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Metagenomics approach to the study of the gut microbiome structure and function in Zebrafish *Danio rerio* fed with gluten formulated diet

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Abstract

In this study, we report the gut microbial composition and predictive functional profiles of zebrafish, *Danio rerio*, fed with a control formulated diet (CFD), and a gluten formulated diet (GFD) using metagenomics approach and bioinformatics tools. The microbial communities of the GFD-fed *D. rerio* displayed heightened abundances of Legionellales, Rhizobiaceae, and *Rhodobacter,* as compared to the CFD-fed counterparts. Predicted metagenomics of microbial communities (PICRUSt) in GFD-fed *D. rerio* showed KEGG functional categories corresponding to bile secretion, secondary bile acid biosynthesis, and the metabolism of glycine, serine, and threonine. The CFD-fed *D. rerio* exhibited KEGG functional categories of bacteria-mediated cobalamin biosynthesis, which was supported by the presence of cobalamin synthesizers such as *Bacteroides* and *Lactobacillus.* Though these bacteria were absent in GFD-fed *D. rerio*, a comparable level of the cobalamin biosynthesis KEGG functional category was observed, which could be contributed by the compensatory enrichment of *Cetobacterium*. Based on these results, we conclude *D. rerio* to be a suitable alternative animal model for the use of targeted

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Conflict of interests

The authors declare no conflicts and financial interest with this study.

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metagenomics approach along with bioinformatics tools to further investigate the relationship between the gluten diet and microbiome profile in the gut ecosystem leading to the gastrointestinal diseases and other undesired adverse health effects.

Keywords

NextGen Sequencing; 16S rRNA; QIIME; PICRUSt; Pathogens

1. Introduction

The Neolithic Revolution from hunter-gatherer survival into agricultural strategies redefined diet (Freeman, 2013), incorporating larger quantities of wheat products, barley, and rye – of which the prolamin fraction is known to contain wheat protein gluten (Béres et al., 2014; Thompson et al., 2005). Wheat gluten is comprised of the water soluble monomeric protein gliadin, and insoluble multimeric glutenin, and is immunostimulatory to patients with genetically predisposed celiac disease (CD), and the etiologic agent of non-celiac gluten sensitivity (NCGS) (Howdle, 2006; Narrowe et al., 2015; Sanz, 2015). The pathophysiology of CD has been extensively probed, of which gluten induced autoimmune responses manifest aberrant intestinal mucosa conditions, characterized by inflammation and lesions, leading to malabsorption at the site of immune response followed by diarrhea and steatorrhea (Murray, 1999; Van Kessel et al., 2011). For NCGS, despite a reported ~6% of the human population affected, little is known of the factors underlying and/or progressing enteropathy, beyond the involvement of gluten (Elli et al., 2015; Sapone et al., 2012). Both CD and NCGS have received attention regarding population shifts and perturbations in intestinal bacteria (microbiota) that may be directly or indirectly correlated with disease states (A Daulatzai, 2015; Lotta et al., 2016; Samsel and Seneff, 2013; Sanz, 2015). Examinations of the microbiota of patients with CD and NCGS have concluded similar increases of harmful gram-negative bacteria, pathobionts and overall microbial dysbiosis (A Daulatzai, 2015; Béres et al., 2014).

To explore the effect of a gluten formulated diet (GFD) on both the composition and the metabolic profile of the microbiota, an examination of a model animal lacking a pre-existing aversion to gluten is warranted. Recently, the zebrafish *Danio rerio* has been established as a model organism to study for various genetic and environmental aspects of health, disease, and embryological development (Barut and Zon, 2000; Elli, and Roncoroni et al,. 2015; Sadler et al., 2013; Siccardi III et al., 2009; Watts et al., 2016). Many anatomical and physiological characteristics of the *D. rerio* digestive system, including disease states, are shared with the mammalian digestive system (Sadler, and Rawls et al., 2013; Wang et al., 2010). Also, the usefulness of this organism is emphasized by the ability to formulate feeds to promote growth, health, or disease in the laboratory setting (Siccardi III et al., 2009; Watts et al., 2016; Watts et al., 2012). Because of this, it is possible to formulate a gluten diet to elucidate the effect of the microbial composition in the gut ecosystem of laboratory raised *D. rerio*.

In recent years, NextGen sequencing (NGS) technologies have made it possible to describe the microbiota occurring in the gastrointestinal tracts of host organisms, by targeting the 16S rRNA genes of the collective bacterial genomes (microbiome) as they change in response to diet (Chen et al., 2014; Savarese et al., 2014; Turnbaugh et al., 2009; Umu et al., 2015). It is proposed that dietary gluten will alter the microbiome in the *D. rerio* gut ecosystem, resulting in microbial populations that are pathobionts and/or metabolically unfavorable to maintain and promote host health and physiology. In this study, we determined the microbial compositions in the gut ecosystem of *D. rerio* fed with a gluten formulated diet (GFD) or a control formulated diet (CFD), using NextGen Illumina Miseq targeting the V4 region within 16S rRNA gene. In addition, we have compared the gut microbiota-driven predictive metabolic profiles of the GFD- and CFD-fed *D. rerio* using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (ver.1.0.0), based on the16S rRNA gene sequence datasets.

2. Materials and methods

2.1. Culture of D. rerio and Sample Preparation

D. rerio (wild type strain AB) (Zebrafish International Resource Center, Eugene, OR), were cultivated from embryos in the NORC Aquatic Animal Research Core at the University of Alabama at Birmingham (UAB). *D. rerio* (28 days old) were maintained at 28 °C in 2.8 L tanks on a recirculating rack system (Aquaneering, Inc., San Diego, CA) and at a density of five fish per Liter. The aquaculture water was purified through a 5 μm sediment filter, and subsequently passed through reverse-osmosis mediated by charcoal, followed by ion exchange resin (Kent Marine, Franklin, WI). Synthetic sea salt (Instant Ocean, Blacksburg, VA) was added to maintain a conductivity of 1500 IS/cm. The water pH of 7.4 was sustained through periodic adjustment using sodium bicarbonate. One group of *D. rerio* was fed CFD, containing marine fish protein and other ingredients (fish meal is the standard protein source in most lab animal diets including zebrafish), and another group of *D. rerio* was fed GFD, containing wheat gluten as the primary source of protein (Table 2). All other ingredients were included at identical concentrations in both diets (note that the fish meal protein source contains slightly more fat than the wheat gluten protein source). The animals were fed three times per day. Excess food particles in the culture were removed from the tank via siphoning every other day. After 12 weeks, both the CFD (n=2) and the GFD (n=2) samples (biological replicates) of *D. rerio* were euthanized and guts were removed intact, flash frozen in liquid nitrogen, and preserved at −80°C until used for DNA extraction and preparation for NGS. All experiments with the vertebrate animal (*D. rerio*) were conducted according to the guideline elaborated in “Guide for the Care and Use of Laboratory Animals,” which was published by the National Research Council of the National Academies. The protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham under the Animal Project Number (APN) IACUC-08196.

2.2. DNA Extraction and 16S rRNA Amplicon Library Preparation

Metacommunity DNA was extracted in triplicates (technical replicates) from each CFD and GFD sample using the Zymo Research Fecal DNA isolation kit (Irvine, CA; catalog

#D6010) and then combined into single samples to perform NGS. For amplicon library preparation from the purified DNA, uniquely barcoded oligonucleotide primers (Forward primer V4: 5′-AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3′; and Reverse primer V4: 5′-CAAGAGAAGACGGCATACGAGATNNNNNNAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3′) (Eurofins Genomics, Inc., Huntsville, AL) were used for PCR to amplify the V4 region of the 16S rRNA gene (Kumar et al., 2014). All PCR reagents and cycling parameters were used as described previously (Kumar et al., 2014).

2.3. Illumina Miseq, Sequence Processing, and Bioinformatics Workflow

The PCR amplified V4 segment of the16S rRNA were subjected to NGS (250 bp, paired end) using Illumina Miseq™ platform (Kozich et al., 2013; Kumar et al., 2014). The raw FASTQ sequence files were used for quality checking using FastQC (Andrews, 2010), and ambiguous sequences were filtered or trimmed by using FASTX toolkit (Gordon and Hannon, 2010). Then, overlapping regions of the paired-end reads were merged, and then the chimera sequences were removed by USEARCH (Edgar, 2010).

The processed sequences were then clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity using UCLUST (Edgar, 2010), and then representative sequences were aligned by PyNAST (Caporaso et al., 2010). The taxonomic identifications were assigned to each representative sequence through the Ribosomal Database Project (RDP) classifier (Wang et al., 2007), trained using the Greengenes (ver. 13.8) (McDonald et al., 2012) at 60% confidence threshold. Stack column bar graphs representing the relative abundances of the bacterial taxa in CFD and GFD samples were generated from the filtered OTU table (>0.0005% abundance).

2.4 PICRUSt Analyses for Predictive functions

PICRUSt (ver 1.0.0) (Langille et al., 2013) was used to reveal the predictive functions of the microbial communities from each sample. To determine the predicted functions of each sample, the seq.fasta files were used to assign OTUs by closed reference OTU approach against the GreenGenes database (ver 13.5) at a 97% identity. The resultant OTU table was then normalized, and “predict_metagenomes.py” and “categorize_by_functions.py” commands were used to predict functions by referencing the assigned GreenGenes Ids of the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) database (Kanehisa and Goto, 2000; Kanehisa et al., 2014). The predicted functions were merged into hierarchical categories (Level 1, Level 2, and Level 3) in all samples.

2.5. Statistical Analysis of the Bacterial Diversity and their Predicted Functions

The OTU tables were used to calculate alpha (rarefaction curve and Shannon diversity (Shannon et al., 1964) and beta diversity (principle coordinate analysis (PCoA) plot through QIIME (ver. 1.8.0) (Caporaso et al., 2010). In order to calculate beta diversity, microbial communities between samples were compared using UniFrac metrics (Lozupone et al., 2006). The PCoA plot and a jackknife were created based on the weighted UniFrac phylogenetic distances through QIIME (ver. 1.8.0). A similarity percentages procedure

(SIMPER) analysis was also conducted to estimate the contribution of each taxon to the contrast between CFD and GFD samples using the SIMPER function in the vegan package of R statistical software (Oksanen et al., 2013). Then, the “heatmap.2” function in R package (Oksanen et al., 2013) was used to visualize a top 25 most highly abundant taxa, found from the SIMPER analysis, into a heatmap. For the predicted function analysis (PICRUSt, ver. 1.0.0), the output from “categorize_by_functions.py” command was then used for two-group box-plot analysis implemented in Statistical Analysis of Metagenomic Profiles (STAMP, ver. 2.1.3) (Parks et al., 2014). Welch’s t-test was used for two-group comparison of the CFD and GFD samples with confidence intervals set to 95% (0.95).

3. RESULTS

3.1. Total sequence reads, quality trimming, and OTUs information

A total of 563,879 raw sequence reads from four samples (2 samples from CFD and 2 samples from GFD) of *D. rerio* were listed in Table 1. After quality-based trimming and filtering processes, a total 383,220 sequences were used for further bioinformatics analyses. Within these reads, 114,804 sequences clustered into 471 OTUs from CFD1 sample, and 114,916 sequences clustered into 439 OTUs from the CFD2 sample. Similarly, 79,906 sequences clustered into 331 OTUs from the GFD1 sample; and 73,594 sequences clustered into 322 OTUs from the GFD2 sample (Table 1). The observed species (OTUs) rarefaction curves (Supplementary Fig. 1) were generated for each sample, to show that the sequencing depth was sufficient to produce stable and unbiased estimates of species richness.

3.2. Microbial diversity in CFD and GFD samples

The relative abundances of microbial taxa found to the most resolvable level (up to family or genus) in four samples elaborated in Fig. 1. Proteobacteria and Actinobacteria were commonly found in both CFD and GFD samples. However, Firmicutes and Bacteroidetes were present at higher abundances in the CFD samples, whereas Planctomycetes, Fusobacteria, and Verrucomicrobia appeared as more abundant in the GFD samples (Fig. 1). A comparative analysis of the bacterial distribution at the lower taxonomic levels in CFD and GFD samples showed that, *Bacteroides*, Desulfovibrionaceae, *Bifidobacterium*, Lachnospiraceae, *Oscillospira,* and *Ruminococcus* were found only in CFD samples, whereas *Rhodobacter*, Legionellales, Pirellulaceae, *Luteolibacter*, and *Pseudoxanthomonas* in GFD samples (Fig. 1). Interestingly, BLAST analysis (Altschul et al., 1990; Morgulis et al., 2008) of most of sequences representing the Legionellales identified as *Legionella anisa* (99% identity with E-value of 1e –126), *Legionella norrlandica* (99% identity with E-value of 1e –126) and uncultured *Legionella* sp. (Identity: 97–99%, E-value: 1e–126 – 6e–115). A detailed list of the distribution of taxonomic groups at the genus and the family levels for CFD and GFD sample have been elaborated in Fig. 1.

The Shannon diversity index revealed a low microbial diversity in GFD samples compared to the CFD samples (Table 1). The PCoA plot (Fig. 2a) and Jackknife (Fig. 2b) showed a closely related microbial community within each sample. The jackknife analysis also supported the PCoA plot clustering by showing the robustness of the differences between the CFD and the GFD samples. A SIMPER analyses showed that changes in the abundance of

top 25 taxa contributed to 62% of the contrast between CFD and GFD samples, suggesting a distinct microbial community in GFD-fed *D. rerio* samples. In addition, a heatmap representing the SIMPER results showed low intra-sample variation (Fig. 3). The relative abundance for each bacterial taxon was shown by the color intensity with the legend elaborated in Fig. 3.

3.3. Predicted metabolic functions using PICRUSt

The results of the predicted metabolic functions of the microbial communities visualized by STAMP represented at Level 3 are as follows: bile secretion, secondary bile acid biosynthesis, glycine, serine and threonine metabolism, cobalamin biosynthesis, carbohydrate metabolism, and starch and sucrose metabolism (Fig. 4). In general, GFD samples showed a heightened relative abundance of the KEGG Level 3 categories corresponding to bile secretion (*P*-value = 2.47e–3), secondary bile acid biosynthesis (*P*-value = 0.061), and glycine, serine and threonine metabolism (*P*-value = 0.011) (Figs. 4a–c). In contrast, CFD samples showed a higher abundance of pathways related to cobalamin biosynthesis (*P*-value = 0.018), carbohydrate metabolism (P-value = 0.104), and starch and sucrose metabolism (P-value = 0.028) (Figs. 4d–f). Detail information of each KEGG metagenomics category, as well as other KEGG categories identified using PICRUSt (ver. 1.0.0) were listed in Supplementary Table 1.

4. Discussion

This study has elucidated the distinct community composition and predictive KEGG metagenomic functional profiles of GFD-fed *D. rerio* gut microbiome, as compared to *D. rerio* fed with CFD. Since this study performed NGS approach, the plateau in a rarefaction curve (Supplementary Fig. 1) was used to suggest adequate sample replicates to generate a meaningful experimental outcome for this study. In general, the PCoA plot (Fig. 2a), Jackknife (Fig. 2b) and heatmap (Fig. 3) that accounts for changes in the relative abundances between communities revealed a low intra-sample variation.

The CFD samples displayed a generally beneficial gram-positive microbial profile (Figs. 1 and 3), representing Clostridiales, *Allobaculum*, *Ruminococcus, Lactobacillus*, and *Bifidobacterium* (Duncan et al., 2007; Stefka et al., 2014; Tojo et al., 2014; Zhang et al., 2013), along with *Bacteroides* – a gram-negative taxon that is known to produce cobalamin as a metabolite at high efficiency, and has been observed in the gut of goldfish, common carp, Nile tilapia, and ayu (Tsuchiya et al., 2008). In contrast, the resultant microbiota following a GFD showed a microbial profile with elevated gram-negative taxa (Figs. 1 and 3) including *Cetobacterium* and *Rhodobacter*. In addition, bacteria such as *Legionella* and *Mycobacterium* were also identified and the BLAST analysis of the representative OTU sequences and showed sequence identities with previously reported pathogenic *L. anisa* and *L. norrlandica*, and *Mycobacterium fortuitum* (98% identity with E-value of 1e –121) sequences, respectively (Newton et al., 2010; Sanderson and Hermon-Taylor, 1992). These results corroborated previously reported microbiota profiles in children with CD, which have shown to contribute to decrease beneficial gram-positive taxa such as *Bifidobacterium*, and

elevated occurrences of gram-negative pathogenic bacteria, including *Legionella* (Kopečný et al., 2008; Morgulis et al., 2008; Sanderson and Hermon-Taylor, 1992).

Interestingly, the GFD-fed *D. rerio* gut microbial profile showed a heightened abundance of *Rhodobacter*, members of which are known to assimilate bile to cholesterol, creating bile acid (Afrose et al., 2010; Salma et al., 2007). Such functional categories were supported in our study by PICRUSt analysis, which showed heightened bile secretion functional category in KEGG of the GFD samples, presumably contributing to a high gut bile acid environment (Salma et al., 2007). As a potential response to the increased primary bile acid, pathways related to an elevated secondary bile acid biosynthesis functional KEGG category were also found in the GFD-fed *D. rerio* microbiota samples. This supports previously reported study where the *Rhodobacter* bacteria-exclusive pathways deconjugate primary bile acids (Ridlon and Hylemon, 2006; Ridlon et al., 2014), are implicated in damaging intestinal mucosa (Vuoristo et al., 1988), and are considered co-mutagenic and co-carcinogenic in the onset of colorectal cancer (Hill, 1990; Nagengast et al., 1995; Valko et al., 2001). In addition, the KEGG categories of secondary bile acid biosynthesis by members of *Rhodobacter*, *Legionella*, and *Mycobacterium* found in our GFD-fed *D. rerio* samples were in accordance with results reported elsewhere (Kanehisa and Goto, 2000; Kimura et al., 2014).

Conversely, the CFD-fed *D. rerio* microbiota displayed a high abundance of *Bacteroides*, *Bifidobacterium*, and *Lactobacillus*, which were absent in the GFD-fed *D. rerio*, members of which have been previously reported to suppress secondary bile acid through the assimilation of cholic acid, and reversion to primary bile acid (Ridlon and Hylemon, 2006). Additionally, the GFD-fed *D. rerio* gut microbial community displayed relatively heightened metabolic functional KEGG categories related to glycine, serine, and threonine metabolisms. Under specific circumstances, serine and threonine precede glycine in the one-carbon metabolic pathway, and hyperactivity of these pathways has been implicated in oncogenesis (Amelio et al., 2014; Locasale, 2013; McKnight, 2014). Interestingly, dietary restrictions of the amino acids serine and glycine have been shown to impede tumor development (Maddocks et al., 2013; Tavana and Gu, 2013). Thus increased productions of these amino acids by gut bacteria observed in this study provide a predictive outlook of the potentially developing pathogenic profile of the GFD induced gut microbiota.

As compared to the CFD-fed *D. rerio*, a marked increase in *Cetobacterium* was observed in the GFD-fed *D. rerio*, a genus that has been identified in the gut microbiota of sea mammals (Foster et al., 1995), fathead minnows *Pimephales promelas* (Narrowe et al., 2015), carp *Cyprinus carpio* (Van Kessel et al., 2011), and in previous culture independent microbiome investigations of *D. rerio* (Roeselers et al., 2011). *Cetobacterium*, and particularly *C. somerae*, are known to synthesize cobalamin in fish without dietary sources of the vitamin (Van Kessel et al., 2011), signifying a compensatory enrichment of the taxa in response to either malabsorption of the vitamin, or its deficiency in the diet (Dahele and Ghosh, 2001). Importantly, members of *Cetobacterium* are bile resistant (Duncan, and Louis, 2007; Finegold et al., 2003), which may explain their elevated appearance in a high bile gut environment of GFD samples. Nevertheless, cobalamin biosynthesis KEGG pathways were observed to be heightened in the CFD-fed *D. rerio*, suggesting an overall decrease in beneficial cobalamin producing bacteria in GFD-fed fish.

In a previous study (Bonder et al., 2016), the effect of a gluten-free diet on the gut microbiome in humans with no preexisting gastrointestinal disorders showed changes in the gut microbiome structure and microbial metabolic pathways involved in carbohydrate and starch metabolisms. In support of their observations, the CFD-fed zebrafish in our study showed a noteworthy occurrence of Clostridiaceaes, Clostridiales, Coriobacteriaceae, and *Collinsella*, as well as a low abundance of *Veillonella*, *Ruminococcus*, and *Roseburia*. To further support the predicted functional attributes performed by Bonder et al. (2016) on the gluten free microbiome, our PICRUSt results showed a heightened relative abundance of functional categories related to sugar metabolisms (Figs. 4e–f) in the CFD samples. Interestingly, Clostridiales was found to be the most dominant of the aforementioned bacteria in our CFD samples, members of which are known to perform a variety of anaerobic carbohydrate metabolisms including Alpha- and Beta-linked saccharides hydrolysis (Xia et al., 2015), thereby implicating this taxon in the increased abundance of carbohydrate metabolisms in CFD-fed zebrafish samples.

Both GFD- and CFD-fed *D. rerio* in our study showed gut microbial taxa that were conserved between both sample types, a result which corroborates aspects of the previously suggested core gut microbiota of domesticated and recently caught wild *D. rerio* (Roeselers et al., 2011). Of the described core phyla, Proteobacteria and Firmicutes were observed in both sample groups in this study, though to varying degrees of relative abundances. Specifically, Proteobacteria was more heightened in the GFD samples as compared to the CFD-fed *D. rerio*, which showed a higher observance of Firmicutes. Another described core phylum, Fusobacteria, although commonly observed between GFD and CFD samples, was more heightened in GFD samples. Lastly, members of the phylum Actinobacteria, as well as family Aeromonadaceae and genus *Shewanella* were shared among both sample types in this study. Overall, the GFD and CFD samples shared core microbial taxa that seem to be persistent with the previously described core microbiota of *D. rerio* (Roeselers et al., 2011).

Diet-induced variations in gut microbial population have been observed in human (David et al., 2014) as well as proxy mammalian (Turnbaugh et al., 2009), and other vertebrate and invertebrate model organisms (Newton et al., 2013), including *D. rerio* (Oka et al., 2010; Watts et al., 2016) to predict circumstantial deviations from healthy reference microbiome communities. Such investigations have also been conducted to understand the changes in the gut microbiome influenced by gluten in context to pre-existing diseases such as CD and NCGS. (A Daulatzai, 2015; Béres et al., 2014; Lotta, and Katri, 2016; Samsel and Seneff, 2013).

Although fecal samples are generally accepted for microbiome investigations in humans, recent reports have indicated that by including tissue biopsy techniques from multiple regions of the gastrointestinal tract, it is possible to achieve a more comprehensive and appropriate representation of the microbial communities contributing to gut tissue health (Huse et al., 2014, Bashir et al., 2016 ; Momozawa et al., 2011). In accordance with these previous reports, we have sampled the entire zebrafish gut tissue that included the internal digesta in our study.

This study for the first time demonstrated a uniform and distinct gut microbial profile in *D. rerio* fed with GFD, which contrasted their counterparts fed with CFD. By using a 16S rRNA gene-based metagenomics approach, we have determined the differences in the bacterial community compositions between GFD- and CFD-fed *D. rerio* using bioinformatics software such as QIIME and R, and most importantly were able to use this taxonomic information to predict KEGG functional profiles of those bacterial communities through PICRUSt and STAMP. From this approach, we have corroborated previous studies of gluten-related aberrant gut microbiome in humans with disease profiles (CD and NCGS), and thereby conclude *D. rerio* to be a suitable alternative vertebrate model organism for the investigations of diet-induced variations of gut microbiome, particularly as it relates to diseases or pathophysiology influenced by gluten enriched diet regimen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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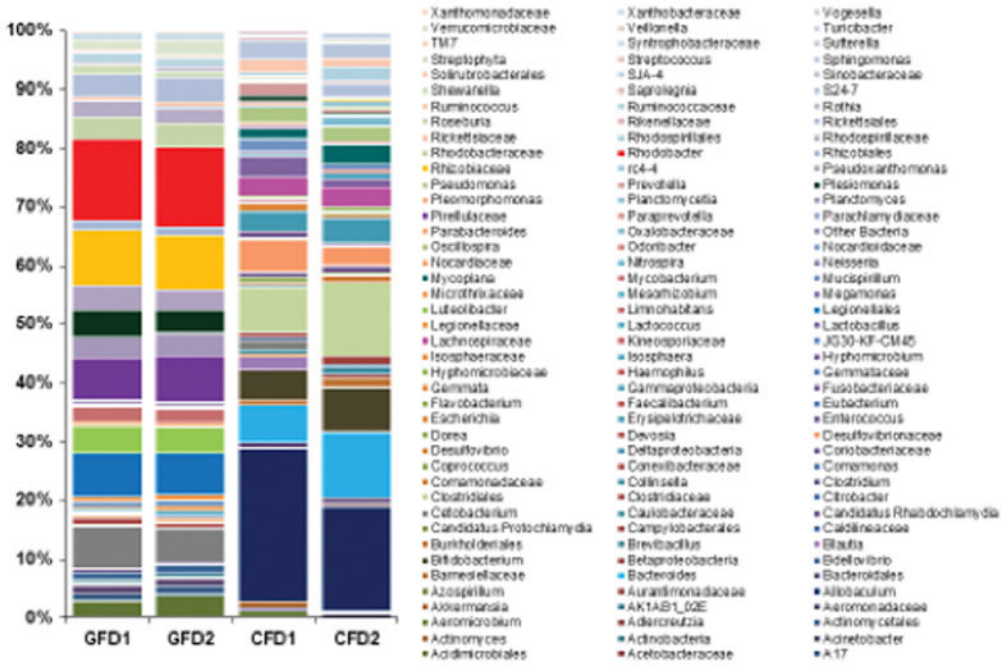


Fig. 1. Stacked column bar graph showing the distribution and abundances of bacterial communities in *D. rerio* fed with control formulated diet (CFD) and gluten formulated diet (GFD)

The figure shows the relative abundances that were analyzed using QIIME (v1.8.0), and the graph was generated using Microsoft Excel software (Microsoft, Seattle, WA) (GFD1 and GFD2 = Gluten formulated diet sample 1 and 2; CFD1 and CFD2 = Control formulated diet sample 1 and 2).

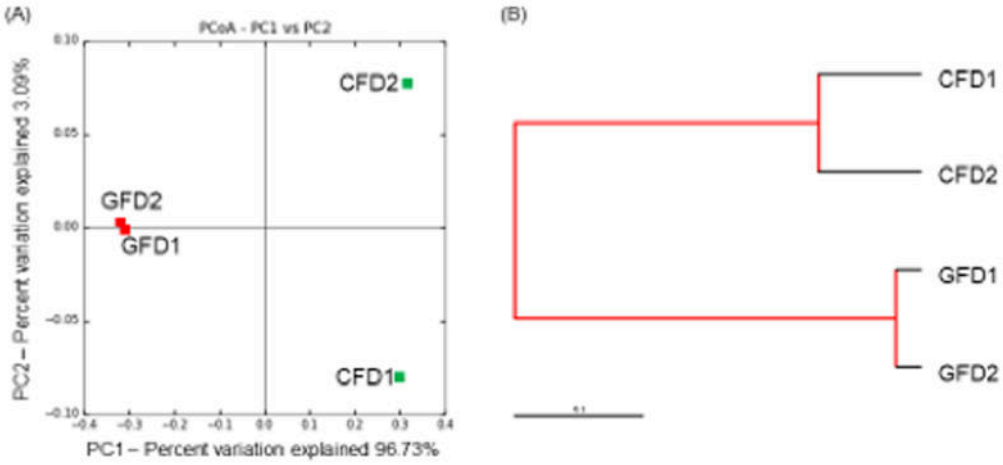


Fig. 2.

(A) Principle Coordinate Analysis (PCoA) plot, and (B) Jackknife, generated by QIIME (v1.8.0) representing the relationship between the composition of the gut bacterial communities in *D. rerio* fed with control formulated diet (CFD) and gluten formulated diet (GFD). (GFD1 and GFD2 = Gluten formulated diet sample 1 and 2; CFD1 and CFD2 = Control formulated diet sample 1 and 2).

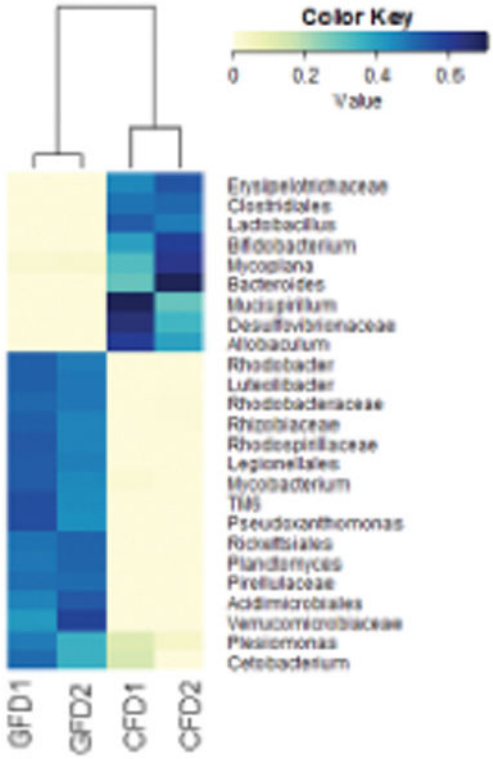
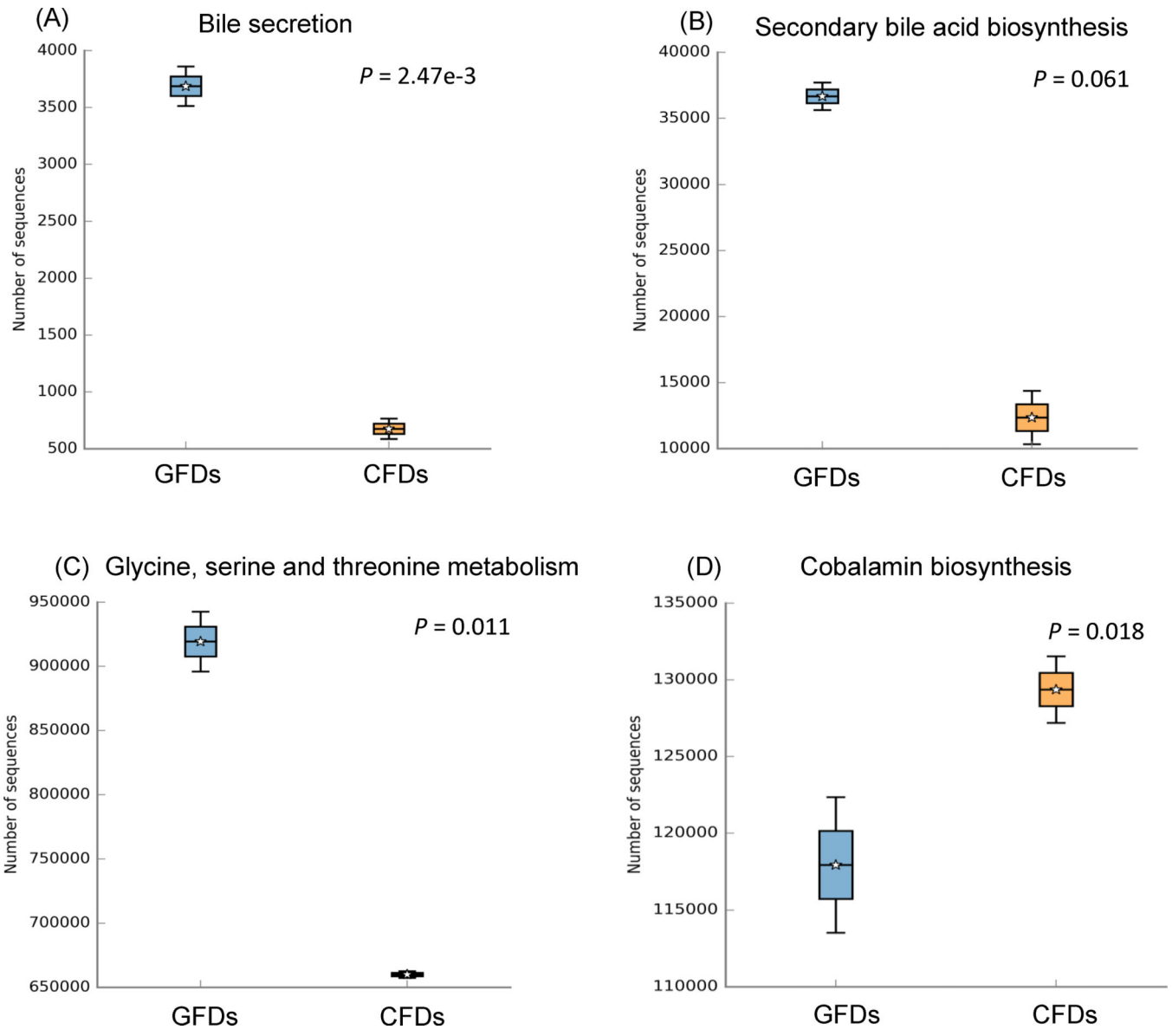


Fig. 3. Heatmap showing the gut microbial compositions at the family level of *D. rerio* fed with control formulated diet (CFD) and gluten formulated diet (GFD)

The heatmap was generated based on the SIMPER (similarity percentages procedure) result, which represents the contribution of each taxon of the *D. rerio* gut samples. The SIMPER analysis and heatmap visualization were performed using the vegan package of R statistical software. (GFD1 and GFD2 = Gluten formulated diet sample 1 and 2; CFD1 and CFD2 = Control formulated diet sample 1 and 2).



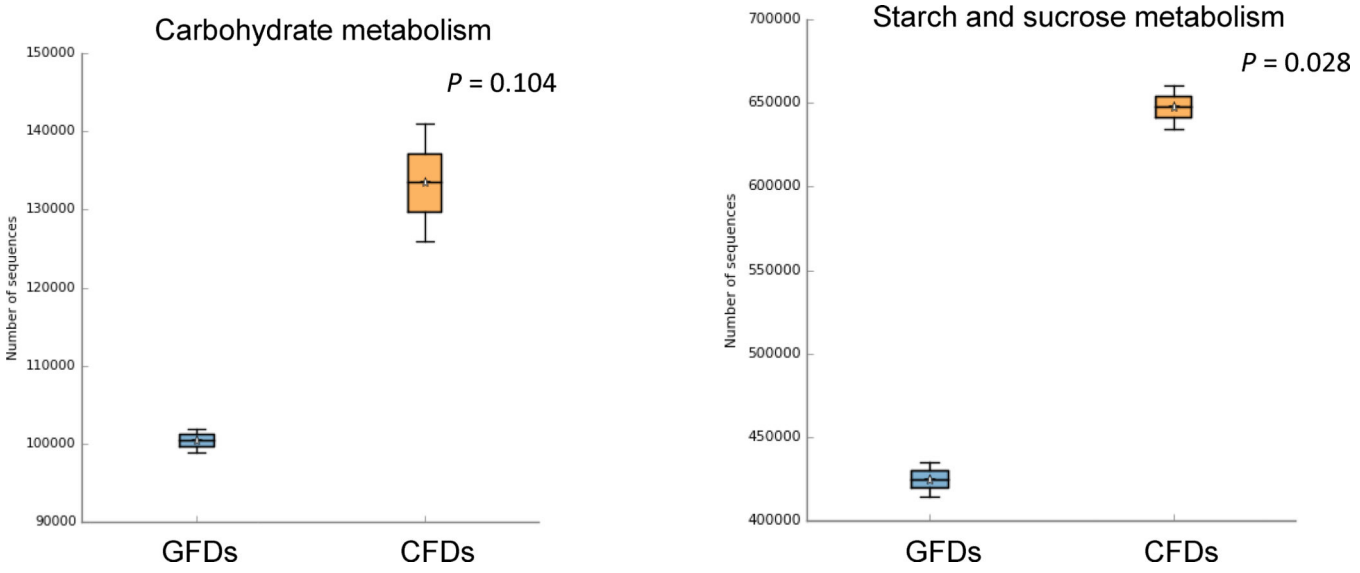


Fig. 4. Box-plot showing the predictive functions of bacterial communities in *D. rerio* fed with control formulated diet (CFD) and gluten formulated diet (GFD)

The plot was generated from the STAMP analysis based on the PICRUSt results. For group comparison, Welch's t-test was used to calculate variance between the two groups (i.e. CFD and GFD). The P-value for the total variance of the two groups is listed in the Box-plots. (GFDs = Gluten formulated diet sample 1 and 2; CFDs = Control formulated diet sample 1 and 2).

Table 1

Raw and trimmed sequence reads following NextGen sequencing of the V4 segment of the 16S rRNA gene. The number of OTUs and calculated alpha diversity of four gut microbiome samples of *D. rerio* fed with control formulated diet (CFD, $n = 2$) and gluten formulated diet (GFD, $n = 2$) are listed.

	CFD1	CFD2	GFD1	GFD2	Total
Number of raw sequences	146,253	153,866	138,447	125,313	563,879
Number of sequences after trimming and filtering processes	114,804	114,916	79,906	73,594	383,220
Number of OTUs	471	439	331	322	1563
Shannon diversity	5.880	6.252	5.382	5.496	–

Table 2

A description of the diet compositions used in this study. Diet composition and proximate analysis of the control formulated diet (CFD. containing fish meal as the primary protein source. The Scoular Company-C.P.S.P.90) and the gluten formulated diet (GFD. containing wheat gluten as the primary protein source. MP Biomedicals) are elaborated in this table.

	Amount included (g/100 g total)	
Ingredient[a]	**CFD**	**GFD**
Fish protein hydrolysate (82%)	59.00	–
Wheat gluten (80%)	–	60.00
Dextrin	13.85	13.85
Soy lecithin	4.00	4.00
Canthaxanthin	2.31	2.31
Ascorbylpalmitate	0.04	0.04
Vitamin premix BML-2	4.00	4.00
Mineral mix BTm	3.00	3.00
Betaine	0.15	0.15
Potassium phosphate monobasic	1.15	1.15
Alginate	5.38	5.38
Cholesterol	0.12	0.12
Menhaden oil	4.67	4.67
Corn oil	2.33	2.33
Diet proximate analysis	CFD	GFD
Moisture (%)[b,c]	10.97, 10.72	9.58, 9.45
Fat(%)[c,d]	15.86, 15.65	11.10, 10.90
Fiber (%)[c]	1.77, 1.67	1.73, 1.85
Protein (%)[c,e]	45.30, 45.19	49.50, 49.09
Ash (%)[c]	8.37, 8.34	5.95, 5.98

[a] Content by percentage of dry matter.

[b] Content by percentage, as fed.

[c] Duplicate measures (analysis by MVTL Laboratories, Minnesota).

[d] Fat by ethyl ether extraction.

[e] Protein = N × 6.25.