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Assessment of Various Standard Fish Diets on Gut Microbiome of Platyfish *Xiphophorus maculatus*

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Abstract

Diet is an external factor that affects the physiological baseline of research animals. It can shape gut microbiome, which can impact the host. As a result, dietary variation can challenge experimental reproducibility and data integration across studies when not appropriately considered. To control for diet-induced variation, reference diets have been developed for common biomedical models. However, such reference diets have not yet been developed for non-traditional model organisms, such as *Xiphophorus* species. In this study, we compared two diets designed for zebrafish, a commercial zebrafish diet (Gemma, GEM), and a proposed zebrafish reference diet developed by the Watts laboratory at the University of Alabama at Birmingham (WAT) to the *Xiphophorus* Genetic Stock Center custom diet (CON) to evaluate the influence of diet on the *Xiphophorus* gut microbiome. *Xiphophorus maculatus* were fed the three diets from two to six months of age. Feces were collected and the gut microbiome was assessed using 16S rRNA sequencing every month. We observed substantial diet-driven variation in the gut microbiome. Our results indicate that diets developed specifically for zebrafish can affect the gut microbiome composition and may not be optimal for *Xiphophorus*.

Introduction

Dietary variations drive phenotypical variations in laboratory animals. These diet-influenced phenotypes include growth, reproduction, and survival metrics. Development of standard diets for rodent model organisms demonstrated that such diets minimized the dietary contribution to variation in experimental results and, thus, enhanced data reproducibility.

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Conflict of Interest:
The authors do not have conflict of interest.

Although standardized diets were developed for many additional model organisms since 1970's, such diets have not yet been established for most aquatic model organisms.

Xiphophorus are a commonly used model organism that have been involved in evolution, behavior, metabolism, development, reproduction, sex determination, disease etiology, toxicology, and genetics research [1]. However, there is not a standardized reference diet established for *Xiphophorus*. Our previous study comparing two zebrafish reference diets to a custom diet in *Xiphophorus* fish demonstrated that the reference diets tailored for zebrafish inhibited *Xiphophorus* fish growth and decreased fecundity [2]. Although it has been demonstrated that diet can influence gut microbiome, dietary impact on *Xiphophorus* gut microbiome has not been assessed [3–6]

The gut microbiome consists of millions of microbes that include archaea, bacteria, fungi, protozoa, and viruses [7]. The variety of microbe species, host cell types, and microbiome-host interactions make the gastrointestinal tract one of the most complex organ systems. The microbes of the gut microbiome can coordinate with the various components of this complex organ to impact host physiology in diverse ways (e.g., B vitamins production [8]; short-chain fatty acid production [9]; neurotransmitter gamma-aminobutyric acid production [10]). An imbalance in the composition of the gut microbiome is associated with many disorders (e.g., inflammatory bowel syndrome [4]; autism, Alzheimer's, and Parkinson's disease [4]; obesity and type 2 diabetes [5]). Fish gut microbiome studies have been limited to a few commercial species (i.e., catfish, bass, salmon) and the biomedical model organism *Danio rerio* (zebrafish). Vatsos et al. suggested that the gut microbiome is a variable in fish experiments because it affects host homeostasis [11]. It has been shown that the gut microbiome is influenced by multiple environmental factors including water salinity [12] and antibiotics administration [13]. However, how diet influences the health conditions of other aquatic biomedical models is relatively understudied.

We hypothesize that diet impacts the *Xiphophorus* gut microbiome profile. To test this hypothesis, we compared the gut microbiome of *Xiphophorus* fed with a custom feeding regime (CON) developed by the *Xiphophorus* Genetic Stock Center (XGSC), Gemma diet made by Skretting Zebrafish (GEM), and a zebrafish reference diet developed by the University of Alabama, Birmingham (WAT). Gaining further insight into how diet affects the gut microbiome of *Xiphophorus* fish is indispensable in establishing a *Xiphophorus* reference diet that ensures reproducible experimental results and enables inter-laboratory comparisons.

Materials and Methods:

Research Animal

All animals included in the present study belong to a subgroup of the animals used in an earlier study [14]. Specifically, this study was conducted in accordance with the ethical guidelines for animal research approved by the Institutional Animal Care and Use Committee at Texas State University (protocol #7234). Platyfish (*Xiphophorus maculatus* strain *JPWild*) were obtained from XGSC at Texas State University, San Marcos, TX. Three 37.8 L tanks per dietary treatments were used for each species. Environmental temperature

was at a constant 25°C and fish were maintained on a 13-hour light: 11-hour dark cycle. At 1 month of age, platyfish were separated into 3 tanks per diet (9 tanks total) at a density of n = 10 fish/tank. From two to six months of age, five fish were randomly selected from different aquariums of each dietary group every month for microbiome analysis, and returned to the original aquarium following the fecal sample collection.

Dietary Treatments

Per dietary group, fish were fed twice daily Monday to Saturday (0800 h and 1600 h) and once on Sunday (0800 h) at 3% body weight (BW) per day. The 3% body weight was determined by previous studies focusing on assessing dietary impact on overall wellness of zebrafish [15]. Weight of different diet or feeding regime was all determined by dry material weight. Per aquarium and feeding, we provided food equivalent to 1.5% of the total fish weight (the sum of all fish weight in the aquarium). However, we did not account for interindividual food intake variation. The three dietary treatments that were tested are: an in-house feeding regime by XGSC consisting of Ziegler flakes (Zeigler Bros, Inc., Gardners, PA), live *Artemia nauplii* (BIO-MARINE®, Hawthorne, CA), and Gordon's beef liver paste (Control, Table 1) [16]; Skretting Gemma Micro150 for juvenile and 300 for adult fish (Skretting Zebrafish, Westbrook, ME; GEM; Table 1); and a zebrafish reference diet developed by the University of Alabama, Birmingham (WAT, Table 1). Ziegler flakes, GEM pellets, and WAT pellets were ground to a fine powder prior to feeding.

Fish were fed according to their life phase: the juvenile phase was defined as 1 to 3 months of age, and the adult phase was 4 to 6 months of age. For Control group juveniles, fish received 50% of dietary allowance as Ziegler Aquatox flake and 50% as live *Artemia nauplii*. Adult Control fish received 25% of dietary allowance as Ziegler Aquatox flake, 50% as live *Artemia nauplii*, and 25% as beef liver paste. For GEM, both juvenile and adult fish were fed the same formulation at the full allotted amount. The WAT diet had two different formulations for juvenile and adult fish and were fed to different growth phases of the two species accordingly. Experiment design is outlined in Fig. 1a.

Flake food portions were controlled using custom, 3-D printed, feeding spoons at the Zebrafish International Resource Center (ZIRC; https://www.thingiverse.com/thing:2855202). Spoon volumes were adapted to the specific densities of flake diets and number of fish per tank.

Proximate and elemental analysis of diets was performed by Eurofins Inc. commercial laboratory for GEM, WAT, and Ziegler flakes. Nutritional contents were obtained from the manufacturer for *Artemia nauplii* and estimated from the USDA food database for beef liver paste [2]. Proximate and elemental analysis results are listed in Supplementary Table S1.

Feces collection and fecal DNA isolation

On fecal collection day, fish were fed at 8:00 am and were subsequently placed in an individual watch glass. Fish were monitored hourly for defecation between 8:30 am to 5:00 pm, and fecal matter was immediately collected using a micropipette with wide-bore tips and stored at −80°C. Fish were returned to their original aquarium following fecal sample

collection. Fecal DNA was isolated using DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), and quantified using Qubit (Thermo Fisher Scientific, Waltham, MA).

16S ribosomal RNA library preparation and next generation sequencing

Polymerase chain reaction (PCR) was performed using MiSeq primers targeting the 16S rRNA v4 region to produce sequencing libraries [17]. Reaction mixtures for each sample included 4 μL 5X SuperFi II Buffer (Thermo Fisher Scientific, Waltham, MA), 0.4 μL Platinum SuperFi II DNA Polymerase (Thermo Fisher Scientific, Waltham, MA), 8.2 μL UltraPure distilled water, 0.4 μL dNTP mix (10 mM) (Applied Biosystems, Waltham, MA), 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), and 5 μL of diluted DNA (5 ng of DNA in 5μL) for a total volume of 20 μL. Samples were placed in a thermocycler using the following parameters: initial denaturation at 95°C for 3 min, 35 cycles of 98°C for 20 s, 60°C for 15 s, and 72°C for 30 s, then a final step at 72°C for 5 min. A second round of PCR was performed using 5 μL of product from the first PCR and unique forward and reverse primer combinations for each sample. The second round of PCR followed the same parameters, with the number of cycles reduced from 15 cycles.

Amplicon libraries (2x250bp) were sequenced with the Illumina MiSeq platform at the Department of Biology at Texas State University. Sequencing reads were filtered and trimmed in R using the DADA2 pipeline [18]. In total, there were 2,247,096 sequencing reads generated with an average of 32,101 reads per sample. A total of 1,231 amplicon sequence variants (ASV) were identified and 726 of those ASVs had at least two counts. Samples with less than 3,000 reads were removed from analysis and the rest of the sequences were rarefied to 3,662 reads (n=69). Abundance plots at the phylum and genus levels were generated in R using phyloseq, ggplot2, and tidyverse packages [19–21] (raw data in Supplement Table S1–S2). Shannon's diversity index and the distance-based redundancy analysis (dbRDA) plot were calculated and visualized using R packages vegan and ggplot2 [20, 22]. Kruskal-Wallis rank sum test was used to analyze statistical significance for alpha diversity. The adonis function in vegan was used to compute permutational multivariate analysis of variance (PERMANOVA) of the microbiome beta-diversity. Linear discriminant analysis (LDA) and effect size (LEfSe) was performed using MicrobiomeAnalyst to determine differentially abundant taxa between groups (i.e., dietary treatments and ages) [23, 24]. Taxa with a False Discovery Rate-adjusted (FDR-adjusted) value of P<0.05 were considered statistically significant.

Results

Assessment of *X. maculatus* gut microbiome

We profiled the gut microbiome using 16S rRNA sequencing. The gut microbes were categorized at phylum (Fig. 1b) and genus level definition (Fig. 1c). We calculated the relative abundance of 51 genera.

Dietary treatments and age do not affect alpha diversity

We first assessed alpha diversity, which is a measure of the diversity within a sample and can be quantified using Shannon's Diversity Index [25–27]. We calculated Shannon's

diversity index for the gut microbiome of each fish in all dietary groups. Shannon diversity H-values were 2.12 ± 0.11, 2.10 ± 0.14 and 1.85 ± 0.17, respectively (Supplementary Figure S1). Kruskal-Wallis analyses indicated alpha diversity scores between dietary treatments were not significantly different ($P>0.05$). Other alpha diversity metrics were also calculated (Supplementary Table S4), but none were statistically different across dietary treatment.

Shannon's diversity index was also calculated based on fish age (i.e., 2, 3, 4, 5, and 6 months; Supplemental Figure S2; Supplementary Table S5) for all fish, regardless of diet. Kruskal-Wallis rank sums test indicated alpha diversity scores between age groups were not statistically different from one another ($P>0.05$).

Gut microbiome composition is affected by dietary treatment and age

We measured the beta diversity of microbiome composition between dietary treatment and age groups. Our PERMANOVA analysis indicates that gut microbiome composition across dietary groups were statistically different from one another ($P<0.001$; Fig. 2a). Proteobacteria, Firmicutes, and Bacteroidetes were the three most prevalent phylum in all platyfish gut microbiome profiles (Fig. 2b). This is consistent with earlier studies performed on various fish gut microbiomes [28, 29]. Although each diet featured the same top three phyla, the prevalence of Bacteroidetes and Firmicutes was different depending on dietary treatment. The GEM diet demonstrated a higher abundance of Firmicutes compared to the Control and WAT diet (LDA score 2.52, $P<0.05$), whereas Bacteroidetes was more abundant in the WAT diet (LDA score 2.37, $P<0.05$) (Fig. 2b). At the genus level, seven genera were the most abundant across all fish gut microbiomes: *Aeromonas, Pseudomonas, Klebsiella, Exiguobacterium, Flavobacterium, Metabacillus*, and *Cloacibacterium* (Fig. 2c). These results are also consistent with previous studies [11, 30]. Of these genera, *Pseudomonas* was more prevalent in fish receiving the Control diet (LDA score 2.68, $P<0.05$), *Metabacillus* was more prevalent in fish receiving the GEM diet (LDA score 2.11, $P<0.05$), and *Cloacibacterium* was most prevalent in fish receiving the WAT diet (LDA score 2.09, $P<0.05$) (Fig. 2c).

LEfSe was also used to identify differentially abundant taxa in different age groups. At the phylum level, Proteobacteria was strongly associated with the oldest age group (LDA score 2.76, $P<0.05$) and Bacteriodetes (LDA score 2.41, $P<0.05$) was strongly associated with fish that were three months old (Supplemental Figure S3a). At the genus level, *Aeromonas* (LDA score 2.85, $P<0.05$) was strongly associated with fish that were five months old, *Cloacibacterium* (LDA score 2.31, $P<0.05$) was strongly associated with fish that were two months old, and *Flavobacterium* (LDA score 2.3, $P<0.05$) was strongly associated fish that were three months old (Supplemental Figure S3b).

Discussion

In this study, we assessed the influence of diet on the gut microbiome of *X. maculatus*. This study is to expand our understanding of dietary impacts on *Xiphophorus* gut microbiome.

There are two main findings from this comparative diet study. First, dietary treatment did not change the gut microbial complexity of individual fish. Second, dietary treatments

of the zebrafish diets to *X. maculatus* exhibited major change to the gut microbiome compared to the custom *Xiphophorus* diet. In an earlier study, we demonstrated that the two diets formulated for zebrafish, GEM and WAT, negatively impacted growth, survival rate, and fecundity of *Xiphophorus* [2]. Together with findings from the current study, we have a clearer picture of the influence of diet on *Xiphophorus*. The present study did not investigate the causality between gut microbiome changes and decreased growth associated with zebrafish diets. Our findings do however complement previous studies demonstrating the need for the following factors to be adhered to when designing a reference diet for model organisms.

First, open formula for a reference diet is required. Knowing the source of nutritional values is critical because different ingredients may introduce different amounts of nutrients and/or contaminants [31]. For example, soybean meal has been used in fish diets instead of fishmeal as an alternative source of protein. Their different nutrient profiles have been shown to impact growth and health [32]. Second, sources of dietary components need to be reliable and traceable. Diet is an environmental factor that can affect reproduction, growth, disease, and responses to extrinsic variables [31]. Traceable and reliable nutrient sources guarantee reproducibility of the reference diet. Third, live feed (e.g., *Artemia nauplii* and paramecia) should be avoided. Not only do live-feeds not meet the above three requirements, but they also serve as a major carrier of pathogens[33, 34].

Conclusion:

Taken together with findings from our previous study, our results suggest zebrafish reference diets may not be optimal for maintaining other aquatic models, including *Xiphophorus* fish.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement

The raw data that support the findings of this study are available on request from the corresponding author, YL. Derived data supporting the findings of this study are available in supplementary tables.

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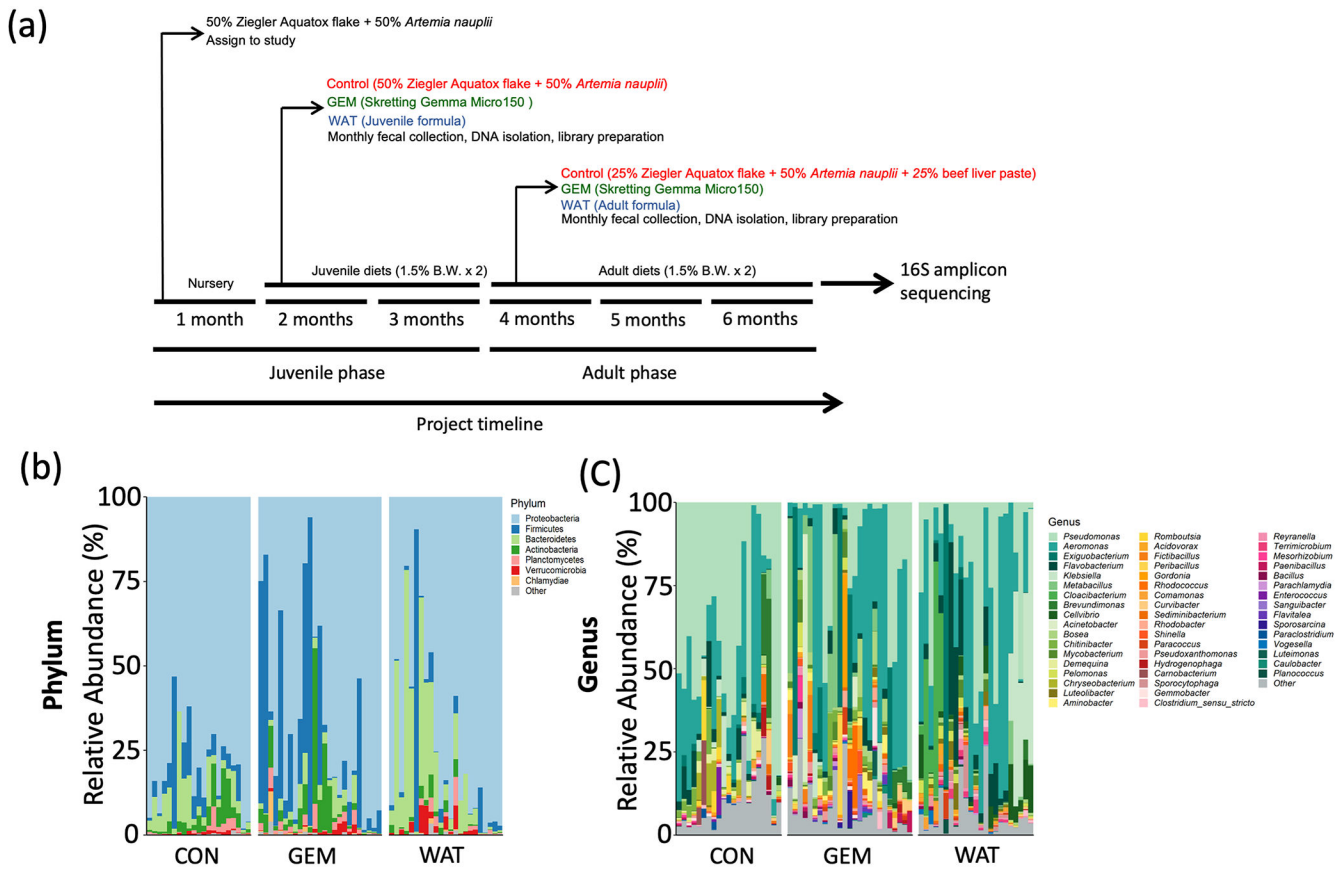

Figure 1. Relative abundance of bacterial phyla and genera in fecal samples of *Xiphophorus* on different feeding regimes

(a) Experiment design and sample collection timeline is displayed. Relative abundance plots were generated at (b) phylum and (c) genus level and grouped by feeding regime. Size of stacked bar represents relative compositions of taxonomy.

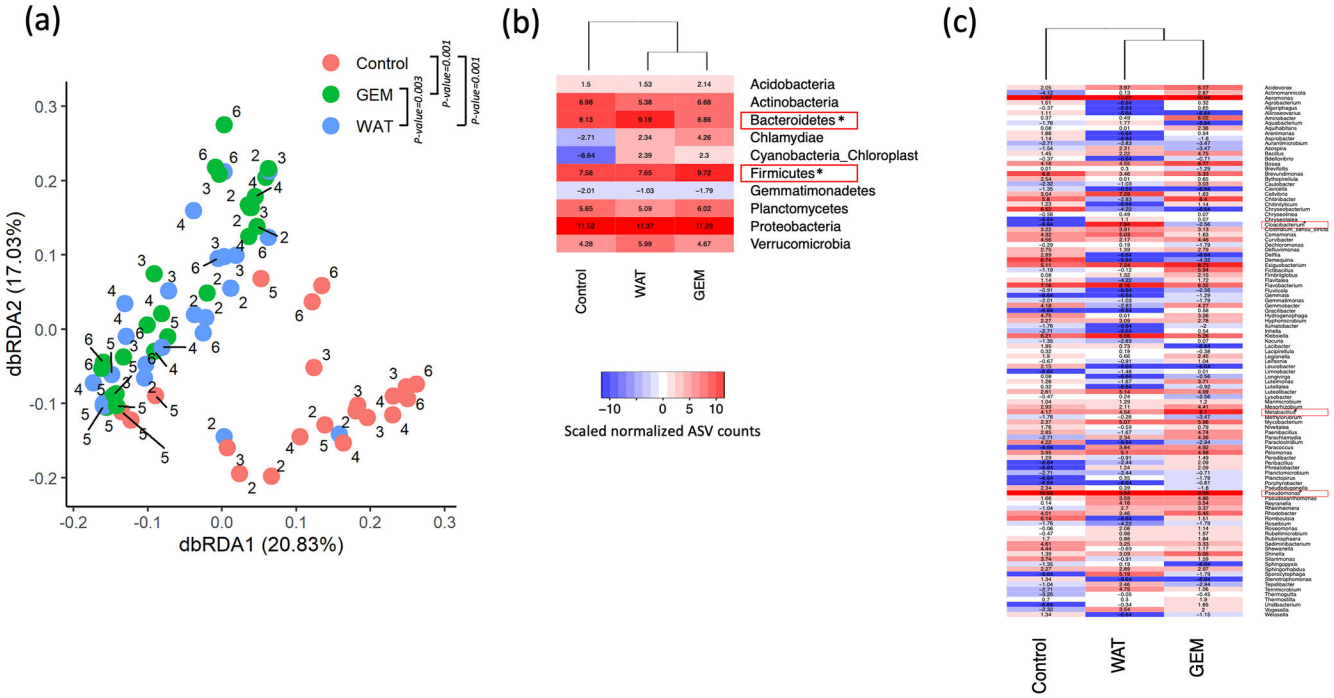

Figure 2. Beta Diversity of bacterial ASVs in fecal samples of *Xiphophorus* between different feeding regimes

(a) The dbRDA plot was based on Bray-Curtis dissimilarity. Different colors indicate dietary treatment and numbers indicate age in months. PERMANOVA results for inter-feeding regime comparisons are shown as *p*-values per comparison group. All *p*-values were corrected for multiple comparisons using Benjamini-Hochberg. Heatmaps show *Xiphophorus* fecal bacterial (b) phyla and (c) genera that were affected by feeding regime. Color and number of each heatmap tile represent scaled normalized ASV counts. Bacterial phylum and genus that were differentially enriched in gut microbiome of different feeding regimes were determined using FDR < 0.05 and LDA ≥ 2.0, and are highlighted using red text box.