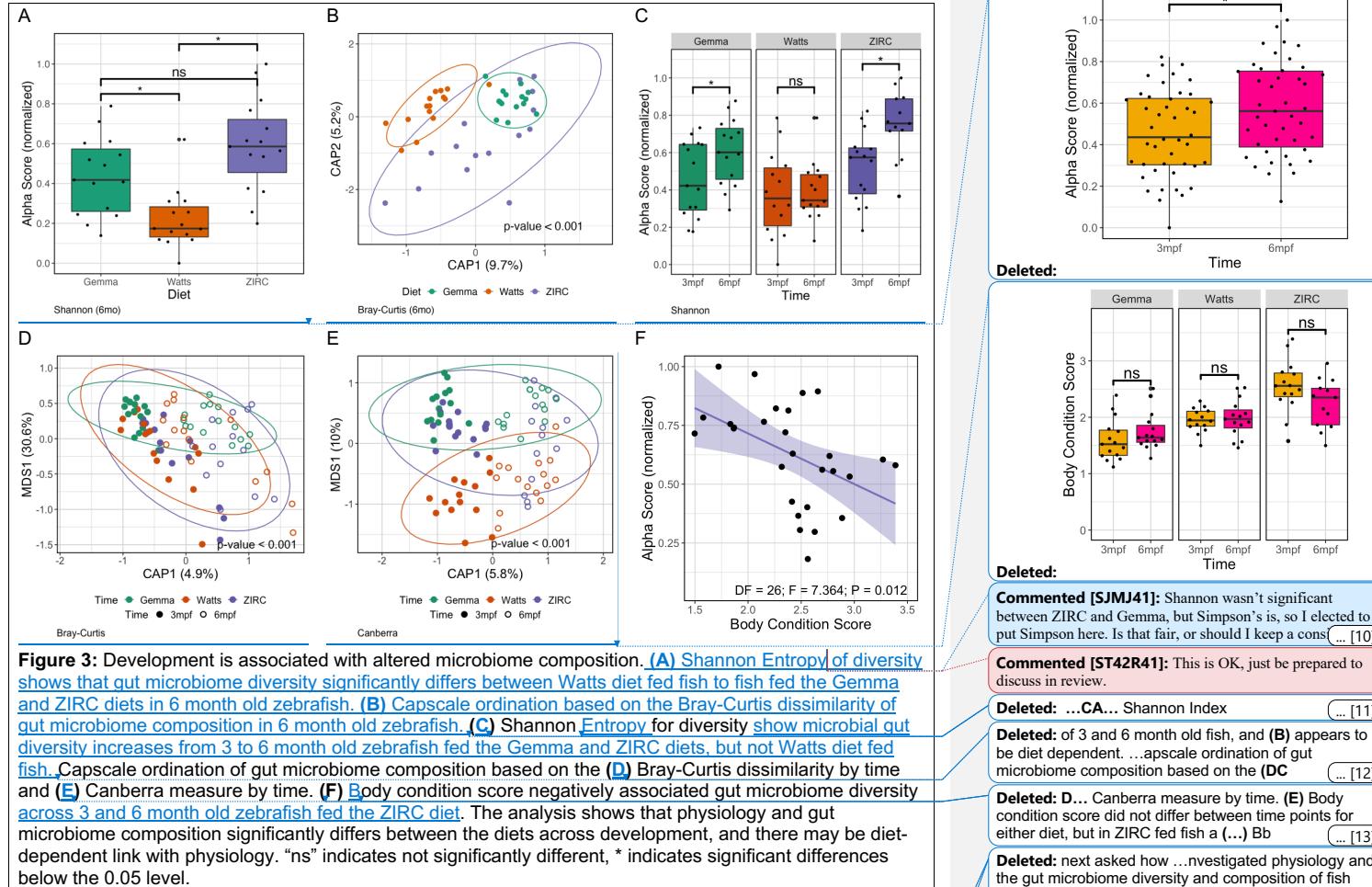
Next, we asked if diet associated with gut microbiome diversity and composition. First, we built generalized linear models (GLM) to determine if diet associated with variation in one of three measures of microbiome alpha-diversity: richness, Simpson's Index, and Shannon Entropy. Using an array of diversity measures allows us to investigate the relative number of taxa in a community (e.g. richness), how diversity may vary across diversity measures that differentially weight the importance of abundant (e.g. Simpson's Index) and rare (e.g. Shannon Entropy) taxa in a community (Microbiome stats textbook). 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. These results suggest gut microbial diversity appears to differ by diet, but these differences vary among abundant and rare taxa.

Next, we used the Bray-Curtis and Canberra dissimilarity metrics to evaluate how diet associates with microbiome community composition. We detected a significant clustering of microbial gut community composition based on diet (PERMANOVA,  $p < 0.05$ ; Figure 2C, Table S1.3.1). To assess variance in microbiome community composition within diets, we measured beta-dispersion. We find the variance microbial gut communities differed across the three diets depending on beta diversity measure. Beta-dispersion levels were significantly different in fish fed the ZIRC diet compared to fish fed the Gemma and Watts diets when measured by Bray-Curtis index (Figure S1.4.1). Beta-dispersion levels differed between fish fed the Gemma diet compared to fish fed the Watts and ZIRC as measured by Canberra measure (Figure S1.4.1). Together these results indicate that fish fed different diets manifest distinct gut microbiome communities at 3 months of age, and some diets display more consistency in composition than other diets.

Finally, to better understand the interactions between the diets and the gut microbiome, we quantified differential abundance using ANCOM-BC. We observed 22 taxa at the genus level were significantly abundant in at least one of the three diets (Figure S1.5.2). Of these taxa, 14 (64%) were found in the phylum Proteobacteria, 5 (23%) in Bacteroidota, 2 (9%) in Firmicutes, and 1 (4%) in Verrucomicrobacteria. Notably, fish fed the Gemma diet were enriched in *Crenobacter*, fish fed the Watts diet enriched for *Luteimonas*, *Cellvibrio*, and genera in *Microscillaceae* and *Chitinibacteraceae* families, and ZIRC diet fed fish enriched for *Cloacibacterium*, *Acinetobacter*, *Pseudomonas* and *Phreatobacter* (Figure S1.5.1). Together these results indicate that diets select for different taxa when fish are 3 months of age.

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### 3. Diet impacts the successional development of the zebrafish gut microbiome

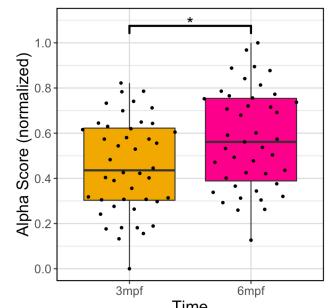


Given the associations we observed above between diet, the gut microbiome and physiology at 3 months of age, we investigated physiology and the gut microbiome diversity and composition of fish fed the three diets at 6 months of age. We found similar trends between diet and physiology at 6 months as we did at 3 months (Supp Fig ###). Linear regression analysis revealed statistically significant main effects of diet on microbial gut diversity in 6 month old zebrafish across all diversity measures ( $p < 0.05$ ; Fig A, Table S####). We also found diet was a significant predictor of microbiome community composition across all beta diversity measures ( $p < 0.05$ ; Fig B, Table S####).

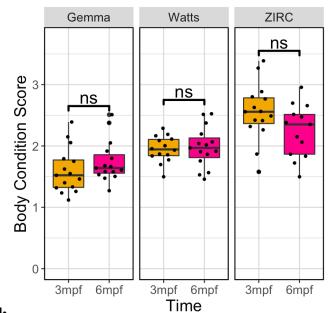
We next sought to determine if diet influences successional development by comparing microbiomes of 3 and 6 month old zebrafish. Linear regression revealed microbial gut diversity was significantly associated with the main effect of time (Supp Fig # C, Table ####). We also observed significant interaction effects between diet

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and time ( $p < 0.05$ ; Fig 3 C, Table S2.2.2.1), across all diversity measures. A post hoc Tukey test showed microbiome diversity was significantly different between 3 and 6 months in fish fed the Gemma and ZIRC diets, fish as measured by the Shannon and Simpson's alpha diversity metrics ( $p < 0.05$ ; Table S2.2.2.3), but the microbial gut diversity of fish fed the Watts diet were temporally stable and did not significantly differ between 3 and 6 months. 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). These results suggest that 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. We used ANCOM-BC to determine if the abundance of taxa associated with a particular diet of zebrafish across their development between 3 and 6 months of age. <Need to add some sentences to talking about interesting taxa>. 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 diet differentially impacts how the successional development of the gut microbiome in zebrafish.

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 between fish 3 and 6 months old. Within each diet, we did not observe a significant association between time and body condition ( $p < 0.05$ ; Fig 2E, Table S2.1.1). However, we observed a significant negative association of body condition score on microbial gut diversity uniquely in ZIRC fed fish ( $p < 0.05$ ; Fig 2F, Table S2.2.1). Conversely, we did not find a significant association between body condition score and microbial community composition or abundance of taxa (Table S2.2.2). These results indicate that there is a link between microbial gut diversity and body condition score across development with fish fed the ZIRC diet, but this is not the case for fish fed the Gemma and Watts diets.

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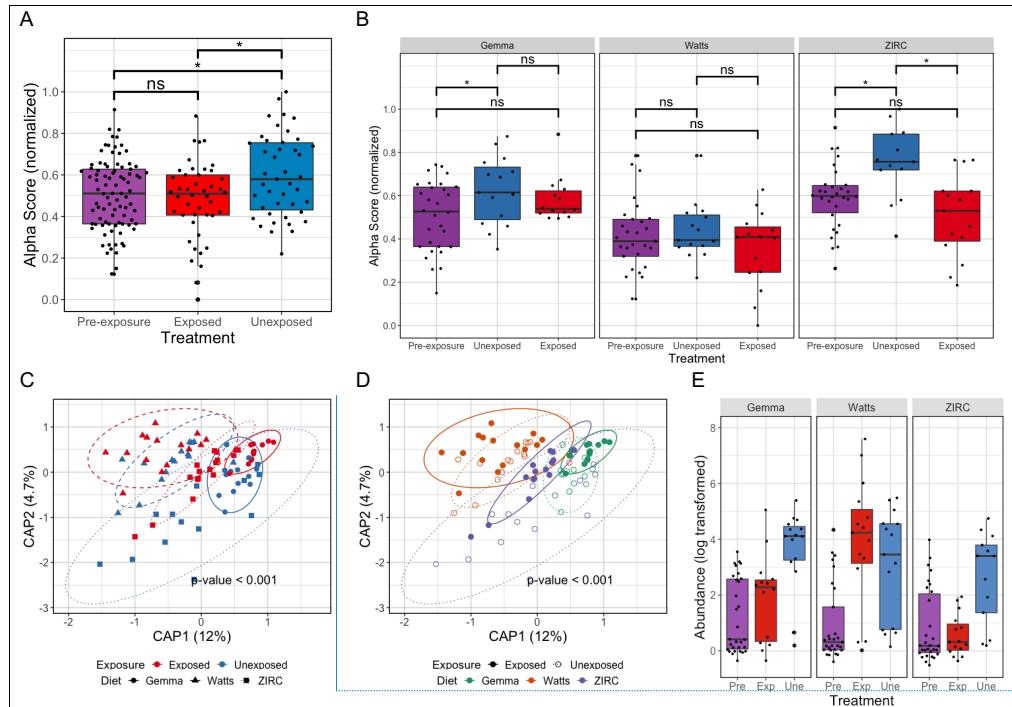
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#### 4. Diet influences gut microbiome's sensitivity to pathogen exposure



**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.

Lastly, we sought to elucidate the potential interactions between the gut microbiome, commonly laboratory diets and exposure to the intestinal pathogen *Mycobacterium chelonae*. 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. We did not find a statistical effect of number of infections or infection burden on physiology or the gut microbiome (Table S####). Instead, exposure to *M. chelonae* best explained the variation in the data. Thus, from this point on all fish up to 129 days old are referred to as "pre-exposed", 214 day old fish not injected with *M. chelonae* are "unexposed" and fished injected with *M. chelonae* are "exposed".

Using linear regression, we find that microbial gut diversity significantly differs between exposure groups as measured by the Observed and Shannon alpha diversity metrics ( $P < 0.05$ ; Table S3.1.2.2), but the statistical effect of diet was greater ( $p < 0.05$ ; Table S3.1.2.2). We did not find a significant interaction effect between diet and exposure. Furthermore, we used post hoc Tukey test to assess whether microbial gut diversity differed between exposure groups within each diet. Unique to ZIRC fed fish, we observed microbiome diversity was

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higher in unexposed fish compared to pre-exposed and exposed fish. Moreover, pre-exposed fish fed the ZIRC diet did not differ in diversity to exposed ZIRC fed fish ( $p < 0.05$ ; Fig 4B, Table S3.1.2.3). These results suggest that fish fed commonly used laboratory diets are differentially sensitive to *M. chelonae* exposure in terms of microbiome diversity. Next, we assessed how pathogen exposure influenced microbial community composition across the diets. PERMANOVA tests as measured by each beta diversity metric found the main effects of diet and pathogen exposure were statistically significant, but diet's statistical effect was greatest ( $P < 0.05$ ; Fig 4C&D, Table S3.2.1). Furthermore, a PERMANOVA test on the interaction of diet and pathogen exposure was statistically significant as measured by Canberra and Sorenson beta diversity metrics, but the statistical effect was less than the main effect of pathogen exposure. These results suggest that while *M. chelonae* exposure may influence the microbial community composition, diet plays a greater role.

Finally, to determine if diet impacted *Mycobacterium* abundance, we used ANCOM-BCI. *Mycobacterium* is a common microbial member of the zebrafish gut microbiome. Furthermore, given the constraints of 16S analysis we can determine the strain level identities of the *Mycobacterium* species present in our samples. Nonetheless, 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). Unique to fish fed the Watts diet, we find that *Mycobacterium* abundance was not significantly different between exposed and unexposed groups. Interestingly, unexposed fish fed the Gemma and ZIRC diets had higher abundance of *Mycobacterium* than exposed fish. Furthermore, we did not see a pathogen exposure effect on body condition score. We also do not find a diet by exposure interaction effect with body condition score. Collectively, these results indicate that zebrafish gut microbiomes vary in their sensitivity following exposure to *M. chelonae* depending on diet, but diet plays a greater influence than *M. chelonae* exposure on the gut microbiome and physiology.

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Deleted: *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). W

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## 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 in zebrafish. 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 fish fed 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 different 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 noted this could impact the gut microbiome of fish. We found that gut microbiome diversity differed by diet, and fish microbiome communities were more similar to fish fed the same diet. 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 microbiome diversity seen 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 and feed samples alongside fecal samples to account for a potential feed microbiome effect. It is 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, 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.

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

We found that gut microbiome diversity increases and composition varies over time. Notably, in ZIRC and Gemma fed fish rare taxa appear more temporally 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 microbiome diversity that they note may be a result of dietary changes. Moreover, previously identified core, keystone taxa had diet dependent changes in abundance over time (Sharpton). Keystone taxa are believed to play important roles in maintaining gut microbiome homeostasis by digesting nutrients, fighting pathogens and communicating with the immune system (Xiao, Others?). 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 microbiome diversity, where fish fed the ZIRC diet had higher gut diversity and lower body condition scores, 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

**Commented [TS97]:** I think you need to provide more clarity about what this prior work did, or your study just sounds redundant with these prior studies.

**Commented [TS98]:** What nutrients? Does the variation considered here overlap with your study?

**Commented [SJM199R98]:** Some overlap in their control diet to Gemma, but otherwise the other diets were very different

**Commented [TS100]:** Did they show it? Be clear.

**Commented [SJM110R100]:** They did not, only speculated.

**Commented [TS102]:** Are you intended to say something that is different from the phrase on the other side of the comma? They sound the same.

**Commented [SJM1103R102]:** Addressing alpha diversity in the first half, and then beta diversity in the second half.

**Commented [TS104]:** But doesn't it seem unlikely given that you are seeing effects in abundance weighted measures?

**Commented [TS105R104]:** The current way this is phrased makes it sound like your results could be entirely an artifact.

**Commented [TS106]:** This point needs to come sooner. By the time the reader gets here, they have assumed that your work is just redundant with prior studies and lost interest in the impact.

**Commented [SJM1107R106]:** Move the sentence [28]

**Commented [TS108]:** How so?

**Commented [SJM1109R108]:** For instance, the p[29]

**Commented [TS110]:** Missing from this discussion [30]

**Commented [SJM1111R110]:** The microbiome p[31]

**Commented [TS112]:** Not clear what you mean here.

**Commented [SJM1113R112]:** What I mean to say [32]

**Commented [SJM1114R112]:** Moreover, once the [33]

**Commented [TS115]:** You like this phase. It works [34]

**Commented [TS116]:** This is an incomplete idea a[35]

**Commented [TS117]:** Over time or throughout aging?

**Commented [TS118]:** The connection between thi[36]

**Commented [TS119]:** This needs to be put somew[37]

**Commented [TS120]:** I don't follow you here.

**Commented [SJM1121R120]:** Gemma increases i[38]

**Commented [TS122]:** Ah - I think you are saying t[39]

**Commented [SJM1123R122]:** I did not know this [40]

**Commented [TS124]:** swing the bat around - how [41]

**Commented [TS125]:** We need more from you her[42]

**Commented [TS126]:** Same comment - it's not ent[43]

**Commented [TS127]:** Have you shown here that th[44]

**Commented [SJM1128]:** Needs significant revisions

**Commented [TS129]:** What does this mean given [45]

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 across development to impact gut microbiome succession, which may manifest differential physiological outcomes.

We find that pathogen exposure inhibited diversification of gut microbiomes, and microbiome community composition was driven primarily by diet rather than pathogen exposure. The gut microbiome diversity of ZIRC fed fish is uniquely sensitive to pathogen exposure, while Gemma and Watts diet were more resistant. Interestingly, *Mycobacterium*'s abundance differed between the diets. Exposed Watts fed fish had more *Mycobacterium*, but Exposed Gemma and ZIRC had fewer relative to controls. 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, others?). We also saw the opposite effect of microbiome communities becoming more similar after exposure to *Mycobacterium* relative to controls. Three 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, these factors could have hindered *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.

**Commented [TS130]:** We can't? Why not? Don't you have both M and F fish here? Why don't you see these trends in other diets then? This argument will be lost on readers without clarification. I'm also not sure it's necessary to make this point here.

**Commented [TS131]:** Try to avoid this.

**Commented [TS132]:** You did? What result shows this?

**Commented [SJM133R132]:** Right. I'm being lazy here saying "pathogen exposure", but what I mean is exposure to *Mycobacterium*. It would be more precise to say *mycobacterium* exposure inhibited diversification of the gut microbiome (Fig 3a)

**Commented [TS134]:** Probably not wise to use Myco as a representative for pathogen exposure here. I recommend being specific so that you aren't accused of over generalizing your results.

**Commented [TS135R134]:** (or anywhere else in the MS)

**Commented [SJM136R134]:** Got it.

**Commented [TS137]:** Personally, I think this is hard to interpret, but worth discussing regardless so long as we note that 16S can't provide insight into what types of Myco are present.

**Commented [TS138]:** This was a good way to link other work. That said, you still want to note above that composition also shows signs of sensitivity to pathogen exposure.

**Commented [TS139]:** compared to what? Remember, readers won't have read gaulke et al.

**Commented [TS140]:** stylistically, I'm not a fan of this approach. it has the tendency to feel like limitations are being swept aside, and - ironically - often allows limitations to carry more weight than they deserve. When discussing limitations, it's always good to mute their potential impact on your conclusions with argumentation or data, when possible.

**Commented [TS141]:** Also something to note in a study design. Important to note that fish injected this way nonetheless display GI evidence of infection.

**Commented [TS142]:** I'm confused about how these factors impact colonization.

**Commented [SJM143R142]:** 1) maybe not because of what you noted in your comment above.

2&3) Perhaps the particular species of *Mycobacterium* we injected is outcompeted by other, pre-existing mycobacterium strains

## Conclusions

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 the microbiome's development and sensitivity to pathogen exposure.

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. 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.

**Commented [TS144]:** I think it's worth underscoring the fact that diet has a bigger effect on microbiome composition than infection and even time (right?). In other words, 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.

**Commented [TS145]:** This seems to contradict the opening sentence of this paragraph.

**Commented [SJMJ146R145]:** True. I was attempting to present a scenario why you'd want consistency in diets (reduce inter-study variability in results), and another where you'd want variation (model human populations)

## 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, Fontaine-les-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, Fontaine-les-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 regimen, 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  $\mu$ L 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  $3.1 \times 10^3$  dose per fish. Day 2 *M. chelonae* inoculum was determined by plating to be  $1.0 \times 10^5$  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

**Commented [SJM147]:** Need Mike to review zebrafish methods

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<sup>3</sup>. [Body condition score is a length normalized metric of weight \(for equation, see Methods\) and serves as a general indicator of health in zebrafish.](#)

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

Commented [ST148]: Might be methods

Deleted:

### 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).

Commented [SJMJ149]: Ask Kristin to review

### 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 Sorenson and 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

Commented [SJMJ150]: Correct?

Commented [SJMJ151]: Correct?

Commented [SJMJ152]: What is the code?

Commented [SJMJ153]: Need to review versions, citations, and packages.

Commented [ST154]: Methods.

the selected model parameters significantly explained the variation in microbiome composition across samples. Differential abundance was measured using ANCOM-BC ( $\nu$ ).

## Supplementary Tables and Figures

### 1) Diet

#### 1.1) Physiology

Wilcoxon Testp. adj: BH. Weight ~ Diet

.y.	group1	group2	n1	n2	statistic	p	p.adj	p.adj.signif
Weight	Gemma	Watts	30	29	528.500	0.158	0.158	ns
	Gemma	ZIRC	30	30	301.500	0.029	0.044	*
	Watts	ZIRC	29	30	225.500	0.002	0.006	**

1.1.1)

Wilcoxon Testp. adj: BH. Weight ~ Sex

.y.	group1	group2	n1	n2	statistic	p	p.adj	p.adj.signif
Weight	F	M	50	39	1,505.500	<0.001	<0.001	****

1.1.2)

Wilcoxon Testp. adj: BH. Body.Condition.Score ~ Diet

.y.	group1	group2	n1	n2	statistic	p	p.adj	p.adj.signif
Body.Condition.Score	Gemma	Watts	30	29	327.000	0.103	0.103	ns
	Gemma	ZIRC	30	30	150.000	<0.001	<0.001	****
	Watts	ZIRC	29	30	167.000	<0.001	<0.001	****

1.1.3)

Wilcoxon Testp. adj: BH. Body.Condition.Score ~ Sex

.y.	group1	group2	n1	n2	statistic	p	p.adj	p.adj.signif
Body.Condition.Score	F	M	50	39	1,631.000	<0.001	<0.001	****

1.1.4)

#### 1.2) Alpha Diversity

glm(Alpha.Score ~ Time), family = quasibinomial)

metric	term	estimate	std.error	statistic	p.value	sig
Observed	(Intercept)	-0.293	0.134	-2.191	0.031	*
	DietWatts	-0.917	0.209	-4.390	<0.001	*
	DietZIRC	0.143	0.189	0.760	0.449	
Shannon	(Intercept)	-0.413	0.149	-2.773	0.007	*
	DietWatts	-0.492	0.221	-2.224	0.029	*
	DietZIRC	0.429	0.208	2.058	0.043	*
Simpson	(Intercept)	-0.283	0.155	-1.821	0.072	
	DietWatts	0.243	0.220	1.101	0.274	
	DietZIRC	0.795	0.222	3.579	<0.001	*

1.2.1)

ANOVA( glm(Alpha.Score ~ Diet), family = quasibinomial )

metric	term	statistic	df	p.value	sig
Observed	Diet	31.207	2	<0.001	*
Shannon	Diet	18.217	2	<0.001	*
Simpson	Diet	13.692	2	0.001	*

1.2.2)

[S2149A](#)

Pairwise Tukey's HSD, p.adj: Dunnett, glm(Alpha.Score ~ Diet), family = quasibinomial)

metric	term	.y.	group1	group2	estimate	std.error	statistic	p.adj	p.adj.signif
Observed	Diet	Alpha.Score	Watts	Gemma	-0.917	0.209	-4.390	0.000	*
	Diet	Alpha.Score	ZIRC	Gemma	0.143	0.189	0.760	0.727	ns
	Diet	Alpha.Score	ZIRC	Watts	1.060	0.208	5.093	0.000	*
Shannon	Diet	Alpha.Score	Watts	Gemma	-0.492	0.221	-2.224	0.067	ns
	Diet	Alpha.Score	ZIRC	Gemma	0.429	0.208	2.058	0.099	ns
	Diet	Alpha.Score	ZIRC	Watts	0.920	0.219	4.202	0.000	*
Simpson	Diet	Alpha.Score	Watts	Gemma	0.243	0.220	1.101	0.513	ns
	Diet	Alpha.Score	ZIRC	Gemma	0.795	0.222	3.579	0.001	*
	Diet	Alpha.Score	ZIRC	Watts	0.552	0.223	2.478	0.035	*

### 1.3) Beta Diversity

metric	term	Df	SumOfSqs	statistic	p.value	sig
Bray-Curtis	Diet	2.00	1.099108	13.949	0.001	*
	Residual	86.00	3.388087			
Canberra	Diet	2.00	3.207838	9.126	0.001	*
	Residual	86.00	15.115341			
Sørensen	Diet	2.00	1.459833	12.970	0.001	*
	Residual	86.00	4.839717			

1.3.1)

### 1.4) Beta-Dispersion

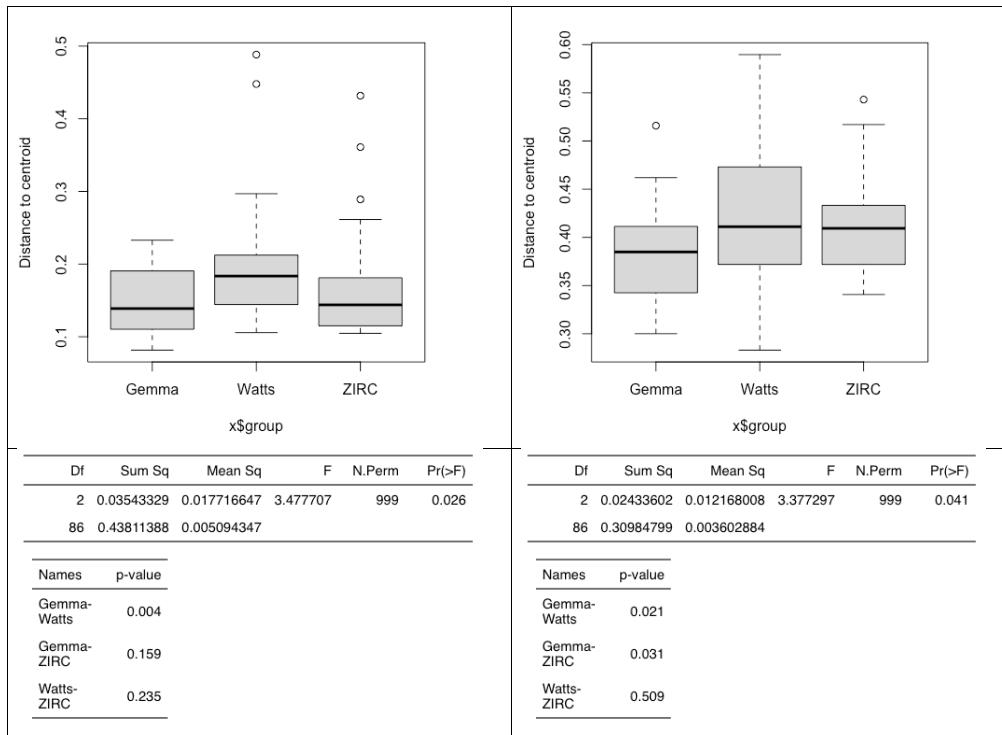
#### 1.4.1) Diet

Bray-Curtis

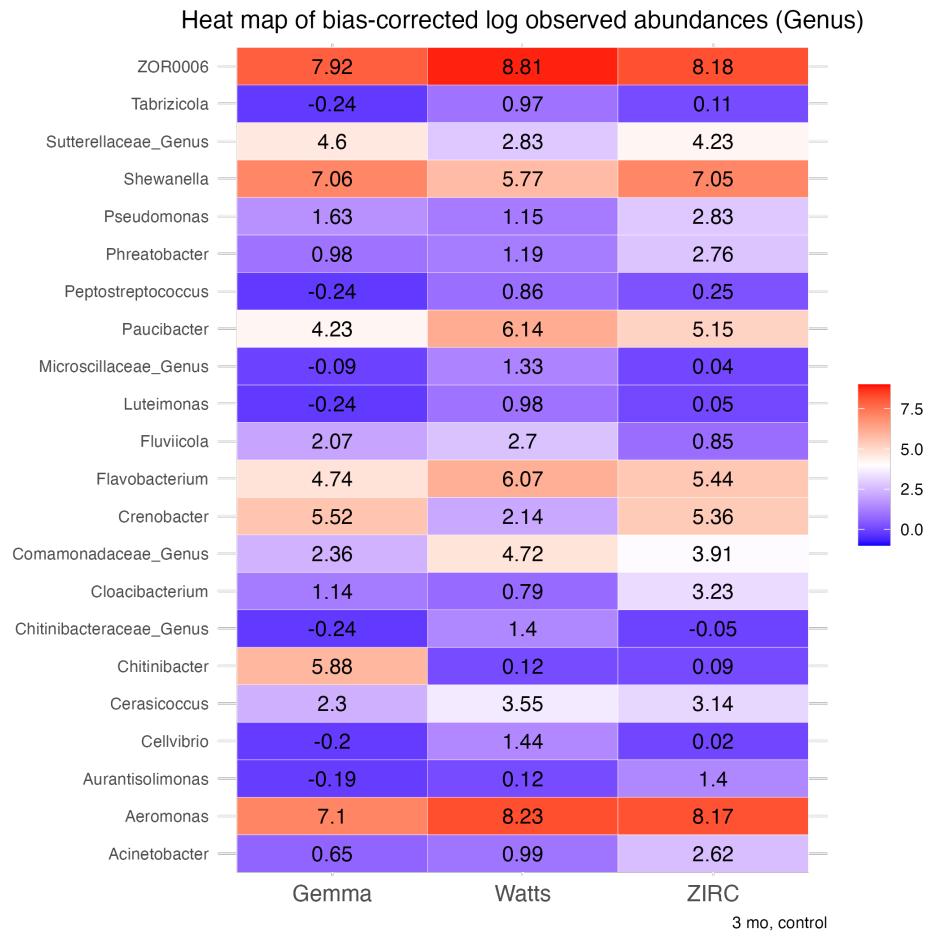
Canberra

Deleted: 

1.2.3)



### 1.5) Differential Abundance



[1.5.1\)](#)

[1.5.2\)](#)

ANCOMBC global test result (3mo, controls), # sig taxa = 22

Taxon	W	p_val	q_val	diff_abn
Chitinibacter	1,421.83	<0.001	<0.001	TRUE
Crenobacter	91.27	<0.001	<0.001	TRUE
Aeromonas	61.69	<0.001	<0.001	TRUE
Shewanella	31.64	<0.001	<0.001	TRUE
ZOR0006	28.42	<0.001	<0.001	TRUE
Comamonadaceae_Genus	27.32	<0.001	<0.001	TRUE
Cellvibrio	26.33	<0.001	<0.001	TRUE
Aurantisolimonas	25.07	<0.001	<0.001	TRUE
Cloacibacterium	24.87	<0.001	<0.001	TRUE
Paucibacter	23.93	<0.001	<0.001	TRUE
Luteimonas	20.47	<0.001	<0.001	TRUE
Microscillaceae_Genus	20.00	<0.001	<0.001	TRUE
Chitinibacteraceae_Genus	19.67	<0.001	<0.001	TRUE
Peptostreptococcus	19.14	<0.001	<0.001	TRUE
Phreatobacter	18.70	<0.001	<0.001	TRUE
Sutterellaceae_Genus	17.58	<0.001	<0.001	TRUE
Flavobacterium	17.28	<0.001	<0.001	TRUE
Tabrizicola	14.86	0.001	0.003	TRUE
Acinetobacter	14.13	0.002	0.003	TRUE
Cerasicoccus	9.38	0.018	0.035	TRUE
Pseudomonas	9.25	0.020	0.035	TRUE
Fluviicola	8.95	0.023	0.039	TRUE



## 2) Development

### 2.1) Physiology

Wilcoxon Testp. adj: BH. Body.Condition.Score ~ Diet:Time

Diet	.y.	group1	group2	n1	n2	statistic	p	p.adj	p.adj.signif
Gemma	Body.Condition.Score	3mpf	6mpf	15	15	78.000	0.161	0.242	ns
Walts	Body.Condition.Score	3mpf	6mpf	14	15	99.000	0.813	0.813	ns
ZIRC	Body.Condition.Score	3mpf	6mpf	15	13	134.000	0.098	0.242	ns

2.1.1)

### 2.2) Physiology ~ Microbiome

glm(Alpha.Score ~ Timepoint), family = quasibinomial

metric	.y.	term	statistic	df	p.value
Observed	Alpha.Score	Body Condition Score (ZIRC)	3.846	1	0.050
Shannon	Alpha.Score	Body Condition Score (ZIRC)	7.372	1	0.007
Simpson	Alpha.Score	Body Condition Score (ZIRC)	9.918	1	0.002

2.2.1)

Distance-based redundancy analysis (dbRDA) ordination. Beta.Score ~ Body.Condition.Score\*Diet

metric	term	Df	SumOfSqs	statistic	p.value	sig
Bray-Curtis	Diet	2.00	0.8945429	8.698	0.001	*
	Body.Condition.Score	1.00	0.0688714	1.339	0.205	
	Body.Condition.Score:Diet	2.00	0.1227086	1.193	0.251	
Canberra	Residual	81.00	4.1651051			
	Diet	2.00	2.1839598	5.004	0.001	*
	Body.Condition.Score	1.00	0.3546246	1.625	0.019	*
Sørensen	Body.Condition.Score:Diet	2.00	0.5359131	1.228	0.092	
	Residual	81.00	17.6756651			
	Diet	2.00	1.1954593	7.229	0.001	*
2.2.2)	Body.Condition.Score:Diet	2.00	0.2402242	1.453	0.064	
	Body.Condition.Score	1.00	0.1100171	1.331	0.168	
	Residual	81.00	6.6975226			

### 2.3) Alpha Diversity

#### 2.3.1) Time

```
glm(Alpha.Score ~ Time), family = quasibinomial)
```

metric	term	estimate	std.error	statistic	p.value	sig
Observed	(Intercept)	0.018	0.120	0.147	0.883	
	Timepoint6mpf	0.337	0.172	1.954	0.054	
Shannon	(Intercept)	-0.181	0.131	-1.375	0.173	
	Timepoint6mpf	0.480	0.188	2.560	0.012	*
Simpson	(Intercept)	-0.370	0.132	-2.812	0.006	*
	Timepoint6mpf	0.440	0.186	2.370	0.020	*

### 2.3.1.1)

```
ANOVA( glm(Alpha.Score ~ Time), family = quasibinomial )
```

metric	term	statistic	df	p.value	sig
Observed	Timepoint	3.834	1	0.050	
Shannon	Timepoint	6.603	1	0.010	*
Simpson	Timepoint	5.651	1	0.017	*

### 2.3.1.2)

```
Pairwise Tukey's HSD, p.adj: Dunnett. glm(Alpha.Score ~ Timepoint), family = quasibinomial)
```

metric	term	.y.	group1	group2	estimate	std.error	statistic	p.adj	p.adj.signif
Observed	Timepoint	Alpha.Score	6mpf	3mpf	0.337	0.172	1.954	0.051	ns
Shannon	Timepoint	Alpha.Score	6mpf	3mpf	0.480	0.188	2.560	0.010	*
Simpson	Timepoint	Alpha.Score	6mpf	3mpf	0.440	0.186	2.370	0.018	*

### 2.2.1.3)

## 2.2.2) Time:Diet

glm(Alpha.Score ~ Diet\*Time), family = quasibinomial)

metric	term	estimate	std.error	statistic	p.value	sig
Observed	(Intercept)	0.344	0.184	1.872	0.065	
	DietWatts	-0.827	0.266	-3.109	0.003	*
	DietZIRC	-0.189	0.258	-0.731	0.467	
	Timepoint6mpf	0.115	0.261	0.440	0.661	
	DietWatts:Timepoint6mpf	0.079	0.372	0.213	0.832	
	DietZIRC:Timepoint6mpf	0.792	0.388	2.042	0.044	*
Shannon	(Intercept)	-0.175	0.203	-0.864	0.390	
	DietWatts	-0.322	0.296	-1.090	0.279	
	DietZIRC	0.276	0.286	0.966	0.337	
	Timepoint6mpf	0.606	0.289	2.096	0.039	*
	DietWatts:Timepoint6mpf	-0.546	0.416	-1.314	0.193	
	DietZIRC:Timepoint6mpf	0.378	0.432	0.874	0.384	
Simpson	(Intercept)	-0.718	0.222	-3.234	0.002	*
	DietWatts	0.387	0.312	1.243	0.218	
	DietZIRC	0.640	0.305	2.100	0.039	*
	Timepoint6mpf	0.693	0.305	2.277	0.025	*
	DietWatts:Timepoint6mpf	-0.770	0.431	-1.786	0.078	
	DietZIRC:Timepoint6mpf	0.142	0.440	0.323	0.748	

2.2.2.1)

ANOVA( glm(Alpha.Score ~ Diet\*Time), family = quasibinomial) )

metric	term	statistic	df	p.value	sig
Observed	Diet	29.866	2	<0.001	*
	Timepoint	6.137	1	0.013	*
	Diet:Timepoint	4.972	2	0.083	
Shannon	Diet	24.198	2	<0.001	*
	Timepoint	9.728	1	0.002	*
	Diet:Timepoint	4.582	2	0.101	
Simpson	Diet	13.541	2	0.001	*
	Timepoint	7.342	1	0.007	*
	Diet:Timepoint	5.077	2	0.079	

2.2.2.2)

### 2.2.2.3)

Pairwise Tukey's HSD, p.adj: Dunnett, glm(Alpha.Score ~ Timepoint), family = quasibinomial)

metric	Diet	.y.	term	group1	group2	estimate	std.error	statistic	p.adj	p.adj.signif
Observed	Gemma	Alpha.Score	Timepoint	6mpf	3mpf	0.115	0.224	0.512	0.608310918	ns
	Watts	Alpha.Score	Timepoint	6mpf	3mpf	0.194	0.274	0.710	0.477616760	ns
	ZIRC	Alpha.Score	Timepoint	6mpf	3mpf	0.907	0.317	2.863	0.004202217	*
Shannon	Gemma	Alpha.Score	Timepoint	6mpf	3mpf	0.606	0.282	2.151	0.031444735	*
	Watts	Alpha.Score	Timepoint	6mpf	3mpf	0.060	0.302	0.199	0.841967493	ns
	ZIRC	Alpha.Score	Timepoint	6mpf	3mpf	0.984	0.325	3.024	0.002490914	*
Simpson	Gemma	Alpha.Score	Timepoint	6mpf	3mpf	0.693	0.324	2.143	0.032142661	*
	Watts	Alpha.Score	Timepoint	6mpf	3mpf	-0.077	0.280	-0.273	0.784806376	ns
	ZIRC	Alpha.Score	Timepoint	6mpf	3mpf	0.836	0.322	2.598	0.009388431	*

## 2.4) Beta Diversity

metric	term	Df	SumOfSqs	statistic	p.value	sig
Bray-Curtis	Diet	2.00	0.8395202	7.992	0.001	*
	Residual	84.00	4.4117078			
Canberra	Diet	2.00	2.2323578	5.063	0.001	*
	Residual	84.00	18.5178049			
Sørensen	Diet	2.00	1.2197670	7.294	0.001	*
	Residual	84.00	7.0234562			

### 2.4.1)

metric	term	Df	SumOfSqs	statistic	p.value	sig
Bray-Curtis	Timepoint	1.00	0.2568805	4.372	0.001	*
	Residual	85.00	4.9943475			
Canberra	Timepoint	1.00	1.2060007	5.245	0.001	*
	Residual	85.00	19.5441621			
Sørensen	Timepoint	1.00	0.8039921	9.186	0.001	*
	Residual	85.00	7.4392311			

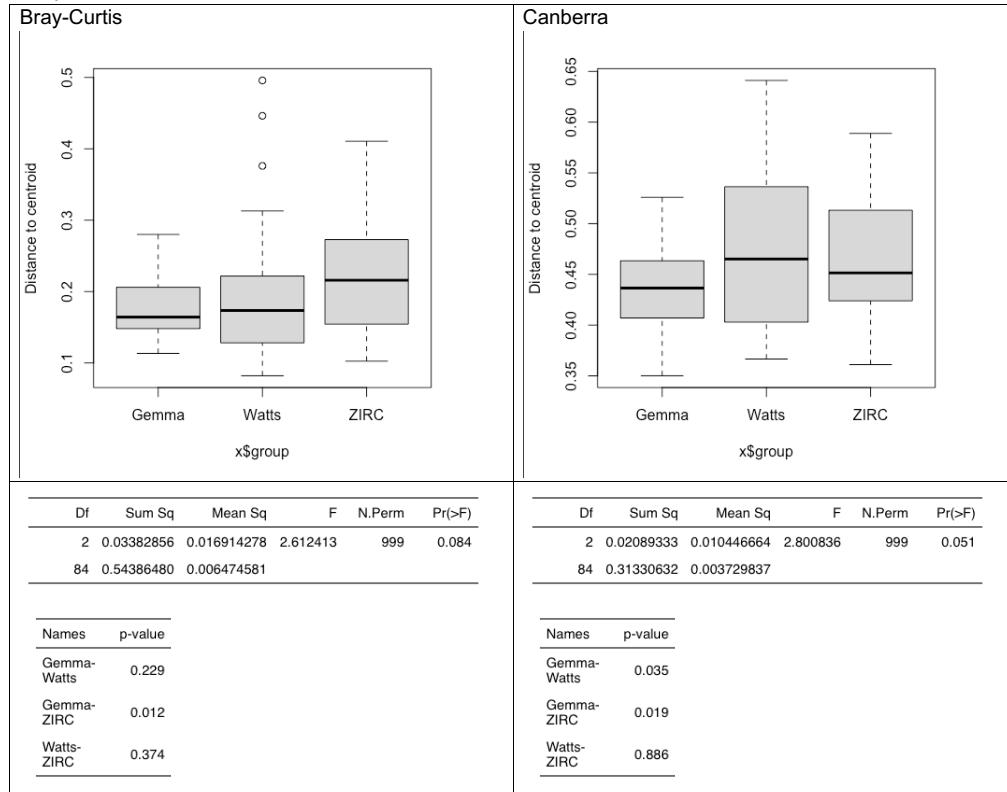
### 2.4.2)

metric	term	Df	SumOfSqs	statistic	p.value	sig
Bray-Curtis	Diet	2.00	0.8395202	8.564	0.001	*
	Timepoint	1.00	0.2635537	5.377	0.001	*
	Diet:Timepoint	2.00	0.1781783	1.818	0.039	*
	Residual	81.00	3.96699758			
Canberra	Timepoint	1.00	1.2121772	6.004	0.001	*
	Diet	2.00	2.2323578	5.528	0.001	*
	Diet:Timepoint	2.00	0.9517175	2.357	0.001	*
	Residual	81.00	16.3539102			
Sørensen	Timepoint	1.00	0.8051774	11.176	0.001	*
	Diet	2.00	1.2197670	8.465	0.001	*
	Diet:Timepoint	2.00	0.3823789	2.654	0.001	*
	Residual	81.00	5.8359000			

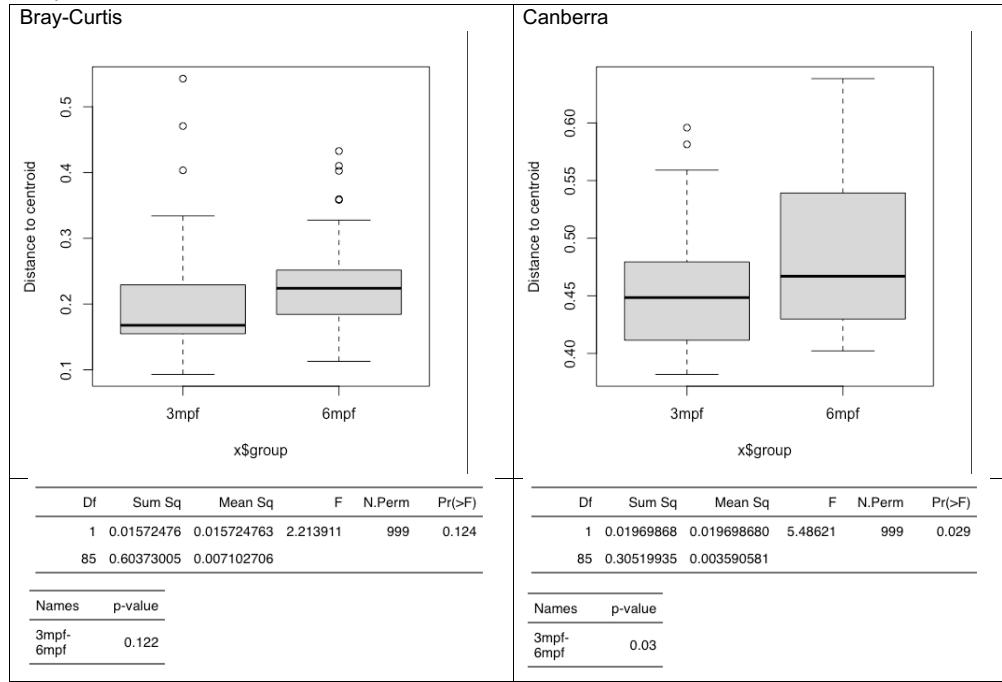
### 2.4.3)

## 2.5) Beta-Dispersion

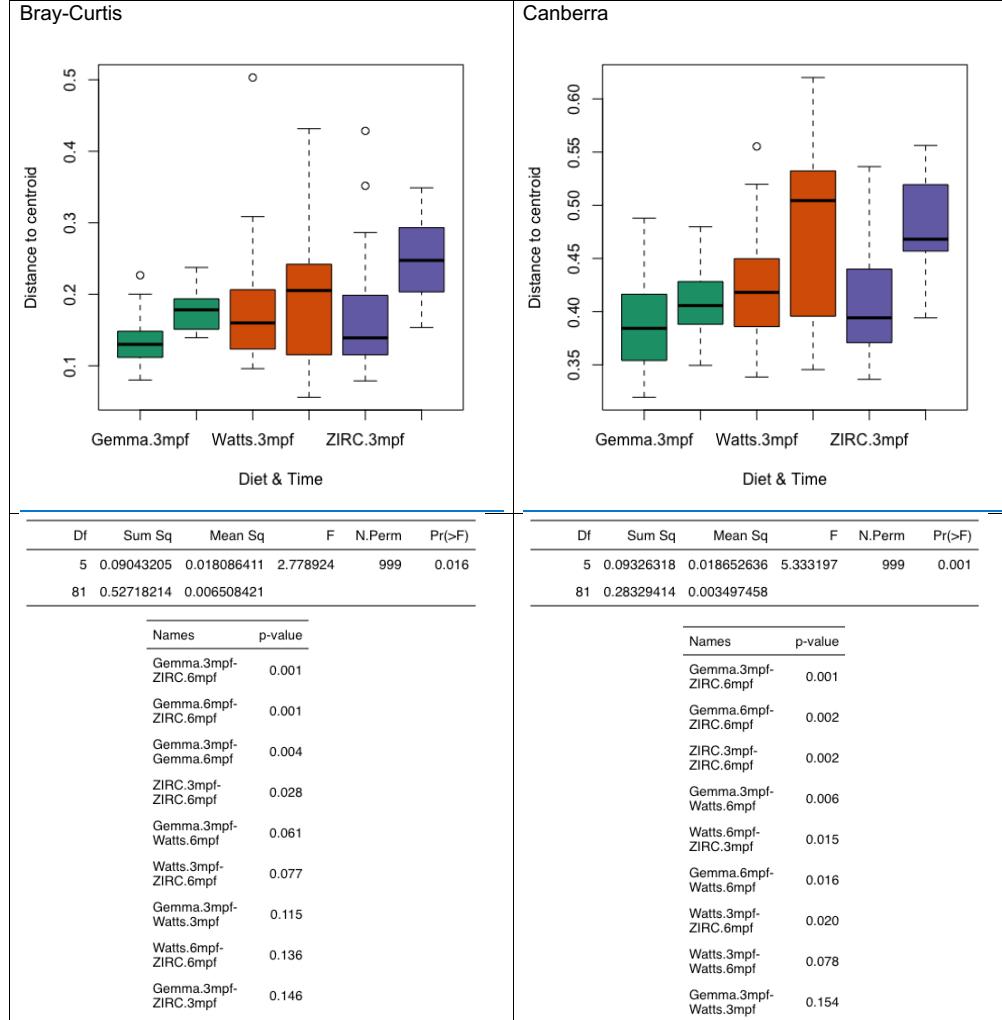
### 2.5.1) Diet



## 2.5.2) Time



### 2.5.3) Diet:Time



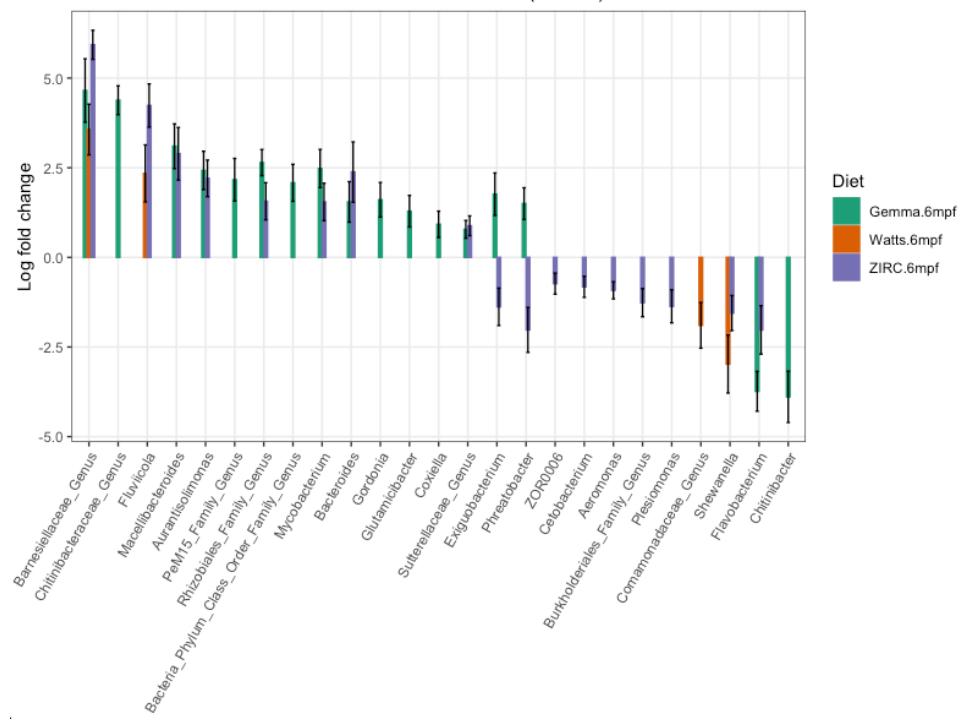
## 2.6 Differential Abundance

Taxon		W	p_val	q_val	diff_abn
	Mycobacterium	19.054	0.002	0.003	TRUE
	Longivirga	50.592	<0.001	<0.001	TRUE
	Glutamicibacter	48.228	<0.001	<0.001	TRUE
Bacteria_Phylum_Class_Order_Family_Genus		22.139	<0.001	<0.001	TRUE
	Bacteroides	16.514	0.005	0.008	TRUE
	Macellibacteroides	42.534	<0.001	<0.001	TRUE
	Aurantisolimonas	44.844	<0.001	<0.001	TRUE
	Fluviicola	54.593	<0.001	<0.001	TRUE
	Flavobacterium	66.064	<0.001	<0.001	TRUE
	Cloacibacterium	19.805	0.001	0.002	TRUE
	ZOR0006	61.705	<0.001	<0.001	TRUE
	Exiguobacterium	29.965	<0.001	<0.001	TRUE
	Peptostreptococcus	41.515	<0.001	<0.001	TRUE
	Cetobacterium	24.718	<0.001	<0.001	TRUE
Rhizobiaceae_Genus		15.964	0.006	0.010	TRUE
	Phreatobacter	32.393	<0.001	<0.001	TRUE
Rhizobiales_Family_Genus		39.652	<0.001	<0.001	TRUE
	Gemmobacter	15.396	0.008	0.013	TRUE
	Aeromonas	41.677	<0.001	<0.001	TRUE
	Shewanella	64.916	<0.001	<0.001	TRUE
	Chitinibacter	711.743	<0.001	<0.001	TRUE
Chitinibacteraceae_Genus		73.999	<0.001	<0.001	TRUE
	Crenobacter	129.621	<0.001	<0.001	TRUE
Comamonadaceae_Genus		25.220	<0.001	<0.001	TRUE
	Paucibacter	22.701	<0.001	<0.001	TRUE
Sutterellaceae_Genus		36.712	<0.001	<0.001	TRUE
	Cellvibrio	35.572	<0.001	<0.001	TRUE
	Plesiomonas	63.616	<0.001	<0.001	TRUE
Gammaproteobacteria_Order_Family_Genus		14.404	0.012	0.019	TRUE
	Acinetobacter	30.056	<0.001	<0.001	TRUE
	Pseudomonas	13.527	0.018	0.026	TRUE
Parachlamydiaceae_Genus		41.188	<0.001	<0.001	TRUE
	Cerasicoccus	13.539	0.018	0.026	TRUE

2.6.1)

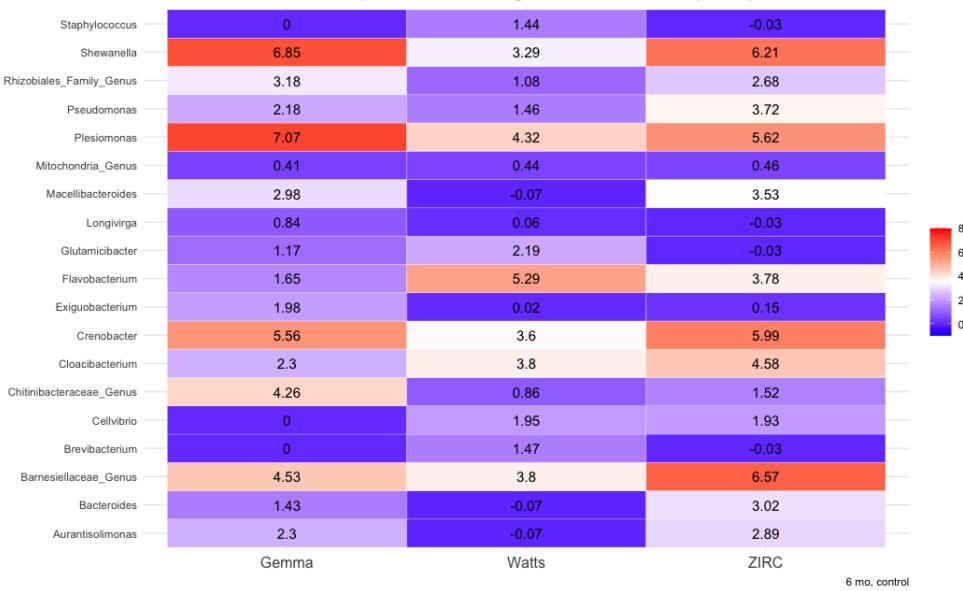
2.6.2)

Waterfall Plot of Diet:Time (Genus)



### 2.6.3)

Heat map of bias-corrected log observed abundances (Genus)



### 3) Exposure

#### 3.1) Alpha Diversity

##### 3.1.1) Exposure

glm(Alpha.Score ~ Exposure, family = quasibinomial)

metric	term	estimate	std.error	statistic	p.value	sig
	(Intercept)	0.055	0.076	0.724	0.470	
Observed	PrePostExpExposed	-0.152	0.133	-1.140	0.256	
	PrePostExpUnexposed	0.347	0.136	2.558	0.011	*
	(Intercept)	0.012	0.078	0.157	0.875	
Shannon	PrePostExpExposed	-0.098	0.135	-0.726	0.469	
	PrePostExpUnexposed	0.385	0.138	2.789	0.006	*
	(Intercept)	-0.271	0.083	-3.264	0.001	*
Simpson	PrePostExpExposed	-0.113	0.145	-0.779	0.437	
	PrePostExpUnexposed	0.341	0.145	2.357	0.020	*

3.1.1.1)

ANOVA( glm(Alpha.Score ~ Exposure, family = quasibinomial) )

metric	term	statistic	df	p.value	sig
Observed	PrePostExp	10.997	2	0.004	*
Shannon	PrePostExp	10.878	2	0.004	*
Simpson	PrePostExp	8.278	2	0.016	*

3.1.1.2)

Pairwise Tukey's HSD, p.adj: Dunnett, glm(Alpha.Score ~ Exposure, family = quasibinomial)

metric	term	.y.	group1	group2	estimate	std.error	statistic	p.adj	p.adj.signif
	PrePostExp	Alpha.Score	Exposed	Pre-exposure	-0.152	0.133	-1.140	0.487	ns
Observed	PrePostExp	Alpha.Score	Unexposed	Pre-exposure	0.347	0.136	2.558	0.028	*
	PrePostExp	Alpha.Score	Unexposed	Exposed	0.499	0.156	3.192	0.004	*
	PrePostExp	Alpha.Score	Exposed	Pre-exposure	-0.098	0.135	-0.726	0.747	ns
Shannon	PrePostExp	Alpha.Score	Unexposed	Pre-exposure	0.385	0.138	2.789	0.014	*
	PrePostExp	Alpha.Score	Unexposed	Exposed	0.484	0.159	3.041	0.007	*
	PrePostExp	Alpha.Score	Exposed	Pre-exposure	-0.113	0.145	-0.779	0.715	ns
Simpson	PrePostExp	Alpha.Score	Unexposed	Pre-exposure	0.341	0.145	2.357	0.048	*
	PrePostExp	Alpha.Score	Unexposed	Exposed	0.454	0.168	2.703	0.019	*

3.1.1.3)

3.1.2) Diet:Exposure

glm(Alpha.Score ~ Diet\*Exposure, family = quasibinomial)

metric	term	estimate	std.error	statistic	p.value	sig
Observed	(Intercept)	0.283	0.116	2.439	0.016	*
	DietWatts	-0.992	0.167	-5.936	<0.001	*
	DietZIRC	0.014	0.163	0.083	0.934	
	ExposureUnexposed	0.140	0.164	0.855	0.394	
	DietWatts:ExposureUnexposed	0.326	0.234	1.393	0.165	
	DietZIRC:ExposureUnexposed	0.140	0.234	0.597	0.551	
Shannon	(Intercept)	0.198	0.126	1.573	0.118	
	DietWatts	-0.721	0.179	-4.019	<0.001	*
	DietZIRC	0.075	0.177	0.423	0.673	
	ExposureUnexposed	0.036	0.177	0.206	0.837	
	DietWatts:ExposureUnexposed	0.218	0.252	0.862	0.390	
	DietZIRC:ExposureUnexposed	0.292	0.254	1.148	0.253	
Simpson	(Intercept)	-0.362	0.144	-2.513	0.013	*
	DietWatts	-0.073	0.203	-0.358	0.720	
	DietZIRC	0.315	0.200	1.573	0.118	
	ExposureUnexposed	0.001	0.202	0.007	0.995	
	DietWatts:ExposureUnexposed	0.063	0.286	0.220	0.826	
	DietZIRC:ExposureUnexposed	0.341	0.285	1.196	0.233	

### 3.1.2.1)

ANOVA( glm(Alpha.Score ~ Diet\*Exposure, family = quasibinomial) )

metric	term	statistic	df	p.value	sig
Observed	Diet	75.452	2	<0.001	*
	Exposure	9.434	1	0.002	*
	Diet:Exposure	1.953	2	0.377	
Shannon	Diet	46.450	2	<0.001	*
	Exposure	3.852	1	0.050	*
	Diet:Exposure	1.438	2	0.487	
Simpson	Diet	16.772	2	<0.001	*
	Exposure	1.377	1	0.241	
	Diet:Exposure	1.623	2	0.444	

### 3.1.2.2)

### 3.1.2.3)

Pairwise Tukey's HSD, p.adj: Dunnett.  $\text{glm}(\text{Alpha.Score} \sim \text{Diet} * \text{Exposure})$ , family = quasibinomial)

metric	Diet	:y.	term	group1	group2	estimate	std.error	statistic	p.adj	p.adj.signif
Observed	Gemma	Alpha.Score	PrePostExp	Exposed	Pre-exposure	0.197	0.177	1.108	0.5067172005	ns
	Gemma	Alpha.Score	PrePostExp	Unexposed	Pre-exposure	0.225	0.174	1.296	0.3951699726	ns
	Gemma	Alpha.Score	PrePostExp	Unexposed	Exposed	0.028	0.205	0.139	0.9893749979	ns
	Watts	Alpha.Score	PrePostExp	Exposed	Pre-exposure	-0.397	0.198	-2.006	0.1097629119	ns
	Watts	Alpha.Score	PrePostExp	Unexposed	Pre-exposure	0.284	0.187	1.518	0.2809402190	ns
	Watts	Alpha.Score	PrePostExp	Unexposed	Exposed	0.681	0.223	3.057	0.0061217113	*
	ZIRC	Alpha.Score	PrePostExp	Exposed	Pre-exposure	-0.239	0.218	-1.097	0.5128253758	ns
	ZIRC	Alpha.Score	PrePostExp	Unexposed	Pre-exposure	0.710	0.252	2.822	0.0129985294	*
	ZIRC	Alpha.Score	PrePostExp	Unexposed	Exposed	0.949	0.280	3.386	0.0020551850	*
Shannon	Gemma	Alpha.Score	PrePostExp	Exposed	Pre-exposure	0.313	0.197	1.584	0.2507345213	ns
	Gemma	Alpha.Score	PrePostExp	Unexposed	Pre-exposure	0.482	0.195	2.470	0.0358379651	*
	Gemma	Alpha.Score	PrePostExp	Unexposed	Exposed	0.169	0.230	0.737	0.7396581311	ns
	Watts	Alpha.Score	PrePostExp	Exposed	Pre-exposure	-0.237	0.217	-1.092	0.5172164942	ns
	Watts	Alpha.Score	PrePostExp	Unexposed	Pre-exposure	0.116	0.212	0.550	0.8456466801	ns
	Watts	Alpha.Score	PrePostExp	Unexposed	Exposed	0.353	0.247	1.431	0.3231294133	ns
	ZIRC	Alpha.Score	PrePostExp	Exposed	Pre-exposure	-0.345	0.217	-1.589	0.2476774623	ns
	ZIRC	Alpha.Score	PrePostExp	Unexposed	Pre-exposure	0.751	0.254	2.963	0.0084755914	*
	ZIRC	Alpha.Score	PrePostExp	Unexposed	Exposed	1.096	0.282	3.892	0.0003129239	*
Simpson	Gemma	Alpha.Score	PrePostExp	Exposed	Pre-exposure	0.284	0.249	1.138	0.4890374427	ns
	Gemma	Alpha.Score	PrePostExp	Unexposed	Pre-exposure	0.547	0.242	2.261	0.0610152077	ns
	Gemma	Alpha.Score	PrePostExp	Unexposed	Exposed	0.264	0.282	0.934	0.6170860681	ns
	Watts	Alpha.Score	PrePostExp	Exposed	Pre-exposure	-0.127	0.230	-0.550	0.8456815725	ns
	Watts	Alpha.Score	PrePostExp	Unexposed	Pre-exposure	-0.049	0.229	-0.214	0.9750433810	ns
	Watts	Alpha.Score	PrePostExp	Unexposed	Exposed	0.078	0.264	0.294	0.9531833954	ns
	ZIRC	Alpha.Score	PrePostExp	Exposed	Pre-exposure	-0.476	0.241	-1.974	0.1172492161	ns
	ZIRC	Alpha.Score	PrePostExp	Unexposed	Pre-exposure	0.657	0.263	2.502	0.0324305223	*
	ZIRC	Alpha.Score	PrePostExp	Unexposed	Exposed	1.133	0.299	3.794	0.0004999882	*

### 3.2) Beta Diversity

Distance-based redundancy analysis (dbRDA) ordination.  $\text{Beta.Score} \sim \text{Exposure}$

metric	term	Df	SumOfSqs	statistic	p.value	sig
Bray-Curtis	PrePostExp	1.00	0.1424569	2.285	0.029	*
	Residual	85.00	5.2981891			
Canberra	PrePostExp	1.00	0.5430310	2.236	0.001	*
	Residual	85.00	20.6384862			
Sørensen	PrePostExp	1.00	0.3442625	3.562	0.001	*
	Residual	85.00	8.2148177			

### 3.2.1)

Distance-based redundancy analysis (dbRDA) ordination. Beta.Score ~ Diets

metric	term	Df	SumOfSqs	statistic	p.value	sig
Bray-Curtis	Diet	2.00	0.8277048	7.536	0.001	*
	Residual	84.00	4.6129411			
Canberra	Diet	2.00	2.2980939	5.111	0.001	*
	Residual	84.00	18.8834233			
Sørensen	Diet	2.00	1.2544358	7.213	0.001	*
	Residual	84.00	7.3046444			

### 3.2.2)

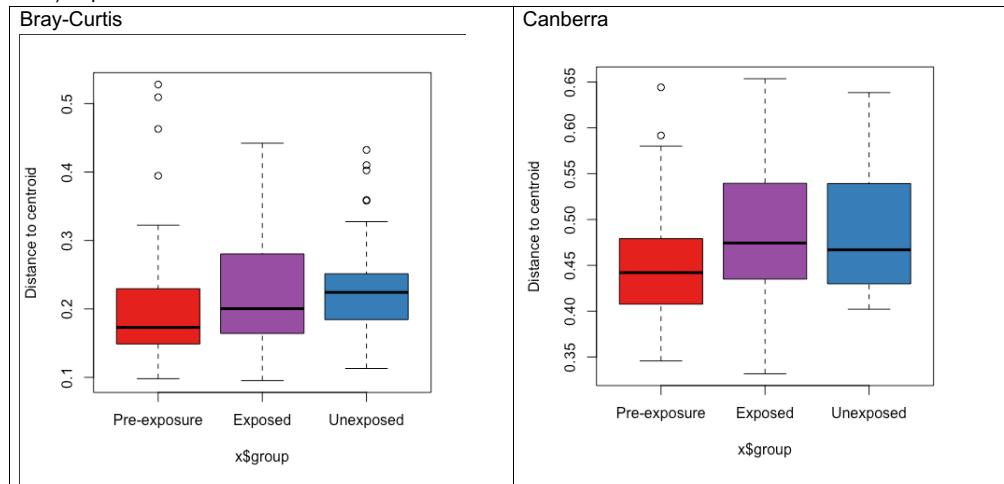
Distance-based redundancy analysis (dbRDA) ordination. Beta.Score ~ Diets

metric	term	Df	SumOfSqs	statistic	p.value	sig
Bray-Curtis	Diet	2.00	0.8277048	7.770	0.001	*
	PrePostExp	1.00	0.1487798	2.793	0.012	*
	Diet:PrePostExp	2.00	0.1496117	1.404	0.150	
Canberra	Residual	81.00	4.3145496			
	Diet	2.00	2.2980939	5.302	0.001	*
	PrePostExp	1.00	0.5498407	2.537	0.001	*
Sørensen	Diet:PrePostExp	2.00	0.7777731	1.794	0.001	*
	Residual	81.00	17.5558095			
	Diet	2.00	1.2544358	7.676	0.001	*
	PrePostExp	1.00	0.3486593	4.267	0.001	*
	Diet:PrePostExp	2.00	0.3376534	2.066	0.001	*
	Residual	81.00	6.6183317			

### 3.2.3

## 3.3) Beta-Dispersion

### 3.3.1) Exposure



Df	Sum Sq	Mean Sq	F	N.Perm	Pr(>F)
2	0.03124468	0.015622342	2.464166	999	0.088
173	1.09678687	0.006339808			

Names	p-value
Pre-exposure-Exposed	0.128
Pre-exposure-Unexposed	0.051
Exposed-Unexposed	0.799

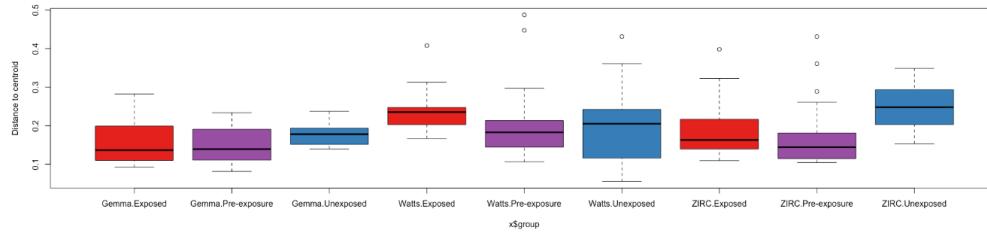
Df	Sum Sq	Mean Sq	F	N.Perm	Pr(>F)
2	0.04742029	0.023710144	6.02015	999	0.003
173	0.68135428	0.003938464			

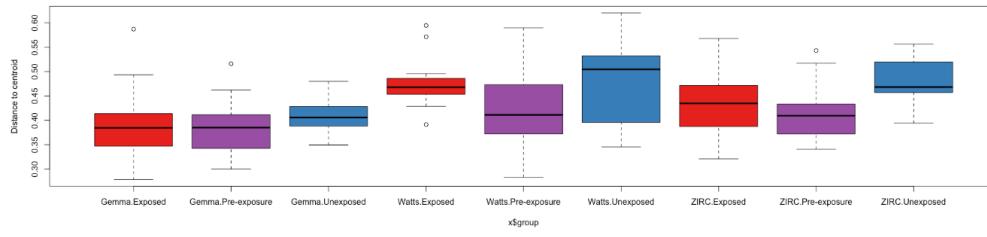
Names	p-value
Pre-exposure-Exposed	0.012
Pre-exposure-Unexposed	0.002
Exposed-Unexposed	0.741

### 3.3.2) Diet:Exposure

#### Bray-Curtis



#### Canberra



#### Bray-Curtis

Df	Sum Sq	Mean Sq	F	N.Perm	Pr(>F)
8	0.1710853	0.021385661	4.247249	999	0.001
167	0.8408750	0.005035179			

#### Canberra

Df	Sum Sq	Mean Sq	F	N.Perm	Pr(>F)
8	0.1775982	0.022199773	5.662318	999	0.001
167	0.6547429	0.003920616			

Names	p-value	sig	Names	p-value	sig
Gemma.Exposed-ZIRC.Unexposed	0.001	*	Gemma.Pre-exposure-Watts.Exposed	0.001	*
Gemma.Pre-exposure-Watts.Exposed	0.001	*	Gemma.Pre-exposure-Watts.Unexposed	0.001	*
Gemma.Pre-exposure-ZIRC.Unexposed	0.001	*	Gemma.Pre-exposure-ZIRC.Unexposed	0.001	*
Gemma.Unexposed-ZIRC.Unexposed	0.001	*	Gemma.Unexposed-Watts.Exposed	0.001	*
Gemma.Exposed-Watts.Exposed	0.002	*	Watts.Exposed-ZIRC.Pre-exposure	0.001	*
Gemma.Unexposed-Watts.Exposed	0.003	*	Gemma.Unexposed-ZIRC.Unexposed	0.002	*
ZIRC.Pre-exposure-ZIRC.Unexposed	0.004	*	Gemma.Exposed-Watts.Exposed	0.003	*
Watts.Exposed-ZIRC.Pre-exposure	0.005	*	ZIRC.Pre-exposure-ZIRC.Unexposed	0.003	*
Gemma.Pre-exposure-Watts.Pre-exposure	0.008	*	Gemma.Exposed-ZIRC.Unexposed	0.006	*
Gemma.Pre-exposure-ZIRC.Exposed	0.014	*	Watts.Unexposed-ZIRC.Pre-exposure	0.007	*
Gemma.Pre-exposure-Watts.Unexposed	0.016	*	Gemma.Pre-exposure-ZIRC.Exposed	0.015	*
Gemma.Pre-exposure-Gemma.Unexposed	0.019	*	Gemma.Unexposed-Watts.Unexposed	0.015	*
Watts.Pre-exposure-ZIRC.Unexposed	0.046	*	Gemma.Pre-exposure-Watts.Pre-exposure	0.018	*
ZIRC.Exposed-ZIRC.Unexposed	0.059		Gemma.Exposed-Watts.Unexposed	0.019	*
Watts.Exposed-ZIRC.Exposed	0.075		Watts.Exposed-Watts.Pre-exposure	0.019	*
Watts.Exposed-Watts.Pre-exposure	0.094		Watts.Pre-exposure-ZIRC.Unexposed	0.030	*
Gemma.Exposed-Watts.Pre-exposure	0.102		Gemma.Pre-exposure-ZIRC.Pre-exposure	0.031	*
Watts.Unexposed-ZIRC.Unexposed	0.138		Watts.Pre-exposure-Watts.Unexposed	0.049	*
Gemma.Exposed-ZIRC.Exposed	0.144		Gemma.Pre-exposure-Gemma.Unexposed	0.067	
			Watts.Exposed-ZIRC.Exposed	0.074	
			ZIRC.Exposed-ZIRC.Unexposed	0.100	

### 3.4) Differential Abundance

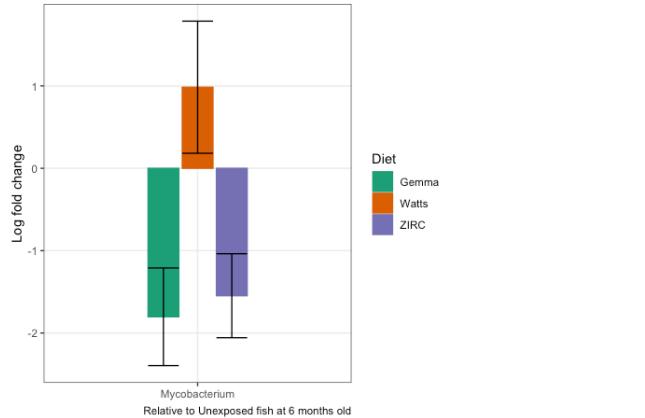
	Taxon	W	p_val	q_val	diff_abn
3.4.1)	Mycobacterium	26.596	<0.001	<0.001	TRUE

ANCOM-BC: Log Fold Change Abundance

Diet	Taxon	LFC	SE	P.value	direct	sig
Gemma	Mycobacterium	-1.802717	0.5926287	0.002350897	Negative LFC	*
Watts	Mycobacterium	0.984404	0.8020263	0.219673719	Positive LFC	
ZIRC	Mycobacterium	-1.547301	0.5099201	0.002410155	Negative LFC	*

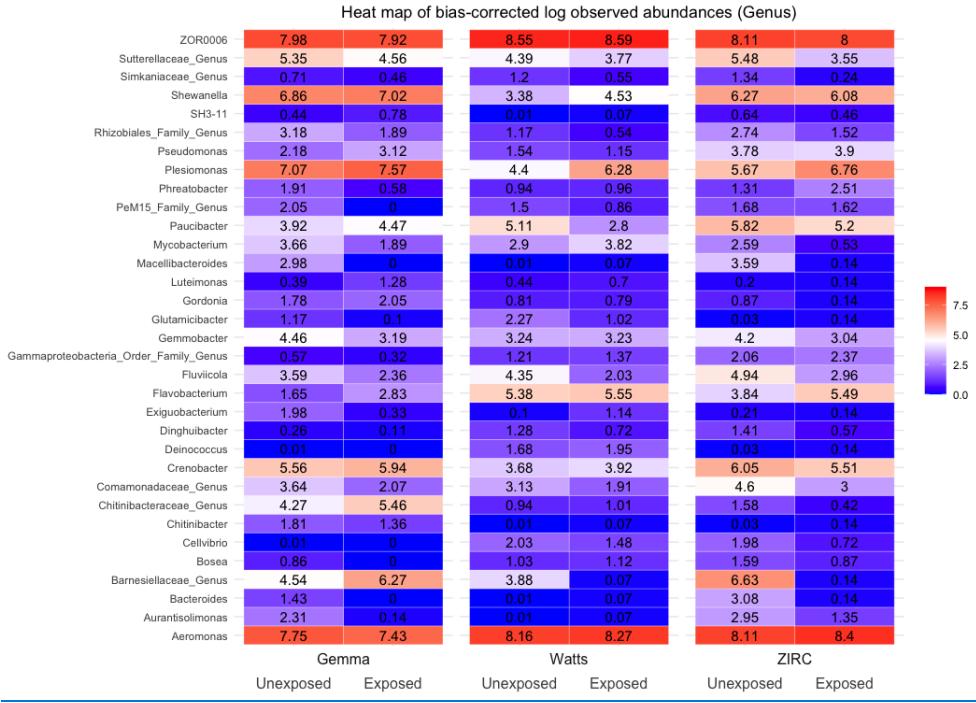
3.4.2)

Log-fold Change of Mycobacterium in Exposed Fish



3.4.3)

### 3.4.4)



**Page 5: [1] Commented [SJMJ30R29]** Sieler Jr, Michael James 10/17/22 10:52:00 AM

These different diversity measures allow us to probe the gut microbial diversity from a variety of facets. For instance, does diversity differ among more abundant, rarer or all taxa?

**Page 5: [2] Commented [SJMJ31R29]** Sieler Jr, Michael James 10/17/22 10:54:00 AM

Should I include a sentence noting this?

**Page 5: [3] Commented [SJMJ32R29]** Sieler Jr, Michael James 10/17/22 10:59:00 AM

Should I do a similar thing with beta-diversity metrics?

**Page 5: [4] Deleted** Sieler Jr, Michael James 10/20/22 9:38:00 AM

**Page 5: [5] Deleted** Sieler Jr, Michael James 10/17/22 4:49:00 PM

**Page 5: [6] Deleted** Sieler Jr, Michael James 10/17/22 4:53:00 PM

**Page 5: [7] Deleted** Sieler Jr, Michael James 10/17/22 11:11:00 AM

**Page 5: [8] Commented [SJMJ39R38]** Sieler Jr, Michael James 9/29/22 9:08:00 AM

Forgot to include table in SupFig

**Page 5: [9] Deleted** Sieler Jr, Michael James 10/17/22 5:40:00 PM

**Page 6: [10] Commented [SJMJ41]** Sieler Jr, Michael James 8/31/22 5:02:00 PM

Shannon wasn't significant between ZIRC and Gemma, but Simpson's is, so I elected to put Simpson here. Is that fair, or should I keep a consistent metric throughout and reference the significance in a table or suppl figure?

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**Page 6: [11] Deleted** Sieler Jr, Michael James 10/19/22 12:11:00 PM

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**Page 6: [13] Deleted** Sieler Jr, Michael James 10/19/22 12:08:00 PM

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**Page 7: [18] Commented [TS59]** Thomas Sharpton 9/29/22 5:49:00 AM

It's important to use all of the words necessary to ensure accurate grammar. Else you will be accused of being a lazy writer.

**Page 7: [19] Commented [TS60]** Thomas Sharpton 9/29/22 5:51:00 AM

So watts changes over time in composition, but beta-dispersion is consistent? That's interesting.

**Page 7: [20] Commented [TS62]** Thomas Sharpton 9/29/22 5:48:00 AM

I think I see what you are trying to get at, but this is a confusing way to phrase it. Perhaps unpack this a little bit more. Brevity is important, but clarity is critical.

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Page 7: [21] Deleted Sieler Jr, Michael James 10/20/22 11:03:00 AM

Page 7: [22] Commented [TS63] Thomas Sharpton 9/29/22 5:52:00 AM

The way this is phrased it sounds like you are discussing each diet's development.

Page 7: [23] Commented [SJM64R63] Sieler Jr, Michael James 9/29/22 9:48:00 AM

Got it. I'll reword this accordingly

Page 7: [24] Commented [TS66] Thomas Sharpton 9/29/22 5:58:00 AM

Variation in BCS between 3 and 6 months? It's not clear what is being discussed here.

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Page 7: [26] Commented [TS70] Thomas Sharpton 9/29/22 5:56:00 AM

Why not just state this in the prior sentence?

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**Page 7: [27] Deleted** Sieler Jr, Michael James 10/20/22 10:26:00 AM

**Page 7: [27] Deleted** Sieler Jr, Michael James 10/20/22 10:26:00 AM

**Page 10: [28] Commented [SJMJ107R106]** Sieler Jr, Michael James 9/29/22 9:18:00 AM

Move the sentiment in this sentence to the top near the “previous research” sentence?

**Page 10: [29] Commented [SJMJ109R108]** Sieler Jr, Michael James 9/29/22 9:27:00 AM

For instance, the protein difference between the highest and lowest protein content of the diets in our study was 13%, but in other studies it was at most 44%. Lipids: 3% vs 18%, Carbohydrate: 11% vs 70%, Ash: 9% vs 17%

**Page 10: [30] Commented [TS110]** Thomas Sharpton 9/29/22 5:53:00 AM

Missing from this discussion: why does this research matter? What have you learned? What does this knowledge effect? What is the impact of the work. You must spell it out - readers will not do it for you.

**Page 10: [31] Commented [SJMJ111R110]** Sieler Jr, Michael James 9/29/22 9:32:00 AM

The microbiome plays an important role in maintaining host health through nutrient digestion and supporting the immune system. We've shown here that subtle differences in diet composition significantly alter the gut microbiome structure, and in the case of ZIRC fed fish their physiology. Given that zebrafish studies do not employ a consistent diet regime across studies our results indicate that this could be producing non-protocol induced variability in study outcomes, especially in studies targeting the microbiome. Therefore, we suggest that zebrafish facilities and researchers consider diet as a confounding factor in their studies to minimize the impact of diet on study outcomes and improve ability for cross-study comparisons.

---

Why does it matter: Zebrafish studies don't control for diet and use very different diets. Our study shows that different diets result in different microbiome outcomes and in some instances physiological outcomes. The variance in the microbiome may be introducing non-protocol induced variation in study outcomes if diet isn't controlled or if similar diets aren't used across studies.

What does this knowledge effect?

The knowledge gained from this study should inform zebrafish facilities and researchers to maintain consistent diets across studies, particularly in microbiome-targeted studies. Given that the microbiome plays an important role in host health, different diets may support a microbiome that is more resilient to perturbations (e.g. pathogen exposure).

The impacts of the work:

The impact of this work is diet, even those that differ subtly, influences the microbiome of zebrafish.

**Page 10: [32] Commented [SJMJ113R112]** Sieler Jr, Michael James 9/29/22 9:57:00 AM

What I mean to say is that stochastic processes of environmental drift/dispersal of microbes into the gut microbiome of ZF becomes less influential as the host ages, while deterministic factors become more important. ZF gut microbiome assembles and stabilizes making it less likely that a microbe from the environment will colonize the gut if similar microbes are not already established there.

**Page 10: [33] Commented [SJMJ114R112]** Sieler Jr, Michael James 9/29/22 10:00:00 AM

Moreover, once the host has reached adulthood their physiology does not alter substantially until senescence allowing microbes to establish themselves along the gut epithelial cells.

Impacts of the immune system development, adaptive immunity coming online will impact gut microbes.

<b>Page 10: [34] Commented [TS115]</b>	<b>Thomas Sharpton</b>	<b>9/29/22 6:04:00 AM</b>
You like this phase. It works, but don't overuse it. TBH, I only like it in abstracts or introductions.		
<b>Page 10: [35] Commented [TS116]</b>	<b>Thomas Sharpton</b>	<b>9/29/22 6:06:00 AM</b>
This is an incomplete idea and not novel. What is more complete and novel is that you found that the successional development of the gut microbiome differs depending upon diet.		
<b>Page 10: [36] Commented [TS118]</b>	<b>Thomas Sharpton</b>	<b>9/29/22 6:06:00 AM</b>
The connection between this idea and the other side of the comma is confusing to me.		
<b>Page 10: [37] Commented [TS119]</b>	<b>Thomas Sharpton</b>	<b>9/29/22 6:08:00 AM</b>
This needs to be put somewhere near the top of the paper around study design.		
<b>Page 10: [38] Commented [SJM121R120]</b>	<b>Sieler Jr, Michael James</b>	<b>9/29/22 10:04:00 AM</b>
Gemma increases in feed size. Watts alters nutrient content but same ingredients. ZIRC goes from Larval diet to Adult diet, and adds two new diets into their "Adult Mix".		
<b>Page 10: [39] Commented [TS122]</b>	<b>Thomas Sharpton</b>	<b>9/29/22 6:10:00 AM</b>
Ah - I think you are saying that the feed changes at 4 months, and that these are the ways in which the feed changes. I was unaware of the switch at 4 months - I thought it happened earlier. So this is a bit of a confounding factor and we need to give this important detail a bit of thought. Are we actually looking at successional dynamics here?		
<b>Page 10: [40] Commented [SJM123R122]</b>	<b>Sieler Jr, Michael James</b>	<b>9/29/22 10:03:00 AM</b>
I did not know this either until I reread Colleens methods. There is a similar issue in Stephens 2016 and Xiao 2020/21 where feed changes appear to line up with variance in gut microbiome		
<b>Page 10: [41] Commented [TS124]</b>	<b>Thomas Sharpton</b>	<b>9/29/22 6:10:00 AM</b>
swing the bat around - how does this connect to the argument you are making and what's the implication?		
<b>Page 10: [42] Commented [TS125]</b>	<b>Thomas Sharpton</b>	<b>9/29/22 6:11:00 AM</b>
We need more from you here. Otherwise, it's hard to see the point you are making.		
<b>Page 10: [43] Commented [TS126]</b>	<b>Thomas Sharpton</b>	<b>9/29/22 6:12:00 AM</b>
Same comment - it's not entirely clear where you are going here.		
<b>Page 10: [44] Commented [TS127]</b>	<b>Thomas Sharpton</b>	<b>9/29/22 6:12:00 AM</b>
Have you shown here that this is the case?		
<b>Page 10: [45] Commented [TS129]</b>	<b>Thomas Sharpton</b>	<b>9/29/22 6:13:00 AM</b>
What does this mean given that it's only ZIRC fish?		