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

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**Abstract**

Despite the long-established importance of zebrafish as a model organism and their increasing use in microbiome-targeted studies, there is a lack of consensus regarding husbandry practices involving diet. ~~Diet is known to influence the structure of the gut microbiome and physiology of zebrafish~~. Given, the microbiome’s important role in mediating host physiology and the potential for diet to drive variation in microbiome composition, efforts to evaluate zebrafish diets should consider their impact on the microbiome. To clarify the impact of diet on the zebrafish gut microbiome, we grew NNN fish on one of three different common laboratory diets and measured how the microbiome’s successional development varies as a function of diet. We also exposed fish to *Mycobacterium chelonae,* a prolific intestinal pathogen of zebrafish, to discern if dietary background impacts the microbiome’s sensitivity to infection. We found that 3 month old zebrafish gut microbiomes stratify by diet, and these effects accumulated across development that resulted in diet-dependent differences in the microbiome and physiology at 6 months of age. Furthermore, we found that different diets modulated the gut microbiome’s sensitivity to exposure to *M. chelonae.* pathogen exposure depended on diet. Our results demonstrate that variation in husbandry practices around diet impacts the composition of the gut microbiome. Collectively, our results indicate that researchers should carefully consider the role of diet in their zebrafish microbiome studies and that diet should be controlled for when integrating microbiome data across studies.

**Introduction**

Despite zebrafish’s long-established importance as a model organism and their increasing use in microbiome-targeted studies, key knowledge gaps remain about how diet influences their microbiome. In contrast to mice, zebrafish do not have a standard reference diet (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 zebrafish gut microbiome communities differ between commonly used laboratory diets, and if these differences persist throughout development.

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

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

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

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

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| **Figure 1:** Effects of fish fed one of three diets (Gemma, Watts, or ZIRC) on physiology and microbiomes of zebrafish. **(A)** Weight of ZIRC significantly differs from Watts and Gemma. Gemma and Watts do not differ from each other. **(B)** Body condition score is a length normalized measure of weight. ZIRC fed fish have significantly higher body condition scores from Gemma and Watts diets. **(C)** 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. | |

<Results>

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

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

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

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

<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, we our results demonstrate that the gut microbiomes of 3-month-old zebrafish differ by diet, and highlights the importance of minor nutritional differences ability to affect the microbiome and physiology of zebrafish.

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

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| **Figure 2:** Development is associated with altered microbiome composition. **(A)** Shannon Index for diversity of 3 and 6 month old fish, and **(B)** appears to be diet dependent. Capscale ordination of gut microbiome composition based on the **(C)** Bray-Curtis dissimilarity by diet and **(D)** Canberra measure by time. **(E)** Body condition score did not differ between time points for either diet, but in ZIRC fed fish a **(F)** body condition score negatively associated gut microbiome diversity. The analysis shows that physiology and gut microbiome composition significantly differs between the diets across development, and there may be diet-dependent link with physiology. “ns” indicates not significantly different, \* indicates significant differences below the 0.05 level. | | |

<Results>

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

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

<Discussion>

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 temporarily sensitive, and abundant taxa are sensitive to diet. Watts fed fish experienced less variation over time. At four months of age, all fish were switched from juvenile to adult diets. ZIRC adult feed incorporates several new feeds and minor changes to nutritional composition. Gemma adult feed changes in size but not nutrition. Watts feed decreases in lipid content, but the ingredients remain the same. Changes in nutritional composition can alter the microbiome (Rawls). Xiao and Stephens observed similar increases in gut 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 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.

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

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

<Results>

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

<Discussion>

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.

**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.

**Supplementary Tables and Figures**

**1) Diet**

**1.1) Physiology**

1.1.1)Table

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**1.2) Alpha Diversity**

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**1.3) Beta Diversity**

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**1.4) Beta-Dispersion**

1.4.1) Diet

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| Bray-Curtis | Canberra |
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**2) Development**

**2.1) Physiology**

2.1.1)Table

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**2.2) Physiology ~ Microbiome**

2.2.1) Table

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**2.3) Alpha Diversity**

2.3.1) **Time**

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2.3.1.2) Table

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2.2.1.3) Table

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2.2.2) **Time:Diet**

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**2.4) Beta Diversity**

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**2.5) Beta-Dispersion**

**2.5.1) Diet**

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**2.5.2) Time**

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**2.5.3) Diet:Time**

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| Bray-Curtis | Canberra |
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**2.6 Differential Abundance**

2.6.1)Table

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2.6.2) Chart, bar chart

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**3) Exposure**

**3.1) Alpha Diversity**

**3.1.1) Exposure**

3.1.1.1) Table

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3.1.1.2) Table

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3.1.1.3) Table

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3.1.2) Diet:Exposure

3.1.2.1) Table

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**3.2) Beta Diversity**

3.2.1) Table

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3.2.2) Table

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3.2.3Table

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**3.3) Beta-Dispersion**

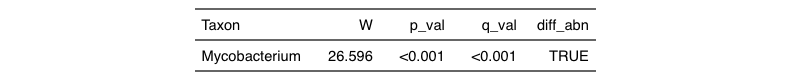
3.3.1) Exposure

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| Bray-Curtis | Canberra |
|  |  |

3.3.2) Diet:Exposure

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

**3.4) Differential Abundance**

3.4.1)

3.4.2) Table

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3.4.3) Chart, box and whisker chart

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