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Sustained changes in digestive physiology and microbiome across sequential generations of zebrafish fed different diets

Samantha C. Leigh [a,*], Caitlyn Catabay [b], Donovan P. German [b]

[a] Department of Biology, California State University Dominguez Hills, Carson, CA 90747, USA
[b] Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697, USA

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ABSTRACT

Alterations to ratios of protein and fiber in an organism's diet have been shown to structurally and functionally alter its individual digestive physiology. However, it is unclear how these dietary changes may affect phenotypic changes across generations. We utilized feeding trials, morphological analyses, enzyme activities, and 16S rRNA sequencing of the gut microbiome of zebrafish (*Danio rerio*) to determine how variations to fiber and protein concentrations, kept consistent across sequential generations, affect phenotypic changes. Our results show that Parental (P) and first generation (F_1) fish did not differ from each other in terms of their intestine length, intestine mass, enzyme activity levels, and microbial community composition for any of the three experimental diets (high-protein/low-fiber, moderate-protein/fiber, and low-protein/high-fiber). However, each of the three experimental diets for the P and F_1 fish, as well as the ancestral diet fish, did have distinct microbial community structure from one another. This indicates that there is a strong dietary effect on digestive physiology and gut microbial community and that these effects are consistent when the diet is kept homogenous across generations.

1. Introduction

Vertebrates are an exceptionally diverse group, and thus, they consume a vast variety of different food items (Stevens and Hume, 1995; Karasov and Hume, 1997; Karasov and Douglas, 2013; Karasov and Martínez del Rio, 2007). Given their diverse diets, different taxa exhibit variations in their digestive morphology (including differences in gut length, mass, and structure; German and Horn, 2006; Wagner et al., 2009; German et al., 2010a, 2010b; He et al., 2013; Leigh et al., 2018a; Herrera et al., 2022) as well as in their digestive function (such as production of digestive enzymes, activity of nutrient transporters, and microbial activity; Buddington et al., 1987; Harpaz and Uni, 1999; Krogdahl et al., 2003; German et al., 2004, German et al., 2010a, 2010b; He et al., 2013; Day et al., 2014; Kohl et al., 2016; Clements et al., 2017; Verri et al., 2017; Leigh et al., 2018a; Parris et al., 2019; Wehrle et al., 2020; Leigh et al., 2021; Herrera et al., 2022). Specifically, variations in the amount of protein and fiber in an individual organism's diet have been shown to have an effect on the structure and function of the digestive system (e.g. Sabat et al., 1998; Karasov and Martínez del Rio, 2007; German et al., 2014; Ribeiro et al., 2015; Król et al., 2016; Leigh et al., 2018a; Herrera et al., 2022). For example, a diet high in protein is typically characterized by a short digestive tract, lower epithelial intestinal surface area, high levels of protein degrading enzymes, and a less diverse microbial community structure (Reimer, 1982; Linder et al., 1995; Kramer and Bryant, 1995; Sabat et al., 1998; Levey et al., 1999; German et al., 2004; German and Horn, 2006; Liu et al., 2016; Leigh et al., 2018a; Herrera et al., 2022). Conversely, a diet high in fiber is typically characterized by a long digestive tract, higher epithelial surface area, increased enterocyte volume, and a more diverse microbial community structure (Olsson et al., 2007; Santigosa et al., 2008; Wagner et al., 2009; Lin and Luo, 2011; Li et al., 2014; Kohl et al., 2016; Yaghoubi et al., 2016; Liu et al., 2016; Leigh et al., 2018a). These differences are related to intake and transit time, with high-fiber diets requiring high-intake, which leads to more rapid transit of material through the gut (Karasov and Martínez del Rio, 2007; German, 2011). The opposite is true for high-protein diets (e.g., Fris and Horn, 1993).

When an animal transitions to a new diet, there can be incredible plasticity of the digestive system. Such phenotypic changes of the digestive tract in response to alterations of the diet has been observed in numerous terrestrial (e.g. Sabat et al., 1998; Levey et al., 1999; Karasov and Martínez del Rio, 2007; Karasov and Douglas, 2013; Kohl et al., 2016) and aquatic systems (e.g. Choat and Clements, 1998; Grossel

* Corresponding author.
E-mail address: sleigh@csudh.edu (S.C. Leigh).

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et al., 2011; He et al., 2013; Gunter et al., 2013; Leigh et al., 2018a, 2018b; Herrera et al., 2022). Whether these structural and functional changes remain consistent in subsequent generations consuming the same diet is unclear. Generally, studies focus on altering the diet of either the Parental (P) or the first filial generation (F_1), but do not keep the altered diet consistent for both generations and track changes through F_1 and beyond (Fontagné-Dicharry et al., 2017; Xu et al., 2019; Hou and Fuiman, 2020; Zarantoniello et al., 2021). Furthermore, previous studies have historically compared different protein sources to one another rather than comparing high-protein to high-fiber diet types. For example, zebrafish (*Danio rerio*) fed varying proportions of fish meal protein and insect protein did not exhibit any significant differences in growth or gut health (i.e. intestinal inflammation) between the P and F_1 generations (Zarantoniello et al., 2021). However, plant-based diets differing in their methionine levels fed to the P generation of rainbow trout (*Oncorhynchus mykiss*) affected growth and low-density lipoprotein levels in the blood of the F_1 fish (Fontagné-Dicharry et al., 2017). None of these studies compared high-fiber to high-protein diets, kept the diet perturbations consistent across sequential generations, nor did they examine dietary induced changes in digestive tract size (length and mass), digestive enzyme production, nor alterations to the gut microbiome.

In this study, we used zebrafish (*Danio rerio*) as a vertebrate model to determine how variations of fiber and protein concentrations in the diet affect phenotypic changes across generations (Fig. 1). In Leigh et al. (2018a), we saw variations in gut structure and function of fish fed varying levels of protein and fiber across just one generation (an ancestral generation, "A" and a parental generation, "P"). Fish from the P generation on the high-fiber diet (also known as the "herbivore" diet) exhibited the longest guts with the largest intestinal epithelial surface area and enterocyte cellular volumes. The fish on the high protein diet (or "carnivore" diet) exhibited the shortest guts as well as the smallest intestinal epithelial surface area and enterocyte cellular volumes, but also had the largest terminal body mass. Fish on the carnivore diet generally exhibited low digestive enzyme activities. This was true for carbohydrate-degrading enzymes (amylase and maltase), a protein-degrading enzyme (aminopeptidase), as well as a lipid-degrading enzyme (lipase). Elevated lipase activities in the P generation fish on the herbivore diet were also observed, perhaps suggesting a lipid scavenging mechanism in those fish consuming high-fiber foods (Heras et al., 2020). Are these patterns maintained in the F_1 generation? Moreover, it is possible that enteric microbes were playing a role in the patterns of gut structure and function that we observed previously (Leigh et al., 2018a).

Hence, we continued the feeding trial of Leigh et al. (2018a), providing the F_1 generation fish the same carnivore, omnivore, and herbivore diets (Table 1), and examined the effects of protein and fiber content on gut size and digestive enzyme activities, and compared the observed patterns with the A and P generation fish (Fig. 1). Uniquely, we performed 16S rRNA sequencing of the gut microbiome for the A, P, and F_1 generation fish, thus allowing us to observe how the microbiome has shifted across generations following a dietary shift. We would expect the F_1 generation to exhibit similar, if not further exaggerated, gut structure and function characteristics to those of the P generation (i.e., F_1 fish on the herbivore diet would still exhibit the longest gut length compared to the fish fed the carnivore or omnivore diets). In terms of microbial diversity, we would expect the P and F_1 fish on the herbivore diet to possess the most diverse community structure when compared to the other two diet types. Typically, the zebrafish gut microbiome is dominated by Pseudomonodota and Actinomycetota (Ma et al., 2020). Known herbivorous fish have been shown to possess microbes such as those in the phyla Bacillota (such as family Clostridiaceae), and Bacteroidota among others (Clements et al., 2007; Sullam et al., 2012; Liu et al., 2016; Campos et al., 2018), and known carnivores tend to align more with what is typically observed in zebrafish, possessing microbes such as various Proteobacteria and Actinomycetota (Menke et al., 2014; Givens et al., 2015). Providing zebrafish with a high fiber diet may lead

Table 1
Artificial diets used in this study for F_1 fish as well as in Leigh et al., 2018a for P fish.

Ingredient	Percent (by mass) of each ingredient in each diet		
	Carnivore	Omnivore	Herbivore
Casein	27.5	20.0	5.0
Soybean meal	27.5	20.0	5.0
Wheat flour	6.0	6.0	6.0
Corn starch	5.5	5.5	5.5
Rice bran	5.0	5.0	5.0
Corn oil	3.3	3.3	3.3
Menhaden oil	3.3	3.3	3.3
Cod liver oil	3.4	3.4	3.4
Cellulose	15.0	30.0	60.0
Methyl cellulose	1.5	1.5	1.5
Vitamin premix	1.0	1.0	1.0
Vitamin C	0.4	0.4	0.4
Mineral premix	0.6	0.6	0.6

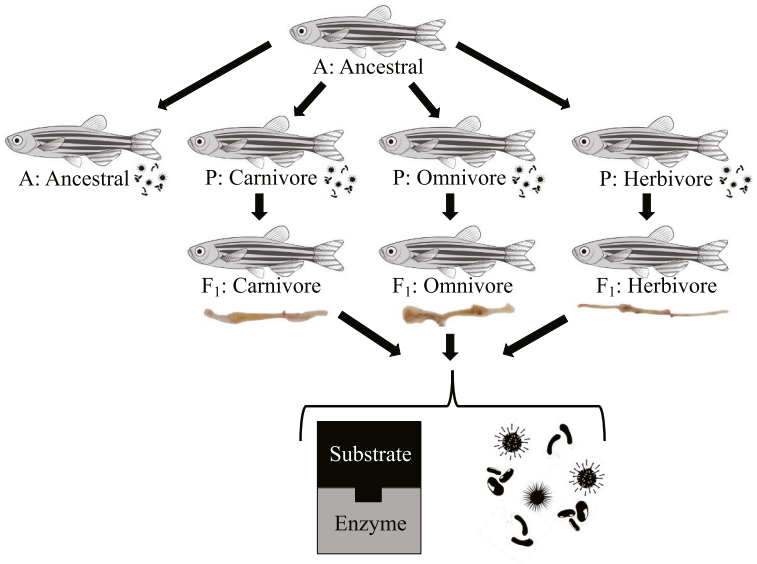

Fig. 1. Depiction of experimental design. Ancestral (A) zebrafish were spawned and their offspring, the Parental (P) generation were divided into three experimental diet groups (carnivore, omninvore, and herbivore). The P generation fish were spawned and their offspring (F_1) were reared on the same diet as their parents. Intestines from fish from each generation (A, P, and F_1) and from each diet group underwent enzyme assays and 16 s rRNA sequencing. Zebrafish illustration by A. Dingeldin.

to changes in their microbiome that share more similarity to a community typically observed in an herbivorous fish. Overall, our goal is to determine if there is a dietary effect on digestive physiology and gut microbial community that is kept consistent when the diet is kept homogenous across generations.

2. Materials and methods

2.1. Fish and feeding experiments

As described in Leigh et al. (2018a), two hundred and forty *Danio rerio* "wild type" larvae were maintained at University of California, Irvine. At 15 days post hatch (DPH), the fish were divided into four groups ($n = 60$ fish per group): ancestral (which was given the same diet they had been consuming in captivity for >100 generations; details in Leigh et al., 2018a), and three experimental diet groups that made up the Parental (P) generation (Fig. 1). These three P diets were "carnivore" (high-protein/low-fiber), "omnivore" (mix of protein and fiber), and "herbivore" (low-protein/high-fiber; Table 1). Once they reached adulthood (>5 months), $n = 26$ individuals fed the ancestral diet and $n = 20$ individuals from each of the variable protein:fiber diets of the P generation were collected from the separate tanks after a feeding event and were used for analyses (Leigh et al., 2018a). All remaining fish from the three P diets that were not used for those initial analyses (reported in Leigh et al., 2018a) were used as mates to generate generation F_1.

At 15 DPH, F_1 fish were fed a mixture of rotifers and their respective diets (the same experimental diets as the P generation; Table 1). By 20 DPH, the fish were only fed their respective diet, and by 50 DPH, fish were transferred to the same re-circulating system of 75.6-L aquaria (30 fish per aquarium, two tanks per diet type) connected to common filtration, including a sump, biological, particulate, activated carbon, and UV filtration. Each tank had the same lighting conditions, and because the water in the system was recirculating through a shared sump, all fish experienced the exact same conditions (except for diet) regardless of tank. Furthermore, we found the fish performed better and grew faster when housed in groups as opposed to individually; indeed, housing conditions (individual vs group) affect experimental results with *D. rerio* (Parker et al., 2012). This design precluded us from measuring digestibility of the different diets in individual fish. The system contained deionized water supplemented with appropriate salts, and fish were under a 12 L:12 D light cycle. The water temperature was maintained at 23 °C with a submersible heater for the duration of the experiment and the temperature and chemical conditions (pH, ammonia concentrations) of the tank system was monitored daily to confirm that they did not vary during the experimental period. The tanks were scrubbed, debris and feces siphoned out, and 20% of the water changed every three days.

The variable protein and fiber diets created in the laboratory (carnivore, omnivore, and herbivore) were composed of varying concentrations of protein sources (casein and soybean meal), carbohydrates (wheat flour, corn starch, rice bran, and cellulose), lipids (corn oil, menhaden oil, and cod liver oil), vitamins, minerals, and methyl cellulose as a binder (Table 1). The ingredients were mixed with water to make a paste, then pressed through a pasta maker (Newsome et al., 2011), dried at 60 °C, and ground back down to a particle size (~1 mm) suitable for zebrafish. The variable diets were designed to be nearly isocaloric, but vary mostly in the protein:fiber ratio. The fish were fed twice daily to satiation. Once they reached adulthood (>5 months), 20 individuals from each of the variable protein:fiber diets were collected from the separate tanks after a feeding event and were used for analyses.

Individual fish were euthanized in buffered water containing 1 g L^{-1} tricaine methanesulfonate (MS-222, Argent Chemicals Laboratory, Inc., Redmond, WA, USA), measured [standard length (SL) ± 1 mm], weighed [body mass (BM) ± 0.5 g], and dissected on a chilled (~4 °C) cutting board. Whole GI tracts were removed by cutting at the esophagus and at the anus and processed in a manner appropriate for specific analyses (see below). For each fish, the whole GI tract was weighed, and the intestine length was measured [intestine length (IL) ± 1 mm]. Relative intestine length (RIL = IL x SL^{-1}) and digestive-somatic index (DSI = intestine mass x body $mass^{-1}$) were determined. Due the fragility and small size of the fish at the start of the experiment, we did not measure initial body size and therefore, could not calculate growth rate. However, terminal fish size at the conclusion of the experiment was recorded and compared among the fish on the different diets.

2.2. Dietary composition

The proportions of nutrients in the diets fed to *Danio rerio* in this study are reported in Leigh et al. (2018a). Proximate analyses were performed following the methods of the Association of Official Analytical Chemists (AOAC, 2006). Total fat was determined by acid hydrolysis followed by extraction in petroleum ether, and total protein was determined by Kjeldahl extraction. Ash was determined by drying the diets at 105 °C (dry matter), and then combusting them at 550 °C for three hours. The remaining content was ash (the proportion that combusted was organic matter, OM). Soluble carbohydrate was calculated as the nitrogen-free extract, or the proportion of the diet that wasn't analytically determined as moisture, protein, fat, crude fiber, or ash.

2.3. Gut microbiome sample processing

The sample DNA was isolated from whole gut for the ancestral fish ($n = 3$), the P fish ($n = 2$ for each of the 3 experimental diets for a total of 6 samples), and the F_1 fish (n = 3 for each of the 3 experimental diets for a total of 9 samples) using the Zymobiomics DNA mini kit from Zymo Research. 16S rRNA amplicon PCR was performed targeting the V4 - V5 region (selected based on previous literature; Caporaso et al., 2012; Walters et al., 2016) using the EMP primers (515F [barcoded] and 926R; Caporaso et al., 2012; Walters et al., 2016). A mock community (ZymoBIOMICS® Microbial Community Standard) was extracted and all downstream analyses run along with the intestinal samples as a control (Supplemental Fig. S1). The libraries were sequenced at the UC Irvine Genomics High Throughput Facility using a miseq v3 chemistry with a PE300 sequencing length. Sequencing resulted in 1,289,779 paired end reads passing filter of which (x% are PhiX) with and overall Q30 > x%. The raw sequences were imported into qiime2 (qiime2.org; the "Moving Pictures Tutorial" guided our analyses: https://docs.qiime2.org/2019.10/tutorials/moving-pictures/). After initial sample quality check and trimming (DADA2 in qiime2) all samples showed significant numbers of reads (the lowest being 4700 reads). From the sequences the first 5 bp were trimmed and the forward reads were truncated at 299 bp and the reverse reads were truncated at 242 bp. Both single-end and paired-end reads were evaluated, but only forward single-end read results are reported. The sequences were assigned a taxonomic classification with SILVA SSU Ref NR99 v138 database (Quast et al., 2013). Sequences were confirmed using the Basic Local Alignment Search Tool (BLAST; blast.ncbi.nlm.nih.gov/Blast.cgi). This process, combined with the quality checks as described earlier in the methods, resulted in the elimination of no samples, so all were used (ancestral fish: n = 3, P fish: n = 2 for each of the 3 experimental diets for a total of 6 samples, F_1 fish: n = 3 for each of the 3 experimental diets for a total of 9 samples).

2.4. Tissue preparation for digestive enzyme analyses

For fishes designated for digestive enzyme analyses (ancestral diet $n = 20$, 10 males and 10 females; F_1 fish on all other diets $n = 10$, five males and five females), the guts were dissected out, placed on a sterilized, chilled (~4 °C) cutting board, and uncoiled. Following length and mass measurements, each entire intestine was placed in a separate sterile centrifuge vial and frozen in liquid nitrogen. All of the samples were then stored at –80 °C until prepared for analysis (within one month). Intestinal homogenates were prepared in 25 mM Tris-HCl pH

7.5 buffer as described by German and Bittong (2009).

2.5. Assays of digestive enzyme activity

All assays were carried out at 25 °C in duplicate or triplicate using a BioTek Synergy H1 Hybrid spectrophotometer/fluorometer equipped with a monochromator (BioTek, Winooski, VT). All assay protocols generally followed methods detailed in German and Bittong (2009), unless otherwise noted. All pH values listed for buffers were measured at room temperature (22 °C), and all reagents were purchased from Sigma-Aldrich Chemical (St. Louis). All reactions were run at saturating substrate concentrations as determined for each enzyme with gut tissues from the zebrafish. Each enzyme activity was measured in each individual fish, and blanks consisting of substrate only and homogenate only (in buffer) were conducted simultaneously to account for endogenous substrate and/or product in the tissue homogenates and substrate solutions.

α-amylase activity was measured using 1% potato starch dissolved in 25 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$. The α-amylase activity was determined from a glucose standard curve and expressed in U (μmol glucose liberated per minute) per gram wet weight of gut tissue.

Maltase activities were measured following Dahlqvist (1968), as described by German and Bittong (2009). We used 112 mM maltose dissolved in 200 mM phosphate buffer (pH 7.5). The maltase activity was determined from a glucose standard curve and expressed in U (μmol glucose liberated per minute) per gram wet weight of gut tissue.

Trypsin activity was assayed using a modified version of the method designed by Erlanger et al. (1961). The substrate, 2 mM Nα-benzoyl-L-arginine-p-nitroanilide hydrochloride (BAPNA), was dissolved in 100 mM Tris-HCl buffer (pH 7.5). Trypsin activity was determined with a p-nitroaniline standard curve, and expressed in U (μmol p-nitroaniline liberated per minute) per gram wet weight of gut tissue.

Aminopeptidase activity was measured using 2.04 mM L-alanine-p-nitroanilide HCl dissolved in 200 mM sodium phosphate buffer (pH 7.5). Aminopeptidase activity was determined with a p-nitroaniline standard curve, and activity was expressed in U (μmol p-nitroaniline liberated per minute) per gram wet weight of gut tissue.

Lipase (nonspecific bile-salt activated) activity was assayed using 0.55 mM *p*-nitrophenyl myristate (in ethanol) in the presence of 5.2 mM sodium cholate dissolved in 25 mM Tris-HCl (pH 7.5). Lipase activity was determined with a p-nitrophenol standard curve, and expressed in U (μmol p-nitrophenol liberated per minute) per gram wet weight of gut tissue.

2.6. Statistical analyses

Appropriate sample sizes were determined via power analysis based on previous studies. A sample size of 8 or greater was deemed appropriate for enzyme assays (German et al., 2004; German and Bittong, 2009). Comparisons of intestine length, intestine mass, and enzyme activity levels were made between P and F_1 fishes on the same diets (i.e., generational comparison) with unpaired *t*-tests using a Bonferroni corrected *p*-value of 0.0167. Activity levels of each enzyme were compared among the F_1 fish on the various diets (herbivore, omnivore, carnivore) and the ancestral fish with ANOVA, followed by a Tukey's HSD with a family error rate of $p < 0.05$. Prior to all significance tests, a Levene's test for equal variance was performed to ensure the appropriateness of the data for parametric analyses. All tests were run using Rstudio software (version 1.0.136). To analyze microbial community composition, alpha diversity (Faith's phylogenetic diversity) significance was determined using a Kruskal-Wallis pairwise test ($p < 0.05$). Beta diversity (Bray-Curtis dissimilarity) significance was determined using a PERMANOVA ($p < 0.05$) with 999 permutations and a sequencing depth of 4000. Taxa with abundances of zero were not included in these analyses. We followed the qiime2 "Moving Pictures Tutorial" to demultiplex and control the quality of sequences. All statistical tests used to analyze 16S rRNA sequencing results were run in qiime2. The mock community controls were verified to confirm that the kit extracted all of the relevant microbial taxa (including gram positive and negative bacteria; Supplemental Fig. S1). R studio (v. 1.0.136) was also used to run an indicator species analysis (De Cáceres and Legendre, 2009) to determine the particular microbial taxa that may dominate the community of a particular diet type or a particular generation.

3. Results

There are no significant differences between P and F_1 fish in terms of intestine length (Table 2), intestine mass (Table 2), and enzyme activity levels (Fig. 2) for any of the three experimental diets (carnivore, omnivore, and herbivore; unpaired t-tests using a Bonferroni corrected p-value of 0.0167). F_1 fish on the omnivore diet had significantly higher amylase activity compared to the ancestral diet fish ($p < 0.001$; Fig. 2). F_1 fish on the omnivore diet also had significantly higher maltase, trypsin, and aminopeptidase activities compared to the ancestral diet fish, as well as F_1 fish on the carnivore and herbivore diets ($p < 0.001$; Fig. 2). F_1 fish on the herbivore diet had significantly higher lipase activity compared to the ancestral diet fish, as well as F_1 fish on the carnivore and omnivore diets ($p < 0.001$; Fig. 2).

In terms of microbial abundance, the top 10 most abundant ASVs (amplicon sequence variants) present in the samples were *Pseudomonas alcaligenes*, *Aeromonadaceae* sp., *Flavobacterium succinicans*, *Aeromonadaceae* sp., *Legionellaceae* sp., *Myobacterium* sp., *Shinnella granuli*, *Rhizobiceae* sp., *Rhodobacter* sp., and *Rickettsiales* sp. (Fig. 3). A full list of the ASVs identified and their occurrence in each sample can be found in Supplemental Table S1 as well as Supplemental Fig. S2. There were no significant differences between the P and F_1 fish in terms of their alpha (Faith's phylogenetic diversity; $p = 0.6$) and beta (Bray–Curtis dissimilarity; $p = 0.4$) microbial diversity. For fish on the same diets, P and F_1 fish did not show significantly different community structure compared to each other (Fig. 4; PERMANOVA: $p = 0.436$). The ancestral diet fish showed distinct community structure compared to both P and F_1 fish (Fig. 4; $p < 0.001$). Fish on herbivore, omnivore, and carnivore diets showed significantly distinct microbial community structure when compared to each other for both P ($p = 0.003$) and F_1 fish ($p = 0.002$). Species indicator analyses revealed that the main driver of gut microbiome community differences of the ancestral diet fish compared to the other three diet types was *Shinella granuli* ($p < 0.01$). Community difference for the carnivore diet fish (for both the P and F_1 generations) was driven by *Flavobacterium succinicans* ($p < 0.01$). Community difference for the omnivore diet fish (for both the P and F_1 generations) was driven by an unidentified *Mycobacterium* sp. ($p < 0.01$). The most abundant

Table 2
Mean (with 95% confidence interval in parentheses below each mean) final intestine length (mm), intestine mass (g), and body mass (g) for P and F_1 fish on the three experimental diets (carnivore, omnivore, and herbivore).

Generation/Diet	Intestine Length (mm)	Intestine Mass (g)	Body Mass (g)
P/Carnivore	24.54 (22.03–27.06)	0.058 (0.046–0.070)	0.790 (0.708–0.873)
F_1/Carnivore	25.25 (24.12–33.02)	0.06 (0.049–0.075)	0.764 (0.712–0.832)
P/Omnivore	27.69 (25.47–29.91)	0.035 (0.024–0.046)	0.638 (0.558–0.718)
F_1/Omnivore	28.9 (25.34–39.60)	0.037 (0.026–0.045)	0.642 (0.502–0.741)
P/Herbivore	33.23 (30.83–35.63)	0.035 (0.023–0.047)	0.512 (0.470–0.554)
F_1/Herbivore	34.5 (29.76–41.33)	0.038 (0.026–0.049)	0.499 (0.401–0.587)

Values reported for P fish are from Leigh et al., 2018a. There are no significant differences for intestine length, intestine mass, or body mass between P and F_1 fish on each of the three experimental diets (unpaired t-tests using a Bonferroni corrected *p*-value of 0.0167).

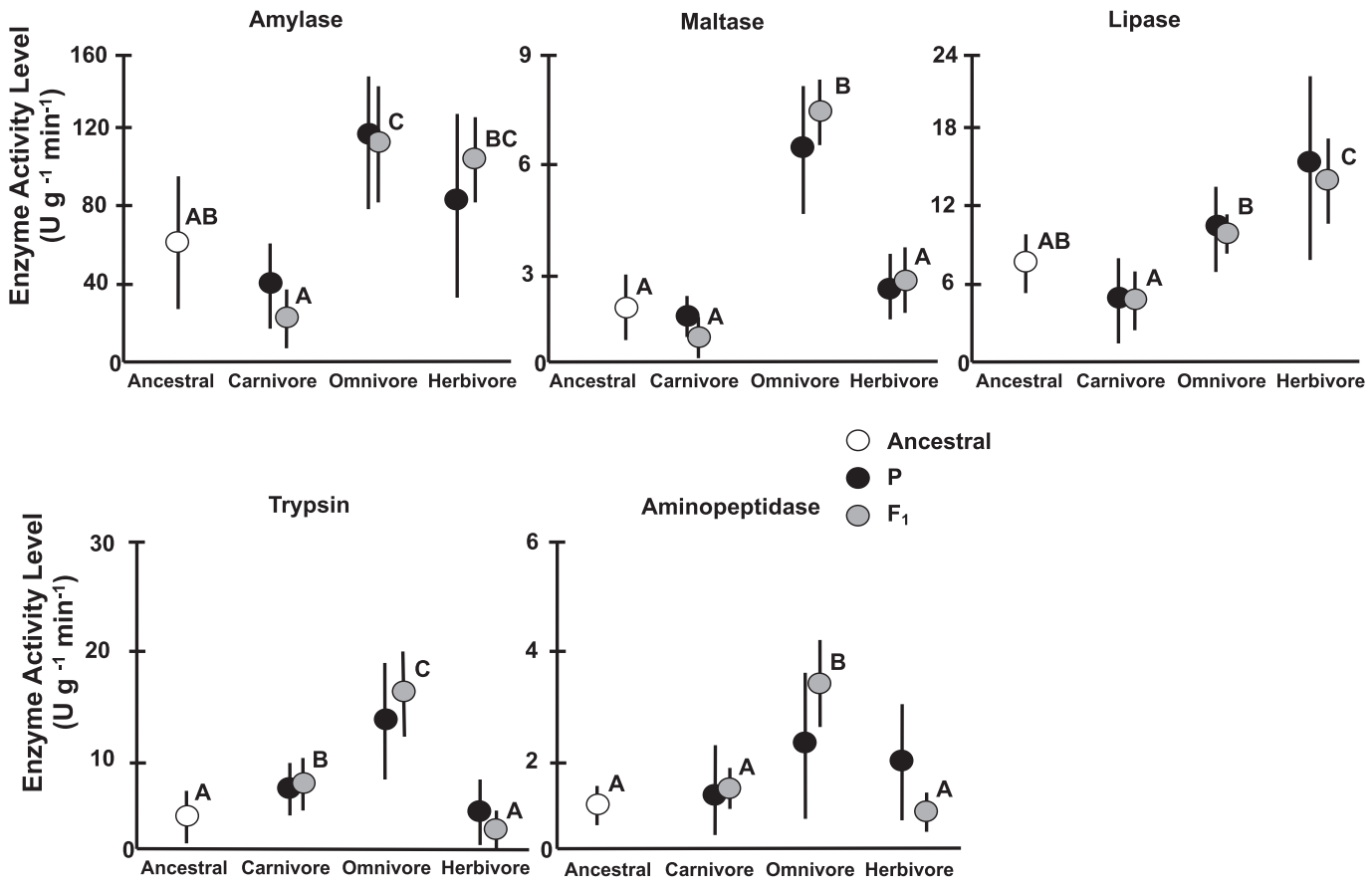

Fig. 2. Amylase, maltase, lipase, trypsin, and aminopeptidase activities in the digestive tracts of ancestral (A) *D. rerio* ($n = 20$) as well as P ($n = 14$ fish per diet type) and F_1 ($n = 10$ fish per diet type) fish (A and P data from Leigh et al., 2018a). Values are means and error bars are standard deviation. No significant differences were found between P and F_1 fish for any of the diets with any of the enzymes (*t*-tests with Bonferroni corrected error rate of $p < 0.0167$). Activity levels of each enzyme were compared among the F_1 fish and the P fish fed the different diets with ANOVA, followed by a Tukey's HSD with a family error rate of $p < 0.05$. Data points sharing a superscript letter for a specific enzyme are not different from one another.

taxa found in the gut microbiome of the herbivore diet fish (for both the P and F_1 generations) were also *Flavobacterium succinicans* and *Mycobacterium* sp., but both taxa were found in significantly greater abundances in the herbivore diet fish (average = 26,953 occurrences and 20,467 occurrences respectively) as compared to the omnivore diet fish (average = 10,749 occurrences for the *Mycobacterium* sp.) and carnivore diet fish (average = 11,430 occurrences for *Flavobacterium succinicans*). Additionally, twenty taxa of interest were selected based on the fact that they were nearly completely absent in the ancestral diet fish, but were consistently present in the experimental diet fish (Fig. 5). When only these taxa of interest are included in analyses, the herbivore diet for both the P and F_1 fish was dominated by *Clostridium butyricum* ($p < 0.01$), the omnivore diet for the F_1 fish was dominated by *Candidatus Amoebophilus* ($p = 0.012$) and the carnivore diet for the F_1 fish was dominated by an unidentified *Clostridium* ($p = 0.022$; Fig. 5).

4. Discussion

We have shown that there is a strong dietary effect on digestive morphology, physiology, and gut microbial community composition and that these effects are consistent across generations when the diet remains homogenous. When altering the ratio of protein and fiber in the diet of *D. rerio*, we observed changes in digestive tract size (length and mass), digestive enzyme production, and alterations to the gut microbiome that remained consistent across sequential generations.

With respect to digestive morphology, a high-fiber diet ("herbivore" diet) is typically characterized by a longer intestine length (Olsson et al., 2007; Santigosa et al., 2008; Wagner et al., 2009; Lin and Luo, 2011; Li et al., 2014; Kohl et al., 2016; Yaghoubi et al., 2016; Liu et al., 2016) and this was observed in comparisons of the ancestral (A) generation to the parental (P) generation of zebrafish in Leigh et al. (2018a). We expected that this would also be true for the F_1 fish kept on the same diet as the P generation given that their overall fiber and protein intake was the same as that of the P generation, and this was in fact the case, with the herbivore diet fish exhibiting average intestine lengths of ~34.5 mm (~33.23 mm for the P generation), compared to ~25.25 mm for the carnivore diet fish (~24.54 mm for the P generation); i.e., the herbivore diet fish intestines were 36% longer than the carnivore diet fish. Typically, herbivores need to consume more food by volume in order to meet their metabolic demands (e.g. Karasov and Martínez del Rio, 2007). As such, a longer gut means that more food can be processed per unit of time (Penry and Jumars, 1987). The opposite is true when naturally herbivorous fishes are fed high protein diets: the gut of the herbivorous *Xiphister mucosus* got shorter when the fish were fed a carnivorous diet in the laboratory, but not as short as closely-related, natural carnivores (Herrera et al., 2022). As cyprinids, zebrafish have a relatively simple digestive tract design (no stomach, no hind gut chambers, etc.; Ulloa et al., 2011) and therefore an increase in overall gut length is a simple solution to accommodate higher intake of food on a high-fiber diet (Sibly, 1981; German et al., 2010a, 2010b). The fact that this result remained consistent between the P and F_1 fish indicated a clear dietary effect rather than a generational effect. We would need to extend our trials many more generations to test what the limits to increasing gut length would be in *D. rerio*. For instance, *Nocomis leptocephalus* (also a

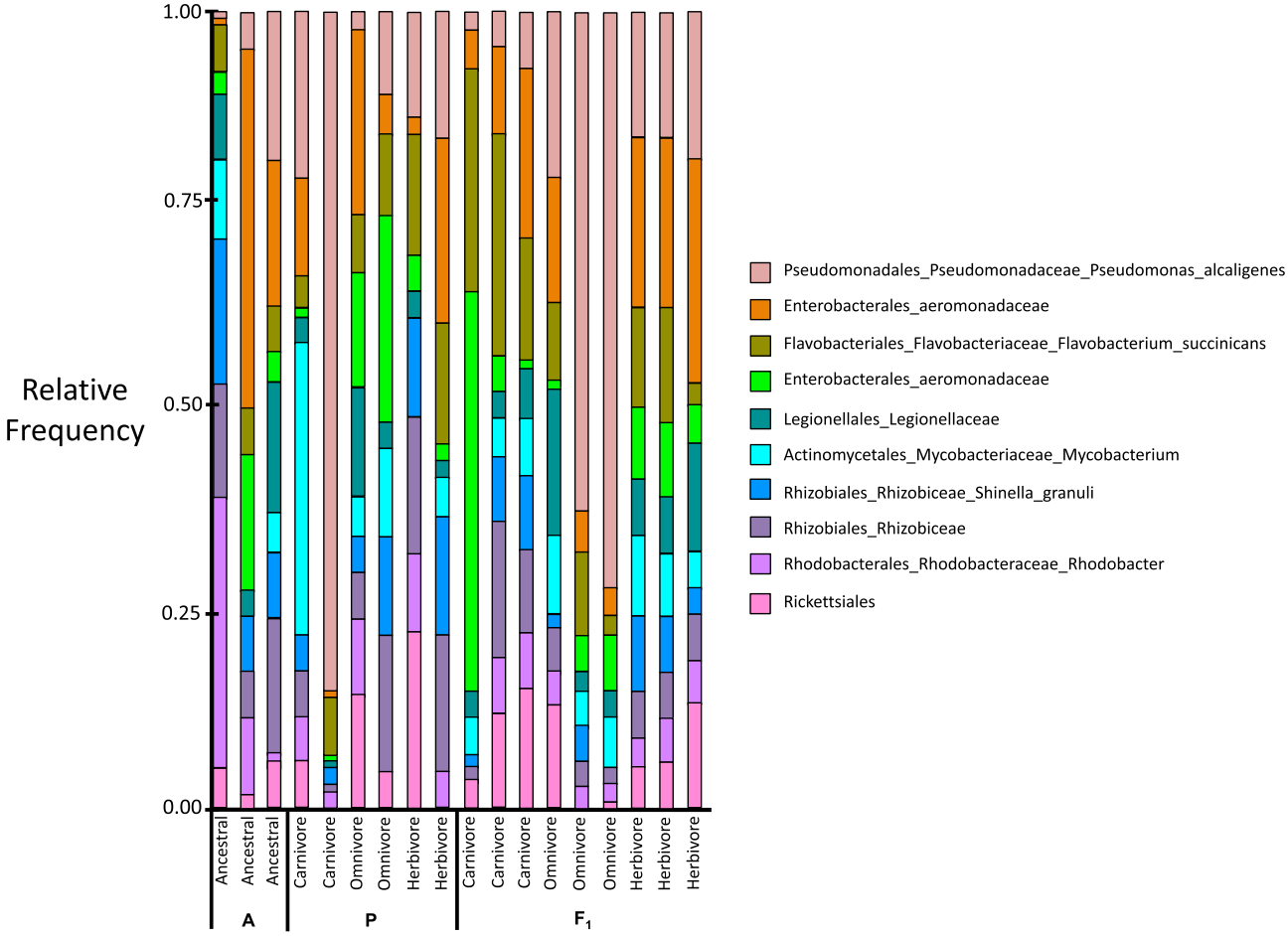

Fig. 3. Taxonomy bar plot for whole guts of ancestral (A) diet fish ($n = 3$), P fish (n = 2 per diet type: carnivore, omnivore, and herbivore), and F_1 fish (n = 3 per diet type) depicting the relative frequency of each bacterial taxa detected from 16 s rRNA sequencing results. Only the top 10 taxa are included in the figure. All taxa can be found listed in Supplemental Table S1, as well as shown in a taxonomy bar plot Supplemental Fig. S2.

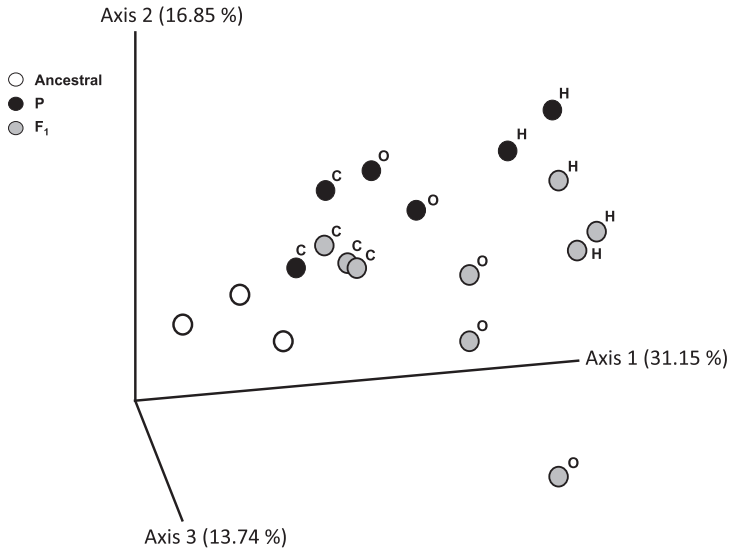

Fig. 4. Bray–curtis PCoA plot depicting microbial community diversity for whole guts of ancestral diet fish (n = 3), P fish (n = 2 per diet type: herbivore [H], omnivore [O], and carnivore [C]), and F_1 fish (n = 3 per diet type: herbivore [H], omnivore [O], and carnivore [C]). 61.74% of the variance is explained by the first three axes. P and F_1 did not show significantly different community structure compared to each other (for fish on the same diets; PERMANOVA: $p = 0.436$). The ancestral diet fish showed distinct community structure compared to both P and F_1 ($p < 0.001$). Fish on herbivore, omnivore, and carnivore diets showed significantly distinct microbial community structure when compared to each other for both P ($p = 0.003$) and F_1 fish ($p = 0.002$).

member of family Cyprinidae, like *D. rerio*) has different diets depending on river drainage in the southeast United States (German et al., 2010a). Herbivorous populations of this species have guts that are twice as long as carnivorous fish from separate drainages. However, the *N. leptocephalus* gut lengths regardless of population or diet are at least five-fold lower than herbivorous minnows in the genus *Campostoma* that are sister to the *Nocomis* and have similar diets to the herbivorous *N. leptocephalus* population (German et al., 2010a). How many

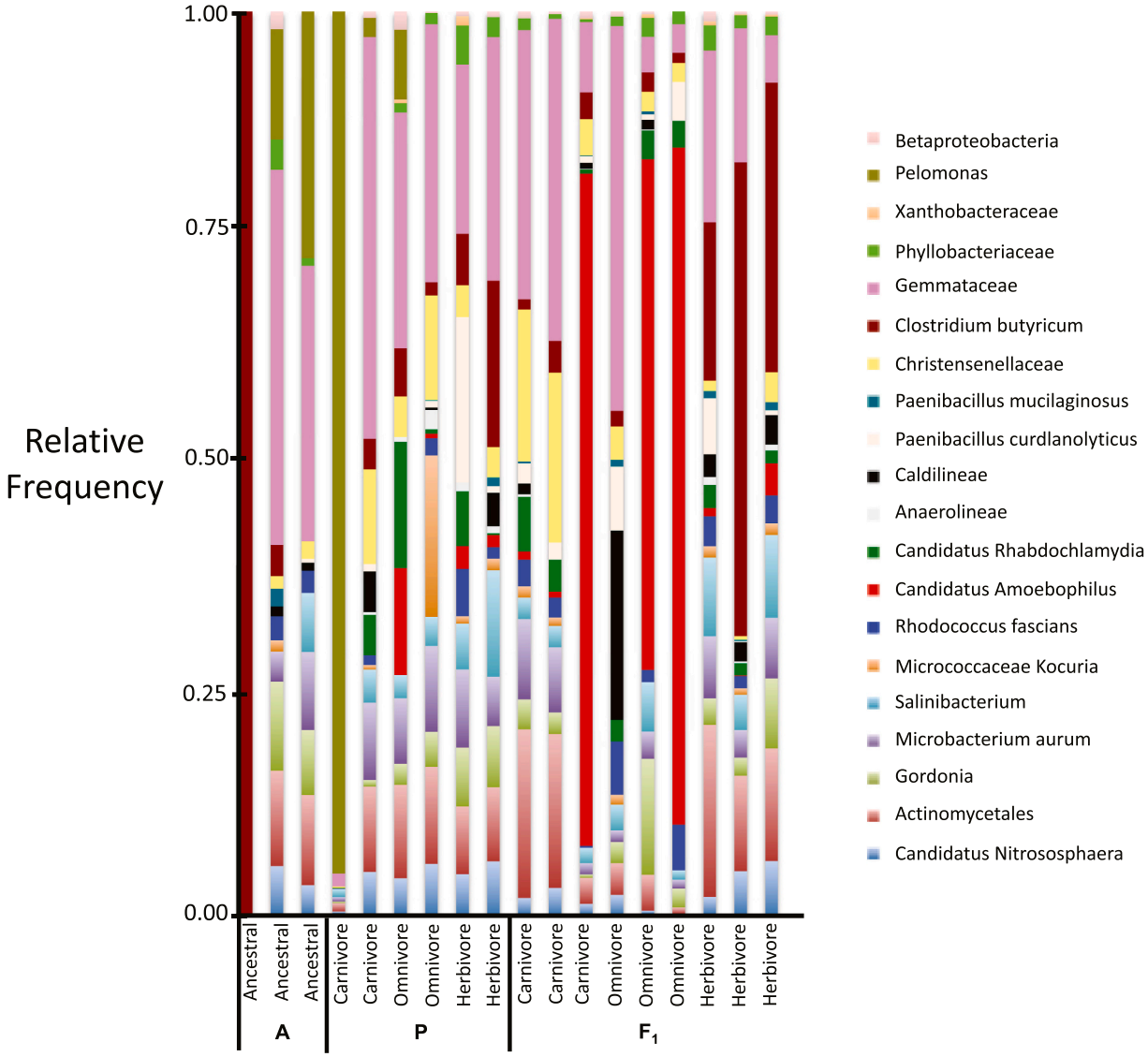

Fig. 5. Taxonomy bar plot for whole guts of ancestral (A) diet fish (n = 3), P fish (n = 2 per diet type: carnivore, omnivore, and herbivore), and F_1 fish (n = 3 per diet type) depicting the relative frequency of each bacterial taxa detected from 16 s rRNA sequencing results. Only 20 taxa of interest are included in the figure. Taxa were selected based on their limited presence in the A fish and common presence in the experimental diet fish. All taxa can be found listed in Supplemental Table S1, as well as shown in a taxonomy bar plot Supplemental Fig. S2.

generations of diet shifting resulted in the doubling in gut length in *N. leptocephalus* and why did this species not achieve the much longer gut lengths observed in *Campostoma* species? Perhaps there are limits to how much something can change in a given time span. Or, other aspects, such as the cartilaginous lower lip of *Campostoma* (Page and Burr, 1991), or differences in pharyngeal teeth (German et al., 2010a) that may allow for higher intake by *Campostoma*, additionally impact gut length in these species. Some traits (e.g., thermal limits; Morgan et al., 2020) do have upper thresholds that cannot be changed. There is evidence that laboratory adapted zebrafish show less plasticity than wild-caught fishes (Morgan et al., 2022).

The patterns of the P generation for digestive enzyme activities held up in the F_1 generation as well. We did not observe any enzyme activity differences between the P and F_1 fish. Rather, we observed the same dietary effects that were noted in Leigh et al. (2018a). For instance, the omnivore diet fish, for both the P and F_1 fish exhibited elevated amylase, maltase, trypsin, and aminopeptidase levels compared to the A fish. This fits within what is called an "optimal protein" content model (Simpson et al., 2004) as discussed in detail in Leigh et al. (2018a), which states that a protein concentration that is potentially closer to some optimum based on the organism's metabolic demands results in the most efficient gut performance in zebrafish (rather than simply that a high-protein diet equates to higher enzyme production). Clearly, diet is the driver of these biochemical differences rather than the generation (Fig. 2). Differences in the enteric microbes present in the guts of the experimental diet fish are also likely playing a role in these patterns of gut structure and function across diets, given that the microbes likely have a hand in the breakdown of nutrients necessary for growth, metabolism, protein production, etc. (e.g. Ghanbari et al., 2015; Herrera et al., 2022).

The top 10 microbial taxa observed in the zebrafish gut in this study were common intestinal denizens observed in previous investigations in this fish species (e.g., Roeselers et al., 2011; Ma et al., 2020; Wang et al., 2021) and other freshwater fishes held in captivity (Giatsis et al., 2015), or in tadpoles (Zhang et al., 2020). As the indicator species of the ancestral diet fish, *Shinella* is intriguing. These Alphaproteobacteria are facultatively anaerobic and found in a variety of environments, including guts and sewage sludge reactors (Qiu et al., 2016). They show a variety of potential pathways and could be abundant based on the

dietary composition of the commercial zebrafish diet, although this genus was detected in wild-type zebrafish guts (Roeselers et al., 2011), and in the zebrafish skin mucus-associated microbiome (Wakeman et al., 2021). Interestingly, the indicator taxa for the carnivore diet fish (*Flavobacterium succinans*) and omnivore diet fish (*Mycobacterium* sp.) were each indicator species for the herbivore diet fish, but they had double the abundance in the fish consuming the herbivore diet in comparison to the fish consuming the other diets. Each of these taxa are common in zebrafish. *Flavobacterium succinans* is known to be associated with fish disease, but are also found in numerous environments, including guts, where they can participate in phosphate acquisition (Poehlein et al., 2017). *Mycobacterium* species are equally as widespread and can engage in many metabolic pathways (Whipps et al., 2012). Although specific *Mycobacterium* species are often associated with disease (Whipps et al., 2012), none of those taxa (e.g., *M. haemophilum* and *M. marinum*) were detectable in our dataset, and this genus is commonly observed in zebrafish in many environments (Roeselers et al., 2011). In terms of each of these taxa increasing in abundance in response to increased fiber in the diet, this study may be the first observation of this, and we cannot speculate what it means or whether *Flavobacterium* and *Mycobacterium* are participating in the digestive process. The fish did not appear to be ill in any way (Leigh et al., 2018a): they achieved the same sizes in the P and F_1 generations across the same amount of time (Table 2), and bred when we gave them the chance. Thus, we do not speculate that intestinal *Flavobacterium* and *Mycobacterium* are indicative of disease, at least in any obvious way, in this study.

While we acknowledge the fact that sample size was low for the microbial analyses, microbiome variation among the fish on the different diets is obvious (Fig. 3 & Fig. 5). These fish were completely bathed in the same water, so the only difference among them was their diet. In the PCoA, it is obvious that the fish on the different diets varied along the first PC axis the most, but the different generations, although not statistically different, appear to be starting to vary along PC3, which should be explored further with additional generations (Fig. 4). Intergenerational transmission of microbiomes are known in mammals (Schulfer et al., 2018; Wang et al., 2022), with some potential in elasmobranchs (Mika et al., 2021). Hybrid *Coregonus* fish had microbiomes that appeared to be in between the two parental species, showing influences of each (Belkova et al., 2017). Here, the dietary differences were clearly maintained among generations.

More detailed statistics on specific taxa revealed that each diet led to more abundance of specific ASVs. For the herbivore diet fish, *Clostridium butyricum* was more abundant than in the fish consuming the other diets (Fig. 3). This is intriguing because this bacterial species is a known producer of the short chain fatty acid (SCFA) butyrate (Cassir et al., 2016), which causes increased proliferation of enterocytes (Scheppach, 1994; Scheppach et al., 1997) and positively impacts immunity (Chang et al., 2014). The herbivore diet fish have higher mucosal surface area (Leigh et al., 2018a), and perhaps *C. butyricum* plays a role in this process (Ma et al., 2020). With their simple intestine, *D. rerio* are not known to be reliant on gastrointestinal fermentation to meet a large proportion of their daily energetic needs, and in fact have relatively low SCFA concentrations in their guts (Ma et al., 2020) in comparison to those fishes that are reliant on fermentation in the digestive process (e.g., Mountfort et al., 2002; Clements et al., 2017; Pardesi et al., 2022). However, microbes play other roles than just providing the host with SCFA (Moran et al., 2019), and taxa like *C. butyricum* may play important roles in gut health (Cassir et al., 2016). This species is clearly associated with the "herbivore" diet in this study. The omnivore diet fish had a *Candidatus* Amoebophilus apparent in their guts. These endosymbiotic organisms may be intra-cellular (Ponnusamy et al., 2018), but any function they may play in the *D. rerio* gut is unknown. Equally as puzzling is the unknown *Clostridium* present in the carnivore diet fish. One Archean, *Candidatus* Nitrosophaera, was more abundant in the fish fed the various formulated diets than on the ancestral diet. These microbes oxidize ammonia and play key roles in nitrogen turnover in gut environments (Lehtovirta-Morley, 2018). Perhaps something inherent in the formulated diets made ammonia more available to these taxa than in the ancestral diet. Overall, the zebrafish fed the high-fiber diet, although possessing an enteric microbial community that is different than the other diets, does not have a community that resembles naturally herbivorous fishes (e.g., Moran et al., 2005; Pardesi et al., 2022), particularly other cyprinids, like grass carp (Wu et al., 2012; Hao et al., 2017). Thus, across two generations on different diets, the microbiome did indeed change, but not necessarily in a way to match fishes with natural diets resembling the formulated laboratory diets used here.

Given that these changes across diets were observed over the course of just one generation (A to P) and maintained for a second generation (F_1), future work should focus on experimental evolution of these phenotypic traits by including additional generations on the experimental diets to observe whether permanent and irreversible changes to gut function and structure are possible on experimental evolutionary timescales, or if the zebrafish digestive tract is flexible at the individual level to changes to the dietary fiber and protein content. The questions remain: how quickly can an animal's gut sufficiently accommodate a diet varying in its proportions of macronutrients? And, how many generations are required before populations on this new diet show some fitness advantage on those diets relative to the ancestral diet populations? The real bottleneck may be reproductive, as we noticed reduced fecundity in our herbivore diet fish relative to the fishes on the other diets (Leigh et al., 2018a). Given that fiber binds to fat (German et al., 1996), and that elevated lipolytic activities were observed in herbivore diet fish in our investigation (Leigh et al., 2018a), and in wild-caught herbivores (e.g., Heras et al., 2020), acquiring enough lipid from a high-fiber, plant-based diet may be the real challenge when herbivory is first evolving in an animal population. What role the microbiome can play in facilitating such a transition remains unknown. Moreover, our starting population sizes mean our fish are inbred. Much larger populations (e.g., Rutledge et al., 2020) would be needed to successfully do experimental evolution with *D. rerio* (Morgan et al., 2020). Finally, if laboratory adapted strains of zebrafish, like we used in this study, show reduced potential for plasticity (Morgan et al., 2022), then perhaps including wild-caught zebrafish in future analyses can reveal just how plastic this fish's gut really is in response to dietary perturbations.

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Data accessibility

All data is presented within the manuscript, figures, supplemental material associated with this manuscript, or on Open Science Framework: https://osf.io/9qsbf/?view_only=80d87f850b904a81a3ca118a5fa33a26.

Author contributions

Conceptualization, SCL and DPG; Methodology, SCL, CC, and DPG; Investigation, SCL and CC; Writing-original draft, SCL and DPG; Writing-reviewing and editing, SCL, CC, and DPG; Funding acquisition, SCL and DPG.

CRediT authorship contribution statement

Samantha C. Leigh: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition. **Caitlyn Catabay:** Methodology, Investigation, Writing – review & editing. **Donovan P. German:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

None.

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