ASSEMBLY OF INTESTINAL MICROBIOTA IS DETERMINED BY HOST DEVELOPMENT, DIET, AND ENVIRONMENT

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ABSTRACT

Sandi Meilien Wong: Assembly Of Intestinal Microbiota Is Determined By Host Development, Diet, And Environment
(Under the direction of John Rawls)

The community of microbes residing in the intestine (gut microbiota) impact and is influenced by host physiology. Additionally, diet has been implicated as a modulator of host-microbiota interactions. However, the impact of prolonged dietary changes, especially in concert with host development, on host-microbiota interactions is largely unexplored. Also unknown is the degree to which gut and environmental microbiota may interact. Improved understanding of how these ecological relationships change over time may lead to more targeted or efficacious means of treating microbiota-associated pathologies such as obesity, malnutrition, and inflammatory bowel diseases.

Here, we use 16S ribosomal DNA sequencing to characterize gut microbiota of fish fed different diets through the host life cycle. To determine the impact of long-term environmental differences, we first compared gut microbiota of rainbow trout fed either fishmeal of grain-based meal feeds combined with different rearing densities over 10 months. Our results show that rainbow trout gut microbiota, which possess a large set of shared bacteria (core microbiota), are resistant to the tested diet and rearing density differences. In zebrafish, we assessed the impact of life-long differences in dietary fat levels on gut microbiota at multiple developmental stages. We observed age-dependent impacts of different dietary fat levels on gut microbiota composition as well as on the degree to which selection and neutral processes impacted microbiota assembly. This suggests that host development is an important determinant of the impact of diet on gut microbiota. Finally, we characterized gut microbiota in zebrafish over the course of three weeks of starvation followed by three weeks of re-feeding. We observed that gut

microbiota of starved fish became increasing different from that of fed fish and that gut microbiota of starved fish were unable to fully recover from starvation within three weeks of refeeding despite restoration of normal growth. Together, these suggest that different long-term environmental differences have different potentials to impact gut microbiota. Future work might characterize whether and how prolonged nutritional differences during discrete developmental windows impact gut microbiota and host physiology later in life.

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LIST OF ABBREVIATIONS AND SYMBOLS

°	Degree
Δ	Deletion
aa	Amino acid
AI-2	Auto-inducer 2
Angptl	Angiopoietin-like 4
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BHC	Benzene hexachloride
BSH	Bile salt hydrolase
BPA	Bisphenol A
Ctrl	Control
DDD	Dichlorophenyl dichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DHA	Docosahexanoic acid
DGGE	Denaturing Gradiet Gel Electrophoresis
dpf	Days post-fertilization
dpR	Days post-refeeding
dpS	Days post-starvation
env, Env	Environment
EPA	Environmental Protection Agency
EPA	Eicosapentanoic acid
FDR	False discovery rate
g	Gram
GALT	Gut-associated lymphoid tissue

GF	Germ-free
GI	Gastrointestinal
F-HD	Fishmeal, high-density
F-LD	Fishmeal, low-density
FAME	Fatty acid methyl ester
FCR	Feed conversion ratio
FDA	Food and Drug Administration
G-HD	Grain-based, high-density
G-LD	Grain-based, low-density
HF	High-fat
kg/m^3	Kilogram per cubic meter
L	Liter
LDA	Linear discriminant analysis
LEfSe	Linear Discriminant Analysis Effect Size
LF	Low-fat
LPL	Lipoprotein lipase
mg	Milligram
min	Minute
mm	Millimeter
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PCB	Polychlorinated biphenyl
PCoA	Principal coordinates analysis
ppm	part per million
QIIME	Quantitative Insights into Microbial Ecology
OD	Optical density

OTU	Operational taxonomic unit
QS	Quorum Sensing
rRNA	Ribosomal RNA
SAA	Serum amyloid A
SCFA	Short-chain fatty acid
SFB	Segmented filamentous bacteria
SL	Standard length
PUFA	Polyunsaturated fatty acid
UV	Ultra violet light
T3SS	Type 3 secretion system
T6SS	Type 6 secretion system
TGC	Thermal growth coefficient
µ	10^{-6}
µl	Microliter
µM	Micro molar
wpf	weeks post-fertilization
WT	Wild-type

CHAPTER ONE

INTERACTIONS BETWEEN GUT MICROBIOTA AND HOST DEVELOPMENT AND DIET

Overview

The surfaces and intestines of animals are colonized by complex communities of microbes (microbiota) shortly after birth, resulting in life-long, dynamic interactions between microbiota and their hosts. Gut microbiota of different compositions have been correlated with different host physiologies, and in some cases have been shown to induce altered host phenotypes. Conversely, different host genotypes and phenotypes have been associated with altered gut microbiota compositions. Thus, host physiology and gut microbiota exert reciprocal influences on each other. Moreover, these reciprocal interactions may be modulated by environmental factors. In particular, food availability and composition may directly impact gut microbiota and host physiology or may impact the relationship between the two. Here, I review interactions between gut microbiota and host physiology as well as the potential influences of different diets and dietary components on host-microbiota interactions.

Introduction

The surfaces of animals are colonized by communities of microbes (microbiota) that intricately interact with host physiology and diet. Here, I review the interactions between microbiota and host physiology and diet. I will focus largely on the impact of nutrition, especially different dietary components, and physiology on the gut microbiota. Most of the research covered here is observed from humans and in the model vertebrates zebrafish and mouse. While human research is most relevant to human health, ethical and practical considerations

prevent many manipulations from being performed. As mammals, mice share many physiological and genetic characteristics with humans and have therefore proven to be suitable models. Furthermore, the gut microbiota of zebrafish, mice, and humans are dominated by the same bacterial phyla, albeit in different proportions and specific membership (Rawls *et al.*, 2006) (Jemielita *et al.*, 2014). However, zebrafish have proven to be a versatile vertebrate model for studying host-microbe interactions for a number of reasons. These include high fecundity and rapid external development, which increases cost effectiveness and statistical power, the ability to derive germ-free larvae for gnotobiotic research (Pham *et al.*, 2008) (Milligan-Myhre *et al.*, 2011), and the optical transparency of larvae for *in vivo* microscopy of host-microbe interactions (Rawls *et al.*, 2007). Furthermore, zebrafish share many physiological similarities with mammals, including the possession of innate and adaptive immunity and many of the same gastrointestinal organs and cell types (Flores *et al.*, 2008) (Ng *et al.*, 2005). These similarities in physiology between zebrafish and mammals make zebrafish a useful tool for studying conserved host-microbe interactions and ecologies.

Gut microbiota compositions have been shown to change along the proximal-distal axis of the gastrointestinal (GI) tract (Wang *et al.*, 2010), and these differences are likely influenced in part by digestive physiology. The digestive process begins once food enters the oral cavity through the mechanical process of chewing and chemical degradation via the secretion of salivary enzymes such as amylase (Lebenthal, 1987) and salivary lipase (Hamosh & Scow, 1973). After swallowing, peristalsis pushes food through the esophagus and, if applicable, into the stomach, signaling the release of digestive enzymes and of gastric acid, which causes a reduction in stomach pH from ~4 to ~2 (Borgström *et al.*, 1957). Digestion continues in the duodenum, jejunum, and ileum, which constitute the proximal, mid, and distal parts of the small intestine, respectively. From the duodenum to the ileum, pH increases from ~6 (duodenum) to ~8 (ileum) (Borgström *et al.*, 1957). In the duodenum, bile and pancreatic digestive enzymes are released (Borgström *et al.*, 1957). These secretions may impact gut microbiota through direct

antimicrobial activity (Hofmann & Eckmann, 2006) or by modifying the bioavailability of dietary nutrients to microbes. The small intestine is the primary site of nutrient absorption (Asche *et al.*, 1989) (Borgström *et al.*, 1957). Moreover, the jejunum is the primary site of fatty acid absorption (Asche *et al.*, 1989) (Borgström *et al.*, 1957). These may create nutrient concentration gradients along the proximal-distal axis of the intestine that could impact microbial ecology. Undigested and incompletely digested food such as indigestible polysaccharides then passes into the colon, where they can be further degraded by microbes. Among the byproducts of these microbial activities are short-chain fatty acids (SCFA), which can be use by colonocytes and other tissues for energy (Clausen & Mortensen, 1995). This is but one example of the impact gut microbiota have on their hosts.

The microbiota and its impact on animal physiology

Microbiota impact animal physiology

Animal evolution has occurred on a planet initially dominated by microbes. Microbiota have therefore likely co-evolved with their animal hosts and have been observed in association with diverse multicellular eukaryotes, from Cnidarians and other invertebrates to vertebrates. Furthermore, bacteria from the Bacteroidetes phylum can induce choanoflagellates to switch from a single-cell planktonic lifestyle to a multicellular rosette form (Alegado *et al.*, 2012). Similarly, in squid, colonization by specific strains of *Vibrio fischeri* is required for the development of the light organ (Montgomery & McFall-Ngai, 1994) (McFall-Ngai & Ruby, 1991). In vertebrates, microbiota play a role in the development of multiple organ systems. These include increases in intestinal epithelial cell proliferation and goblet cell number, differences in the amount and types of mucins produced following microbial colonization (Rawls *et al.*, 2004) (Kleessen *et al.*, 2003) (Bergström *et al.*, 2012), and the development and growth of gut-associated lymphoid tissues (GALTs) such as Peyer's patches and isolated lymphoid follicles (Pabst *et al.*, 2006) (Bouskra *et al.*, 2008). Given the role of gut microbiota in host GI

development, it would be interesting to test whether specific gut microbiota members accelerate or retard GI maturation and function.

Linked to its role in the development of the GI system is the active role that gut microbiota play in host metabolism. For example, colonization of the gut can alter metabolism through the suppression of Angiopoietin-like 4 (Angptl4) (Bäckhed *et al.*, 2004), which inhibits lipoprotein lipases (LPL) (Lee *et al.*, 2009), and inhibition of LPL in turn reduces fat storage in adipocytes. Moreover, different bacteria exert specific effects on host metabolism. For example, lipids can be stored in lipid droplets – intracellular vesicles – and a recent study showed that colonization by Exiguobacterium but not Chryseobacterium or Pseudomonas sp. induced an increase in the size of lipid droplets in intestinal epithelial cells. In contrast, colonization by Chryseobacterium or Pseudomonas but not Exiguobacterium induced an increase in the number of lipid droplets. Additionally, of these three species, only colonization by Exiguobacterium elicited an increase in the size and number of lipid droplets in the liver (Semova *et al.*, 2012), suggesting altered lipid metabolism. The mechanisms by which these different species induce differences in the number and size of intestinal lipid droplets and alter lipid metabolism remain unknown. Bacteria in the gut microbiota, notably those in the genera Clostridium and Lactobacillus, also impact metabolism by regulating the activity of the nuclear receptor FXR, which binds bile salts and also regulates bile acid production and fatty acid metabolism (Reschly *et al.*, 2008) (Li *et al.*, 2013) (Sayin *et al.*, 2013). Moreover, the gut microbiota, through bile salt hydrolase (BSH)-mediated modification of primary bile salts into secondary bile salts that are reabsorbed in the small intestine, is required for FXR-mediated inhibition of bile salt production (Ridlon *et al.*, 2006) (Li *et al.*, 2013) (Sayin *et al.*, 2013). Other mechanisms by which gut microbiota impact metabolism include the production of SCFAs, which are a source of energy for colonocytes, through degradation of fiber (Stevens & Hume, 1998) (Clausen & Mortensen, 1995) and by altering glucose absorption in the gut by promoting increases in the expression of the glucose transporter GLUT2 in the intestine (Mangian &

Tappenden, 2009) (Woting *et al.*, 2014). Because gut microbiota are associated with both increases and decreases host energy harvest (Turnbaugh *et al.*, 2006) (Smith *et al.*, 2013), it would be interesting to determine if some bacteria are able to inhibit the production or absorption of secondary bile salts or SCFAs.

Gut microbiota are intricately entwined with the host immune system, and can directly mediate protection against pathogens. The strength of colonization resistance is emphasized in mouse models of Salmonella pathogenesis, where inflammatory disease only occurs if streptomycin is administered prior to infection (Barthel *et al.*, 2003). However, the particular bacteria and mechanisms mediating colonization resistance against different pathogens remain unknown but may include the bacterial production of anti-microbial compounds, secretion of molecules that regulate Salmonella pathogenesis, and competition for intestinal niches. Gut microbiota are also involved in the development and regulation of the immune system. For example, through a number of mechanisms that likely include bacterial detection by TLR2, TLR4, and TLR5, colonization leads to increased MyD88 and NF-κB signaling (Kanther *et al.*, 2011) (Cheesman *et al.*, 2010). NF-κB is regulated in part by serum amyloid A (SAA) (Deguchi *et al.*, 2013), and both are upregulated in colonized animals (Reigstad *et al.*, 2009) (Kanther *et al.*, 2014) (Rawls *et al.*, 2004) (Rawls *et al.*, 2006) (Kanther *et al.*, 2011). Also upregulated in response to colonization are antimicrobial proteins such as RegIIIgamma, which are regulated by MyD88 (Vaishnava *et al.*, 2011) (Frantz *et al.*, 2012) (Menendez *et al.*, 2013). Interestingly, mono-colonization with wild-type *Pseudomonas aerugionsa* but not non-motile *P. aeruginosa* strains, whether or not a flagellum is produced, recapitulates host innate immune responses to conventionalization (Rawls *et al.*, 2007). This suggests that host immune responses to colonization are induced in part by mechanisms that sense the act of flagellar motility or by bacterial production of molecules specific to sessile phenotypes.

Another component of innate immunity is the mucus layer covering the intestinal epithelium. The mucus layer forms a physical barrier between the microbiota and the surface of

the intestinal epithelium. The type of mucins as well as the thickness of the mucus layer, which in turn impact barrier function, are impacted by colonization status and the composition of gut microbiota (Bergström *et al.*, 2012) (Jakobsson *et al.*, 2015). For example, increased levels of Bifidobacteria have been associated with decreased metabolic endotoxemia and increased intestinal barrier function (Wang *et al.*, 2006) (Griffiths *et al.*, 2004) (Cani *et al.*, 2007). While some of these effects may be mediated by bacterial activation of MyD88 signaling (Frantz *et al.*, 2012), that different gut microbiota compositions are associated with mucus differences and barrier function brings forth the possibility that specific types of bacteria impact the expression of different mucins or glycosyltransferases. Alternatively, these associations raise the possibility that bacteria induce the production of glycoproteins that they can consume.

Moreover, gut microbiota are extremely important in myeloid hematopoiesis, with colonization leading to an increase in innate immune cells (Khosravi *et al.*, 2014). Furthermore, gut microbiota regulate the adaptive immune system, with different bacterial species exerting a myriad of pro-inflammatory and anti-inflammatory effects. These include changes in the amount of IgA in the intestine (Talham *et al.*, 1999), changes in the colonic T cell receptor repertoire (Lathrop *et al.*, 2011), and changes in the numbers of different T cell subsets. For example, colonization by segmented filamentous bacteria (SFB) leads to an increase in the number of inflammatory Th17 cells through the presentation of SFB antigens on MHCII molecules of dendritic cells in the lamina propria (Ivanov *et al.*, 2009) (Goto *et al.*, 2014). In contrast, Clostridia and Bacteroides species, in particular *Bacteroides fragilis* through the production of polysaccharide A (PSA), can promote increases in the number of anti-inflammatory FOXP3+ regulatory T (Treg) cells (Atarashi *et al.*, 2013) (Round & Mazmanian, 2010). Both pro- and anti-inflammatory effects of gut microbiota are likely important in immune homeostasis, and imbalances may increase the risk of intestinal inflammatory diseases or of infection by enteropathogens, respectively. It would be interesting to test these hypotheses by colonizing GF animals with defined communities differing in the relative abundance of the different members

before challenge with enteropathogens or chemical agents that induce colitis. Moreover, the adaptive immune system of zebrafish is thought to be not fully functional until ~4 weeks post-fertilization (wpf) (Lam *et al.*, 2004). Given the impact of gut microbiota on the adaptive immune system and the initial reliance of zebrafish on innate immunity, it would be tempting to use zebrafish to test whether some microbes alter the rate at which the adaptive immune system develops.

Altered microbiota compositions are correlated with alterations in human health and physiology

For the same reasons that gut microbiota are crucial for proper host development and physiology, gut microbiota with altered compositions can also influence the development of sub-optimal health. A growing number of maladies have been associated with altered microbiota compositions, including autism (de Theije *et al.*, 2014), inflammatory bowel disease (IBD) (Frank *et al.*, 2007) (Manichanh *et al.*, 2012), obesity (Ley *et al.*, 2005) (Turnbaugh *et al.*, 2006), malnutrition (Smith *et al.*, 2013), atherosclerosis (Karlsson *et al.*, 2012) (Koeth *et al.*, 2013), and metabolic endotoxemia (Cani *et al.*, 2007). Furthermore, events that impact gut microbiota early in life, such as antibiotic administration to infants, are increasingly correlated with disease phenotypes such as asthma, obesity, and auto-immune diseases in adolescence and adulthood (Cho *et al.*, 2012) (Cox *et al.*, 2014) (Decker *et al.*, 2011). Critically, microbiota transplant studies demonstrate that, for some conditions such as atherosclerosis (Gregory *et al.*, 2014), malnutrition/decreased adiposity (Smith *et al.*, 2013) (Liou *et al.*, 2013), obesity/increased adiposity (Turnbaugh *et al.*, 2006), and colitis (Eun *et al.*, 2014), gut microbiota are causative factors in the development of pathogenesis. It is, however, important to realize that microbiota can also be curative, as has been the case of *Clostridium difficile* infections being successfully treated with fecal transplants from healthy donors (Khoruts & Sadowsky, 2011) (Grehan *et al.*, 2010).

The impact of animal physiology on gut microbiota composition and assembly

Developmental changes may influence gut microbiota assembly

Gut microbiota assembly is determined by a combination of microbial interactions, nutrient availabilities that are host-produced and host-ingested, host digestive and immune physiology, and environmental ecologies. The gut is initially seeded by microbes from the surrounding environment. In mammals, this includes microbes that are transmitted from mother to offspring during gestation (Aagaard *et al.*, 2014) and birth, where the mode of delivery (vaginal or Caesarean section) determines whether the neonate is initially exposed to vaginal or skin microbiota (Dominguez-Bello *et al.*, 2010). Other early colonizers likely reach the gut through ingestion, for example through breastfeeding in mammals or through feeding in fish. Subsequently, the gut is frequently and perpetually exposed to microbes from food and other environmental sources, and some of these microbes have the potential to remodel the microbiota (Reeves *et al.*, 2011) (David *et al.*, 2014).

Gut microbiota are highly variable between hosts at the beginning of life. However, as the host ages, the gut microbiota becomes more similar to that of adults. Moreover, gut microbiota of adults are more similar to each other than to gut microbiota of younger hosts (Yatsunenko *et al.*, 2012) (Koenig *et al.*, 2011) (Stephens 2015 submitted). Despite continued inter-individual variation in adult gut microbiota, this suggests that gut microbiota go through somewhat conserved assembly processes in concert with developmental changes in the host. For example, the vertebrate gut is thought to be initially an aerobic environment and to become anaerobic later in life. Correspondingly, early in life aerobic and aerotolerant bacteria are highly abundant; particularly abundant are Proteobacteria, which are hypothesized to possess more defenses against oxidative stress (Palmer *et al.*, 2007) (Sommer & Backhed, 2013). In contrast, gut microbiota of older animals are characterized by increasing amounts of facultative and obligate anaerobes such as Clostridia, Fusobacteria, and Bacteroides species (Rawls *et al.*, 2004) (Roeselers *et al.*, 2011) (Palmer *et al.*, 2007). Other age-associated physiological

parameters that are likely to influence gut microbiota are changes in gut morphology and GI system development. In mammals, while GI organs are formed *in utero*, the pancreas, intestines, and other digestive organs continue to undergo postnatal development (Le Huerou-Luron *et al.*, 2010). For example, age-associated changes in the gradient of glycoproteins modified by FUT2, a fucosyltransferase, along proximal-distal axis of the intestine have been observed (Nanthakumar *et al.*, 2013) (Marcobal *et al.*, 2013). As fucosyltransferase activity has been identified as a host factor impacting gut microbiota composition (Hooper *et al.*, 1999) (Rausch *et al.*, 2011), it is possible that the developmental changes in FUT2 modification of glycoproteins in the gut influence age-associated changes in gut microbiota. Zebrafish, arguably more so than mammals, also exhibit extensive changes in GI morphology and physiology after initial exposure to the microbial world after hatching. These changes include the opening of the distal end of the intestine to form a patent tube ~2 days after hatching (5dpf) (Ng *et al.*, 2005), the formation of intestinal folds (Oehlers *et al.*, 2010), and the formation of visceral white adipose tissues (Imrie & Sadler, 2010) (Flynn *et al.*, 2009). Changes in the physiology of digestive organs may correlate with changes in the secretion of antimicrobial molecules and hydrolysis of macronutrients into components readily utilized by microbes, yielding another potential link between host development and microbiota assembly. Additionally, changes in diet typically coincide with changes in development, for example weaning and the switch to solid foods in mammals. The mechanisms by which these dietary differences might impact gut microbiota will be discussed later.

Other developmental changes that likely impact gut microbiota are those that occur in the immune system. Zebrafish are a striking example, in which the adaptive immune system is not fully functional until about 4wpf (Lam *et al.*, 2004). While developmental changes in mammalian adaptive immunity are arguably less extreme, the human thymus also goes through postnatal morphological and functional changes (Boehm & Swann, 2013). Interestingly, the difference in the timing of adaptive immune system maturation in zebrafish compared to

mammals may lead to altered gut microbiota assembly processes early in life. More generally, age-associated changes in adaptive immune physiology may lead to gut microbiota changes by altering recognition of specific members of gut microbiota. Additionally, increases in Th17 and Treg cells following gut colonization (Ivanov *et al.*, 2009) (Goto *et al.*, 2014) (Atarashi *et al.*, 2013) (Round & Mazmanian, 2010) may modulate pro- and anti-inflammatory responses in the gut. Subsequent immune responses in the gut could be protective or could potentiate colonization by bacteria. For example, pathogenic strains of *E. coli* and *Salmonella enterica* serotype Typhimurium possess competitive advantages and exhibit increased fitness in the context of gut inflammation (Rivera-Chavez *et al.*, 2013). Furthermore, adipose tissues have been shown to secrete cytokines and modulate the immune system (Exley *et al.*, 2014). This raises the possibility that adipose tissues that form or expand later in life can impact gut microbiota through the immune system. Additionally, as stated above, the adaptive immune system and visceral adipose tissues in zebrafish do not develop until several weeks post-fertilization. It would therefore be interesting to test whether altering the rate of host physiological development would alter gut microbiota assembly.

Physiological parameters can impact microbiota

A variety of additional physiological parameters likely influence gut microbiota composition. These include genetic polymorphisms present in the general population, which have been associated with gut microbiota composition in both mice and humans (Benson *et al.*, 2010) (Goodrich *et al.*, 2014). Host genetic polymorphism could impact gut microbiota composition by altering host physiology. For example, *FUT2* polymorphism has been correlated with gut microbiota differences (Parks *et al.*, 2013) (Rausch *et al.*, 2011). FUT2 is involved in fucosylation of intestinal glycoproteins (Nanthakumar *et al.*, 2013). *FUT2* mutations may therefore alter the landscape of nutrients available to gut microbes and contribute to the genotype-associated differences in gut microbiota. Changes in gut microbiota composition have

also been shown to differ at different times of day (Thaiss *et al.*, 2014). Potential causes of these differences include daily patterns in food intake as well as circadian rhythms in the immune system (Abo *et al.*, 1981) (Kawate *et al.*, 1981) and in gut motility (Hoogerwerf, 2010).

Gut microbiota compositions have been shown to differ between proximal and distal intestinal regions and between luminal and mucosal compartments of the GI tract (Wang *et al.*, 2010). Correspondingly, the physiological characteristics of the GI tract vary along the proximal-distal and luminal-mucosal axes. For example, the stomach is highly acidic, while the pH of the intestines is closer to neutral. The greater acidity in proximal regions of the GI tract may in part explain the lower bacterial density in the stomach and small intestine compared to the colon. Additionally, in mammals Paneth cells are only found in the small intestine (Sandow & Whitehead, 1979). This likely results in differences in antimicrobial peptide production and concentration in the proximal versus distal intestine (Noble *et al.*, 2008) (Ouellette *et al.*, 1999). Similarly, zebrafish also exhibit differences in antimicrobial peptide gene expression along the proximal-distal axis of the intestine (Oehlers *et al.*, 2011). These differences in antimicrobial gene expression may lead to differences in microbial selection along the proximal-distal axis of the intestine. To date, Paneth cells have not been detected in zebrafish but have been observed throughout the intestinal tract of other animals such as amphibians and reptiles (Sandow & Whitehead, 1979). Interestingly, these differences in Paneth cell presence and localization may also contribute to microbiota compositional differences that have been observed in different animals (Ley *et al.*, 2008). Other changes along the proximal-distal axis of the intestines include changes in the concentrations or types of digested nutrients, with decreases in the availability of sugars, amino acids, and lipids following absorption in the small intestine (Asche *et al.*, 1989), and increased mucus thickness in the colon compared to more proximal portions of the intestinal tract (Jakobsson *et al.*, 2015) (Szentkuti & Lorenz, 1995). All of these factors have the potential to exert positive and negative selection on microbiota members and are therefore potential contributors to the differences in gut microbiota in proximal versus distal regions of the

GI tract. Similarly, the composition of the gut microbiota differs between the lumen and the mucosa. The mucosa contains IgA, antimicrobial peptides, and glycoproteins (Rogier *et al.*, 2014) (Meyer-Hoffert *et al.*, 2008) (Frantz *et al.*, 2012), which may diffuse away from the epithelial surface toward the lumen and result in epithelium-lumen concentration gradients. It is therefore possible that differences in both nutrient availability, antibodies, and anti-microbial molecules combine to differentiate the microbiota composition of different intestinal compartments.

Associations between nutritional differences and gut microbiota composition

Differences between gut microbiota of fed and undernourished or fasted animals

Gut microbiota have been shown to be responsive to host feeding status, and these changes may be evolutionarily conserved. For example, studies in zebrafish and pythons have documented post-prandial increases in Firmicutes (Semova *et al.*, 2012) (Costello *et al.*, 2010). Correspondingly, decreases in Firmicutes accompanied by increases in Bacteroidetes and Gammaproteobacteria have been observed during fasting and hibernation in mammals and fish (Carey *et al.*, 2013) (Xia *et al.*, 2014). Similarly, gut microbiota of malnourished children, who may consume both fewer calories and less nutritious food, differs from that of healthy children (Subramanian *et al.*, 2014). Additionally, pre-prandial or fasted gut microbiota have exhibited lower richness and phylogenetic diversity compared to fed gut microbiota (Semova *et al.*, 2012) (Carey *et al.*, 2013) (Crawford *et al.*, 2009).

These microbiota differences between fed and unfed or underfed animals are likely the result of a number of mechanisms including differences inter-microbial interactions and host physiology. For example, members of the genus Bacteroides, which is often relatively more abundant in pre-prandial conditions, tend to thrive in the mucosa. Its member species possess large numbers polysaccharide utilization loci (PULs) (Martens *et al.*, 2008) that may confer a competitive advantage in the harvest of intestinal glycoproteins for energy, especially in the

absence of host-ingested nutrients. The introduction of exogenous nutrient sources expands the types of nutrients available for utilization by microbes as well as increases the total amount of energy available in the gut. The increase in energy availability may relieve competition for limited energy sources, for example host glycoproteins and, potentially, dead cell debris, in the starved gut. Alternatively, feeding may supply a critical nutrient for species that cannot consume host glycoproteins for energy. Ingestion of food may also provide bacteria with energy sources other than host glycans. Simultaneously, the consumption of these newly available substrates can lead to the production of energy sources supporting the growth of other gut microbiota members. For example, byproducts of *Bifidobacterium longum* metabolism of oligofructose support the growth of *Anaerostipes caccae*, which cannot grow with oligofructose as the sole carbon source (Falony *et al.*, 2006). Additionally, *Ruminococcus bromii* utilization of resistant starch 2 and resistant starch 3, forms of starch that are not digested in the small intestine, produces sugars that may support the growth of other gut microbiota members, for example *Eubacterium rectale* and *Bacteroides thetaiotaomicron*, that possess limited ability to consume these starches (Ze *et al.*, 2012). Moreover, the recently sequenced genome of SFB lacks a sialidase gene but possesses genes for uptake and degradation of sialic acid (Sczesnak *et al.*, 2011), suggesting that SFB can metabolize sialic acid but must rely on other gut microbes to liberate sialic acid from host glycoproteins. Similarly, some Bacteroides species have been shown to produce corrinoids, which might then be utilized by other bacteria that do not possess a full corrinoid biosynthesis pathway (Degnan *et al.*, 2014). Notably, these compounds as well as other micro- and macro-nutrients may also be made available to gut microbiota through the ingestion of food.

The effects of short- and long-term fasting and of feeding on host physiology are also numerous. The increases in MUC2 production and antimicrobial defenses in hibernating squirrels (Dill-McFarland *et al.*, 2014) brings forth the possibility that food deprivation induces physiological mechanisms that maintain colonization by commensals and prevent colonization

by opportunistic pathogens or pathosymbionts. Additionally, due to their stressful natures, fasting and starvation may result in increased levels and alterations in the biosynthesis and degradation of stress hormones such as glucocorticoids, which can influence the expression of FUT2 (Nanthakumar *et al.*, 2013), and norepinephrine (NE) (Heitkemper & Marotta, 1985), a GI neutrotransmitter that also activates QS in bacteria (Sperandio *et al.*, 2003). Furthermore, as a siderophore (Paris *et al.*, 2005) that can also regulate the expression of other siderophores (Tapryal *et al.*, 2015) NE may impact gut microbiota by altering iron availability to bacteria (Li *et al.*, 2009) (Doherty *et al.*, 2009). Similarly, upon ingestion of food, blood flow to the gut increases (Reininger & Sapirstein, 1957) (Eliason *et al.*, 2008) (Bohlen, 1998) and digestive hormones, bile, and digestive enzymes are secreted into the GI tract. Host degradation of nutrients may render food more easily consumed by microbes and postprandial increases in blood flow to the gut may impact gut microbiota by increasing oxygen levels in the gut. Moreover, bile, pancreatic lipase, and other digestive secretions directly impact gut microbiota through antimicrobial activities, discussed below.

Associations between gut microbiota and different diets and dietary components

While the act of feeding itself can both directly and indirectly impact gut microbiota, gut microbiota are also influenced by dietary content. Importantly, gut microbiota respond to different diets not just by altering microbial transcription but also by changing the microbial composition (Faith *et al.*, 2011). Gut microbiota composition has been shown to differ between carnivorous, omnivorous, and herbivorous animals (Sullam *et al.*, 2012) (Ley *et al.*, 2008) as well as between humans consuming “Western” diets (high in fat, sugar, and salt) and “rural” diets that tend to be higher in plant content (De Filippo *et al.*, 2010) (Yatsunenko *et al.*, 2012). Moreover, gut microbiota compositions have been shown to change following transitions in diet composition. Such changes have been observed in humans switching from normal to high-fat or high-calorie diets as well as in mice switching between plant-based and high-fat diets

(Turnbaugh *et al.*, 2009) (David *et al.*, 2014) (Turnbaugh *et al.*, 2008) (Ravussin *et al.*, 2012). Similarly, host development is accompanied by both dietary changes and changes in gut microbiota composition. For example, the types of human milk oligosaccharides have been shown to differ over the course of the first year lactation (Marcobal *et al.*, 2013) (Chaturvedi *et al.*, 2001). Given that bacterial species possess different specificities for specific oligo- and polysaccharides (Marcobal *et al.*, 2013) (Martens *et al.*, 2014), these changes in breastmilk composition may in turn impact developing gut microbiota. Additionally, the switch from breastfeeding to consumption of solid foods is one that includes not only dietary composition changes but also changes in the solidity of the contents entering the GI tract, which may promote the proliferation of bacteria that prefer insoluble substrates (Martens *et al.*, 2014) (Leitch *et al.*, 2007). Many of these diet-associated differences in gut microbiota are likely the product of the impact of specific dietary components on host physiology or directly on gut microbes. Component-specific effects will be discussed below.

Carbohydrates

Carbohydrates consist of simple sugars such as mono-,di- and oligosaccharides as well as digestible starch and fiber, which exhibit large variations in polymerization. Simple sugars, whether directly ingested or produced from digestion of digestible starches, is absorbed in the small intestine (Asche *et al.*, 1989). In contrast, indigestible fiber progresses to the colon where it may be fermented by microbes (Stevens & Hume, 1998). This raises the possibility that dietary fiber may be more likely to impact colonic microbes while dietary simple sugars may have greater influence on microbes in the small intestine. Additionally, sugars such as fucose, sialic acid, and N-acetylglucosamine may be linked, as single units or as a chain, to protein, forming glycoproteins, which include mucins. Importantly, different carbohydrates may differentially impact the population density of specific subsets of bacterial species within the gut. For example, in animals fed HF diets, animals that also received oligofructose supplementation

had greater numbers of Bifidobacterium cells, a decrease in the number of Enterobacteriaceae cells, but no difference in the total amount of Bacteroides cells in the gut (Cani *et al.*, 2007). Similarly, another study comparing the effects of adding inulin or fructo-oligosaccharides to the diet observed that inulin but not fructo-oligosaccharides was associated with increases in combined caecal levels of Bacteroides, Prevotella, and Porphyromonas. In contrast, supplementation with fructo-oligosaccharides but not inulin was associated with an increase in caecal Bifidobacterium levels, and both inulin and fructo-oligosacchares were associated with decreased levels of Clostridium Cluster XI in the caecum (Koleva *et al.*, 2012). The differential effects of different types of carbohydrates are likely due in part to differences in the specific sugar transporters or glycoside hydrolases encoded within the genome of different bacterial species. For example, the *B. thetaiotaomicron* genome contains a beta2-6-fructanase gene, BT1760, that is required for *B. thetaiotaomicron* to grow in minimal media containing levan. In contrast, other Bacteroides species that do not possess an orthologous beta2-6-fructanase gene were shown to be unable to grow on levan (Sonnenburg *et al.*, 2010). Moreover, multiple Bacteroides species encode a large numbers of PULs (McNulty *et al.*, 2013) (Martens *et al.*, 2008), where different PULs contain different glycoside hydrolase genes that are upregulated in response to different polysaccharides (Martens *et al.*, 2008). Similarly, during growth on starch *E. rectale* upregulates proteins predicted to be ABC transporters as well as starch-degrading glycoside hydrolases with different specific activities for different types of starch (Cockburn *et al.*, 2015). A comprehensive review of microbial degradation of glycoproteins, starches, and complex carbohydrates as well as the bacterial genetics of different carbohydrate specificities can be found in (Martens *et al.*, 2014).

Aside from its role as a nutrient source for microbes, dietary carbohydrates can also impact microbiota through direct or indirect impacts on host physiology. For example, changes in GI transit time, which can result in changes in gut microbiota composition, have been observed with consumption of different types of fiber (Kashyap *et al.*, 2013). Additionally,

addition of inulin and oligofructose to the diet has been associated with changes in rat intestinal morphology, including higher villi and deeper crypts. Moreover, rats given the dietary inulin and oligofructose also exhibited an increase in intestinal sulfomucins compared to control rats, which exhibited greater amounts of sialomucins (Kleessen *et al.*, 2003). Interestingly, changes in intestinal morphology have also been observed in response to SCFAs, which are byproducts of microbial fermentation of fiber (Stevens & Hume, 1998). SCFAs can induce colonic serotonin production, which alters GI motility and digestive secretions (Reigstad *et al.*, 2014) (Gershon & Tack, 2007) (Mawe & Hoffman, 2013), which can in turn impact gut microbiota in a feedback loop.

Proteins, peptides, and amino acids

Protein digestion occurs primarily in the stomach and proximal small intestine, and amino acids are primarily absorbed in the jejunum (Borgström *et al.*, 1957). Similar to carbohydrates, the amount and type of protein in the diet has also been correlated with differences in gut microbiota. For example, a recent study reported that rats fed a high-protein diet exhibited decreases in the abundance of *Clostridium coccoides*, *Faecalibacterium prausnitzii*, and *Clostridium leptum* in the caecum and feces. Interestingly, the authors also noted that, compared to rats fed a normo-protein diet, rats fed the high-protein diet exhibited increased microbiota diversity in the caecum but lower microbiota diversity in the feces (Liu *et al.*, 2014). Additionally, different protein sources, and therefore possibly also different ratios of amino acids, within diets have been associated with differences in gut microbiota. For example, different gut microbiota compositions have been observed in rainbow trout fed fishmeal versus soy- or grain-based diets (Wong *et al.*, 2013). Importantly, dietary proteins and amino acids function not only as energy sources for microbes but also as sources of essential amino acids for both the host and auxotrophic microbes. Amino acids from dietary and host proteins can be incorporated into microbial protein (Libao-Mercado *et al.*, 2009), and bacteria with amino acid

auxotrophies residing in the gut may rely on host diet to satisfy those nutritional requirements. Examples of gut microbes with amino acid auxotrophies include SFB, which lacks complete biosynthetic pathways for several amino acids but encodes amino acid transporters (Sczesnak *et al.*, 2011), and multiple Lactobacillus species exhibiting arginine auxotrophy (Bringel & Hubert, 2003). Therefore, the fitness of these microbes may be decreased in the guts of hosts consuming diets deficient in their essential amino acids.

Gut microbiota are also impacted by host physiological responses to consumption of proteins or to diets high in protein. A major waste product of host protein metabolism is urea, which can be excreted into the gut (Wickersham *et al.*, 2008). In the gut, microbes can hydrolyze urea and utilize the resulting ammonia for amino acid biosynthesis (Potrikus & Breznak, 1981) (Wickersham *et al.*, 2008) (Libao-Mercado *et al.*, 2009) (Atasoglu *et al.*, 1998). Moreover, the ammonia produced can be used by microbes to produce nitric oxide (NO) (Vermeiren *et al.*, 2009), which both impacts intestinal physiology (Zani & Bohlen, 2005) (Gribovskaja-Rupp *et al.*, 2014) and exerts antimicrobial activity. Similarly, animals can synthesize NO from arginine (Zani & Bohlen, 2005) but may also use arginine to produce spermine (Blachier *et al.*, 1991), which induces the maturation of the intestinal immune system (ter Steege *et al.*, 1997), leads to strengthened barrier function (Viana *et al.*, 2010), and acts as an anti-inflammatory agent (Zhang *et al.*, 1999). Similarly, addition of glutamine and/or whey protein to the diet has been found to improve intestinal permeability in patients with Crohn's disease (Benjamin *et al.*, 2012). Accordingly, high-protein and casein hydrolysate diets have been associated with reductions in levels of pro-inflammatory cytokines and T-cell activation in the intestine, reduced levels of anti-microbial molecules such as ROS, RNS, and RegIIIgamma in the ileum, increased mucin levels and goblet cell proliferation in the gut, and altered gut microbiota composition (Emani *et al.*, 2013) (Lan *et al.*, 2015).

Fats and lipids

The third major nutrient class in animal diets is lipid, which is primarily consumed as mono-, di-, and triacylglycerides or as free fatty acids. Notably, lipids also include fat soluble vitamins such as Vitamins A, D, E, and K and their precursors and metabolites, some of which will later be discussed with respect to vitamins and minerals, and phospholipids and cholesterol, which are components of cellular membranes. Fatty acids are typically categorized based on length and saturation of the acyl chain and, if applicable, on the location of the double bond. Diets with increased levels of fat have been correlated with changes in gut microbiota (Cani *et al.*, 2007) (Turnbaugh *et al.*, 2006) (David *et al.*, 2014) (Zhang *et al.*, 2012). Moreover, diets with different relative amounts of different types of fats have also been shown to impact gut microbiota. For example, in mice fed HF diets with different PUFA:saturated fat ratios, where a diet containing palm oil had a lower ratio of PUFAs to saturated fats than diets containing olive oil or safflower oil, the diet containing palm oil was associated with lower gut microbiota diversity and a higher Firmicutes:Bacteroidetes ratio (de Wit *et al.*, 2012). Similarly, a study comparing saturated, omega-3, and omega-6 fatty acids found that a diet rich in saturated fat was associated with the greatest decrease in the relative abundance of Bacteroidetes while the diet rich in omega-6 fatty acids was associated the greatest decrease in the relative abundance of Porphyromonadaceae (Liu *et al.*, 2012). These indicate that the extent of lipid saturation as well as double bond location in unsaturated lipids can differentially impact gut microbiota.

As with proteins and carbohydrates, the impact of fat on gut microbiota is both direct and, through host responses to lipids, indirect (Yao & Rock, 2015). Both Gram-negative and Gram-positive bacteria can utilize exogenous lipids, and bacterial active transport of exogenous lipids has been described in multiple Gram-negative species (Zalatan & Black, 2011) (Nunn *et al.*, 1986) (Weimar *et al.*, 2002) (DiRusso *et al.*, 1999) (Krulwich *et al.*, 1987). Characterization of the active transport of exogenous medium- and long-chain fatty acids across the cell wall has been well-characterized in *E. coli*. In this species, lipid transport is mediated by the monomeric

integral outer membrane protein FadL and by the fatty acyl coenzyme A synthetase FadD (Nunn *et al.*, 1986) (Black & DiRusso, 2003). Following esterification of Coenzyme A to the acyl chain, exogenous lipids are oxidized through beta-oxidation to produce ATP or incorporated into membrane lipids (reviewed in (Dirusso & Black, 2004)) (O'Connell *et al.*, 1986) (DiRusso *et al.*, 1993) (Yao & Rock, 2015). Additionally, medium- and long-chain fatty acids can regulate degradation, biosynthesis, and further uptake of lipids by binding FadR, a transcription factor that represses transcription of *fadL* (DiRusso *et al.*, 1993) (O'Connell *et al.*, 1986) (Fujihashi *et al.*, 2014). Furthermore, bacteria can use acetyl-CoA, which is produced during beta-oxidation of fatty acids, to synthesize acyl-homoserine-lactones (AHLs) (Hoang *et al.*, 2002), which are secreted as quorum sensing (QS) signals and can be detected by members of the same or different bacterial species (Riedel *et al.*, 2001) (Smith & Ahmer, 2003). Whether and how inter-species QS is a mechanism by which gut microbial ecology is modulated remains largely unexplored.

Notably, lipids can also impact gut microbiota by influencing host digestive and immune physiology. HF diets have resulted in increased levels of amino acids in the portal vein, increased expression of amino acid transporters in the intestine, and reduced nitrogen in feces (Do *et al.*, 2014). This raises the possibility that in animals consuming HF diets, gut microbiota may experience a selective environment where nitrogen sources are limiting. Lipids can also impact the immune system through multiple mechanisms and contribute to both pro- and anti-inflammatory programs. For example, omega-3 and omega-6 fatty acids, which possess double bonds starting 3 or 6 carbons, respectively, from the end of the acyl chain, are only obtained through diet and are precursors for host production of eicosanoids, leukotrienes, and arachidonic acid (Rubin & Laposata, 1992). This suggests that consumption of different levels omega fatty acids may impact inflammatory tone.

Moreover, just as lipids can bind bacterial transcription factors, in the host they have been shown to act as ligands for nuclear receptors such as PPARalpha, PPARgamma, LXR,

and HNF4a (Yoshikawa *et al.*, 2002) (Kliewer *et al.*, 1997) (Oswal *et al.*, 2013) (Yuan *et al.*, 2009). Similarly, the ligands for TLR2 and TLR4 are lipoteichoic acid and lipopolysaccharide, respectively. This, as well as associations between fatty acids and alterations in TLR2 and TLR4 signaling (Lee *et al.*, 2004) (Lee *et al.*, 2001), bring forth the possibility that fatty acids may regulate TLR activity. Importantly, these lipid-binding receptors have been shown to regulate digestive and immune physiology, with physiological responses varying with fatty acid chain length and saturation. For example, one study observed that diet supplementation with some lipids, for example alpha-linoleic acid but not linoleic acid resulted in a decrease in levels of SAA (Rallidis *et al.*, 2003), a potent inducer of inflammatory responses (Sandri *et al.*, 2008). Similarly, another study replacing corn oil with medium-chain triglycerides observed an increase in HNF4a expression and in the expression of genes involved in bile synthesis (Li *et al.*, 2013), which HNF4a has been shown to positively regulate (Blazquez *et al.*, 2013). Bile, which is released into the intestine to facilitate lipid absorption, possesses antimicrobial properties (Hofmann & Eckmann, 2006) (Begley *et al.*, 2005). Interestingly, different vertebrates produce different forms of bile. For example, humans and mice produce C24 bile acids and fish produce bile alcohol sulfates (Krasowski *et al.*, 2005). These differences in bile structure may contribute to differences in host response to fat ingestion or in gut microbiota composition between animal species. Additionally differences in bile structure may manifest as differences in gut microbe ability to deconjugate bile salts. Bile deconjugation leads to host reabsorption of bile, which can then bind and activate FXR in the ileum (Wang *et al.*, 1999) (Parks *et al.*, 1999) (Makishima *et al.*, 1999). Hence potential differences in microbial modificaiton of bile may alter downstream FXR activity. FXR activation results in the inhibition of hepatic bile salt production (Wang *et al.*, 2002) and in ileal expression of genes involved in the production of antimicrobial compounds; for example NO, angiogenin 1 and angiogenin 4, and pro-inflammatory cytokines (Inagaki *et al.*, 2006). In contrast, despite having been shown to lead to the production and accumulation of NO (Tanaka *et al.*, 2014), the lipid-binding nuclear receptor PPARalpha has been implicated in the

induction of anti-inflammatory responses (Buler *et al.*, 2012) (Georgiadi *et al.*, 2012). Similarly, while increased PPARgamma levels have been associated with increases in IFNgamma (Zhang *et al.*, 2012), PPARgamma activation has also been shown to induce anti-inflammatory responses, including decreases in the production NO and pro-inflammatory cytokines such as TNFalpha, IL-6, and IL-1beta and decreases in NFkB signaling (Jiang *et al.*, 1998) (Ricote *et al.*, 1998). These contrasting effects of PPARgamma may be due potential differential responses of different cell types, potential differentials among signals modulating PPARgamma activity, and/or different cellular responses to varying PPARgamma activity levels. Together, these show that dietary fat not only impacts host physiology, which in turn can impact gut microbiota, but also that different lipid species and different lipid-responsive proteins can lead to different host responses to fat ingestion.

Vitamins and minerals

In addition to the impact of the major dietary energy sources discussed above, micronutrients such as vitamins and minerals also impact both host and microbial physiology. Iron is required by both bacteria and animals, and different iron availability to microbes in the gut has been associated with altered enteropathogen load (Jaeggi *et al.*, 2014). Correspondingly, many pathogens regulate the expression of virulence genes in response to iron (Harvie *et al.*, 2005) (Sana *et al.*, 2012) (Gode-Potratz *et al.*, 2010), and increased ingestion of iron has been shown to result in altered gut microbiota compositions, including an increase in enteric pathogens (Jaeggi *et al.*, 2014). It would be interesting to determine the effects of increased or decreased iron availability on gut microbiota composition and activity of well-nourished individuals.

Additionally, Vitamin B12, also called cobalamin, is a cofactor in anabolic and catabolic enzymatic reactions in animals and microbes, including ethanolamine utilization and methionine production (Roof & Roth, 1989) (Butzin *et al.*, 2013) (Degnan *et al.*, 2014). Furthermore, Vitamin

B12 can regulate bacterial gene expression through riboswitch binding (Mellin *et al.*, 2014) (Nahvi *et al.*, 2004). However, animals cannot synthesize cobalamin. Similarly, many bacteria lack a complete enzymatic pathway for *de novo* production of cobalamin and therefore must obtain it or intermediates in the cobalamin synthesis pathways from the environment (Degnan *et al.*, 2014) (Butzin *et al.*, 2013) (Men *et al.*, 2014). Correspondingly, the availability of and ability to transport cobalamin or its derivatives has been shown to influence interactions between different bacterial species, commensal and pathogenic, that colonize the gut (Degnan *et al.*, 2014). Moreover, Vitamin B12 has been suggested to promote T cell proliferation and activity and natural killer cell activity (Sakane *et al.*, 1982) (Tamura *et al.*, 1999). Other vitamins, for example the fat-soluble Vitamin A, can impact gut microbiota by regulating immune and liver physiology. Retinol, a form of Vitamin A, is a ligand for both SAA (Derebe *et al.*, 2014) and the nuclear receptor retinoid x receptor (RXR). Interestingly, SAA is a marker of inflammation (Uhlar & Whitehead, 1999) while RXR can induce anti-inflammatory responses (Park *et al.*, 2004) that, through heterodimer formation with PPARalpha (Keller *et al.*, 1993), may be additive or synergistic with PPAR activation. Just as different fats induce different immune responses, it would be interesting to test whether different retinoids induce pro- or anti-inflammatory effects. In summary, micronutrients are not only compounds for which microbes must compete with each other as well as with the host to obtain, but are also directly involved in transcriptional control of the immune system and digestive physiology by direct binding to nuclear receptors and through interactions with nuclear receptors regulated by other dietary components.

Summary and future Directions

In summary, gut microbiota and host physiology have profound effects on each other, with dietary components capable of directly and indirectly influencing both host and gut microbiota. As such, diet is an important mediator of how host and gut microbiota interact. Because of the intimacy and extent of host-gut microbiota interactions, separating cause from

effect and defining the precise mechanisms by which diet impacts these interactions remain challenging. Nevertheless, comparison of diet-associated changes in host and gut microbiota between different host species reveals several potential conserved themes. 1) Gut colonization induces changes in digestive and immune physiology. 2) Digestive and immune physiology impacts gut microbiota. 3) Diet can directly impact both host physiology and gut microbiota. Furthermore, the feedback occurring between host and gut microbiota during host development and *de novo* gut microbiota assembly may drive the age-associated similarities in gut microbiota assembly between birth and adulthood.

The plethora of physiology- and diet-associated differences in gut microbiota further suggests that gut microbiota composition is the result of selection. However, to date few if any studies have explored the degree to which gut microbiota composition is the result of neutral as opposed to selective processes, where neutral processes include random migration into the community and random death and reproduction events. As microbial community assembly is the result of a combination of neutral and selective processes (Sloan *et al.*, 2006), it is possible that many members of the microbiota are present by chance while colonization by others is the result of increased microbial fitness. Contributions of neutral processes to microbiota assembly may partially explain the large inter-individual variation in gut microbiota between different hosts. Identification of microbial species under positive selection in the gut may constitute a consortium of particularly beneficial microbes. Furthermore, while neutral models of community assembly acknowledge the possibility of microbial migration into the gut, few studies to date have explored microbial exchange between gut and host environment in healthy hosts. Nevertheless, environmental microbes can easily be introduced to the gut through the oral route and microbes present in the diet have been detected in gut microbiota (David *et al.*, 2014). Many mechanisms by which gut microbiota assemble and change, including the extent to which many microbes in the environment or food contribute to gut microbiota, have yet to be characterized.

Microbial interactions influencing microbiota likely include competition for access to niches and nutrients within the intestine and the secretion of metabolites, intra-and inter-species signals, and anti-microbial molecules. Several interactions between a few gut microbiota species have been described. However, interactions between many other species in gut microbiota, including those that currently cannot be cultured or are genetically intractable, and interactions within complex communities have yet to be defined. Similarly, many mechanisms by which microbial pathogens interact with the immune system are well described. However, most host-microbe interactions are not pathogenic and many are instead beneficial for both host and microbe. Mechanistic information on these positive interactions, including those determining tolerance of gut microbiota by the host immune system, have been limited to a few species. Further work will need to be done to identify other bacterial species and the mechanisms by which they modulate host immune function.

A third player in host-microbe interactions is diet, which can induce pro- and anti-inflammatory responses and impact host digestive physiology and microbial metabolism and interactions. However, receptor and enzyme affinities may differ greatly for dietary molecules that differ only slightly. Similarly, different species of particular macronutrients may exert contradictory physiological effects on host or microbial physiology. For example, some lipids may exert anti-inflammatory effects while others may activate inflammation through TLR activation. Therefore, careful attention to the effects of specific species of macronutrients is warranted. Furthermore, ingested food is typically a mixture of different macromolecules, and how host and microbiota responses to different macronutrient species are titrated remains an open question.

CHAPTER TWO

AQUACULTURED RAINBOW TROUT (ONCORHYNCHUS MYKISS) POSSESS A LARGE CORE INTESTINAL MICROBIOTA THAT IS RESISTANT TO VARIATION IN DIET AND REARING DENSITY[1]

OVERVIEW

As global aquaculture fish production continues to expand, an improved understanding of how environmental factors interact in fish health and production is needed. Significant advances have been made towards economical alternatives to costly fishmeal-based diets, such as grain-based formulations, and defining the effect of rearing density on fish health and production. Little research, however, has examined the effects of fishmeal- and grain-based diets in combination with alterations in rearing density. Moreover, it is unknown whether interactions between rearing density and diet impact composition of the fish intestinal microbiota, which might in turn impact fish health and production. We fed aquacultured adult rainbow trout (*Oncorhynchus mykiss*) fishmeal- or grain-based diets and reared them under high- or low-density conditions for 10 months in a single aquaculture facility, and evaluated individual fish growth, production, fin indices, and intestinal microbiota composition using 16S rRNA gene sequencing. We found that the intestinal microbiotas were dominated by a shared core microbiota consisting of 52 bacterial lineages observed across all individuals, diets, and rearing densities. Variation in diet and rearing density resulted in only minor changes in intestinal microbiota composition despite significant effects of these variables on fish growth, performance, fillet quality and welfare. Significant interactions between diet and rearing density

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were only observed in evaluations of fin indices and relative abundance of the bacterial genus Staphylococcus. These results demonstrate that aquacultured rainbow trout can achieve remarkable consistency in intestinal microbiota composition, and suggest the possibility of developing novel aquaculture strategies without overtly altering intestinal microbiota composition.

INTRODUCTION

As aquaculture's contribution to global food fish consumption continues to increase (FAO, 1950), alternatives to fishmeal as the traditional protein source in aquaculture feeds need to be researched, refined, and adopted for sustainable industry growth (Barrows & Hardy, 2001) (Gatlin *et al.*, 2007). Much research has focused on all-plant-protein diets, and their impact on fish performance (Gaylord *et al.*, 2007), palatability (Stickney *et al.*, 1996), digestibility (Gaylord *et al.*, 2008), water quality (Davidson *et al.*, 2013), intestinal inflammation (Krogdahl *et al.*, 2003), and the community of microorganisms residing in the intestine (microbiota) (Mansfield *et al.*, 2010). Overall, significant advances have been made to alternative protein diet formulations in recent years, such that growth performance of fish fed grain-based diets has been reported to be comparable to that of fish fed traditional fishmeal-based diets (Davidson *et al.*, 2013) (Barrows *et al.*, 2007). Limited research, however, has examined the effects of grain-based feeds in combination with alterations in fish rearing density. Provided that a given aquaculture system's carrying capacity can support increases in fish biomass, larger harvests can, in theory, be attained by increasing rearing density as fish are raised to market size. Inappropriately high rearing densities, however, can have negative effects on fish production, and are commonly associated with decreased growth, decreased feed intake, reduced feed efficiency, and greater fin erosion (Ellis *et al.*, 2002). Whether these density-associated changes in performance and welfare are consistent when fish are fed either fishmeal- or grain-based diets remains unclear. Moreover, it remains unknown whether interactions between fish rearing density and diet

composition impact the composition of intestinal microbiota. This gap in our knowledge is significant because processes such as intestinal inflammation, dietary energy harvest, and behavior in other vertebrate species are due in part to alterations in intestinal microbiota composition (Rawls *et al.*, 2006) (Turnbaugh *et al.*, 2006) (Turnbaugh *et al.*, 2008) (Vijay-Kumar *et al.*, 2010) (Nayak, 2010) (Diaz Heijtz *et al.*, 2011) (Archie & Theis, 2011) (Semova *et al.*, 2012).

A fundamental challenge in host-associated microbial ecology is determining the extent to which microbial lineages in a given host are shared among other hosts. Previous studies have shown that a subset of microbial lineages harbored by an individual host might also be found in many or all other individual hosts, a concept often referred to as a "core microbiota". This term can be variably defined based on taxonomic level or the degree of ubiquity and abundance among individual hosts within a given experimental condition, a given environment, or a given host species (Shade & Handelsman, 2012) (Li *et al.*, 2013). Although detection of a core microbiota is strongly affected by sample number, sampling depth, and many genetic and environmental factors, these factors can be addressed through careful experimental design. The relatively consistent environmental, dietary, and husbandry parameters inherent to aquaculture facilities provide attractive opportunities to explore the potential for core microbiota in animal hosts. As new strategies for aquaculture enhancements are developed, it will be important to determine whether core microbiotas occur in aquaculture settings and whether such cores are affected by husbandry variation.

Our current information on the gut microbiota of rainbow trout (*Oncorhynchus mykiss*) is derived from analysis of culturable microorganisms (Ringø *et al.*, 1995) (Kim *et al.*, 2007) (Dimitroglou *et al.*, 2009) and culture-independent studies using fingerprinting and sequencing of 16S rRNA and other microbial genes (Mansfield *et al.*, 2010) (Kim *et al.*, 2007) (Huber *et al.*, 2004) (Navarrete *et al.*, 2010) (Navarrete *et al.*, 2012). These studies revealed that rainbow trout gut microbiota is dominated by the bacterial phyla Proteobacteria and Firmicutes, the same

phyla that dominate the intestines of many other fishes (Nayak, 2010) (Roeselers *et al.*, 2011) (Sullam *et al.*, 2012). In contrast to these methodologies, high-throughput pyrosequencing of 16S rRNA genes permits unbiased identification of rare as well as abundant bacterial members of the gut microbiota at low cost per sequence. The gut microbiota of aquacultured trout has previously been analyzed by pyrosequencing of the *cpn60* gene (Mansfield *et al.*, 2010) (Desai *et al.*, 2012) but not the more commonly studied 16S rRNA gene. In this study, we tested whether long-term differences in rearing density and diet, alone and in combination, lead to alterations in animal performance, welfare, fillet quality, or gut microbiota using 16S rRNA gene pyrosequencing.

MATERIALS AND METHODS

Experimental treatments, fish performance data collection, and processing attributes

All experiments involving rainbow trout were conducted in compliance with the requirements of the Animal Welfare Act (9CFR) and were approved by The Freshwater Institute's Institutional Animal Care and Use Committee.

A flow-through fish culture system consisting of 12 circular 500 L tanks was employed in this study using water from a spring source with approximately constant 12.5 ^{o}C temperature. Eyed rainbow trout eggs were procured from Troutlodge, Inc. (Sumner, WA); hatched alevins were then transferred to two of the twelve flow-through tanks for introduction to feed. Fishmeal-based starter feed was used for all fish during this acclimation period. When fish reached approximately 10 g, they were re-combined into one tank, and then randomly distributed in equal numbers to all 12 flow-through tanks. Fish were subsequently fed either the experimental fishmeal- or grain-based feeds (Table 2.1) for the remainder of the study, and were reared at one of two density ranges [either 20-40 kg/m^3 (low density) or 40-80 kg/m^3 (high density)]. As tanks approached the maximum density (40 or 80 kg/m^3) for their specific treatment, fish were culled to reduce densities back to lower end levels (20 or 40 kg/m^3). The diet and density

treatments were randomly allocated within the 12-tank system, such that each of the four diet / density treatment groups was replicated in three study tanks. Monthly length and weight assessments were made for each tank over the 10-month study to update their biomass increase and guide density adjustments. All sampled fish were first anesthetized (75 mg/L tricaine methanesulfonate (MS-222) (Tricaine-S; Western Chemical Inc., Ferndale, WA) prior to collection of performance data. Dead animals were removed and recorded daily to assess cumulative survival. Feed was administered by an in-house designed computer operated program to identical feeders for all 12 experimental tanks, with feeding events approximately once per hour. Daily feed levels were determined using standardized feed charts for rainbow trout; however, minor adjustments to daily feeding amounts were occasionally made based on visual observations of increased appetence or satiation. Overall thermal growth coefficients (TGC) and feed conversion ratios (FCR) were calculated for each tank at the end of the study period, based on the final performance data, and compared between treatments as follows:

$$TGC = ((\text{Final mean weight}^{1/3} - \text{Initial mean weight}^{1/3}) / (\text{Days during interval} * \text{mean temperature})) \times 1000$$

$$FCR = \text{Feed}_{cumulative} / \text{Biomass gain}$$

where weight is in grams, length is in mm, and temperature is in ºC. At study's end (312 days-post hatch), 5 randomly selected fish were removed from each tank, euthanized with an overdose (200 mg/L) of MS-222, eviscerated, and processed to yield butterfly fillets. The butterfly fillet is produce when the head, viscera, and vertebral column and ribs have been removed. Dress yield (%) (i.e., head-on gutted yield) was calculated as eviscerated weight / whole weight * 100. The pectoral girdle, belly flaps (approximate 1 cm strips along the ventral

midline), and skin were removed from the butterfly fillet. These fillets were weighed and fillet yield (%) was calculated as fillet weight / whole weight X 100).

16S rRNA gene sequencing and analyses

At 312 days post-hatch (after 214 days under study treatment protocols), three fish/tank (2-3 tanks/treatment combination; 33 fish total) were randomly selected and euthanized with 200 mg/L MS-222 (Western Chemical Inc., Ferndale, WA), and uniform 5 cm mid-intestine segments were carefully resected, flash frozen in liquid nitrogen, and stored at -80°C. Intestinal samples were shipped overnight on dry ice to the Core for Applied Genomics and Ecology, University of Nebraska (Lincoln, NE). Total genomic DNA was extracted from intestinal samples using Qiagen (Valencia, VA) Stool Kits. From the resulting DNA the V_1-V_3 region of bacterial 16S rRNA genes was amplified using F8 and R518 primers tagged with the A and B Roche 454 Titanium sequencing adapters. The F8 primers were modified to contain an 8 base barcode unique to each sample (Table S2.1). Pyrosequencing was performed by pooling all samples into a single region of a 2-region Titanium PicoTitre plate. Sequence data were filtered and analyzed with QIIME (Caporaso *et al.*, 2010) using default parameters with the following exceptions: we removed sequences with ≥50 consecutive bases possessing an average quality score of <25 or with lengths <150 or >1000 bases. Sequences were then grouped by trout sample based on their barcode; we used the QIIME denoiser algorithm (Caporaso *et al.*, 2010) to denoise the sequences. The denoised sequences were binned by the UCLUST method into Operational Taxonomic Units (OTUs) using a threshold of 97% or higher sequence identity. Representative sequences from each OTU were then aligned to the Greengenes core set (version gg_otus_4feb2011/taxonomies/greengenes_tax_rdp_train.txt) using PyNast (Caporaso *et al.*, 2010). The representative sequences from each OTU were also taxonomically classified using the RDP Classifier program (Wang *et al.*, 2007). Consensus lineages were assigned at each taxonomic level if ≥90% of the sequences in the OTU agreed with the classification. We also

used the QIIME ChimeraSlayer algorithm to identify and exclude from subsequent analysis any OTUs with chimeric representative sequences. Additionally, OTUs assigned to phylum Cyanobacteria were considered potential plant chloroplast contaminants and removed from the analysis. After the above filtering steps, a total of 185,216 high-quality bacterial 16S rRNA gene sequences remained for analysis. OTUs and their consensus lineages are tabulated in Table S2.4. To determine the relative abundance of each bacterial taxon, OTUs were binned according to their consensus lineage (Table S2.3). To assess the degree of dissimilarity between the gut microbiota of different samples, we conducted weighted and unweighted UniFrac analyses using 1345 sequences from each sample. UniFrac distance matrices were graphically represented using Principal Coordinates Analysis (PCoA). Additionally, we calculated non-phylogenetic distances between samples by performing binary-Jaccard analyses. To determine the bacterial diversity within individuals, we calculated Chao1, Shannon diversity indices, and Phylogenetic Distance values for each sample (Table S2.2). LEfSe software (Segata *et al.*, 2011) was used to identify discriminatory bacterial groups between conditions using sequences that had been taxonomically classified with RDP Classifier in QIIME. Taxa identified as discriminatory between two conditions were further subjected to two-way ANOVA followed by Bonferroni post-test using GraphPad Prism software. All analyses were performed using default parameters. These sequence data have been submitted to MG-RAST under accession number 4509015.3.

Fin quality assessments

During the final sampling event, 25 fish from each tank were anaesthetized and measured for fork length. Then, using digital microcalipers, the maximum length (i.e. the longest ray) of the following fins was measured to the nearest 0.1mm: left and right pectoral, left and right pelvic, dorsal, ventral, and the top and bottom poles of the caudal fin. Fin indices (Kindschi,

1987) for all eight measured fins or fin components were then calculated by dividing their individual lengths by the fork length.

Fillet quality and contaminants analyses

Fillet samples collected for processing attribute evaluation were sent to West Virginia University (Morgantown, WV) for the following assessments: cook yield, instrumental texture, proximate composition, and fatty acid profiles. Standard laboratory methods were used to determine fillet cook yield and texture (Aussanasuwannakul *et al.*, 2010). Analyses of fillet moisture, fat, protein, and ash were performed according to AOAC approved methods (Chemists, 1990). Total lipids were extracted from muscle according to Bligh and Dyer (Bligh & Dyer, 1959). Fatty acid analysis was performed on powdered muscle and minced visceral adipose tissue. Fatty acids were methylated using the method described by Fritshe and Johnston (Fritsche & Johnston, 1990). Nonadecanoic acid (19:0) was used as an internal standard. Fatty acid methyl esters (FAMEs) were quantified using a Varian CP-3800 Gas Chromatograph (Varian Analytical Instruments; Walnut Creek, California, U.S.A.) equipped with a flame ionization detector. FAMEs were identified based on comparison to retention times of standard FAMEs (SupelcoTM quantitative standard FAME 37; Sigma-Aldrich, St. Louis, Missouri, U.S.A.). Peak area counts were computed by an integrator using the Star GC workstation version 6 software (Varian Inc.) and reported as percent fatty acid.

To determine pesticide and PCB levels, at study's end, 3 fish were randomly selected from each of the 6 high density tanks, euthanized with MS-222, and filleted. These 18 fillet samples were sent fresh on ice to Northeast Analytical Inc. (Schenectady, NY), where they were processed, homogenized, and analyzed. The Soxhlet Extraction Method (EPA Method 3540C) was employed for all fillet samples; analysis for organochlorine pesticides was performed by EPA Method 8081. Analysis for PCB congeners was performed by Comprehensive Quantitative Congener Specific PCB Method (Northeast Analytical Inc. Standard Operating Procedure

NE133_02); a total of 209 PCB congeners were quantified. Pesticides quantified included: aldrin, alpha-chlordane, alpha-BHC, beta-BHC, chlordane, delta-BHC, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, gamma-chlordane, gamma-BHC, heptachlor, heptachlor epoxide, hexachlorobenzene, methoxychlor, p,p'-DDD, p,p'-DDE, p,p'-DDT, toxaphene.

Histopathology evaluations

At study's end, the 5 fish per tank randomly selected for processing attribute assessment also had standardized 3 cm sections of the posterior intestine removed and fixed in 10% neutral buffered formalin (3.7% formaldehyde). Fixed samples were sent to the Washington Animal Disease Diagnostic Laboratory (Pullman, WA) for histopathology evaluation. A 0 to 5 point grading scale was developed to quantify the extent and severity of intestinal inflammation, with 0 representing normal healthy tissue and 5 denoting severe inflammation with loss of mucosal integrity across most or all of the tissue evaluated. All animals displayed at least minimal inflammation, and severe inflammation (i.e., score of 4) was observed in only one fish from the low density, fishmeal diet group.

Statistical analysis

Measurements of final fish performance, health, and yield were assessed for treatment effects using multivariable ANOVA, with diet, density, and diet*density interaction as independent variables. Contaminant data were analyzed with ANOVA for diet effects only. An alpha level of 0.05 was used to determine statistical significance. Relative abundances of bacterial taxa were considered significant by LEfSe (Segata *et al.*, 2011) analysis if the Kruskal-Wallis test yielded an alpha value < 0.05, the pairwise Wilcoxon test yielded an alpha value < 0.05, and the logarithmic LDA effect score reached 2.0. LEfSe results were confirmed if two-way ANOVA yielded a p-value <0.05. Relative abundances of core OTUs were normalized by $\log_{10}$

transformation (Ramette, 2007) prior to determining statistical significance using pair-wise Student's t-tests and a 5% false-discovery rate.

RESULTS

Rainbow trout intestines possess a large core microbiota that persists following long-term alteration in rearing density and diet

We sought to define the effects of diet composition and rearing density on rainbow trout intestinal microbiota through 16S rRNA gene sequencing. Fish were raised together under identical conditions and fed a fishmeal-based diet until averaging approximately 10 g, and then they were randomly distributed to flow-through tanks and reared under high- or low-density and fed either fishmeal- or grain-based diets for 214 days. Genomic DNA was extracted from the mid-intestines of these animals, and their respective bacterial communities were evaluated using 454 pyrosequencing of the V_1-V_3 region of 16S rRNA genes (3 fish/tank, 2-3 tanks/condition; 5612 ± 2671 sequences/fish). We binned the resulting 185,216 16S rRNA gene sequences into 3376 Operational Taxonomic Units (OTUs) defined by 97% pairwise sequence identity and then classified the taxonomy of each OTU. We found that the relative diversity (Table S2.2) and abundance of bacterial classes (Figure 2.1) in the intestine were strikingly similar in most individuals across different diet and rearing density conditions. All bacterial communities were dominated by classes Bacilli (48.6±9.3% of sequences per sample), Alphaproteobacteria (21.8±5.8%), Gammaproteobacteria (17.1±7.6%), Betaproteobacteria (3.8±2.0%), and Clostridia (2.2±1.3%). This strong similarity among all samples at the class level raised the possibility that these rainbow trout intestines harbored a shared set of OTUs, or a core gut microbiota.

To determine the extent to which OTUs were shared across individuals and treatment groups, we first identified the OTUs present in every individual within a given treatment group (operationally defined here as a "treatment core"), and then evaluated the overlap between

different treatment cores to identify those OTUs shared among all sequenced individuals (operationally defined here as the “shared core”; Figure 2.2A). Surprisingly, we found that the majority of OTUs within each treatment core was shared among all four conditions, yielding a shared core of 52 OTUs. This large shared core contained greater than half of the OTUs that appear in each treatment core (Figure 2.2A) and constituted 81.6% of all sequences in this study (Figure 2.2B). In agreement with the overall abundances of bacterial taxa (Figure 2.1), we found that the shared core is composed primarily of the bacterial classes Bacilli, Alphaproteobacteria, and Gammaproteobacteria (Figure 2.2B). As expected from the similarity in overall bacterial composition between samples (Figure 2.1), treatment cores were not markedly different from each other or from the shared core (Figure 2.2C-2.2F, Figure S2.1). Additionally, sequences in OTUs within the shared core constituted 81.8-89.8% of all sequences in each treatment group (Figure 2.2C-2.2F). These results indicate that the tested variations in diet and rearing density did not exert large, long-term alterations on the gut microbiota of rainbow trout.

Variation in diet and rearing density causes minor changes to the rainbow trout gut bacterial community

We next sought to determine whether variations in diet and rearing density evoked any consistent alterations in gut bacterial community composition. Although treatment cores were highly similar to each other and to the shared core (Figure 2.2B-F), we did identify OTUs within each treatment core that were not observed in the shared core (operationally defined here as the “treatment accessory cores”; Figure 2.2G-J). OTUs within each treatment accessory core constituted a small fraction of the sequences within their respective treatment core (3.7-5.3%), but comparison of treatment accessory cores revealed distinct differences between diet and rearing density treatments. Although relative abundances of bacterial classes were similar in both high-density accessory cores, we observed a relative increase in Clostridia abundance and

diversity in the accessory core of the fishmeal high-density treatment (Figure 2.2G,I; Figure S2.1). In contrast, we observed pronounced differences between both low-density accessory cores compared to each other and to the high-density accessory cores. For example, the grain-based low-density accessory core displayed marked increases in the abundance and diversity of class Bacilli compared to other accessory cores (Figure 2.2J; Figure S2.1). These results suggest that the tested variations in diet and rearing density are sufficient to induce specific alterations in the diversity and proportional abundance of relatively rare members of the gut microbiota.

We next sought to determine whether the different diet-by-rearing density treatments were sufficient to evoke alterations in overall composition of gut bacterial communities. To do so, we compared diversity between samples from different treatment groups (i.e., beta diversity). Principal Coordinates Analysis (PCoA) of weighted UniFrac distances (an evaluation of community structure) showed that samples clustered together, regardless of diet or rearing density (Figure 2.3A,B) and consistent with our observation of a large shared core microbiota. In contrast, PCoA of unweighted UniFrac distances (an evaluation of community membership which does not consider abundances) showed slight clustering of samples from the same treatment group (Figure 2.3C-2.3D). In accord, binary-Jaccard analysis (a non-phylogenetic measure of community similarity) revealed that microbial communities from individual samples within the same treatment group were more similar to each other than to those from other treatment groups (Figure 2.3E).

We next determined whether the similarity of gut bacterial communities within each treatment group is associated with differential abundance of specific bacterial taxa using LEfSe software (Segata *et al.*, 2011) followed by 2-way analysis of variance (ANOVA). This analysis identified several taxa within phylum Firmicutes that were significantly discriminatory for diet type. The relative abundance of family Lactobacillaceae and its included genus Lactobacillus were significantly enriched in fish fed grain-based diet under both density conditions (Figure

2.4A,B). Although there was no significant effect of diet on the relative abundance of family Streptococcaceae, the included genus Streptococcus was enriched in fish fed grain-based diet and was the only taxon to display a significant interaction between diet and density conditions (Figure 2.4C,D). The relative abundance of family Staphylococcaceae and its included genus Staphylococcus were significantly enriched in fish fed grain-based diet with the major effect being observed in the low-density condition (Figure 2.4E,F). In contrast, the relative abundance of family Clostridiales and its included genus Clostridia were significantly affected by diet with a trend towards increased relative abundance in fishmeal-fed animals (Figure 2.4G,H). Together, these results indicate that the tested diet and rearing density combinations caused consistent alterations in a limited number of bacterial community members, and that differences between treatments were sufficient to create treatment-specific bacterial community profiles in these animals.

Long term alteration in diet and rearing density do not impact intestinal histopathology

Because grain-based diets have previously been associated with intestinal inflammation in fish (Baeverfjord & Krogdahl, 1996) (Bakke-McKellep *et al.*, 2000), we next sought to determine if alterations in diet and rearing density were sufficient to alter intestinal histopathology. All animals displayed at least a minimal level of intestinal inflammation, but intestinal inflammation was not affected by treatment ($P>0.05$; Table S20). These data suggest that the tested diets and rearing densities were not sufficient to significantly alter severity of intestinal inflammation.

Rainbow trout performance, survival, and fin condition are significantly affected by diet and rearing density

Additionally, we determined if variation in diet and rearing conditions impacted fish performance and health. By study's end, statistically significant differences in fish weight were

detected, with higher weights being observed in fishmeal diet groups relative to grain-based diet groups. Rearing density, however, did not significantly affect final fish weight (Table 2.2; Figure 2.5). Despite lower final weights, however, fish fed grain-based diets were better able to utilize dietary energy for growth, as indicated by the significantly greater feed efficiency (i.e., lower feed conversion rates) in these groups (Table 2.2). Again, no significant association between density and feed conversion was determined. Survival was generally high (>96%) among all treatment groups; however, significantly higher survival was observed in grain-based diet treatment groups (Table 2.2).

Because fin erosion is an established indicator of fish welfare under culture conditions (St-Hilaire *et al.*, 2006) (Adams *et al.*, 2007), we measured fin indices (i.e., the length of the longest ray of each rayed fin relative to the fork length) as a means of evaluating fish welfare. Although no major fin erosion was noted qualitatively on any of the sampled fish, fin indices were significantly higher in grain-based diet treatment groups for all measured fins (Table 2.3) indicating healthier fins overall in these groups. Statistical interaction between diet and density was observed when modeling these main effects and their associations with indices for the pectoral (left and right), dorsal, and pelvic (left and right) fins. In these cases, the overall trend was an increase in fin index when fish were fed grain-based diets but a decrease in fin index associated with the increased density treatment. Together, these data show that the tested alterations in diet and rearing density were sufficient to independently and interactively modify rainbow trout health and performance.

Rainbow trout diet and rearing density alter processing attributes and product quality

Fish from the grain-based diet treatment groups had significantly greater dress yield compared to fish from the fishmeal diet groups (Table 2.2). There was a small but statistically higher level of % protein in fillets from fish fed grain-based diet, and these fish also contained significantly higher fillet levels of eicosadienoic acid and total Omega-6 fatty acids. However,

fish fed fishmeal-based feed had fillets with significantly higher levels of EPA, DHA, and total Omega-3 fatty acids. No statistical differences were noted between treatment groups for fillet contaminants (Table S2.21). Among all pesticides examined, only DDE and PCBs were detected. Levels of DDE and total PCBs in both treatment groups were very low, and as measured, would be of little or no concern to human health [maximum DDE levels detected were >750x lower than FDA limits (5 ppm) for the edible portions of fish; PCB levels were >250x lower than FDA limits (2 ppm)] for food fish. No density effects ($P<0.05$) were noted for any of the processing and product quality parameters investigated (Table 2.2). These results indicate that the tested variations in diet, not rearing density, had marked impact on yield and nutrient content.

DISCUSSION

Diet composition and rearing density have been identified as environmental factors that can impact health and physiology of rainbow trout. Furthermore, diet type is known to impact the composition of the intestinal microbiota in a variety of animal species. The study reported here is the first to test whether diet and rearing density interact in rainbow trout to impact gut microbiota composition, health, and fish performance metrics. Our results reveal consistent effects of diet composition on fish growth and product quality, and novel interactions between diet and rearing density on fish welfare. Despite these marked changes in fish health and yield, the tested alterations in diet and rearing density were not sufficient to significantly alter an unexpectedly large core microbiota in the intestines of aquacultured rainbow trout. As discussed below, these results have important implications for aquaculture of rainbow trout and other finfish, as well as our understanding of vertebrate gut microbial ecology.

Characterization of the microbial lineages ubiquitous in any habitat is an important step towards understanding the determinants of microbiota membership and the respective roles of its members, and developing effective approaches for managing and manipulating that microbial

ecosystem. Deep sequencing of 16S rRNA genes from the intestines of humans, mice, and zebrafish sampled from different populations and geographic locations have suggested that very few bacterial OTUs are common among all individuals from a given host species, and that they represent a minor portion of overall community membership (Shade & Handelsman, 2012) (Roeselers *et al.*, 2011) (Durban *et al.*, 2012). In contrast, we found that all of the individual aquacultured rainbow trout analyzed in this study possessed very similar intestinal bacterial communities dominated by a large shared core microbiota comprised of 52 OTUs. Moreover, the relative abundances of most of these shared OTUs were largely unaffected by tested alterations in diet or rearing density. Since the sequencing depth of this study was not sufficient to saturate diversity in any sample (Figure S2.2), the size of this shared core microbiota may be even larger than our data indicate.

The factors underlying the large size of this shared core gut microbiota remain unknown and represent an important subject for future research. The aquacultured trout studied here were raised under identical husbandry conditions prior to the onset of the experimental manipulations, and it is possible that early colonization events are strong determinants of bacterial community composition, greatly dampening the impact of the experimental manipulations. It is also possible that the large core microbiota might be due to rearing these animals in flow-through tanks without water recirculation, likely limiting environmental variation among tanks and individuals during the experimental manipulations. Importantly, the aquacultured trout analyzed here were obtained from a single commercial supplier and raised in a single aquaculture facility thereby limiting the environmental and host genetic variation and increasing the likelihood of similar microbiota membership. Previous studies have suggested that gut microbiota composition can vary markedly among domesticated zebrafish and mice from different vivarium facilities (Roeselers *et al.*, 2011) (Friswell *et al.*, 2010) (Trust & Sparrow, 1974). We therefore expect that comparisons of gut microbiota from rainbow trout obtained from different aquaculture facilities or caught in the wild would reveal a smaller shared core

microbiota than that reported here. Previous evaluations of gut microbiota composition in wild rainbow trout identified many of the bacterial genera that we observed in the shared core in this study (e.g. Aeromonas, Acinetobacter, Escherichia, Pseudomonas, Streptococcus, and Enterococcus) (Cahill, 1990). These and many other genera observed within the shared core reported here have also been identified in culture-independent and culture-based evaluations of gut microbiota composition in aquacultured trout and other salmonids (Mansfield *et al.*, 2010) (Ringø *et al.*, 1995) (Roeselers *et al.*, 2011) (Desai *et al.*, 2012) (Austin & Al-Zahrani, 1988) (Nieto *et al.*, 1984) (Sugita *et al.*, 1996) (Holben *et al.*, 2002) (Ashraf & Shah, 2011) (Kim *et al.*, 2012) (Rumsey *et al.*, 1994). However, these previous reports did not identify these genera in all animals within the respective studies. This could be due at least in part to the limited sampling depths and the inherent limitations of the respective culture-based and culture-independent methods utilized in these studies.

To provide a more robust frame of reference for interpreting our observations, we compared our results with the only other published study that used deep sequencing to evaluate gut microbiota composition in aquacultured rainbow trout (Desai *et al.*, 2012). This previous study by Desai and colleagues differed from ours in several ways including the specific intestinal region analyzed (luminal contents of distal intestine vs. whole mid-intestine in our study), the bacterial gene targeted for deep sequencing (*cpn60* vs. *16S* in our study), the source of tank water (recirculating vs. flow-through in our study), and other aspects of animal husbandry. We detected no bacterial species or genera that were present in all animals across both studies, however many of the genera within the shared core that we report here were frequently detected in the animals analyzed by Desai and colleagues. Of the 52 OTUs that comprise the shared core in our study, 26 were confidently assigned to a specific genus by RDP Classifier. Of those 26 genera, 8 (Weissella, Acidovorax, Citrobacter, Aeromonas, Enterococcus, Lactococcus, Pseudomonas, Klebsiella) were observed by Desai and colleagues in at least half of the trout sampled in their study and they observed an additional 10 genera (Erwinia,

Leuconostoc, Escherichia/Shigella, Streptococcus, Veillonella, Acinetobacter, Bacillus, Sphingomonas, Chryseobacterium, Pantoea) in at least one animal (Desai *et al.*, 2012). This suggests that the shared core microbiota observed in our study is not a "true" core microbiota possessed by all rainbow trout. Although we have operationally defined core microbiota as those OTUs possessed by 100% of samples in a group, others have suggested that the criteria for core microbiota can be relaxed to include OTUs present in a less than 100% of samples or deeper taxonomic levels (Li *et al.*, 2013) (Olli *et al.*, 1995). Using this relaxed definition, the frequent detection of several bacterial genera in rainbow trout from diverse populations and locations suggests that these genera may be members of a true core microbiota shared by many or all aquacultured rainbow trout. Additional studies are needed to directly compare individual animals from different aquaculture facilities and from wild fish to determine whether a true core microbiota exists in rainbow trout, and to determine how variations in husbandry techniques and animal provenance impact the composition of the gut microbiota in rainbow trout.

Despite the dominance of the shared core we observed, analysis of the accessory core microbiotas – the set of OTUs present in all individuals in at least one experimental group but not in the shared core – revealed several significant differences between experimental conditions. For example, fish fed grain-based diet were enriched for the genera Lactobacillus and Streptococcus compared to those fed fishmeal-based diet. The relative abundance of the Streptococcus genus was the only one in the study to display a significant statistical interaction between diet and density, where the effect of diet was greater in fish raised at high density. Moreover, the relative abundance of one Streptococcus OTU - the only OTU in the shared core microbiota with a statistically significant variation among treatment groups - was increased in both groups fed the grain-based diet (Figure S2.3). Lactobacillus and Streptococcus genera contain species that are used as probiotics in mammals and fish (Olli *et al.*, 1995) (Gaylord *et al.*, 2006). These diet-dependent differences in gut microbial community structure raise the

possibility that minority members may contribute to the physiological differences, such as growth rate, that we observed between fish raised on the fishmeal-based versus grain-based diet.

Early studies report an association between alternative protein diets and decreased fish growth (Buttle *et al.*, 2001) (Ellis *et al.*, 2008) (Latremouille, 2003), likely in response to intestinal inflammation brought about by dietary anti-nutritional factors (Chemists, 1990) (Buttle *et al.*, 2001). Subsequent research demonstrates improved performance with newer plant-based diet formulations (Wong *et al.*, 2013) (Barrows *et al.*, 2007) (Barrows & Lellis, 1999) with reduced anti-nutritional factors (Wedemeyer, 1996). We did not detect differences in intestinal inflammation between treatment groups, and furthermore the grain-based feed treatment groups, despite slower growth, had better feed conversion than groups fed a fishmeal-based diet. Fin condition is an indicator of fish welfare (Turnbull *et al.*, 2005), but its etiology is a complex, multifactorial process (Brockmark *et al.*, 2007). We found that fin indices were significantly better in the grain-based diet treatment groups. Barrows and Lellis (Barrows & Lellis, 1999) suggest an association between fin health and elements within the protein and/or mineral fraction(s) of diets; however, in our study it is difficult to identify specific dietary components' impacts on fin condition. Lower fin indices were found in the high density treatment groups, as has been previously noted by others (Ramette, 2007) (Wedemeyer, 1996) (Turnbull *et al.*, 2005) (Brockmark *et al.*, 2007), underscoring the importance of maintaining an appropriate density range.

In summary, we find that variations in rearing density and diet composition within the context of a single aquaculture facility are sufficient to interactively alter rainbow trout growth, performance, fillet quality and welfare. However, these tested variations in rainbow trout husbandry had only minor effects on gut microbiota composition, and did not markedly alter a surprisingly large core microbiota shared among all animals in the study. Although the shared core microbiota we observed in this cohort of aquacultured rainbow trout may not be a “true”

core microbiota shared among all aquacultured or wild rainbow trout, our results do reveal that rainbow trout gut microbiota composition can achieve remarkable consistency within the context of a single aquaculture facility. This should encourage additional research and implementation of alternative diets and husbandry practices for trout production by reducing concerns over potential impact on the structure and function of the gut microbiota.

TABLES

Table 2.1. Nutritional composition of the fishmeal and grain-based experimental diets utilized

Ingredient	Fishmeal Diet (g/kg)	Grain-based Diet (g/kg)
Fish meal [1]	312.7	---
Blood meal [2]	74.7	---
Soy protein concentrate [3]	---	289.1
Corn gluten meal [4]	---	251.7
Soybean meal [5]	192.4	---
Wheat gluten meal [5]	---	46.5
Wheat flour [6]	284.0	---
Menhaden oil [7]	112.0	167.4
Vitamin premix [8]	7.5	7.5
Lysine	---	11.1
Methionine	---	2.8
Taurine	---	5.0
Dicalcium phosphate	---	36.5
Trace mineral premix [9]	1.0	1.0
Choline CL	2.0	2.0
Ascorbic acid [10]	2.0	2.0
Astazanthin [11]	---	0.2
Total protein (%)	*41.0*	*47.5*
Total fat (%)	*15.1*	*18.0*

[1] Omega Proteins, Menhaden Special Select, 628 g/kg protein
[2] IDF Inc., 832 g/kg protein
[3] Solae, Pro-Fine VF, 693 g/kg crude protein
[4] Cargill, 602.0 g/kg protein
[5] ADM Inc., 480 g/kg protein
[6] Manildra Milling, 120 g/kg protein
[7] Omega Proteins Inc.
[8] United States Fish and Wildlife Service (USFWS) #30, contributed per kilogram of diet: vitamin A (as retinol palmitate), 10,000 IU; vitamin D_3, 720 IU; vitamin E (as DL-%-tocopherol-acetate), 530 IU; niacin, 330 mg; calcium pantothenate, 160 mg; riboflavin, 80 mg; thiamin mononitrate, 50 mg; pyridoxine hydrochloride, 45 mg; menadione sodium bisulfate, 25 mg; folacin, 13 mg; biotin, 1 mg; vitamin B_{12}, 30 ug
[9] USFWS #3, contributed in mg/kg of diet; zinc, 37; manganese, 10; iodine, 5; copper, 1
[10] Rovimix Stay-C, 35%, DSM Nutritional Products
[11] Carophyl Pink, DSM Nutritional Products

Table 2.2. Fish performance and processing and fillet quality attributes for each diet / density treatment group at study's end

	Fishmeal Diet				Grain-based Diet			
Parameter	High Density		Low Density		High Density		Low Density	
Fish performance:								
Final weight (g) †	925	± 12	807	± 26	663	± 40	691	± 46
Survival (%) †	96.4	± 0.5	97.1	± 0.7	97.9	± 0.2	97.6	± 0.5
FCR (overall) †	1.18	± 0.02	1.15	± 0.03	1.02	± 0.06	1.10	± 0.01
TGC (overall)	2.46	± 0.04	2.45	± 0.04	2.42	± 0.07	2.33	± 0.08
Processing attributes:								
Dress yield (%) †	86.1	± 0.4	85.4	± 0.5	87.7	± 0.4	88.9	± 0.4
Fillet index (%)	49.8	± 0.5	49.7	± 0.6	50.3	± 0.3	50.8	± 0.5
Fillet attributes:								
Cook yield (%)	84.3	± 0.6	84.8	± 0.5	85.0	± 0.4	84.4	± 0.5
Texture (Kramer g/g wgt)	340	± 20	334	± 18	316	± 11	344	± 11
Proximate analysis:								
Moisture (%)	70.6	± 0.3	70.4	± 0.2	70.4	± 0.2	70.8	± 0.3
Fat (%)	8.8	± 0.4	8.7	± 0.3	9.1	± 0.3	8.8	± 0.4
Protein (%) †	20.2	± 0.2	20.4	± 0.1	20.6	± 0.1	20.7	± 0.1
Ash (%)	1.2	± 0.0	1.3	± 0.0	1.3	± 0.0	1.3	± 0.0
Fatty acids (mg/g tissue):								
ALA (C18:3n3)	0.75	± 0.23	0.84	± 0.19	0.86	± 0.27	1.18	± 0.04
EPA (C20:5n3) †	3.41	± 0.13	3.05	± 0.17	2.10	± 0.09	2.19	± 0.07
DHA (C22:6n3) †	11.3	± 0.65	10.3	± 0.44	8.92	± 0.23	10.0	± 0.77
Total Omega-3 †	15.5	± 0.68	14.1	± 0.34	11.9	± 0.31	13.8	± 0.57
DGLA (C20:3n6)	1.13	± 0.17	1.01	± 0.26	1.00	± 0.24	1.13	± 0.16
Eicosadienoic acid † (C20:2n6c)	9.65	± 0.24	9.55	± 0.20	13.2	± 0.24	13.7	± 0.14
Total Omega-6 †	10.8	± 0.13	10.6	± 0.25	14.2	± 0.16	14.8	± 0.23

† Parameters with a statistically significant difference between diet treatment groups using ANOVA ($p<0.05$); no statistical differences were determined between density treatment groups, and no statistical interactions between treatments were detected.

Table 2.3. Indices of rainbow trout fins in each treatment group

	Fishmeal Diet		Grain-based Diet	
Fin	High Density	Low Density	High Density	Low Density
Pectoral (left) †*	0.104 ± 0.002	0.108 ± 0.001	0.114 ± 0.001	0.117 ± 0.002
Pectoral (right) †*	0.106 ± 0.001	0.107 ± 0.001	0.115 ± 0.001	0.118 ± 0.002
Dorsal†*	0.088 ± 0.003	0.088 ± 0.005	0.091 ± 0.003	0.095 ± 0.004
Pelvic (left) †*	0.088 ± 0.001	0.088 ± 0.001	0.090 ± 0.003	0.096 ± 0.003
Pelvic (right) †*	0.097 ± 0.001	0.097 ± 0.001	0.102 ± 0.001	0.104 ± 0.001
Ventral†	0.097 ± 0.001	0.099 ± 0.002	0.102 ± 0.001	0.104 ± 0.002
Caudal (upper) †	0.105 ± 0.001	0.109 ± 0.002	0.109 ± 0.001	0.113 ± 0.001
Caudal (lower) †	0.103 ± 0.002	0.105 ± 0.001	0.109 ± 0.001	0.112 ± 0.002

† Fins with statistically significant differences between diet treatment groups ($p<0.05$); no statistical differences were determined between the density treatment groups.
* Fins with statistically significant interaction ($p<0.05$) between diet and density treatments.

FIGURES

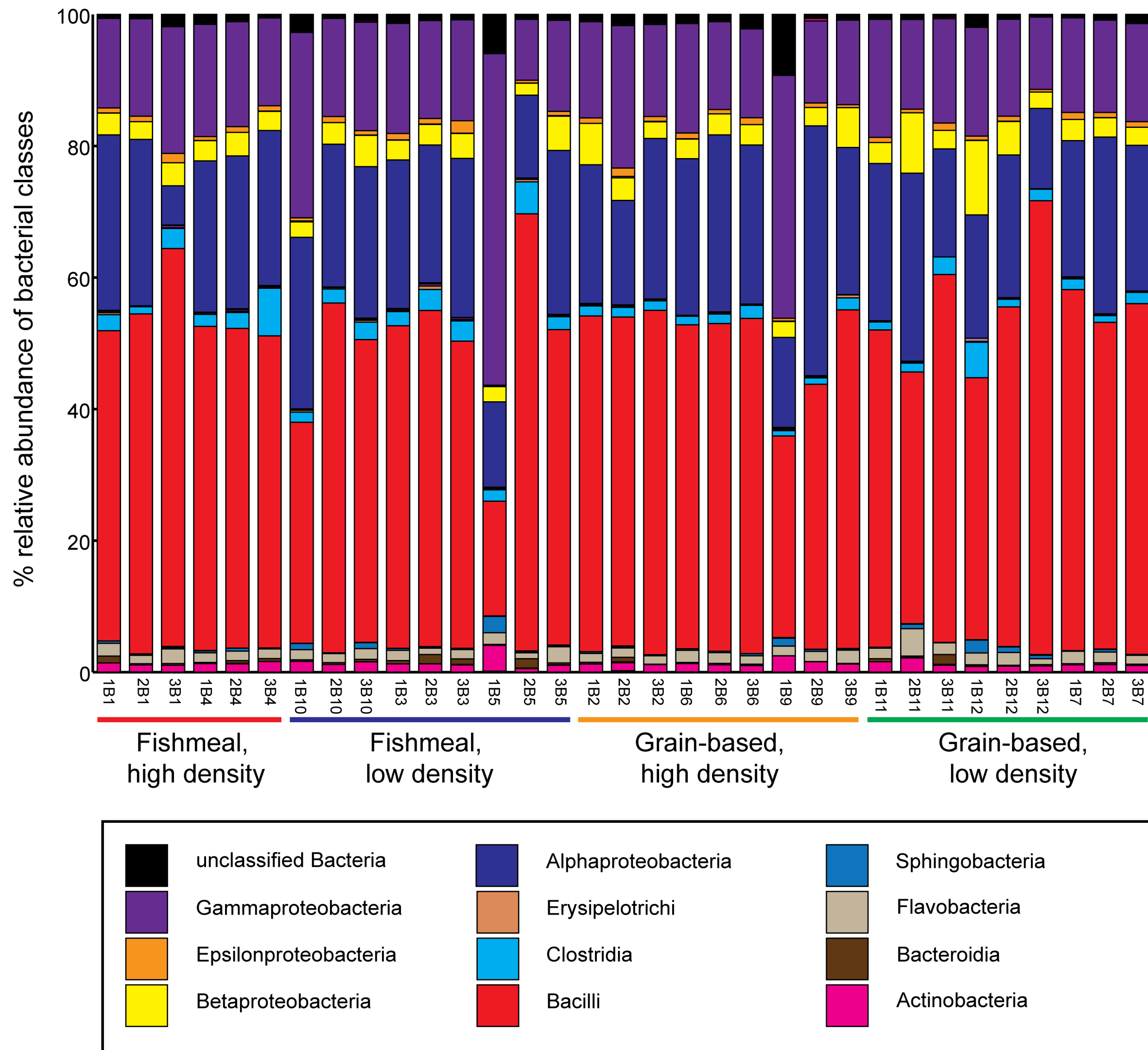


Figure 2.1. 16S rRNA gene sequences reveal similarities between intestinal microbiotas of rainbow trout raised under different diet and density conditions. Relative abundances of bacterial classes in each sample. Labels under each column are sample names corresponding to individual fish. Numerical suffixes indicate the tank number; numerical prefixes identify biological replicates drawn from a given tank; the letter "B" acts as a delimiter to separate tank number from fish number. Figure legend entries are only included for taxa constituting 0.005% or more in at least one sample. For each treatment condition, samples were taken from 2-3 different tanks, and 3 biological samples were analyzed per tank.

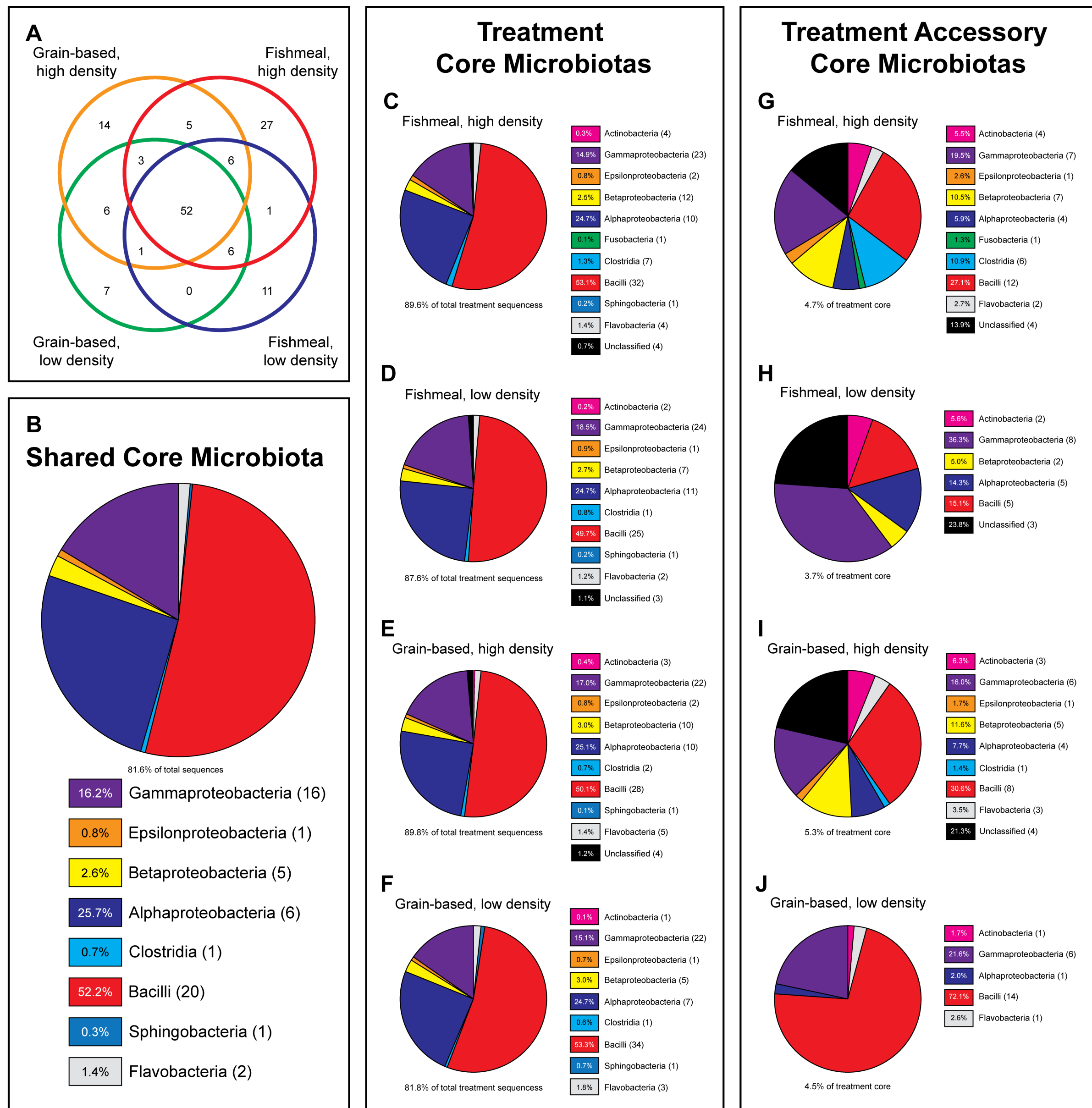
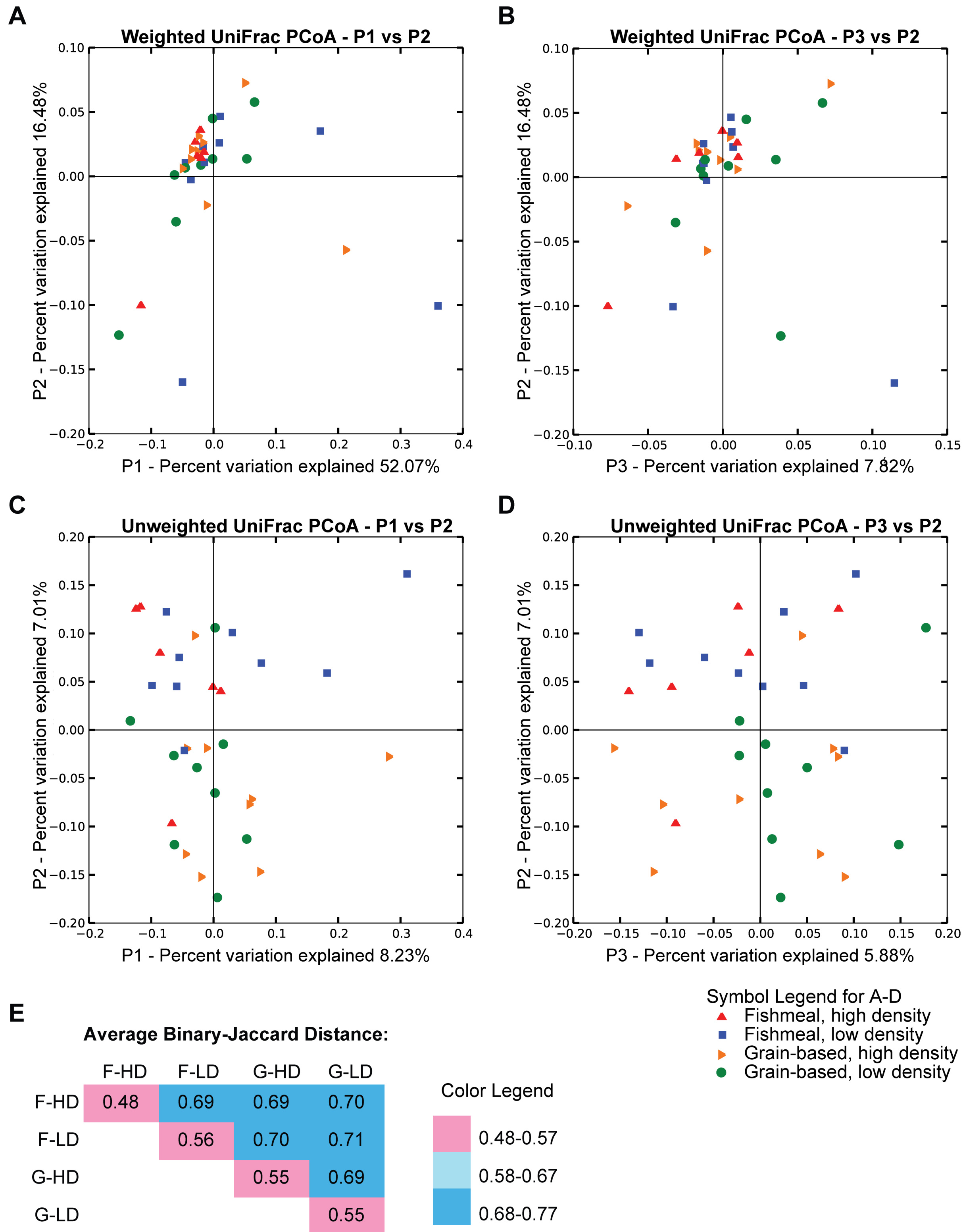


Figure 2.2. Core microbiotas shared between rainbow trout raised under different diet and density conditions. (A) Number of OTUs (97% sequence identity) shared in all individuals within each specific treatment condition (treatment cores) and within all treatment conditions (shared core). Numbers indicate the number of OTUs shared by overlapping circles. Of the 3376 OTUs identified in this study, 52 comprised the shared core and an additional 87 OTUs were included in one or more treatment cores. **(B)** Composition of the shared core microbiota (the 52 OTUs present in all individuals) for all treatment conditions. The relative abundances of the bacterial classes present are shown in the chart legend; numbers in parentheses following legend labels denote the number of OTUs in the core microbiota belonging to the corresponding bacterial class. **(C-F)** Composition of the treatment core microbiotas for each for each of the four treatment groups. The relative abundances of the bacterial classes present are shown in the chart legend; numbers in parentheses following legend labels denote the number of OTUs in the core microbiota belonging to the corresponding bacterial class. Text below the pie charts denote the contribution of the core microbiota to the entire microbiota of trout in each treatment

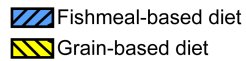
condition. **(C)** Fishmeal, high density. **(D)** Fishmeal, low density. **(E)** Grain-based, high density. **(F)** Grain-based, low density. **(G-J)** Composition of the accessory core microbiota for each treatment condition (i.e., OTUs present in each individual of a given treatment condition but not in each individual in all treatment conditions). The relative abundances of the bacterial classes present are shown in the chart legend; numbers in parentheses following legend labels denote the number of OTUs in the core microbiota belonging to the corresponding bacterial class. Text below the pie charts denote the contribution of the core microbiota to the entire microbiota of trout in each treatment condition. **(G)** Fishmeal, high density. **(H)** Fishmeal, low density. **(I)** Grain-based, high density. **(J)** Grain-based, low density. See also Tables S6-S13.



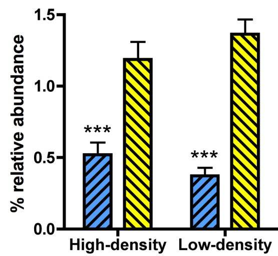
	F-HD	F-LD	G-HD	G-LD
F-HD	0.48	0.69	0.69	0.70
F-LD		0.56	0.70	0.71
G-HD			0.55	0.69
G-LD				0.55

Figure 2.3. Beta diversity estimates of the rainbow trout intestinal microbiota. (A-D) Use of UniFrac to measure phylogenetic distances between the gut microbiota of individual trout from all treatment groups. Red triangles denote individuals from the fishmeal, high density treatment group. Blue squares denote individuals from the fishmeal, low density treatment

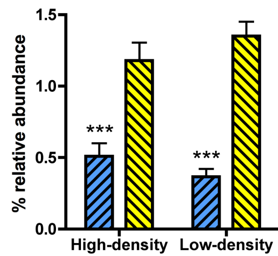
group. Orange triangles denote individuals from the grain-based, high density treatment group. Green circles denote individuals from the grain-based, low density treatment group. **(A-B)** Weighted UniFrac principal coordinate analysis (PCoA) plotted against the PC1 versus PC2 axes (A) and the PC2 versus PC3 axes (B). **(C-D)** Unweighted UniFrac PCoA plotted against the PC1 versus PC2 axes (C) and the PC2 versus PC3 axes (D). **(E)** Average binary-Jaccard (non-phylogenetic) distances between the gut microbiota of individuals in the same treatment group and between individuals from different treatment groups. "F-HD": fishmeal, high density; "F-LD": fishmeal, low density; "G-HD": grain-based, high density; "G-LD": grain-based, low density.



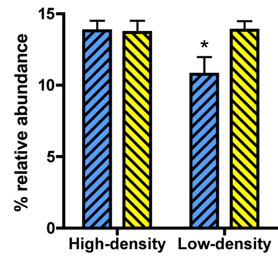
A **Lactobacillaceae**
diet: P<0.0001
density: n.s.
interaction: n.s.



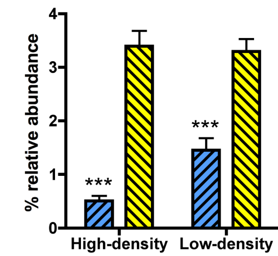
B **Lactobacillus**
diet: P<0.0001
density: n.s.
interaction: n.s.



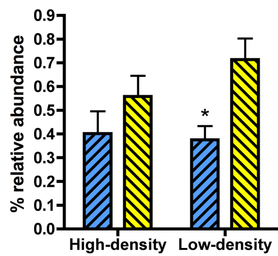
C **Streptococcaceae**
diet: n.s.
density: n.s.
interaction: n.s.



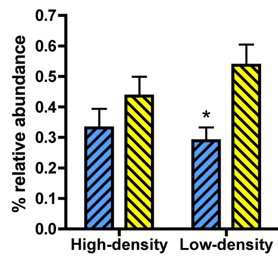
D **Streptococcus**
diet: P<0.0001
density: n.s.
interaction: P<0.05



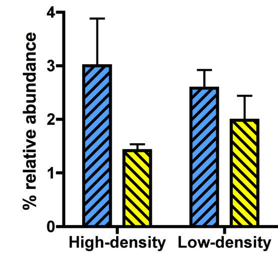
E **Staphylococcaceae**
diet: P<0.01
density: n.s.
interaction: n.s.



F **Staphylococcus**
diet: P<0.01
density: n.s.
interaction: n.s.



G **Clostridiales**
diet: P<0.05
density: n.s.
interaction: n.s.



H **Clostridia**
diet: P<0.05
density: n.s.
interaction: n.s.

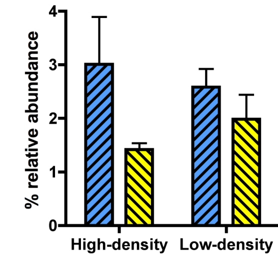


Figure 2.4. Bacterial taxa identified as discriminatory between experimental conditions. Bacterial taxa identified by LEfSe as discriminatory between experimental conditions were subjected to 2-way ANOVA and Bonferroni post-tests. The taxa that were confirmed as significant by 2-way ANOVA are shown here (with the exception of panel C). Data are plotted as mean percent relative abundance ± SEM, with the ANOVA P-value summary for diet, density, and interaction between diet and density shown above each graph. The taxa shown are **(A)** family Lactobacillaceae and **(B)** included genus Lactobacillus, **(C)** family Streptococcaceae and **(D)** included genus Streptococcus, **(E)** family Staphylococcaceae and **(F)** included genus Staphylococcus, and **(G)** family Clostridiales and **(H)** included genus Clostridia. Asterisks indicate significant differences between low-density and high-density samples from the same diet condition identified by Bonferroni post-test (*, P<0.05; ***, P>0.001). See also Tables S14-S19.

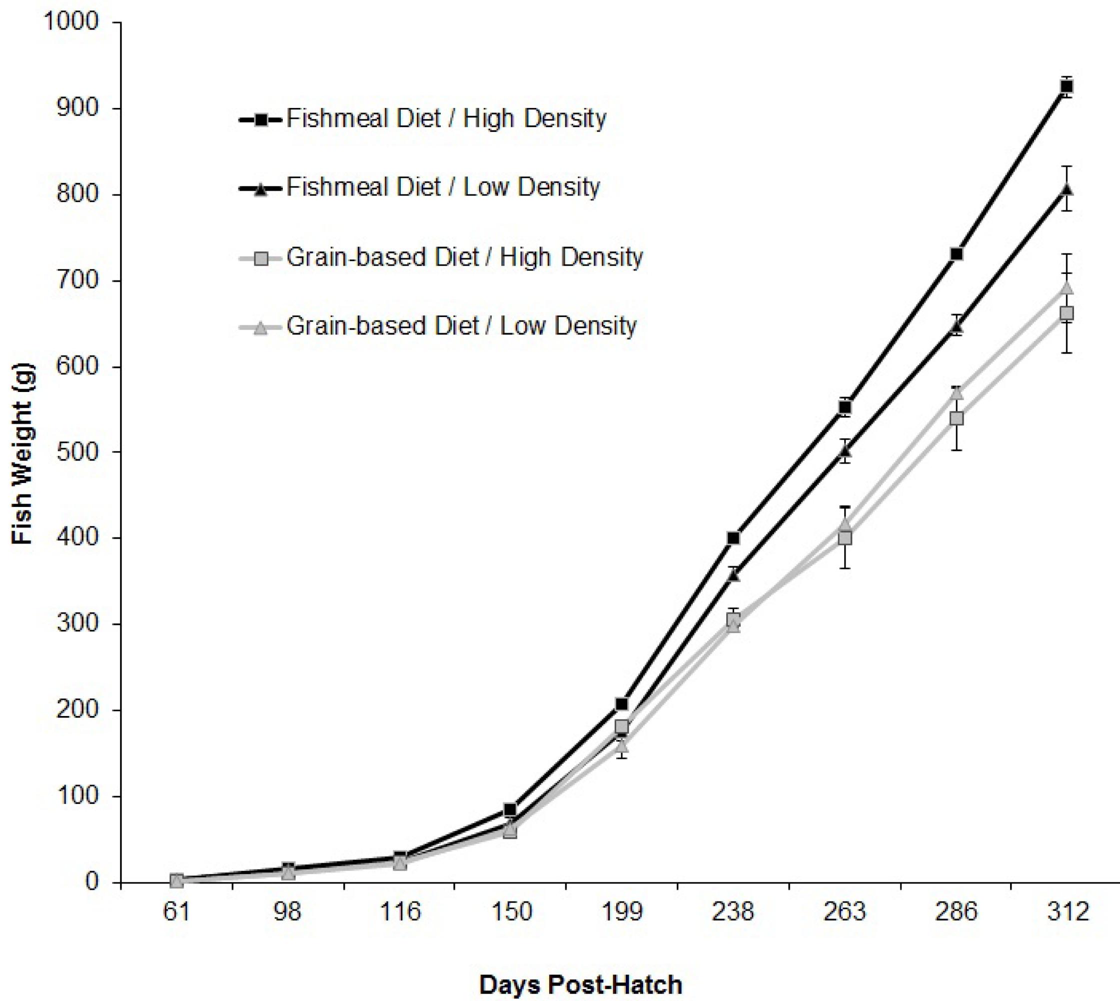


Figure 2.5. Average rainbow trout weight for the duration of the experiment. Data points represent means of 20-60 trout sampled at each monthly growth performance assessment up to 312 days post-hatch. Error bars represent standard errors.

SUPPLEMENTAL FIGURES

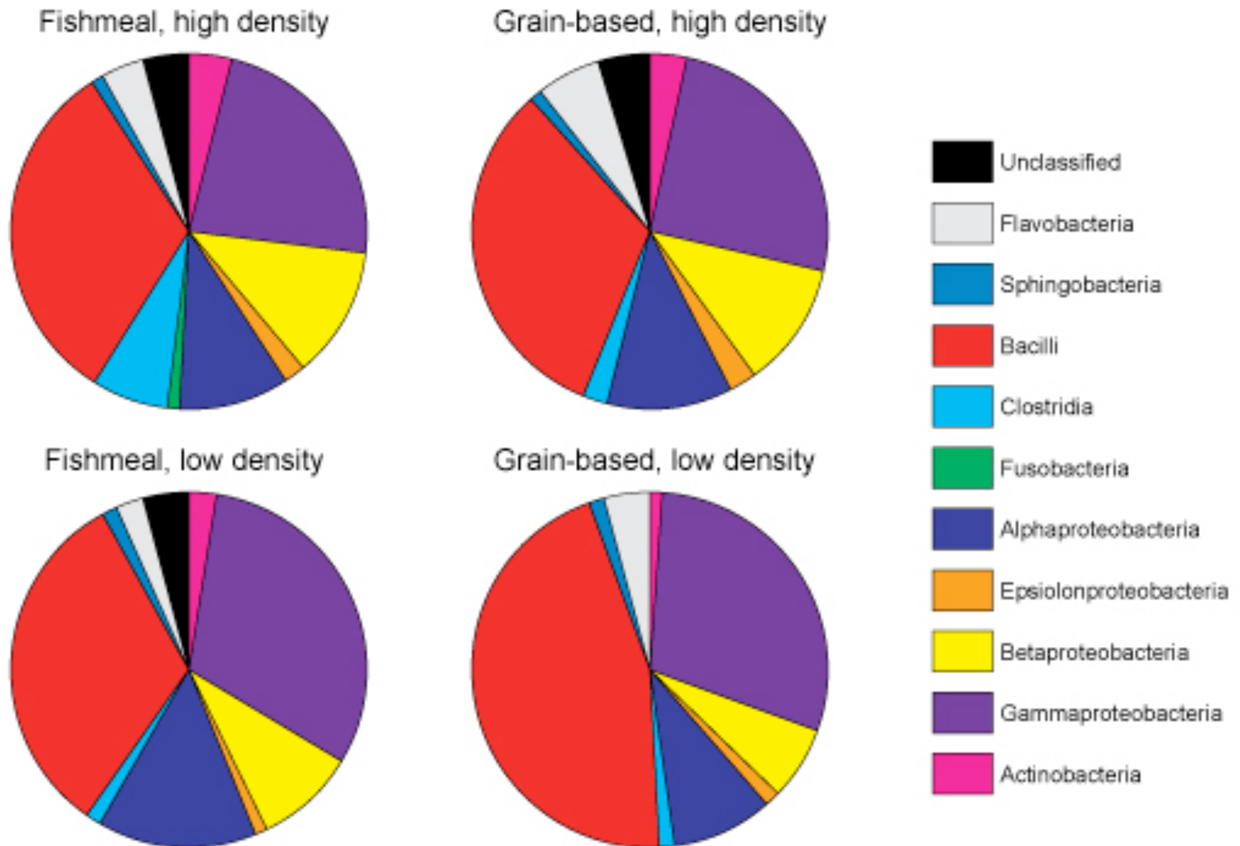


FIGURE S2.1. Diversity of rainbow trout treatment core microbiotas. Percentage of the treatment core OTUs from each bacterial class.

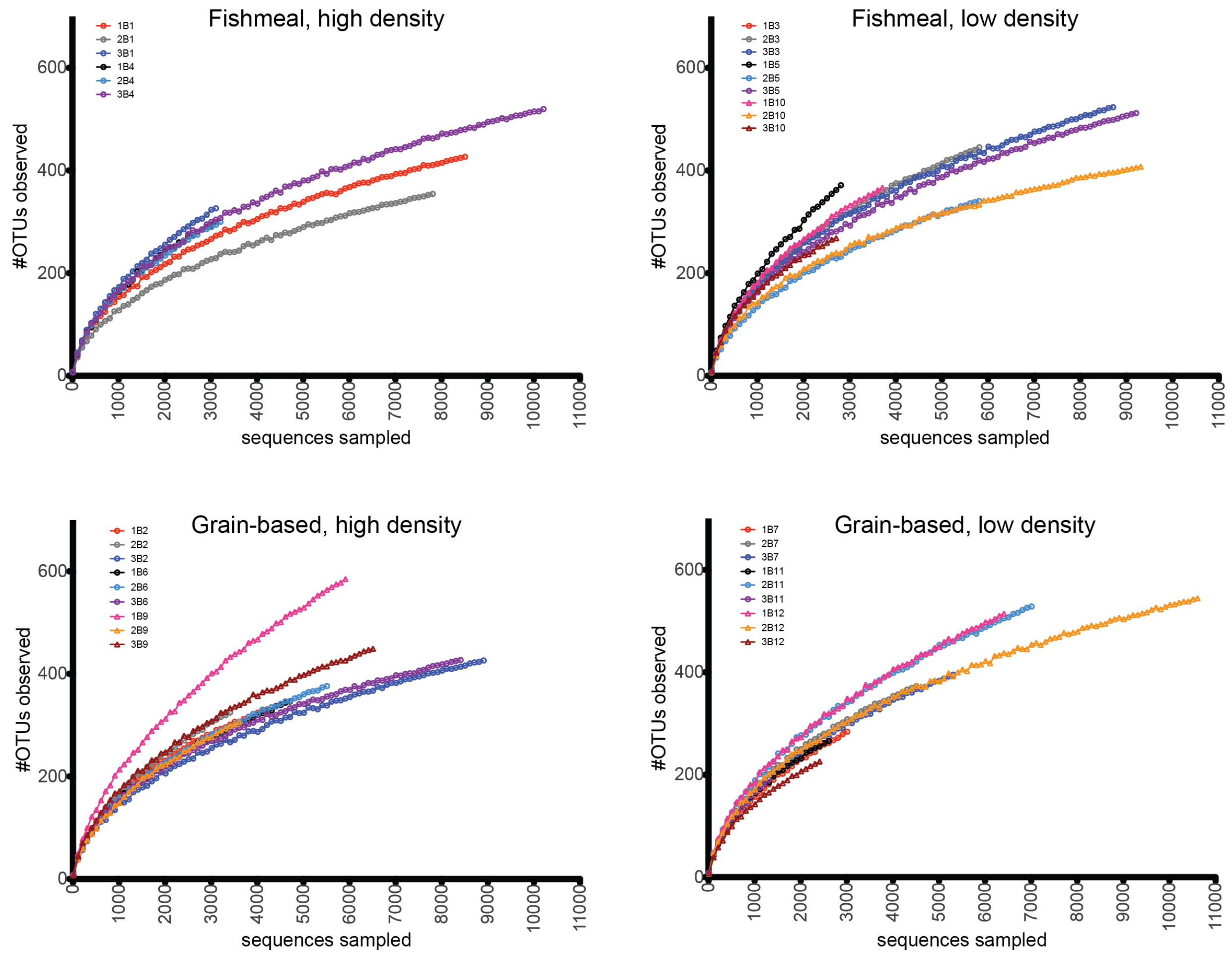


Figure S2.2. Alpha rarefaction of 16S sequences from rainbow trout intestines. The number of unique observed 16S sequences for each sequenced fish at sampling depths freom 10-11000 sequences. Each curve in each panel represents a single animal. Curves are truncated when all sequences in an individual have been sampled.

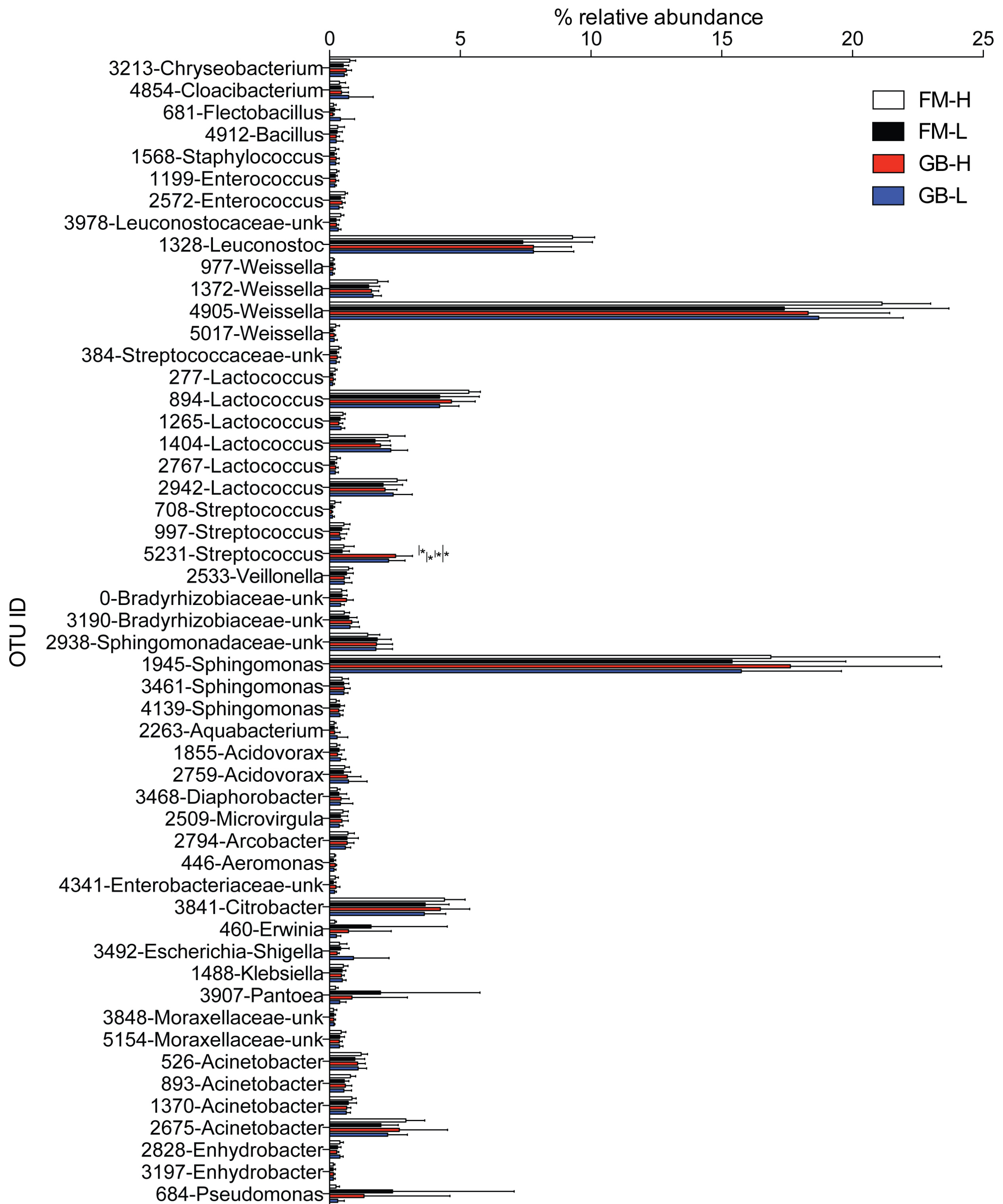


Figure S2.3. Average relative abundances of 52 shared core OTUs in each treatment. "OTU ID" indicates and arbitrarily assigned OTU number followed by the most detailed taxonomic classification for the OTU. For statistical comparison between

treatment groups, OTU counts were standardized by sample and normalized by log10 transformation. Relative abundances between treatment groups were compared by Student's t-test with a 5% FDR. Asterisks indicate statistically significant differences between treatment groups.

CHAPTER THREE

LIFE-LONG DIFFERENCES IN DIETARY FAT DIFFERENTIALLY INFLUENCE MICROBIOTA ASSEMBLY IN THE ZEBRAFISH GUT AND ENVIRONMENT[2]

OVERVIEW

Gut microbiota influence the development and physiology of their animal hosts, and these effects are determined in part by gut microbiota composition. Reciprocally, gut microbiota composition can be affected by introduction of microbes from the environment, changes in the gut habitat during development, and acute dietary alterations. However, little is known about the relationship between gut and environmental microbiotas, or how host development and life-long dietary differences impact the assembly of gut microbiota. We sought to explore these relationships using zebrafish because they are constantly immersed in a defined environment and can be fed the same diet for their entire lives. We conducted a cross-sectional study in zebrafish raised for their entire lives on high-fat, control, or low-fat diets, and used bacterial 16S rRNA gene sequencing to define microbial communities in gut and environment at different developmental ages. Gut and environmental microbiota compositions rapidly diverged following the initiation of feeding, and became increasingly different as zebrafish grew under a constant diet. Different dietary fat levels were associated with distinct gut and environmental microbiota compositions at different ages, and differential contribution of neutral processes to gut microbiota assembly. In addition to alterations in individual bacterial taxa, we identified putative assemblages of bacterial lineages that co-varied in abundance as a function of age, diet, and location. These results reveal life-long complex and dynamic relationships between dietary fat levels, the gut microbiota, and the environmental microbiota.

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[2] Co-authors: W. Zac Stephens, Adam R. Burns, Keaton Stagaman, Lawrence A. David, Brendan J.M. Bohannan, Karen Guillemin, and John F. Rawls.

IMPORTANCE

The ability of gut microbial communities to influence host health is determined in part by the composition of those communities. However, little is known about the relationship between gut and environmental microbiotas or how life-long differences in dietary fat impact gut microbiota composition. We addressed these gaps in knowledge using zebrafish, because their environment can be thoroughly sampled and they can be fed the same diet for their entire lives. We found that microbial communities in the gut changed as zebrafish aged under constant diet, and became increasingly different from microbial communities in their surrounding environment. Further, we observed that the amount of fat in the diet had distinct age-specific effects on gut and environmental microbial community assembly.

INTRODUCTION

Beginning at birth, the intestinal tracts of animals are colonized by microbes acquired from the surrounding environment (Aagaard *et al.*, 2014) (Decker *et al.*, 2011) (Dominguez-Bello *et al.*, 2010) and assemble into communities as hosts age (Rawls *et al.*, 2004) (Koenig *et al.*, 2011) (Stephens, Burns, Stagaman, et al., submitted for publication). The resulting gut microbiota influences diverse aspects of host development and physiology (McFall-Ngai *et al.*, 2013) which can vary as a function of gut microbiota composition (Rawls *et al.*, 2006) (Smith *et al.*, 2013) (Turnbaugh *et al.*, 2006). Alterations to microbiota during early life stages are associated with effects on gut microbiota composition and host phenotypes at adult stages (Cho *et al.*, 2012) (Decker *et al.*, 2011) (Russell *et al.*, 2012) (Dominguez-Bello *et al.*, 2010). An improved understanding of processes governing gut microbiota assembly during early life stages is therefore warranted. Gut microbiota assembly typically occurs in the context of host development and age-associated diet alterations, with ample opportunities for microbial exchange between the gut and environment. However, the relative contribution of these factors to gut microbiota assembly has remained elusive. In addition, the extent to which host

development in combination with diet contribute to selection and neutral processes during gut microbiota assembly remains unresolved.

Diet can be a potent selective force, as feeding status and diet composition have been correlated with different gut microbiota compositions (Costello *et al.*, 2010) (Semova *et al.*, 2012) (Turnbaugh *et al.*, 2008) (David *et al.*, 2014) (Carmody *et al.*, 2015). Dietary fat is a key nutrient class often associated with changes in gut microbiota (Turnbaugh *et al.*, 2008) (David *et al.*, 2014) (Zhang *et al.*, 2012) and is a rich source of energy and substrates that potentially influence both gut and environmental microbial ecologies. However, most of the prior studies examining the impact of different levels of dietary fat on gut microbiota have focused on relatively short-term diet alterations (David *et al.*, 2014) (Zhang *et al.*, 2012) (Cani *et al.*, 2008), and have been conducted in mammals where nursing limits experimental capacity for diet manipulations during critical early postnatal stages (Cox *et al.*, 2014). To date, no studies have examined the impact of differences in dietary fat level throughout life (i.e., from first feed to adulthood) on the process of gut microbiota assembly. It also remains unknown whether dietary fat levels contribute to selection and neutral processes during gut microbiota assembly. Additionally, the impact of life-long differences in dietary fat level on microbiota assembly in the host's environment, and on the relationship between gut and environmental microbiotas, remains unexplored.

The zebrafish (*Danio rerio*) permits a unique analysis of relationships between the gut microbiota, the surrounding environmental microbiota, and diet composition. In this experimental model system, the animal host is fully immersed in an aqueous medium in which the microbes are relatively well-mixed and the microbial environment can be well surveyed. Additionally, the high fecundity of zebrafish allows for high biological replication. We recently showed that gut microbiota in zebrafish subjected to standard husbandry and age-associated diet changes undergo compositional alterations, increased interindividual variation (Stephens, Burns, Stagaman, et al., submitted for publication), and increased selective pressure as fish age

(Burns, Stephens, Stagaman, et al., submitted for publication). However, zebrafish can be raised on a single diet for their entire lives, allowing rigorous control over the exogenous nutrient environment. Here we report the first analysis of microbiota assembly in the zebrafish gut and environment in the context of constant life-long diet composition. This study is also the first to use zebrafish to study the effects of a life-long high- or low-fat diet on microbiota of the gut and surrounding environment. We further compared environmental microbiota from tanks with or without fish to evaluate the degree to which gut microbiota influence environmental microbiota assembly. In addition to studying individual bacterial taxa, we identified groups, or assemblages, of bacteria that co-vary in abundance and may therefore be under similar ecological pressures.

RESULTS

Gut and environmental microbiota compositions quickly diverge early in animal development

To compare microbial community assembly in the gut and environment under constant long-term exposure to different levels of dietary fat, we raised zebrafish under controlled conditions on one of three sterilized diets: low-fat (LF), control (Ctrl), or high-fat (HF) diet (Table S3.1). We performed deep sequencing of bacterial 16S rRNA genes from gut samples and three types of samples from their tank environment at an early pre-feeding stage (5 days post-fertilization or dpf) and three subsequent fed stages during zebrafish development (10, 35, and 70 dpf) (Figure S3.1, Table S3.2). Previous studies reported age-dependent changes in zebrafish gut microbiota when animals were progressed through conventional changes in dietary regimens (Rawls *et al.*, 2004) (Stephens, Burns, Stagaman, et al., submitted for publication). To test whether such changes still occurred when diet was held constant, we compared gut microbiota from fish fed a constant life-long diet. We found that community membership varied between ages (Fig. 3.1A), community richness (according to Chao1 estimate) increased with age (Pearson $r = 0.25$, $p < 0.0001$), (Fig. 3.1C, and community

evenness (as measured by the Shannon index) remained constant except for a transient decrease at 10dpf (Pearson r = -0.03, p = 0.67), (Fig. 3.1B). Moreover, age-associated changes in the prevalent bacterial taxa in the zebrafish gut in this study (Fig. S3.2) largely reflected those described in previous studies (Rawls *et al.*, 2004) (Stephens, Burns, Stagaman, et al., submitted for publication). For example, Gamma-, Beta-, and Alphaproteobacteria were abundant at all ages, with Firmicutes classes displaying transient enrichment at early stages (5, 10, and 35dpf). Together, these results reveal a dietary regimen-independent association between fish development and gut microbiota composition.

In-depth information on the relationship between gut and environmental microbiotas is lacking. We therefore first tested whether the presence of zebrafish is associated with changes in their environmental microbiotas. Given the opportunity for microbial exchange between zebrafish and environmental microbiotas, we hypothesized that the composition of environmental microbiota from tanks with fish would be different from that of tanks without fish. In support, we observed significant fish-dependent differences in beta-diversity, measured by pairwise Bray-Curtis dissimilarities, at early but not later time points (Fig. 3.1D). This was accompanied by a decrease in the number of bacterial taxa that were differentially abundant based whether or not fish were present (Table S3.3). This suggests that fish alter the microbiota composition of their surrounding environment. We next compared gut and environmental microbiotas and found that environmental microbiota evenness and richness were higher than gut microbiotas at all time points (Fig. 3.1B,C). Additionally, assessment of beta diversity (Fig. 3.1D) revealed compositional differences between gut and environmental microbiotas at each age. Considering all ages together, variation among individual guts was significantly higher than variation among individual environmental samples (unpaired t-test with Welch's correction $p<0.0001$). These results indicate that the compositions of zebrafish gut and environmental microbiotas rapidly diverge early in host development even when diet is held constant. They further highlight the large degree of variation between gut microbiota in different individual

hosts, some of which may be attributed to the age-associated variation discussed above (Fig. 3.1A).

To determine whether age impacted the degree of inter-individual variation between gut microbiota samples, we compared inter-individual Bray-Curtis dissimilarity values of each age group. We found that inter-individual variation in gut microbiota composition was higher at 5dpf than at any other age (ANOVA $p<0.001$, all Bonferroni pair-wise post-tests $p<0.0001$). This was salient when comparing the relative abundance of bacterial classes from individual guts at each age (Fig. S3.3). Interestingly, gut and environmental microbiotas also displayed greater similarity at 5dpf than at any other age (Fig. 3.1D). Environmental microbiotas at this early age formed two clusters as defined by PCoA of Bray-Curtis distances, with water and floor clustering separately from wall samples (Fig. 3.1E). Some gut microbiotas at this early age clustered with water/floor or wall communities, while other gut microbiotas were separate from environmental microbiotas (Fig. 3.1E). This raises the possibility that developing gut microbial communities are initially seeded from distinct environmental sources. These gut-environmental microbiota clusters further suggest that there may be distinct bacterial taxa that co-occur as assemblages (Fauth *et al.*, 1996) within individual zebrafish guts and associated environmental microbial communities.

To operationally identify groups of co-occuring bacteria, we used established methods (David *et al.*, 2014) to cluster bacterial OTUs observed in this study into 145 assemblages (Fig. S3.4, Table S3.4). In many of these assemblages, the observed phylogenetic diversity of OTUs was lower than expected (Fig. 3.2A). In contrast, no assemblage had observed phylogenetic diversity significantly higher than expected. This suggests that patterns of bacterial co-occurrence were strongly linked to phylogenetic relatedness. Analysis of these assemblages across gut and environmental microbiotas revealed striking geographic and temporal patterns (Fig. 3.2, Table S3.5). For example, several assemblages were more abundant in the gut than environment, suggesting relatively increased fitness in the gut habitat (Fig. 3.2B). One of these,

Assemblage #4, was gut-enriched at all ages and less phylogenetically diverse than expected (Fig. 3.2A), containing 9 of the 11 OTUs from our dataset in the order Aeromonadales (Table S3.4) which is commonly observed in zebrafish guts and aquatic environments (Roeselers *et al.*, 2011) (Stephens, Burns, Stagaman, et al., submitted for publication) (Janda & Abbott, 2010). Further, Assemblage #4 contained the only OTU observed in all gut microbiota samples in this study (Aeromonadales OTU#839072). Other assemblages exhibited transient enrichment in the gut. For example, Assemblage #75 was gut-enriched only at 10dpf and 35dpf, was less phylogenetically diverse than expected, and was rich in Clostridia (phylum Firmicutes) and Bacteroidetes OTUs (Table S3.4). Several other assemblages, such as Assemblage #139, were enriched in environmental microbiotas compared to gut at all timepoints. 5 of the 9 OTUs in Assemblage #139 are in order Sphingobacteriales, with 4 of these in family Chitinophagaceae (Table S3.4). This suggests that members of this bacterial family may have relatively low fitness in the zebrafish gut.

Focusing on changes in gut or environmental enrichment may mask changes in relative abundance in one or both types of samples. Therefore we proceeded to compare changes in assemblage relative abundance between successive time points in the gut (Fig. 3.2C). In some cases, consistent environmental enrichment was concomitant with progressive decreases in gut relative abundance, and vice versa. For example, Assemblage #135 was always environmentally enriched but progressively decreased in relative abundance in the gut and environment over the course of the experiment. This assemblage contains many OTUs from Sphingomonadales (Alphaproteobacteria) and Sphingobacteriales (Bacteroidetes) (Table S3.4), suggesting that bacteria that produce sphingolipids may have increased fitness in the environment compared to gut.

Dietary fat density impacts environmental microbiota compositions

Having explored diet-independent factors influencing microbiota, we next tested whether differences in dietary fat level influenced environmental and gut microbiota assembly. We first

examined the impact on the environmental microbiota. We found that dietary fat level does not significantly impact microbial evenness or richness in the water column, on the tank floor, or on the tank wall at any age (data not shown). In contrast, Bray-Curtis distances indicated that dietary fat level significantly impacted environmental microbiota composition at 10dpf and 70dpf (Table 3.1). Interestingly, the impact of dietary fat was generally larger in water compared to the tank floor, and in the water the impact was generally more significant when fish were present (Table 3.1). These results suggest that dietary fat has differential effects on microbiota in distinct locations of the tank environment and that this is influenced by the presence of fish.

We next tested if there were differences in the relative abundances of the bacterial assemblages in the environmental communities from low-fat (LF) versus high-fat (HF) tanks. We found that dietary fat level was associated with differentially abundant assemblages at all 3 fed ages (Table S3.5, Fig. 3.2D). At 10dpf, 11 assemblages were significantly enriched in HF environmental microbiotas from tanks containing fish, and 5 assemblages were enriched in the LF environments. HF-enriched Assemblage #142 consisted entirely of OTUs from Proteobacteria (Table S3.4), 3 of which are in the family Pseudomonadaceae, which was identified by LEfSe as indicative of HF environmental microbiotas (Table S3.3). Moreover, LEfSe identified a specific OTU (Pseudomonas OTU#72643) within Assemblage #142 (Table 3.3) as indicative of 10dpf HF environmental microbiotas. At 35dpf, only three assemblages exhibited significantly different relative abundances in HF versus LF environmental microbiotas from tanks with fish, and all were enriched in the HF environment (Table S3.5, Fig. 3.2D). At 70dpf, four assemblages were significantly enriched and eight assemblages were significantly depleted in environmental microbiotas from tanks with fish receiving HF diet compared to those receiving LF diet (Table S3.5, Fig. 3.2D). One of the HF-enriched assemblages, Assemblage #97, is composed almost entirely of OTUs from class Betaproteobacteria, which LEfSe identified as indicative of HF environmental microbiotas at 70dpf from tanks containing fish (Table S3.3).

Together, these results reveal that dietary fat level impacts distinct assemblages during environmental microbiota assembly.

Dietary fat density impacts gut microbiota composition

We next tested whether different dietary fat levels influence gut microbiota assembly. We found that community evenness and richness were not significantly different at any age based on the proportion of fat in the diet (data not shown). In contrast, comparison of Bray-Curtis distances revealed that dietary fat level had a significant effect on gut microbiota beta diversity at each age, with significant differences between HF and LF guts at 35dpf and 70dpf, and the largest effect size at 35dpf (Table 3.1). To identify the bacterial groups underlying these differences, we compared the relative abundances of bacterial assemblages from gut microbiotas of HF-fed versus LF-fed fish. At the first post-feeding time point of 10dpf, only 2 assemblages, both enriched in HF guts, were significantly different in relative abundance (Fig. 3.2E, Table S3.5). Interestingly, both of these assemblages were also enriched in environmental microbiotas of HF diet tanks with fish (Fig. 3.2D). At 35dpf, Assemblage #69 was the only assemblage enriched in HF guts while 13 other assemblages were depleted in HF guts (Fig. 3.2E, Table S3.5). Assemblage #69 contained just three OTUs including one Janthinobacterium OTU and one Pseudomonas OTU#141564 (Table S3.3). In accordance, LEfSe analysis identified that Pseudomonas OTU and the entire Janthinobacterium genus as indicative of HF gut microbiotas at this age (Table S3.5). At 70dpf, eight assemblages were enriched and seven assemblages were depleted in HF guts compared to LF guts (Fig. 3.2E, Table S3.5). For example, Assemblage #19 is relatively enriched in the guts of HF-fed fish, and contains an OTU from Firmicutes class CK-1C4-19 as well as 2 Fusobacteria OTUs from the genus Cetobacterium (Table S3.4). Use of LEfSe to compare 70dpf gut microbiotas from HF and LF-fed fish also identified Fusobacteria, a phylum that has been associated with adult zebrafish gut microbiotas (Rawls *et al.*, 2004) (Rawls *et al.*, 2006) (Roeselers *et al.*, 2011) (Stephens, Burns, Stagaman, et al., submitted for publication), suggesting that HF diet may accelerate the

establishment of these characteristic “adult” bacteria. In contrast, Assemblages #49 and #54, which were depleted in HF compared to LF guts, are entirely or predominantly composed of Alphaproteobacteria, particularly the order Rhizobiales (Table S3.4). This suggests that a variety of Rhizobiales members experience a competitive advantage in the guts of zebrafish fed diets containing less fat.

Dietary fat levels influence the role of neutral processes in community assembly and incur selection on different bacteria

We next sought to determine whether differences in dietary fat level were also associated with differences in the degree to which selective versus neutral processes drive microbiota assembly. To do so, we defined the metacommunity as the combination of OTUs from all samples within the experimental group being assessed. We first fit the gut data from each diet treatment at each time point to a neutral model of assembly (Sloan *et al.*, 2006). This revealed diet- and age-dependent differences, as well as an interaction between age and diet, in the degree to which neutral processes could explain gut microbiota compositions (Fig. 3.3A). Specifically, at 10dpf, increasing dietary fat levels were associated with an increased contribution of neutral processes to microbiota assembly. In contrast, at 35dpf and 70dpf, increasing dietary fat levels were associated with a decreased contribution of neutral processes to microbiota assembly (Fig. 3.3A).

To determine whether selection on OTUs changed in association with different levels of dietary fat, we re-defined the metacommunity at each time point as the combination of microbial communities from all guts – regardless of diet – sampled at that specified time point. We defined OTUs under positive selection if they were more prevalent than predicted, OTUs under negative selection if they were less prevalent than predicted, and OTUs neutral if their prevalence was within model predictions. Interestingly, OTUs under positive selection in the gut were more likely to be in a gut-enriched assemblage at later time points, while more OTUs under negative selection in the gut were in gut-enriched assemblages at all time points (Fig 3.3B). Strikingly, at

each time point we observed a large overlap between OTUs that were not detected in HF guts but neutral in LF guts and vice versa (Fig 3.3C-E). These OTUs may have been undetected due to either negative selection or to random sampling of very rare OTUs. Because we could not discern between these two possibilities, we focused subsequent comparisons on OTUs present in both HF and LF communities. Within these remaining OTUs, we identified substantial overlaps in the OTUs under positive selection in both HF and LF guts. While we observed less overlap between OTUs under positive selection in HF or LF guts and neutral in the other diet group (Fig. 3.3C-E, Table S3.6), these data suggest that the level of dietary fat can substantially alter the impact of neutral processes on specific OTUs during gut microbiota assembly.

We next attempted to determine whether specific bacterial taxa were more likely to contain OTUs under positive selection in HF or LF guts. At 10dpf, Clostridia and Erysipelotrichi OTUs were under positive selection in LF but not HF guts (Fig. 3.3F). Conversely, at 70dpf, Bacilli, Erysipelotrichi, and Clostridia OTUs were under positive selection in HF but not LF guts (Fig. 3.3F). Interestingly, these bacterial classes are in the phylum Firmicutes, which is often positively correlated with increased caloric intake or dietary fat (Costello *et al.*, 2010) (Jumpertz *et al.*, 2011) (David *et al.*, 2014) (Hildebrandt *et al.*, 2009). However, in our study OTUs in those taxa exhibited age-dependent correlations with dietary fat and caloric density. Similarly, we observed that Bacteroidia OTUs were under positive selection in LF but not HF guts at 10dpf and vice versa at 35dpf. In contrast to Firmicutes, Bacteroidia has been anti-correlated with high-fat diets, adiposity, and/or diabetes (Ridaura *et al.*, 2013) (Turnbaugh *et al.*, 2006) (Cani *et al.*, 2008). These observations further highlight that different members of the same taxon are differentially selected for or against in the zebrafish gut based on the amount of fat in the diet and the age of the fish. Interestingly, the general trend is that fat-associated bacterial groups – both positively and negatively correlated with obesity – in fish were more likely to be under positive selection (Fig. 3.3F, Table S3.5) in older fish fed the HF diet but in 10dpf fish fed the LF diet.

DISCUSSION

This study provides the most detailed analysis to date of the life-long relationship between an animal's gut microbiota and the microbiota of its surrounding environment. This is also the first description of the *de novo* assembly of a vertebrate gut microbiota in the context of a life-long unchanging dietary composition. Moreover, our results provide the first description of how life-long diets that are high or low in fat impact both gut and environmental microbiota assembly processes. This study held diet constant, in contrast to our recent report on *de novo* zebrafish gut microbiota assembly in which animals were raised on conventional diets that were altered with developmental progression (Stephens, Burns, Stagaman, et al., submitted for publication). Here we provide definitive evidence that development-associated changes in gut microbiota occur independent of changes in diet composition. With diet held constant, we found that gut and environmental microbiotas became increasingly divergent over time, but also that fish impacted the microbiota of their environment. Further, we found that different levels of dietary fat led to distinct effects on gut and environmental microbiotas, and differences in the contribution of neutral processes on gut microbiota assembly.

Of the factors contributing to variation in the gut microbiota, host development has proven to be a strong correlate in diverse animal species, including mammals and fish. Despite inter-individual variation in microbiota composition observed at all ages, both mammalian and zebrafish gut microbiota undergo broadly similar taxonomic changes with age. For example, human infant microbiotas are abundant in Bifidobacteria but eventually become abundant in Bacteroidetes and Firmicutes following the introduction of solid foods (Koenig *et al.*, 2011). Zebrafish larvae are abundant in Firmicutes and Proteobacteria, but adult gut microbiota are dominated by Fusobacteria (Roeselers *et al.*, 2011) (Rawls *et al.*, 2006) (Stephens, Burns, Stagaman, et al., submitted for publication). Our recent cross-sectional study described the changes occurring in the zebrafish gut microbiota at multiple developmental stages in the

context of standard diet transitions (Stephens, Burns, Stagaman, et al., submitted for publication). In the present study where diet was held constant, we observed similar taxonomic changes in gut microbiota as the zebrafish aged. Our previous studies also reported that community richness decreased with host age (Stephens, Burns, Stagaman, et al., submitted for publication) and that fit to a neutral model of microbiota assembly decreased with age (Burns, Stephens, Stagaman, et al., submitted for publication). Additionally, the earliest time point from Stephens et al – a pre-feeding larval stage – yielded the largest number of distinguishing taxa (Stephens, Burns, Stagaman, et al., submitted for publication), indicating that the greatest differences between stages were between this pre-fed stage and all other stages. In accord, previous studies have reported profound post-feeding changes in gut microbiota composition (Costello *et al.*, 2010) (Semova *et al.*, 2012). It is therefore possible that the onset of feeding may be a strong driver of change in gut microbiota. In contrast to Stephens et al, we observed an increase in microbial richness as well as an increase in fit to a neutral model of assembly as the fish aged. Importantly, unlike Stephens et al, we held diet composition constant throughout the course of this study. This underscores a confounding factor in prior studies exploring the relationship between host development and microbiota assembly: the changes in diet that typically occur at developmental milestones (e.g., weaning in mammals, feed transitions in fish husbandry). Importantly, different diets can impact gut microbiota directly or indirectly through diet-induced alterations to host physiology (Emani *et al.*, 2013) (Kleessen *et al.*, 2003) (Kashyap *et al.*, 2013) (Libao-Mercado *et al.*, 2009), which can in turn influence host development and physiology. We therefore speculate that observed differences between this study and Stephens et al may be due to use of constant and variable diet respectively. In the context of constant diet, sources of selection in the gut may be established earlier in life. Indeed, our results suggest that gut microbiota have a relatively poor fit to neutral models during early life stages.

While the simple act of feeding on exogenous dietary nutrients may incur a strong influence on gut microbiota, altering the composition of the diet sources can also impact

microbiota of both the gut and the environment. Lipids are a major macronutrient class and are used by both microbes and animals as both an energy source and as critical components of cellular structure. Lipids may act as a source of direct microbial selection through, for example, differential capabilities for lipid metabolism (Zalatan & Black, 2011) and can also exert indirect effects through the modulation of host immunity and physiology (Miles & Calder, 2015) (Rallidis *et al.*, 2003). For example, bile is released in response to fat consumption and possess anti-microbial properties (Hofmann & Eckmann, 2006) and can be metabolized by some microbes via bile salt hydrolases (Ridlon *et al.*, 2006). Host immunity and gastrointestinal physiology are also impacted by host development (Davidson *et al.*, 2004) (Bates *et al.*, 2006). Moreover, while zebrafish are born with functional innate immunity, adaptive immunity is thought to not be fully functional until about 28dpf (Lam *et al.*, 2004). Therefore developmental stage, especially in zebrafish, may exert a large impact on how different levels of dietary fat directly or indirectly impact gut microbiota assembly.

Indeed, we observed such effects of development on the correlation between dietary fat levels and gut microbiota. For example, the relative abundances of Bacteroidetes, Clostridia, and Erysipelotrichi have been reported to be differentially abundant in the guts of mammals fed diets differing in the amount of fat (Ridaura *et al.*, 2013) (Turnbaugh *et al.*, 2006) (Cani *et al.*, 2008) (David *et al.*, 2014). In our study, these taxa also exhibited diet-associated differences in relative abundance in the gut, but we found that the dietary condition with the higher relative abundance also depended on the age of the fish (Table S3.4). Similarly, the abundance of *Akkermansia muciniphila* in mammals has been negatively correlated with increased adiposity and diabetes (Liou *et al.*, 2013) (Everard *et al.*, 2013). However, we found that whether there were diet-associated differences in how well the prevalence of *A. muciniphila* fit neutral model predictions depended on the age of the fish (Table S3.6). More broadly, the age of the fish determined whether dietary fat levels were directly or inversely correlated with fit to the neutral model of assembly (Fig. 3.3). The underlying age-dependent processes remain unclear, but

could include the maturation of the adaptive immune system which occurs between 10dpf and 35dpf, the maturation of the intestine and other digestive organs during metamorphosis (Parichy *et al.*, 2009) (Ng *et al.*, 2005), and establishment of an anaerobic niche. Together, these results emphasize the need to consider developmental context when studying microbiota responses to diet and other perturbations.

There are ample opportunities for microbial exchange between gut and environmental communities via host ingestion and excretion, but the degree to which these influence each other remained unclear. In the context of dietary manipulations, one might expect to see differences in the gut reflected in the environment and vice versa. A recent study revealed that human inhabitants profoundly altered the microbiota of their homes by serving as a microbial source, but found little reciprocal impact of the home microbiota on humans (Lax *et al.*, 2014). This study also observed that specific parts of homes had different microbiota compositions, indicating that location and substrate are determinants of environmental microbiota composition. Similarly, we found that environmental microbiota differed depending on whether or not fish were present and that the impact of dietary fat changed depending on tank site. Whether or not fish were in the tank also impacted the diet-dependent differences in environmental microbiota (Fig. 3.1, Table 3.1). Changes in environmental microbiota may result from proliferation of bacteria that can metabolize animal waste products such as urea or that withstand molecular insults such as oxidation from animal metabolites. These are possible etiologies for the divergence we observed between environmental microbiotas from tanks with versus without fish at earlier time points (Fig. 3.1) and from the greater impact of dietary fat levels on environmental microbiota from tanks with fish compared to tanks without fish (Table 3.1). Despite these differences and in contrast to Lax and colleagues, we found that the zebrafish environmental microbiota was relatively stable, with inter-sample variation between environmental microbiotas of all time points much smaller than inter-individual variation between gut microbiotas (Fig. 3.1). These differences between microbiota of fish and human environments may be due to the

relatively low nutrient availability and the aggressive use of cleaning agents on surfaces in human dwellings. Our results suggest that most members of the environmental microbiota in a tank are relatively impervious to the presence of fish or dietary fat variation, but that a small contingent of environmental microbes is highly sensitive to these factors.

While the presence of fish can alter the environmental microbiota, environmental microbes seed gut microbiota. A probable result, which we recently described (Stephens, Burns, Stagaman, et al., submitted for publication), is that gut microbiota most resemble environmental microbiota at birth. Such phenomena have been reported in humans, where gut microbiota of infants born by C-section contain skin microbes and that of infants born naturally contain vaginal microbes (Decker *et al.*, 2011). Intriguingly, despite high inter-individual variation between 5dpf gut microbiotas, in this study we also observed that different subsets of 5dpf gut microbiotas resembled the microbiota of different tank sites (Fig. 3.1). This suggests that the microbiota of these guts may have been seeded from those respective tank sites. While differences in the initial seeding could potentially lead to differences in gut microbiota assembly and alter host physiology later in life, we observed decreased inter-individual differences in the gut microbiota at the fed 10dpf time point (Fig 3.1). This suggests the differences observed at 5dpf were superceded by changes associated with feeding. Further work is needed to test whether assemblages of co-occurring bacteria remain linked under different conditions, for example new diets or altered host physiology, or whether rearrangements of functional networks occur.

MATERIALS AND METHODS

Zebrafish Husbandry

All zebrafish experiments were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals using protocols approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Unless stated otherwise, all fish were maintained at 28.5^{o}C on a 14-hr light cycle on a

recirculating zebrafish aquaculture system (Z-Mod, Marine Biotech). 6 adult pairs of zebrafish (Tubingen strain), all from one sib-ship, were allowed to mate naturally and collectively laid ~1800 fertilized eggs. All embryos were mixed and split evenly among 9 sterile Petri dishes containing fresh conditioned water obtained from the recirculating zebrafish aquaculture system (system water). Embryos were incubated in the Petri dishes in system water at 28.5^{o}C until 1dpf. At 1dpf, live embryos from each dish were transferred to an autoclaved 8L tank containing 300mL of system water (Fig. S3.1). For the remainder of the experiment, fine mesh was secured over all entries into the tank to limit introduction of undesired material. Embryos were left in 300ml static water until 5dpf, at which the water volume was increased to 500ml/tank. Drip water flow commenced at 6dpf, and fast water flow commenced at 28dpf. At 5dpf, all tanks received one feeding of their assigned diet after sample collection was completed. Starting at 6dpf, each tank received two feedings per day for the remainder of the experiment. Throughout the course of the experiment, all fish remained in the same tank in which they were placed at 1dpf with periodic removal of floc from the tank floor using sterile cell scrapers and sterile serological pipettes (Fig. S3.1).

Dietary Manipulations

Control (Ctrl), high-fat (HF), and low-fat (LF) diets (Table S3.1) were custom formulated and ground to a pellet size of 50–100 um (Ziegler Brothers, Inc.) and then sterilized by irradiation (absorbed dose range 106.5–135.2 kGy; Neutron Products, Inc.). For the duration of the experiment starting at 5dpf, each tank was assigned one of 3 diets: LF, Ctrl, or HF. 3 fish-free tanks per diet were also maintained in parallel with identical husbandry conditions (Fig. S3.1). From 5dpf to 27dpf, each tank received 80mg of their assigned diet per feeding; from 28dpf to 40dpf, tanks received 120mg of their assigned diet per feeding; from 40dpf to 49dpf, each tank received 160mg of their assigned diet at each feeding; from 49dpf to 70dpf, each tank received 192mg of their assigned diet at each feeding. For the remainder of the experiment, each tank received 160mg of their assigned diet per feeding.

Sample Collection

At each sampling time point, randomly selected fish were collected and euthanized via tricaine overdose (sterile-filtered tricaine at 0.83mg/ml). Following euthanasia, fish were imaged for subsequent standard length measurements (Parichy *et al.*, 2009). Intestinal tracts were dissected from each fish, placed into a tube containing sterile lysis buffer (20mM Tris-HCl (pH 8.0), 2mM EDTA (pH 8.0), 1% Triton X-100) and sterile 0.1mm beads (BioSpec Products catalog #11079101z), and immediately frozen in a dry ice-ethanol bath. Samples were stored at -80°C until sample processing.

At each sampling time point, environmental microbiota samples were gathered from the upper water column, the lower water column/floor deposits (floc), and the tank walls. For the upper water column, 50ml tank water was filtered on a 0.22um filter (MO BIO Laboratories, Inc. catalog #14880). For the floc, tank floors were scraped with sterile cell scrapers to loosen floc, and 8ml from the tank floor floc was filtered on a 0.22um filter (MO BIO Laboratories, Inc. catalog #14880). Filters were then extracted using flame-sterilized forceps and cut in half using flame-sterilized scissors and stored at -80°C until sample processing. For wall samples, sterile cell scrapers were used to make one vertical scrape on the tank wall and swirled in 10ml sterile PBS. Scraped debris was allowed to settle, and then 200ul of suspended debris was placed into 2ml tubes containing sterile lysis buffer and sterile 0.1mm beads before freezing in dry ice-ethanol and storage at -80°C.

Samples were named as follows: tank ID followed by time point (5, 10, 35, or 70), followed by sample ID (‘m’ for water column, ‘s’ for tank floor, ‘w’ for tank wall, and remaining letters in alphabetical order for guts) (Table S3.2). For example: sample 2H35d indicates that the sample was the 4th gut sample from the 2nd HF tank at 35dpf.

Molecular Biology

Genomic DNA was extracted from intestinal tract and tank wall samples using QIAmp DNA micro kits (Qiagen, modified as previously described (Stephens, Burns, Stagaman, et al.,

submitted for publication)). For water samples, genomic DNA was extracted from one half of each filter using QIAmp DNA micro kits (Qiagen #56304, (Stephens, Burns, Stagaman, et al., submitted for publication)) and from the second half using PowerWater® kits (MO BIO Laboratories, Inc., catalog #14900). Amplification of the v4 region of 16S rRNA gene was performed using 2-step PCR amplification process as previously described (Stephens, Burns, Stagaman, et al., submitted for publication) and sequencing was performed on an Illumina HiSeq 2000 Sequencing System at the High Throughput DNA Sequencing and Genomics Facility at the University of Oregon.

Bioinformatic Analysis

Preprocessing of raw sequence data was performed as previously described (Stephens, Burns, Stagaman, et al., submitted for publication) prior to de-multiplexing. We used QIIME v. 1.6.0 (Caporaso *et al.*, 2010) to de-multiplex the reads using default parameters with the following changes: reads less than 199bp were discarded and 2 primer differences were allowed in the sequences. We then used QIIME to cluster the reads, using open-reference UCLUST (Edgar, 2010), into operational taxonomic units (OTUs) against greengenes 2012 (October update) at the 97% sequence identity level and assigned taxonomic classifications to each OTU using RDP classifier v2.2. We further required that the OTU be detected in at least 5 samples for inclusion in the analyses.

Additionally, we used QIIME to assess alpha- and beta-diversity in our dataset. For alpha diversity analyses of all samples except tank walls (excluded here due to lower sequencing depths), the data were rarified such that each sample included in the analyses contained 10100 sequences per sample. For alpha diversity analyses including tank wall microbiota samples, we rarified to 100 sequences per sample to ensure adequate sample numbers. We calculated Shannon indices and Chao1 values to evaluate alpha diversity. For beta-diversity analyses, we rarified the samples to 1000 sequences per sample with the goal of retaining at least 18 gut samples/condition at each age and generated Bray-Curtis, Canberra,

unweighted and weighted UniFrac distance matrices. We used PCoA to visualize beta-diversity distances and calculated effect sizes of variables using ANOSIM. To identify bacterial taxa with statistically significant differences in relative abundance between different experimental groups, we employed the LEfSe module (version 1.0) (Segata *et al.*, 2011) available on the Huttenhower lab Galaxy instance (http://huttenhower.sph.harvard.edu/galaxy/). For all comparisons, we used the default parameters (Kruskal-Wallis test alpha < 0.05, pair-wise Wilcoxon text alpha < 0.05, and LDA score >= 2.0 for significance) with the following exception: when comparing gut microbiota from fish of different ages, we employed the all-against-all option in order to identify taxa for which there was a significant difference in relative abundance between at least 2 ages.

Identification of Bacterial Assemblages

Using the OTU table generated by open-reference UCLUST clustering at 97% sequence identity, where each OTU included was required to have been observed in at least 5 samples, we clustered OTUs into assemblages using established methods (David *et al.*, 2014). Briefly, using custom Python scripts and SciPy, OTU counts were normalized as described by David and colleagues (David *et al.*, 2014). The OTUs were then ranked based on their absolute abundances, and we retained the top OTUs that together comprised 95% of all reads and normalized the OTU read count [in a way that maintained 'subcompositional coherence']. We then used SparCC (Friedman & Alm, 2012) with 10 iterations to generate pair-wise Pearson correlation values for each OTU and used hierarchical clustering to cluster the OTUs and generate a distance-based tree. We arbitrarily set a depth threshold of 0.729 on the tree to delineate clusters, yielding 145 clusters (referred to here as assemblages). Following the identification of these assemblages, we used STAMP (Parks *et al.*, 2014) and/or Metastats (White *et al.*, 2009) to compare the relative abundance of each assemblage containing at least 3 OTUs between the different sample groups (White's non-parametric t-test, false discovery rate (FDR) threshold of 5%). Heatmaps were generated using Cluster3.0 (de Hoon *et al.*, 2004) and JavaTreeView (Saldanha, 2004).

Neutral Model Methods

We used a rarified OTU table and custom R scripts, published in (Burns, Stephens, Stagaman, et al., submitted for publication), to test for neutral processes in microbiota assembly using the Sloan's Neutral Community Model for Prokaryotes (Sloan *et al.*, 2006). To determine the degree to which neutral processes drove microbiota assembly, for each experimental group assessed we defined the metacommunity as the combination of OTUs from all samples within the experimental group. To determine bacterial drivers of diet-associated variation at each time point, we re-defined the metacommunity for each age as the combination of OTUs from the guts of all 3 diets. OTUs not present in an experimental group but present in the metacommunity for that age were categorized as undergoing negative selection.

TABLES

Table 3.1. ANOSIM effect sizes comparing HF and LF microbiota based on Bray-Curtis distance matrices

			r	p
Gut	10dpf	N/A	0.0313	0.122
	35dpf	N/A	0.0998	0.007
	70dpf	N/A	0.0977	0.028
Environment With Fish	10dpf	all tank sites	0.2621	0.001
		water column	0.8093	0.004
		tank floor	0.3306	0.004
	35dpf	all tank sites	0.0272	0.247
		water column	0.1987	0.05
		tank floor	0.2923	0.074
	70dpf	all tank sites	0.3277	0.007
		water column	0.8315	0.002
		tank floor	0.3160	0.037
Environment Without Fish	10dpf	all tank sites	0.1535	0.004
		water column	0.3287	0.009
		tank floor	0.0500	0.247
	35dpf	all tank sites	0.0223	0.3
		water column	0.1176	0.126
		tank floor	0.1107	0.19
	70dpf	all tank sites	0.2856	0.008
		water column	0.8176	0.002
		tank floor	-0.0063	0.503

FIGURES

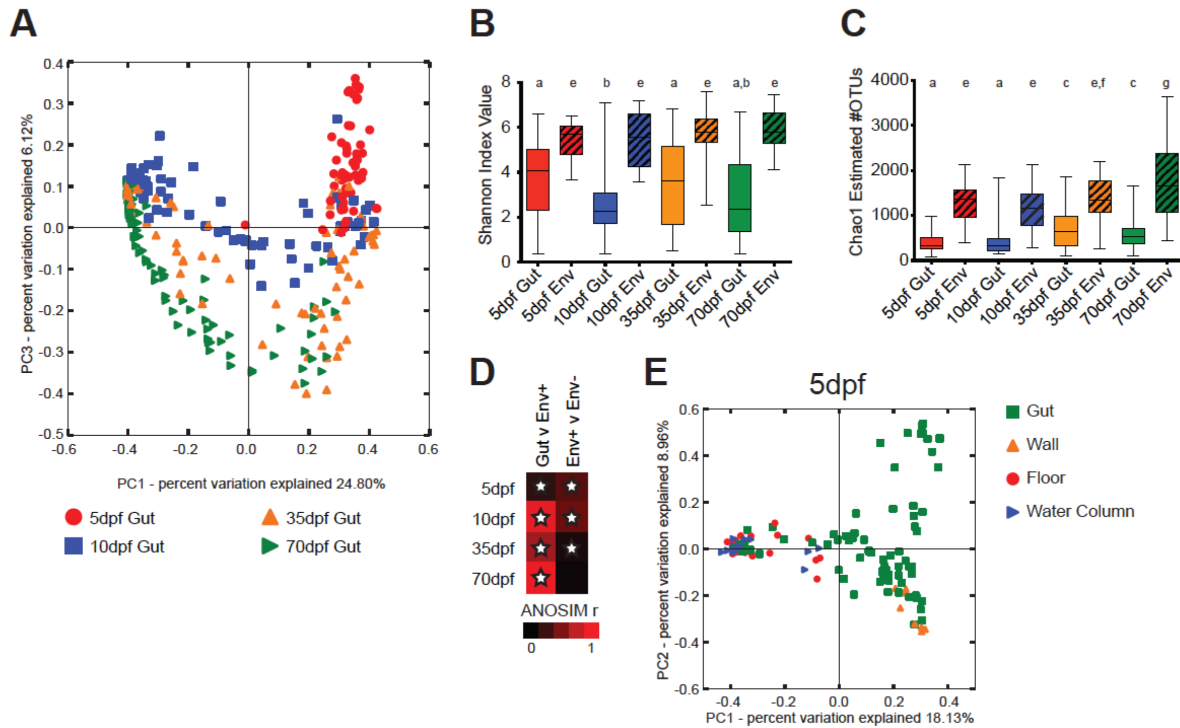
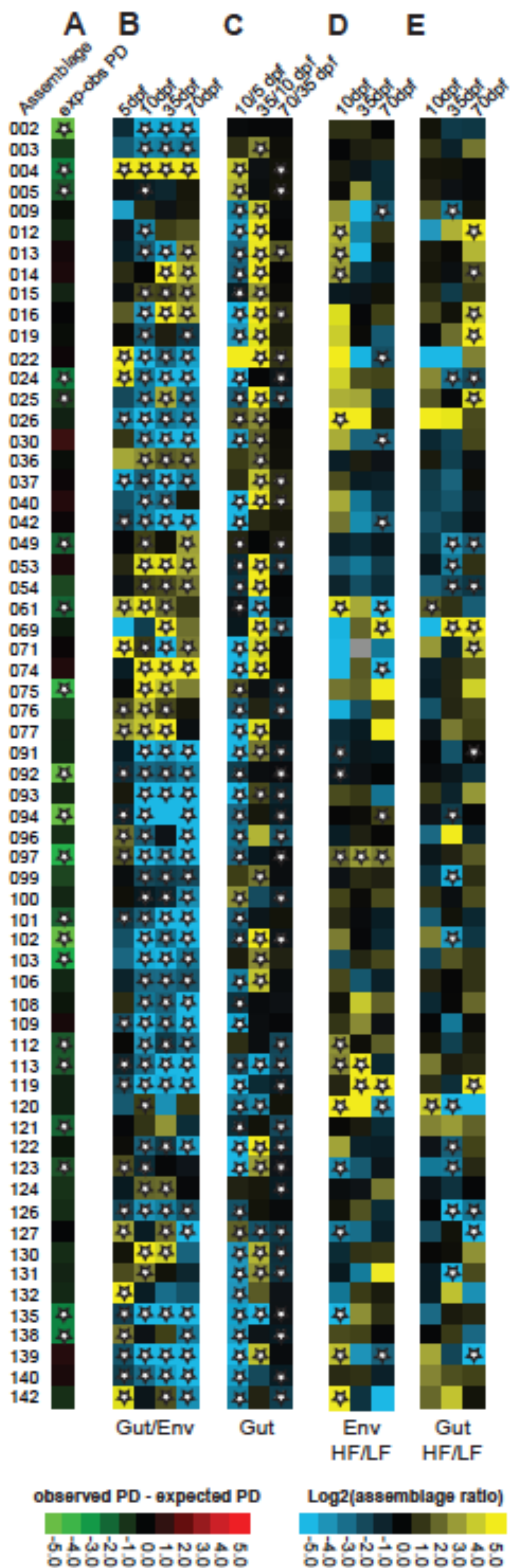


Figure 3.1. Alpha- and beta-diversity between zebrafish gut and environmental microbiotas.
(A) Bray-Curtis dissimilarities between gut microbiota samples visualized by PCoA along the 1st and 3rd axes. Samples colored by age. **(B and C)** Alpha diversity in gut and fish-containing environmental microbiotas at each time point, as measured by **(B)** Shannon index of evenness and **(C)** Chao1 estimate of richness. Statistics comparing gut or environmental microbiota at different ages: ANOVA with Bonferroni post-tests. Groups with the same letters are not significantly different. “a-c” used for gut comparisons. “e-f” used for environment comparisons. Statistics comparing gut and environment calculated by student’s t-test. Shannon diversity was statistically significantly higher in environment than in guts at all ages except 10dpf. Richness was statistically significantly higher in environment than in guts at all ages except 35dpf. **(D)** ANOSIM effect sizes for Bray-Curtis comparisons of gut versus environment with fish (left column) and environments with versus without fish (right column). Stars indicate $p < 0.05$. **(E)** PCoA plot of Bray-Curtis dissimilarities between 5dpf gut and fish-containing environmental samples. Plot colored by sample type.


A
B
C
D
E
Assemblage
exp-obs PD
5dpf
10dpf
35dpf
70dpf
10/5 dpf
35/10 dpf
70/35 dpf
10dpf
35dpf
70dpf
10dpf
35dpf
70dpf
002
003
004
005
009
012
013
014
015
016
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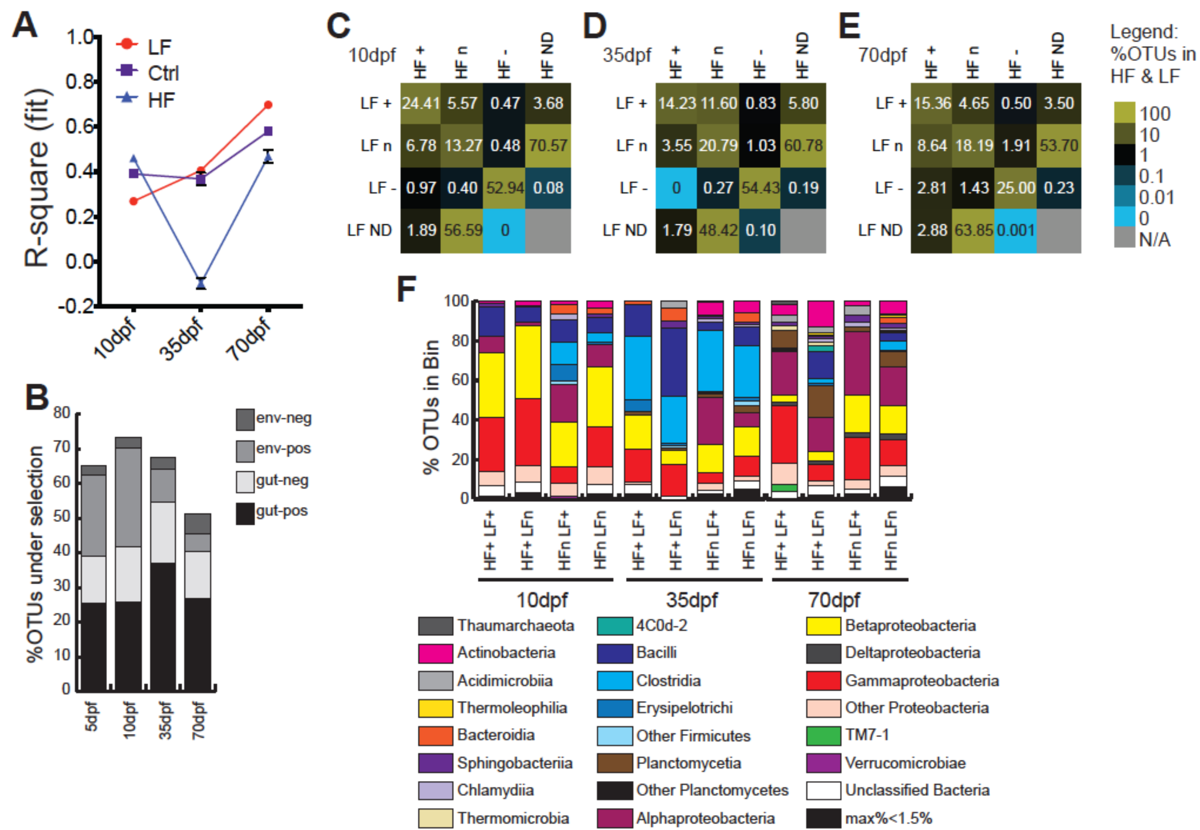
102
103
106
108
109
112
113
119
120
121
122
123
124
126
127
130
131
132
135
138
139
140
142
Gut/Env
Gut
Env HF/LF
Gut HF/LF
observed PD - expected PD
-5.0 -4.0 -3.0 -2.0 -1.0 0.0 1.0 2.0 3.0 4.0 5.0
Log2(assemblage ratio)
-5.0 -4.0 -3.0 -2.0 -1.0 0.0 1.0 2.0 3.0 4.0 5.0

Figure 3.2. Differences in assemblage relative abundance between different experimental groups.

(A) Heatmap showing difference between observed and expected phylogenetic distance (PD) of assemblages with at least 3 OTUs. Stars indicate differences greater than variance in expected PD. **(B-E)** Heatmaps show fold difference in assemblage relative abundance between 2 experimental groups. Stars indicate a statistically significant change according to White's non-parametric t-test followed by FDR correction using a cutoff of 5%. **(B)** Gut versus fish-containing environmental microbiotas at each time point. **(C)** Changes in gut microbiota between 2 consecutive time points. **(D)** HF versus LF fish-containing environmental microbiota at each fed time point. **(D)** HF versus LF gut microbiota at each fed time point.



10dpf	HF +	HF n	HF -	HF ND
LF +	24.41	5.57	0.47	3.68
LF n	6.78	13.27	0.48	70.57
LF -	0.97	0.40	52.94	0.08
LF ND	1.89	56.59	0	

35dpf	HF +	HF n	HF -	HF ND
LF +	14.23	11.60	0.83	5.80
LF n	3.55	20.79	1.03	60.78
LF -	0	0.27	54.43	0.19
LF ND	1.79	48.42	0.10	

70dpf	HF +	HF n	HF -	HF ND
LF +	15.36	4.65	0.50	3.50
LF n	8.64	18.19	1.91	53.70
LF -	2.81	1.43	25.00	0.23
LF ND	2.88	63.85	0.001	

Figure 3.3. Differences in dietary fat levels impact the contribution of neutral and selective processes to gut microbiota assembly. (A) Fit to Sloan neutral model of microbial community assembly for the zebrafish gut microbiota from fish fed HF, Ctrl, or LF diets at each fed time point. Statistics on bootstrapped replicates: 2-way ANOVA p<0.0001. Diet p<0.002. Age p<0.002. Diet-Age Interaction p<0.002. Bonferroni post-tests: 10dpf: all pair-wise diet comparisons p<0.0001. 35dpf: LF vs Ctrl p<0.001, LF vs HF and Ctrl vs HF p<0.0001. 70dpf: all pair-wise diet comparisons p<0.0001. **(B)** % OTUs under selection at each age that are in gut- or environment-enriched assemblages with 3 or more OTUs. Because these exclude OTUs that are not in assemblages and OTUs in assemblages with fewer than 3 OTUs, the bars are not expected to reach 100%. **(C-E)** Heatmaps for each fed time point showing %(#OTUs shared between 2 diet-selection categories) divided by (Total #OTUs in HF and/or LF selection categories). Categories for each diet: OTUs under positive selection (+), under negative selection (-), within model predictions (n), and not detected (ND). Percentages noted in each box. **(C)** 10dpf. **(D)** 35dpf. **(E)** 70dpf. **(F)** For each age and each of the following groups, %OTUs belonging to each bacterial class: under positive selection in HF and LF guts, under positive selection in HF guts but neutral in LF guts, under positive selection in LF guts but neutral in HF guts, and neutral in both HF and LF guts.

SUPPLEMENTAL FIGURES

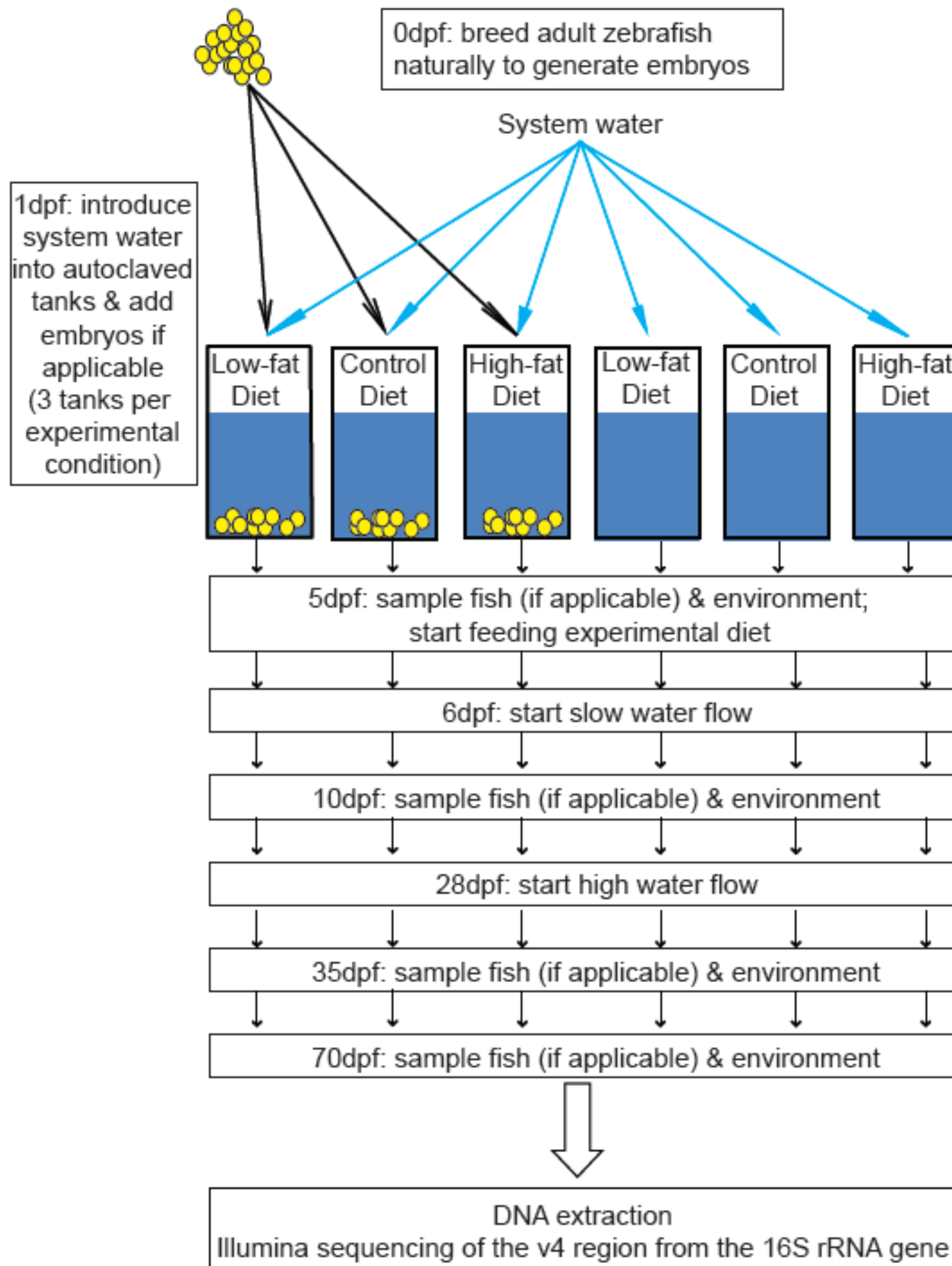


Figure S3.1 Experimental design. ~1800 fertilized embryos were mixed and split into 9 equal groups of at 0 days post fertilization (dpf). At 1dpf, each group of embryos was placed into an autoclaved 6L tank containing 500mL of non-filtered water from the UNC Zebrafish Core Facility. Fish-free tanks were also maintained under identical husbandry conditions as fish-free controls. In total, there were 18 tanks (3 per experimental condition). For the entire duration of the experiment, no tank changes occurred. Slow water flow commenced at 6dpf, and high water flow commenced at 28dpf. Environmental samples and, if applicable, zebrafish intestinal samples were collected for

microbiota analysis at 5dpf, 10dpf, 35dpf, and 70dpf. If applicable, 8 fish/tank were sampled at 5, 10, and 35dpf; at 70dpf, 5-17 fish per tank were sampled. At 5dpf, following sample collection, all tanks received one feeding of their respective experimental diets. For the remainder of the experiment, each tank was fed their corresponding diets twice a day.

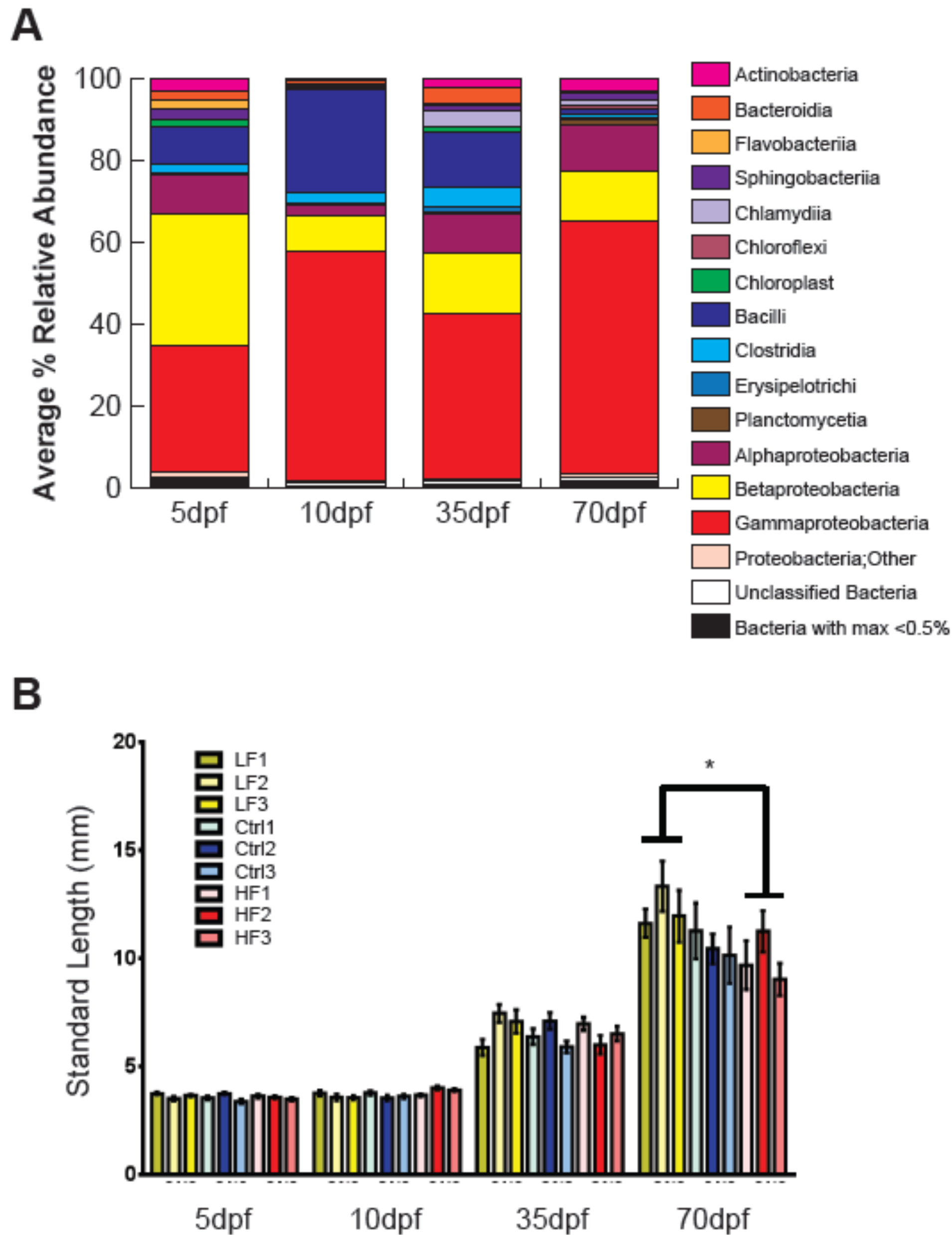
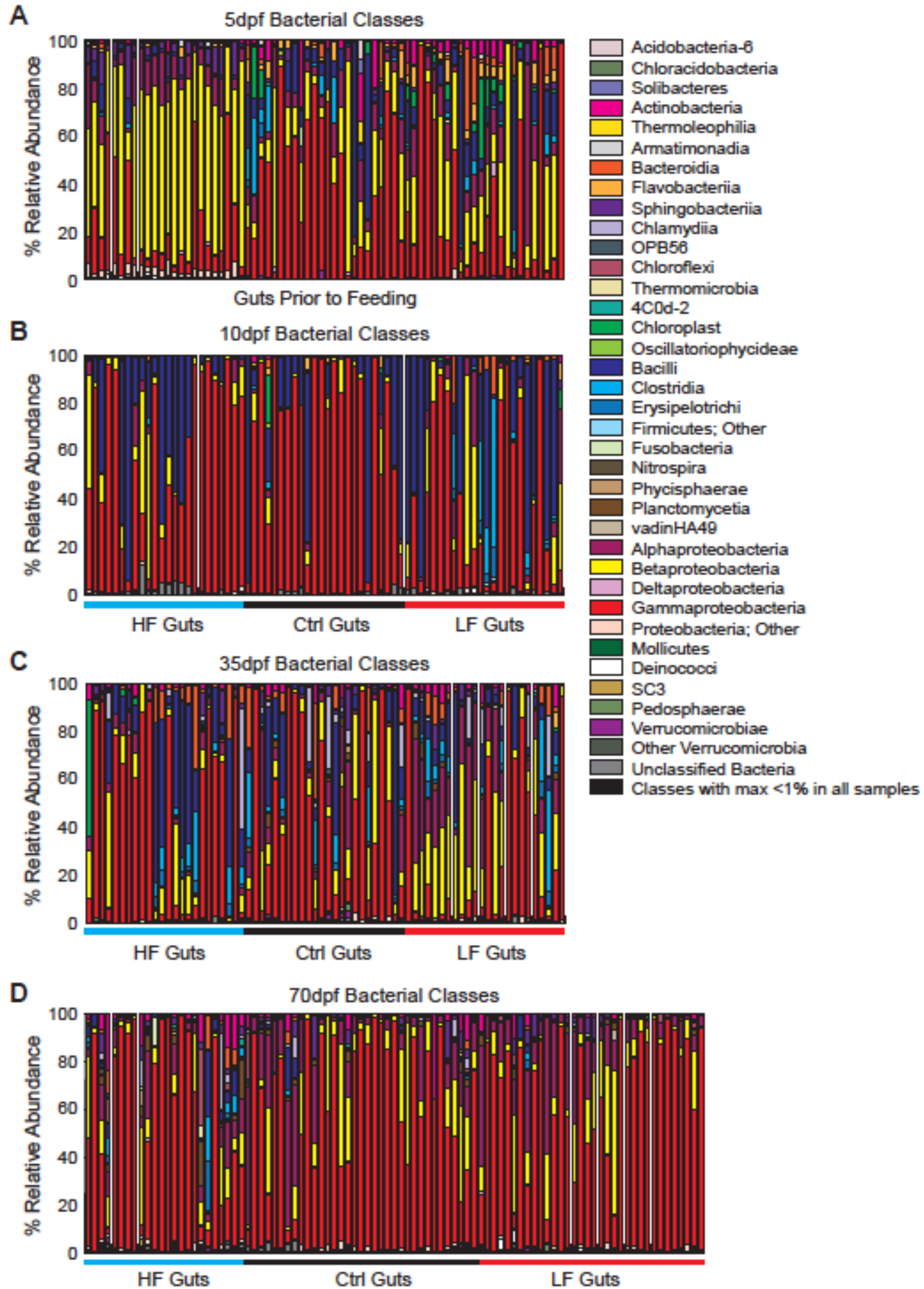


Figure S3.2. Microbiota and SL characterization at each time point. (A) Average relative abundance of bacterial classes in the zebrafish gut at each age. Bacterial classes for which the maximum average relative abundance was less than 0.5% were grouped together. **(B)** Average standard length of zebrafish within each tank at each time point. Statistical comparison of standard length at each age between fish fed different diets, regardless of tank: ANOVA with Bonferroni post-test.


A
5dpf Bacterial Classes
% Relative Abundance
100
80
60
40
20
0
Guts Prior to Feeding
B
10dpf Bacterial Classes
HF Guts
Ctrl Guts
LF Guts
C
35dpf Bacterial Classes
D
70dpf Bacterial Classes
Acidobacteria-6
Chloracidobacteria
Solibacteres
Actinobacteria
Thermoleophilia
Armatimonadia
Bacteroidia
Flavobacteriia
Sphingobacteriia
Chlamydiia
OPB56
Chloroflexi
Thermomicrobia
4C0d-2
Chloroplast
Oscillatoriophycideae
Bacilli
Clostridia
Erysipelotrichi
Firmicutes; Other
Fusobacteria
Nitrospira
Phycisphaerae
Planctomycetia
vadinHA49
Alphaproteobacteria
Betaproteobacteria
Deltaproteobacteria
Gammaproteobacteria
Proteobacteria; Other
Mollicutes
Deinococci
SC3
Pedosphaerae
Verrucomicrobiae
Other Verrucomicrobia
Unclassified Bacteria
Classes with max <1% in all samples

Supplemental Figure 3. Relative abundance of bacterial classes in individual zebrafish guts. Stacked bar charts of the percent relative abundance of bacterial classes, with individual organized along the x-axis by diet. **(A)** 5dpf. **(B)** 10dpf. **(C)** 35dpf. **(D)** 70dpf. Bacterial classes for which the relative abundance was less than 1% in all samples were grouped together.

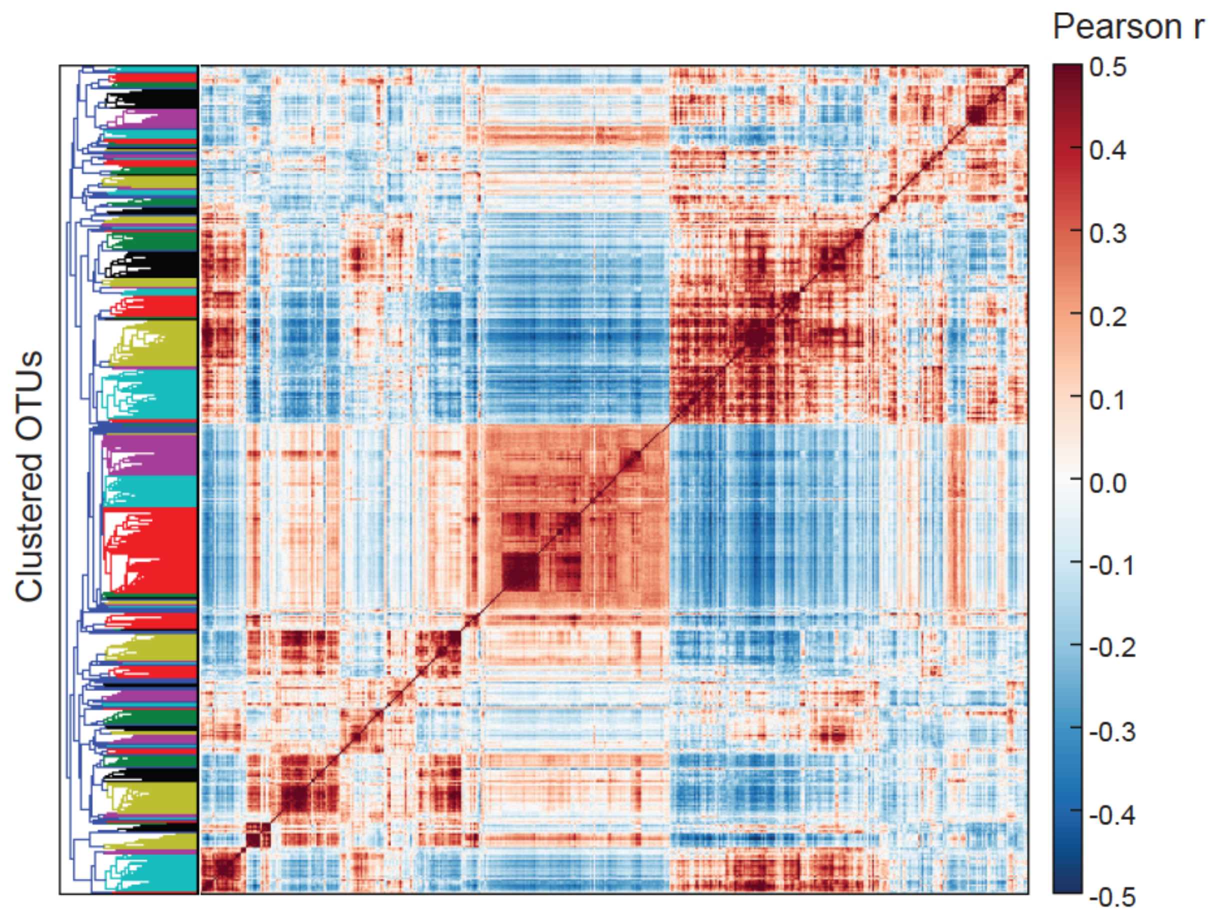


Figure S3.4. Hierarchical clustering of OTUs to identify assemblages.
Heatmap showing Pearson correlation values for each pair-wise comparison of absolute OTU abundances. Distance-based tree was generated by iterative hierarchical clustering, with alternating colors representing changing assemblages.

CHAPTER FOUR

PROLONGED STARVATION INDUCES POTENTIALLY IRREVERSIBLE DIFFERENCES IN ZEBRAFISH GUT MICROBIOTA[3]

OVERVIEW

Undernutrition and starvation are associated with alterations in microbiota that can in turn promote malnutrition-associated physiologies in the host. However, it remains unclear how gut microbiota dynamically respond to prolonged starvation and subsequent restoration of normal feeding. Here we addressed these gaps using a zebrafish model of prolonged starvation and re-feeding. Exogenous nutrition can be completely withheld from adult zebrafish for 3 weeks, resulting in the depletion of adipose stores. We therefore characterized changes in zebrafish gut microbiota over the course of 3 weeks of starvation and 3 weeks of re-feeding. We observed a number of differences in taxonomic relative abundance between fed and starved/re-fed gut microbiota at all starvation and re-feeding time points. These were associated with increasing differences in beta-diversity between fed and starved gut microbiota over the course of starvation. Additionally, fed and re-fed gut microbiota were more similar to each other than fed and starved microbiota at the end of starvation. However, beta-diversity between fed and re-fed gut microbiota remained significantly greater after 3 weeks of re-feeding compared to the beta-diversity observed prior to starvation. Together, these demonstrate increasing changes in gut microbiota with increasing duration of starvation and suggest that, following prolonged starvation, zebrafish gut microbiota does not fully recover within three weeks after the cessation of starvation.

[3] Co-authors: Sol Gomez de la Torre Canny and John F. Rawls.

INTRODUCTION

Malnutrition and undernutrition negatively impact the health millions of people per year with consequences include wasting, decreased immune function, loss of productivity, and impaired brain development (Black *et al.*, 2013) (Subramanian *et al.*, 2014) (Kau *et al.*, 2015) (Prado & Dewey, 2014). Recent studies have shown that these altered physiologies can impact and be impacted by the microbial communities residing in the intestine (gut microbiota) (Smith *et al.*, 2013) (Turnbaugh *et al.*, 2006) (Subramanian *et al.*, 2014). While there are numerous descriptions of the relationships between altered diet compositions and gut microbiota, few studies have focused on how short-term starvation impacts gut microbiota. Even fewer have characterized changes in gut microbiota in response to prolonged starvation (Carey *et al.*, 2013) (Xia *et al.*, 2014). Consistent with observations of post-prandial changes in gut microbiota (Semova *et al.*, 2012) (Costello *et al.*, 2010), these studies have reported that gut microbiota of animals subjected to prolonged nutrient deprivation are relatively depleted of Firmicutes and relatively enriched in Bacteroidetes (Kohl *et al.*, 2014) (Carey *et al.*, 2013) (Xia *et al.*, 2014) (Crawford *et al.*, 2009). However, as the longest duration of starvation in a model vertebrate (mouse) was three days (Kohl *et al.*, 2014), the dynamics of gut microbiota changes over the course of prolonged starvation in model vertebrates remains unknown.

Our information on the ability of gut microbiota to recover after periods of prolonged starvation is also very limited. The reported post-prandial changes in gut microbiota, which include decreases in the relative abundance of Bacteroidetes and increases in the relative abundance of Firmicutes, suggest that gut microbiota changes during starvation are reversible. In general, gut microbiota have been shown to be resilient to many perturbations including changes in diet composition, gastrointestinal infections, and antibiotic treatments (Zhang *et al.*, 2012) (Smith *et al.*, 2013) (David *et al.*, 2014) (Cox *et al.*, 2014). In contrast, chronic *Clostridium difficile* infection is associated with altered gut microbiota compositions and infection can be

recalcitrant to dietary changes and antibiotic treatment (Reeves *et al.*, 2011). These indicate that some changes to gut microbiota are not readily reversed. Additionally, the same types of bacteria, in particular Bacteroidetes species, are associated with lean individuals, starved or undernourished individuals, and individuals exhibiting wasting symptoms (Smith *et al.*, 2013) (Ridaura *et al.*, 2013) (Xia *et al.*, 2014) (Kohl *et al.*, 2014). Moreover, a report analyzing the gut microbiota of co-housed lean and obese mice suggested that bacteria of lean mice were able to invade gut microbiota of obese mice (Ridaura *et al.*, 2013). This raises the possibility that bacteria with increased relative abundance in starved gut microbiota may be resilient to changes in exogenous nutrient availability. This further raises the possibility that bacteria residing in starved guts may drive the formation of different microbiota compositions after starvation ends. In contrast, seasonal changes in gut microbiota of ground squirrels suggest that gut microbiota changes following prolonged periods of time without food may be reversible (Carey *et al.*, 2013) (Dill-McFarland *et al.*, 2014). Similarly, Costello and colleagues reported post-prandial higher Firmicutes relative abundance and lower Bacteroidetes relative abundance in Burmese pythons (Costello *et al.*, 2010), which typically experience long periods of time without feeding. These studies suggest that gut microbiota may be resilient to prolonged starvation. To our knowledge, these are the only studies to date characterizing gut microbiota changes during recovery from extended periods of time without feeding. However, both ground squirrels and pythons have evolved to withstand long periods of time without feeding. Therefore, gut microbiota dynamics during recovery from prolonged starvation in animals that do not typically experience prolonged starvation remains unknown.

The zebrafish (*Danio rerio*) allows unique exploration of the impact of prolonged starvation on gut microbiota in a model vertebrate. Compared to mammalian models, the high fecundity of zebrafish greatly increases biological replication. Moreover, unlike mammals, food can be completely withheld from adults for prolonged periods without mortality (Flynn *et al.*, 2009) (McMenamin *et al.*, 2013). We previously showed that adipose stores in adult zebrafish

are completely depleted after 3 weeks of starvation (McMenamin *et al.*, 2013). To determine how zebrafish gut microbiota change over a period of prolonged starvation and during recovery from starvation, we characterized zebrafish gut microbiota at multiple time points during 3 weeks of starvation and 3 weeks of re-feeding. This study is the first to characterize gut microbiota changes in a model vertebrate over prolonged starvation of this duration. Moreover, this study is the first to characterize microbiota recovery from prolonged starvation sufficient for the restoration of normal growth. We found that food availability had the strongest effect on gut microbiota during early starvation but that differences between gut microbiota of starved and fed fish increased as starvation continued. Furthermore, we observed that following re-feeding gut microbiota of re-fed and control fish remained more different than that at baseline, suggesting that prolonged starvation may lead to irreversible changes in gut microbiota.

MATERIALS AND METHODS

Animal husbandry and experimental manipulation

All zebrafish experiments were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals using protocols approved by the Institutional Animal Care and Use Committee of Duke University. Unless otherwise stated, all fish were maintained on a 14-hour light cycle at 28 degrees Celsius. All zebrafish used for the experiment were born on the same day from 3 breeding pairs from a single sibship. Fertilized embryos were transferred into Petri dishes containing egg water (6g sea salt, 1.5g calcium sulfate, 0.75g sodium bicarbonate, 10-12 drops methylene blue, 10L water) at a density of 50 embryos/dish and incubated at 28.5 degrees Celsius. At 1 day post-fertilization (dpf), embryos were transferred to 3L tanks containing 500mL water from a recirculating zebrafish aquaculture system (system water). Each tank contained 10 embryos. Fish were then maintained under standard zebrafish husbandry until the start of the experiment at 60dpf. During rearing, zebrafish larvae remained unfed until 5dpf and were given two feedings of AP500 (Zeigler

#384709-01-11) and two feedings of GEMMA Micro 75 (Skretting #B2805) each day until 13dpf. Larvae were then given two feedings of Artemia and two feedings of GEMMA Micro 75 each day until 28dpf. From at 28dpf-56dpf, juvenile zebrafish were given two feedings of Artemia and two feedings of GEMMA Micro 150 (Skretting #B1471) each day. Starting at 56dpf, fish were given two feedings of Artemia twice a day and two feedings of GEMMA Micro 300 (Skretting #B2809) each day. At 60dpf, Zebrafish were then randomly transferred into eight clean 10L tanks at a density of 67 fish per tank, with half the tanks receiving no food for the following 21 days (Fig. 4.1A). Following the 21 days of starvation, feedings for all tanks were allowed to occur as per standard husbandry: two feedings of Artemia hatched from Grade A Brine Shrimp Eggs (Brine Shrimp Direct) per day interspersed with two feedings of GEMMA Micro 300. Importantly over the 21 days of starvation and 21 days of refeeding, we observed no mortality (data not shown).

All fish to be sampled on a particular day were collected prior to the first daily feeding in the fish facility. Samples were collected at 0 days post-starvation (0dpS), 1 dpS, 3dpS, 7dpS, 21dpS, 1 day post-re-feed (dpR), 3dpR, 7dpR, and 21dpR (Fig. 4.1A). At each time point, six randomly selected fish per tank were euthanized by tricaine overdose (0.83mg/ml tricaine). Fish were imaged on a dissecting scope to facilitate subsequent standard length (SL) and height at anterior of anal fin (HAA) measurements (Parichy *et al.*, 2009). Intestinal tracts were then dissected from each fish and placed individually in lysis buffer (20mM Tris-HCl (pH 8.0), 2mM EDTA (pH 8.0), 1% Triton X-100), flash-frozen in a dry ice-ethanol bath, and stored at -80 degrees Celsius until DNA extraction.

Molecular Biology and Bioinformatic Analysis

Genomic DNA was extracted from individual zebrafish intestinal tracts using Qiagen DNeasy Blood and Tissue Kits (Qiagen, modified as previously described (Stephens, et al., 2015, submitted)). Genomic DNA was subsequently used as a template for PCR amplification of

the v4 region of 16S rRNA gene and paired-end sequencing was performed on an Illumina HiSeq 2000 Sequencing System (see Table S4.1 for primers) at the University of Oregon Genomics and Cell Characterization Core Facility. Variable-length spacer sequences that were introduced during PCR amplification to increase read complexity were trimmed from all reads using a perl script written by Dr. Doug Turnbull. QIIME version 1.8.0 (Caporaso *et al.*, 2010). The resulting trimmed sequences were then further processed for analysis. Within QIIME, paired forward and reverse reads were joined and sequences were demultiplexed. Using open-reference UCLUST (Edgar, 2010), we clustered the reads against greengenes (May 2013) into operational taxonomic units (OTUs) of 97% sequence identitiy and assigned taxonomic classifications to each OTU with the RDP classifier v2.2. We further mandated that each OTU be observed in at least 2 samples for inclusion in downstream analyses.

We used QIIME to calculate beta-diversity in our dataset. Beta-diversity between samples, assessed using Bray-Curtis distance matrices, was calculated using a sampling depth of 2000 sequences per sample with the goal of retaining at least 18 gut samples per experimental group at each time point. Effect sizes of variables on Bray-Curtis distances were determined using ANOSIM. To identify bacterial taxa with statistically significant differences in relative abundance between different experimental groups, we employed the LEfSe module (version 1.0) (Segata *et al.*, 2011) available on the Huttenhower lab Galaxy instance (http://huttenhower.sph.harvard.edu/galaxy/). For all comparisons, we used the default parameters (Kruskal-Wallis test alpha < 0.05, pair-wise Wilcoxon text alpha < 0.05, and LDA score >= 2.0 for significance).

RESULTS

Changes in zebrafish size over the course of starvation and re-feeding

At the start of the experiment, 0dpS, we observed no difference in zebrafish size as measured by SL, HAA, or HAA/SL ratio (Fig. 4.1B-D). As expected, zebrafish in fed tanks

continued to increase in SL and HAA over the course of the experiment. In contrast, by 7dpS starved fish exhibited significantly smaller body sizes, as measured by SL and HAA, and remained so for the during of the experiment, despite resuming normal growth rates after the cessation of starvation (Fig. 4.1B-C). Correspondingly, starved/re-fed zebrafish exhibited a lower HAA/SL ratio from 21dpS to 7dpR. However, by 21dpR, the HAA/SL ratio of starved/re-fed and fed fish were indistinguishable (Fig. 4.1C), suggesting that body proportions had been restored by the end of the re-feeding arm of the experiment, albeit at smaller sizes than fed controls.

Starvation quickly leads to altered gut microbiota composition with differences increasing as starvation is prolonged

We sought to determine how zebrafish gut microbiota change over the course of prolonged starvation. We observed that beta-diversity, as measured through comparison of Bray-Curtis distances, between gut microbiota of starved and fed fish increased over the course of starvation (Fig. 4.2A-B). Interestingly, while Bray-Curtis distances between starved and control gut microbiota at 1dpS were indistinguishable from distances at 0dpS (Fig. 4.2A), provision of food had the strongest effect size, as determined using ANOSIM, on Bray-Curtis dissimilarities between fed and starved gut microbiota at 1dpS (Fig. 4.2A-B). ANOSIM is a measure of the degree to which beta-diversity dissimilarities may be attributable to a specified variable. Therefore, while Bray-Curtis dissimilarities between fed and starved gut microbiota were similar at 0dpS and 1dpS, the difference in effect size at these two time points brings forth the possibility that different microbes contributed to the Bray-Curtis dissimilarities. This suggests that food availability influenced gut microbiota at 1dpS but not at 0dpS. These results further suggest that the differences between fed and starved fish are more strongly driven by food availability during early compared to late starvation.

We next characterized the changes in bacterial relative abundance over the course of starvation. Unexpectedly, we observed in fed and starved gut microbiota dynamics over the course of starvation that were broadly similar. Despite these similarities, we noticed differences in the magnitude of the change in relative abundance in fed versus starved gut microbiota (Fig. 4.3A, B). Using LEfSe, we identified a number of bacterial taxa and OTUs with statistically significant differences between fed and starved gut microbiota (Table S4.2). Of the bacterial orders with an average relative abundance of at least 0.5% in at least one experimental group, we observed relative depletions, compared to fed gut microbiota, of the Firmicutes orders Clostridiales, Bacillales, and Lactobacillales in starved gut microbiota, with the strongest depletion at 1dpS (Fig. 4.3C). Interestingly, relative to 0dpS, only fed gut microbiota exhibited significant increases and only starved gut microbiota exhibited significant decreases, in the relative abundance of Firmicutes orders (Fig. 4.3B). These differences are consistent with other studies reporting a decrease in Firmicutes relative abundance during starvation (Xia *et al.*, 2014) (Carey *et al.*, 2013) (Crawford *et al.*, 2009). Additionally, relative to fed gut microbiota, we observed a general increase in the extent of Enterobacteriales depletion over the course of starvation as well as the depletion of Desulfovibrionales at 7dpS. Interestingly, both of these orders have been associated with intestinal inflammatory diseases (Loubinoux *et al.*, 2002) (Carvalho *et al.*, 2012). In contrast, Rhizobiales and, aside from a transient depletion at 1dpS, Vibrionales tended to be enriched in gut microbiota of starved fish (Fig. 4.3B). Like Clostridiales, Bacillales, and Lactobacillales, Vibrionales enrichment in starved guts gradually became less pronounced over the course of starvation (Fig. 4.3B). These data suggest that starvation can alter the magnitude of increases or decreases in relative abundance and that there are different “stages” of starvation microbiota composition.

Differences in gut microbiota persist following re-feeding and despite recovery of body size proportions

We next wanted to determine whether gut microbiota of fish subjected to prolonged starvation recovered upon restoration of feeding. Interestingly, microbial richness remained greater in gut microbiota of fed compared to re-fed fish during early re-feeding whereas we observed no difference in richness at 21dpR (data not shown). We characterized differences in beta-diversity by comparing Bray-Curtis distances between fed and re-fed gut microbiota. Compared to 0dpS, differences between re-fed and fed gut microbiota remained high after 3 weeks of re-feeding (Fig. 4.2A). Interestingly, compared to the distances between 21dpS starved and control gut microbiota, distances between re-fed and control fish decreased early during re-feeding but increased at later re-feeding time points (Fig. 4.2A). This not only suggests that re-introduction of food strongly drives gut microbiota recovery in starved fish but also raises the possibility that prolonged starvation impairs the ability of the gut microbiota to respond to complete restoration of normal feeding.

We next wanted to characterize changes in bacterial relative abundance following the cessation of prolonged starvation. Similar to the starvation arm of the experiment, we observed many similar changes in the relative abundance of bacterial orders when comparing fed and re-fed microbiota (Fig. 4.3A). However, when comparing bacterial order relative abundances during the re-feeding arm of the experiment to last starvation time point (21dpS), we observed almost no statistically significant differences in fed gut microbiota (Fig. 4.3C). In contrast, in re-fed gut microbiota we observed significant decreases in Vibrionales and Fusobacteriales (Fig. 4.3C). We also observed a transient decrease followed by increases in Enterobacteriales, and late increases in the relative abundances of Lactobacillales and Clostridiales (Fig. 4.3C). Correspondingly, at 21dpR we observed significant enrichment of Vibrionales in fed compared to re-fed gut microbiota. Furthermore, while Clostridiales and Lactobacillales were enriched in fed gut microbiota at 21dpS, we found that the relative abundances of these orders were no

longer significantly different between fed and re-fed gut microbiota at 3dpR (Fig. 4.3B). While re-fed and fed gut microbiota were more similar each other than 21dpS starved and fed gut microbiota were to each other (Fig. 4.2A), our results these indicate that three weeks of re-feeding was insufficient for gut microbiota to completely recover from prolonged starvation. Moreover, this incomplete gut microbiota recovery was observed despite apparent host physiological recovery to starvation (Fig. 4.1D).

DISCUSSION

Here, we report the first characterization of gut microbiota changes in zebrafish over prolonged starvation. To our knowledge, this is also the first to characterize gut microbiota composition dynamics for an extended amount of time following the cessation of prolonged starvation in a model vertebrate. We observed a number of differences and increasing beta-diversity between fed and starved gut microbiota over the course of starvation. In contrast, during re-feeding, changes in re-fed gut microbiota were accompanied by increased similarity to fed gut microbiota. Intriguingly, 21dpR fed and re-fed gut microbiota retained greater differences in beta-diversity compared to 0dpS, suggesting incomplete gut microbiota recovery.

Chronic undernutrition detrimentally impacts the health of millions of people and can lead to Kwashiorkor and severe acute malnutrition. Importantly, altered gut microbiota compositions have been observed in people suffering from these conditions (Smith *et al.*, 2013) (Subramanian *et al.*, 2014), suggesting that insufficient caloric intake over prolonged periods of time lead to altered gut microbiota. However, few studies have characterized changes in gut microbiota in response to prolonged starvation, and none have done so in a model vertebrate for longer than 3 days (Xia *et al.*, 2014) (Carey *et al.*, 2013, Kohl *et al.*, 2014). Consistent with these studies, among the differences in the relative abundance of various bacterial taxa we observed decreases in the Firmicutes orders Clostridiales, Bacillales, and Lactobacillales during starvation (Fig. 4.3). This suggests that some of the conservation in vertebrate gut microbiota

response to prolonged starvation that was reported by Kohl and colleagues (Kohl *et al.*, 2014) extends to zebrafish gut microbiota.

In addition to the availability of exogenous nutrients, host physiological responses to starvation likely also drive changes in gut microbiota. For example, starvation has been correlated with decreased numbers of goblet cells and changes intestinal glycosylation in Atlantic salmon (Landeira-Dabarca *et al.*, 2013) (Landeira-Dabarca *et al.*, 2014). Moreover, the lack of exogenous nutrition likely makes intestinal glycoproteins one of the few nutrient sources available to gut microbiota. This would, in turn, select for bacteria, for example Bacteroidales species, capable of consuming endogenous glycoproteins (Marcobal *et al.*, 2013) and lead to an increase in the relative abundance of these bacteria. Correspondingly, compared to fed guts, we observed enrichment of the order Bacteroidales in starved/re-fed guts late in starvation and early during re-feeding (Fig. 4.3C). Additionally, as a source of stress, starvation leads to increases in the production of glucocorticoids. Glucocorticoids are anti-inflammatory and have also been shown to influence intestinal glycosylation (Nanthakumar *et al.*, 2013) and could therefore regulate gut microbiota. Strikingly, we observed many similar changes over the course of starvation and re-feeding in the relative abundance of many bacterial orders in fed and starved gut microbiota. It is possible that these changes were the result of stress induced by the transfer of all fish into new tanks at the start of the experiment. While one might have predicted recovery of gut microbiota in fed fish following recovery from stress, we instead observed continued differences in fed gut microbiota compared to 0dpS (Fig. 4.3A). This, as well as the continued increased in beta-diversity between fed and re-fed gut microbiota after 3 weeks of re-feeding, run counter to reports of microbiota following transitions to and from HF diet or hibernation (Zhang *et al.*, 2012) (Carey *et al.*, 2013). They further bring forth the possibility that some forms of stress may induce prolonged or irreversible gut microbiota changes in zebrafish. Future experiments might test whether other sources of acute or chronic stress result in similar changes in gut microbiota composition.

Physiological development has also been correlated with gut microbiota changes in a number of vertebrates (Koenig *et al.*, 2011) (Stephens 2015 submitted) (Yatsunenko *et al.*, 2012). Most reports correlating development with gut microbiota have focused on changes that occur early in life. However, physiological changes that occur later in life may also impact gut microbiota. For example, as sex steroids and estrogen receptors have been implicated in immune regulation (Lelu *et al.*, 2011) (Kovats, 2015) (Phiel *et al.*, 2005), changes in estrogen and testosterone during puberty may alter regulation of the immune system. Notably, at the start of the experiment, zebrafish had not yet reached the size at which sexual maturity is typically observed (Fig. 4.1B) (Parichy *et al.*, 2009). It is therefore possible that some of the similarities we observed between fed and starved/re-fed gut microbiota were due to changes associated with ongoing development of the reproductive system. Future studies could compare gut microbiota and host tissue transcriptional analyses to determine whether host physiological changes in response to reproductive development and/or starvation and re-feeding correlate with changes in gut microbiota. Host genes that are implicated in analyses might then be validated in animal hosts with genetic analysis. Furthermore, the associations between physiological development, gut microbiota, and undernutrition emphasize the need to understand the long-term impacts of undernutrition during different developmental stages on gut microbiota.

FIGURES

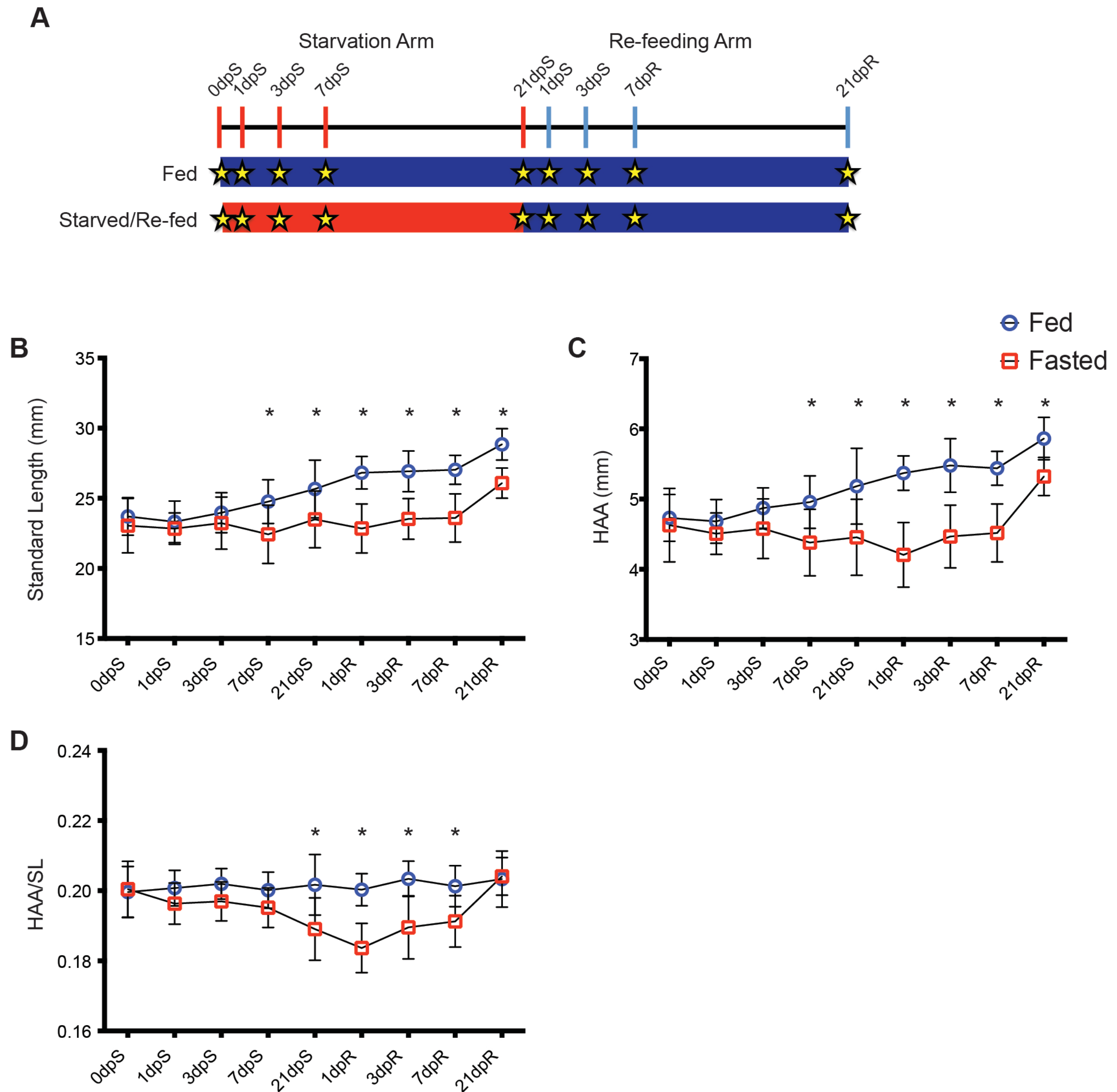


Figure 4.1. Experiment schematic and zebrafish size measurements. (A) Schematic of experimental design. Zebrafish were raised until 60dpf, which was the day starvation commenced (0dpS). Red indicates starvation. Stars denote sampling time points. Time-line marked by sampling time points. dpS, days post-starvation. dpR, days post-re-feeding. **(B-D)**. Size measurements (average +/- SD) of starved/refed and fed fish over the course of the experiment. Statistics: 2-way ANOVA with Bonferroni post-test. Asterisks indicate statistically significant differences between fed and starved/re-fed fish. **(B)** SL (mm). **(C)** HAA (mm). **(D)** HAA/SL.

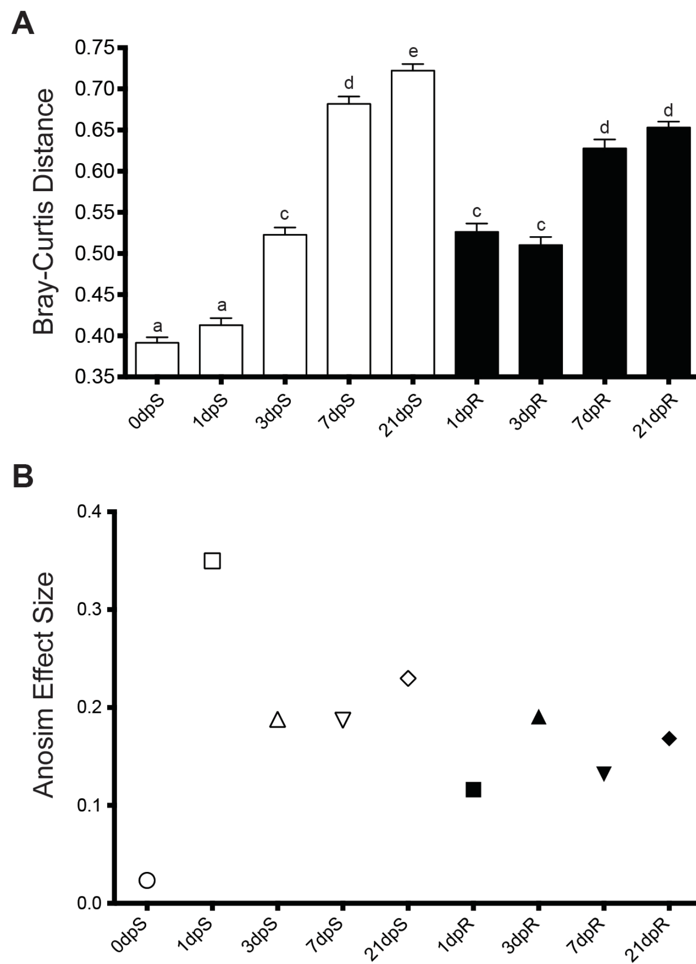


Figure 4.2. Beta-diversity between fed and starved/re-fed gut microbiota. (A) Average Bray-Curtis distances between fed and starved/re-fed gut microbiota. Statistics: ANOVA with Tukey's multiple comparisons test. Bars with the same letter above are not statistically different.. **(B)** Effect sizes, as determined by ANOSIM, based on Bray-Curtis distance matrices for comparsons between fed and starved/re-fed gut microbiota. ANOSIM p-value < 0.05 for all time points except 0dpS.

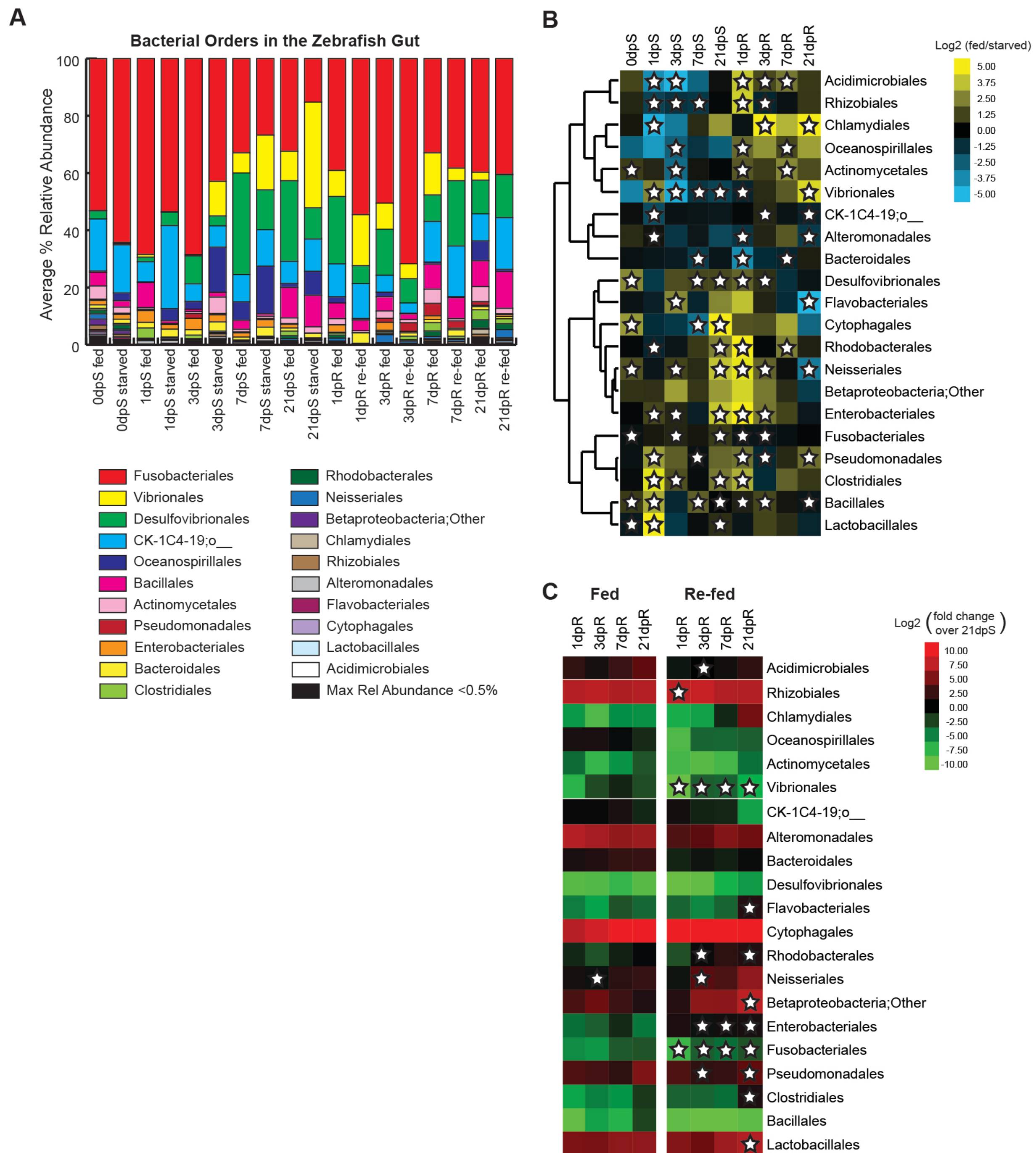


Figure 4.3. Relative abundance of bacterial orders in fed and starved/re-fed fish. **(A)** Average relative abundance of bacterial orders in fed and starved/re-fed guts at each time point. (**B**) Heatmap showing fold difference between fed and starved/re-fed relative abundance for bacterial orders with at least 0.5% relative abundance in at least one time point. Stars indicate LEfSe-identifed statistically significant difference between fed and starved/re-fed. **(C)** Heatmap showing fold-difference compared to 21dpS of bacterial order relative abundance in fed and starved/re-fed guts at each re-fed time point. Stars indicate statistically significant difference after an FDR correction of 5%.

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Gut microbiota are communities of microbes that colonize animal intestines. Interest in gut microbiota has greatly expanded over the last decade due to growing associations between differences in gut microbiota and differences in host physiology (Turnbaugh *et al.*, 2008) (Ley *et al.*, 2005) (Smith *et al.*, 2013) (Frank *et al.*, 2007) (Cani *et al.*, 2008) (Karlsson *et al.*, 2012) (de Theije *et al.*, 2014). The dissertation work has focused primarily on nutritional factors that impact gut microbiota composition. In Chapter Two, I showed that while rainbow trout gut microbiota are largely resilient to differences in rearing density and diet, a few low-abundance bacterial taxa exhibited diet-dependent differences in relative abundance. In Chapter Three, I described the impact of life-long differences in dietary fat on zebrafish gut microbiota assembly at different ages. I found that the impact of dietary fat on gut microbiota was greater in older compared to younger fish. Fat-dependent differences included not just differences in the relative abundance of bacterial taxa but also differences in the relative abundance of co-varying groups of bacteria (assemblages), which are not necessarily phylogenetically related, and differences in the relative role of neutral processes and selective pressures on assembly. Additionally, I observed that whether or not zebrafish were present in the tank as well as differences in dietary fat levels impacted environmental microbiota composition. In Chapter Four, I characterized the microbiota changes that occur in adult zebrafish during prolonged starvation and during recovery from starvation. I found that gut microbiota differ in fed versus starved fish and that gut microbiota during early starvation is different from that following prolonged starvation. I further observed

that gut microbiota of starved fish quickly change following resumption of feeding, but remain different from gut microbiota of fish that had never been starved.

While many studies have explored the impact of different diets on gut microbiota, most dietary manipulations under controlled settings were conducted over short periods of time compared to the lifespan on the animal. Furthermore, none have examined how long-term interactions between diet and development impact microbiota assembly. However, the composition of the gut microbiota is the cumulative result of assembly processes occurring over long periods of time. The works I have reported in Chapters Two and Three are novel in the duration of dietary manipulation and in the examination of the intersection of the associations between host physiological development, continuous differences in dietary fat levels, and gut microbiota assembly. Additionally, much remains unknown about the relationship between prolonged malnutrition or undernutrition and the associated gut microbiota. Mammalian studies have limited fasting to a few days or seasonal hibernation (Carey *et al.*, 2013) (Crawford *et al.*, 2009) (Okada *et al.*, 2013) (Kohl *et al.*, 2014), and studies on the effect of prolonged starvation on gut microbiota are extremely limited (Xia *et al.*, 2014) (Kohl *et al.*, 2014). Not only does Chapter Four contribute to this knowledge base by adding information about the effects of prolonged starvation on zebrafish gut microbiota, but it is also novel in its characterization of the changes in gut microbiota over 3 weeks of re-feeding. Unearthing how gut microbiota respond to prolonged starvation, undernutrition, or malnutrition and whether gut microbiota are resilient to such conditions may yield inroads toward the treatment of malnutrition and wasting diseases.

Future directions

Impact of prolonged nutritional differences on gut microbiota

Prolonged differences in diet composition

Many studies have reported correlations between gut microbiota and diets high in fat and/or sugar. There is also evidence that diets with different types of fat have different impacts

of host physiology. For example, dietary consumption of poly-unsaturated fatty acids (PUFAs) as opposed to saturated fats have been associated with decreased pathology, and diets richer in omega-3 versus omega-6 fatty acids have been associated with decreases or increases, respectively, in pro-inflammatory markers (Hekmatdoost *et al.*, 2013) (De Boer *et al.*, 2014). Moreover, different degrees (or lack thereof) of nuclear receptor specificity for and activation by different lipid molecules have been reported (Kliewer *et al.*, 1997) (Oswal *et al.*, 2013). However, few studies have focused on the effect of different lipid species on gut microbiota. One study reported that dietary supplementation with fish oil (omega-3 fatty acids) led to differences in gut microbiota composition (Yu *et al.*, 2014). Another compared HF and LF diets in which different lipid species constituted the fat in the HF diets. In this study the type of dietary fat, in particular palm oil, altered both phylogenetic diversity and relative abundances of bacteria within the gut (de Wit *et al.*, 2012). Similarly, in Chapter Two I showed that gut microbiota varied in rainbow trout fed fishmeal versus plant-based meal diets (Table 2.1) for 10 months. Because fish typically possess higher omega-3 to omega-6 ratios compared to plants (Hekmatdoost *et al.*, 2013), dietary differences between the fishmeal- and plant-based diets likely included differences in the amount of omega-3 versus omega-6 fatty acids. This suggests that omega-3 and omega-6 fatty acids may differentially influence gut microbiota. Further work with controlled dietary differences may determine whether omega-3 and omega-6 fatty acids exert different effects on gut microbiota. Similarly, because diets often contain a mixture of lipids, it will be important to determine whether varying dietary ratios of different types of fat, for example raising or lowering omega-3 to omega-6 fatty acid ratios or saturated to unsaturated fat ratios, impacts gut microbiota.

Most studies exploring the effect of changing the amount of fat or the type of fat in the diet have performed relatively short diet manipulations in adult animals. However, many people exercise relatively stable dietary patterns over long periods of time. Moreover, gut microbiota are the result of cumulative microbiota assembly processes. It is therefore important to

determine how chronic dietary differences impact gut microbiota. To my knowledge, no prior studies have examined how life-long dietary manipulations impact gut microbiota assembly. In Chapter Three, I showed that life-long differences in dietary fat levels resulted in increasingly different gut microbiota with age. Given the potential for different types of fat to impact both host physiology and gut microbiota, further work will need to be done to determine how long-term differences in dietary lipid profiles impact gut microbiota.

Prolonged nutrient deprivation and restoration

Here, I define nutrient deprivation to include starvation, under-nutrition, and malnutrition. However, in contrast with the abundance of studies exploring how differences in diet composition impact gut microbiota, few studies have directly focused on the effects of prolonged nutrient deprivation on gut microbiota, and the animal hosts studied include non-mammals (Xia *et al.*, 2014) (Kohl *et al.*, 2014) and hibernating mammals (Carey *et al.*, 2013) (Dill-McFarland *et al.*, 2014). These studies have reported increases in the relative abundance of the bacterial class Bacteroidia as well as decreases in the relative abundance of several Firmicutes taxa, including Lachnospiraceae in hibernating squirrels (Carey *et al.*, 2013) (Dill-McFarland *et al.*, 2014), and Lactobacillus in tilapia, quail, and mice (Kohl *et al.*, 2014). Importantly, of the animals in these studies, mice were the only model host used; furthermore, the mice were fasted for a maximum of 3 days (Kohl *et al.*, 2014). In contrast, in Chapter 4 I characterized changes in zebrafish gut microbiota during 3 weeks of starvation followed by 3 weeks of re-feeding. Interestingly, while higher in starved versus fed fish at 7 days post-starvation, the relative abundance of Bacteroidia was more abundant in the fed fish at 21 days post-starvation (Fig 4.3B). In a similar vein, Kohl and colleagues reported that the relative abundance of Fusobacteria in tilapia was higher during early starvation and lower at late starvation compared to the fed baseline (Kohl *et al.*, 2014). These suggest that interactions between microbes or between host and microbe vary over the course of starvation, and microbial changes may be the result of physiological changes as starvation progresses. A plethora of physiological

changes are known to occur over short-term and prolonged starvation (Drew *et al.*, 2008) (Cahill, 1970). For example, in squirrels, *MUC2* expression increases during early hibernation, *TLR4* transcription decreases during hibernation, and *TLR5* expression increasing during hibernation (Dill-McFarland *et al.*, 2014). Additionally, glucocorticoids, which exert anti-inflammatory effects, play a regulatory role in the physiological response to starvation (Simonnet, 1999) and have also been shown to impact intestinal glycoprotein fucosylation (Nanthakumar *et al.*, 2013). These suggest that interactions between the host immune and GI systems and gut microbiota change during starvation. Further work should be done to determine whether the impact of gut microbiota on host immune and GI systems changes during starvation as well as characterize the mechanisms by which host physiological changes promote changes in the relative abundance of different microbes during starvation. It would also be tantalizing to test whether, during starvation, certain microbes are maintained or subdued in the gut to mitigate the effects of starvation or promote quicker recovery during refeeding. These could be tested in gnotobiotic experiments where animals colonized with defined microbiota, with or without a species of interest, are subjected to starvation and re-feeding.

Resilience of gut microbiota to dietary changes

The degree to which and mechanisms by which microbiota are resilient to different perturbations remain an intriguing open question. Gut microbiota have been reported to be resilient to gastrointestinal infection (David *et al.*, 2014), antibiotic insults (Cox *et al.*, 2014), and many dietary alterations, including switches to and from HF diets and, in mice colonized with microbiota from humans suffering from Kwashiorkor, switches to and from ready-to-use diets (Smith *et al.*, 2013) (Zhang *et al.*, 2012) (David *et al.*, 2014). In contrast, in the re-feeding arm of the project I described in Chapter Four, gut microbiota in re-fed fish became more similar to that of control (never starved) fish. However, even after 3 weeks of re-feeding, gut microbiota of these fish remained fairly distinct from that of the control fish (Fig 4.A-B). There are several

possible explanations for such gut microbiota resilience that are not mutually exclusive. 1) Microbiota responses to the nutrient environment are extremely plastic. 2) During *de novo* assembly, microbiota adopt the most stable conformations given their nutrient environment and become highly resilient to perturbations. 3) Some environmental conditions, which may include nutrient deprivation, result in more stable gut microbiota compositions than others. The persistent difference between re-fed and control gut microbiota could be the result of either permanent alterations to gut microbiota or insufficient amounts of time for recovery to be completed. Future studies should extend post-starvation monitoring to distinguish between these two possibilities.

If gut microbiota of re-fed fish remain perpetually different from gut microbiota of fish that had never experienced prolonged starvation, it would be intriguing to identify the mechanisms leading to continued gut microbiota differences. Microbiota transplant studies suggest that animals fed identical diets but colonized with different gut microbiota may maintain the differences observed in the donors' microbiota (Turnbaugh *et al.*, 2006) (Smith *et al.*, 2013). For example, GF mice colonized by microbiota from twins discordant for Kwashiorkor assembled different microbiota communities despite consumption of the same diet (Smith *et al.*, 2013). These suggest that earlier gut microbiota compositions influence subsequent microbiota assembly processes. Therefore, persistent differences between gut microbiota of animals that experienced prolonged starvation and those that did not might occur if the gut microbes of starved animals induce alternative microbiota assemblies. This hypothesis might be tested by comparing gut microbiota assembly in GF animals allowed to become naturally conventionalized to assembly in GF animals colonized by microbiota from starved animals. Alternatively, it is possible that epigenetic changes induced by starvation might lead to long-lasting gut microbiota differences. Epigenetic changes in the promoters of immunity-related genes have been observed following Roux-en y gastric bypass surgery (Benton *et al.*, 2015) (Nilsson *et al.*, 2015), which is similar to starvation in that the surgery results in drastic

reductions in food ingestion. Similarly, DNA methylation has been implicated as a means of regulating host metabolism and immunity during fasting (Tsai *et al.*, 2013). While some epigenetic changes have been shown to readily change in non-diabetic wild-type mice in response to feeding after short-term fasting (Li *et al.*, 2012), it remains to be established whether such changes are reversible after prolonged starvation. It is therefore possible that host epigenetic changes in response to prolonged starvation might alter gut microbiota assembly permanently or for an extended period of time. This hypothesis could be tested by comparing the epigenetic landscape and gut microbiota of animals subjected to prolonged starvation and re-feeding against that of animals that had never been starved. The role of particular genes for which epigenetic regulation changes could then be tested in whole-animal or tissue-specific null mutants.

Interestingly, previous studies have shown that adipose stores in adult zebrafish are completely depleted after 3 weeks of starvation (McMenamin *et al.*, 2013). However, despite the continued gut microbiota differences after three weeks of re-feeding, re-fed fish recovered HAA-to-SL ratios (Fig 4.1D) (see (Parichy *et al.*, 2009)), which are conceptually similar to waist circumference-to-height ratios. Studies examining the impact of gut colonization in gnotobiotic animal models have demonstrated that colonization can promote increased adiposity (Bäckhed *et al.*, 2004) (Turnbaugh *et al.*, 2008). In a similar vein, gut microbiota are likely important mediators of physiological responses to nutrient intake following starvation. A recent study reported that *Lactobacillus murinus*, at least in part through lactate production, promotes increased intestinal epithelial cell proliferation following starvation (Okada *et al.*, 2013). It would be interesting to test whether other bacteria that increase in relative abundance during starvation or bacteria that colonize the guts of re-fed fish contribute to host physiological recovery to starvation.

An interesting extension to the questions posed above is whether repeated feast-famine cycles or repeated dietary transitions lead to increasingly divergent gut microbiota. A recent

study reported that most but not all gut microbiota changes were reversible following repeated transitions between a low-fat, high plant polysaccharide diet and a high-fat, high-sugar diet in mice. Furthermore, diet history was a causative factor in the non-reversible gut microbiota changes (Carmody *et al.*, 2015). Similarly, it is possible that gut microbiota changes will accumulate with additional starvation events. This could be tested in experiments where animals subjected to multiple fasting episodes and gut microbiota are characterized during and after each starvation episode.

Interactions between host development, diet and gut microbiota

Impact of physiological development on gut microbiota

Gut microbiota are impacted by host physiology, and *de novo* gut microbiota assembly occurs in the context of changes in host physiology that occur with development. Accordingly, many studies in diverse organisms have shown gut microbiota composition changes that occur concomitantly with host development (Stephens *et al*, 2015, submitted) (Koenig *et al.*, 2011) (Franzenburg *et al.*, 2013). Moreover, nutrition impacts both host physiology and gut microbiota. It is therefore important to understand how the changes in host physiology during development interact with diet in their influences on gut microbiota assembly. In Chapter Two I showed that associations between different dietary fat levels and gut microbiota composition depended in part on zebrafish age (Fig. 3.1, Fig. 3.2, Fig. 3.3). For example, previous studies have reported increased relative abundance of the bacterial class Clostridia during HF diet consumption (Ridaura *et al.*, 2013). In contrast, I observed that Clostridia were at greater relative abundance in LF guts at 10dpf and were at greater relative abundance in HF guts at 70dpf (Table S3.5). One possible cause for the age-dependent differences is that, until ~4wpf, zebrafish lack a functional adaptive immune system (Lam *et al.*, 2004), which has been shown to impact gut microbiota (Carmody *et al.*, 2015) (Peterson *et al.*, 2007) (Zhang *et al.*, 2015). The magnitude of the impact of the developing adaptive immune system on the zebrafish gut microbiota could be

tested by comparing the gut microbiota from wild-type and *rag1*-/- fish before and after adaptive immunity normally becomes functional.

Many other organ systems, including digestive organs, undergo extensive morphological and functional changes after birth. In zebrafish, these include the development of adipose tissues (Flynn *et al.*, 2009) and changes in intestinal morphology, include the formation of intestinal folds (Flores *et al.*, 2008) (Ng *et al.*, 2005). Similarly, differences in the glycosylation patterns of mammalian intestinal glycoproteins have been shown to change with age (Nanthakumar *et al.*, 2013). Because of the intimate interactions between gut microbiota and host GI and metabolic physiology, developmental changes in digestive physiology could be a factor in the different impact of fat on gut microbiota of older and younger fish. For example, the expansion of adipose tissues between hatching and 28dpf in zebrafish (Flynn *et al.*, 2009) may result in an increase in the secretion of adipokines, which have immunomodulatory properties (Kilroy *et al.*, 2007) and could therefore impact immune influence on gut microbiota. The hypothesis that increased immune signaling from adipose tissue influences gut microbiota assembly could be tested via targeted depletion of adipocyte progenitors. Additionally, the vertebrate gut has been hypothesized to become increasingly anaerobic with age (Albenberg *et al.*, 2014) (Palmer *et al.*, 2007), which will not only select against aerobic microbes but will also alter the mechanisms by which nutrients can be metabolized (i.e., a switch to fermentation). Therefore, oxygen tension is a probable determinant of gut microbiota composition. Not only has oxygen tension been shown to differ between the gut mucosa and lumen, but changes in oxygen tension in the gut have also been correlated with changes in gut microbiota (Albenberg *et al.*, 2014). Because the vasculature is a source of oxygen within the gut, one could indirectly test the impact of oxygen tension on gut microbiota using drugs or intestine-specific mutants that either inhibit or promote angiogenesis. Similarly, the role of specific glycoproteins or antimicrobial peptides secretion in mediating the impact of dietary fat on gut microbiota at different ages could be tested using genetic null mutants.

Impact of nutritional differences during specific developmental windows

The correlations between physiological development and gut microbiota bring forth the possibility that certain developmental windows may be especially influential in microbiota assembly. Gut microbiota perturbations during these windows have been correlated with altered host physiology later in life (Dominguez-Bello *et al.*, 2010) (Decker *et al.*, 2011) (Cho *et al.*, 2012), and altered gut microbiota are associated with altered host physiologies. These bring forth the possibility that perturbations to gut microbiota during critical host developmental windows may lead to altered gut microbiota later in life. However, the long-term impacts of gut microbiota alterations early in life on gut microbiota in adulthood are understudied. Such perturbations might include under-nutrition or malnutrition limited to childhood and differences in the amount of dietary fat during or prior to puberty. These gaps in knowledge could be filled by subjecting animals to nutritional differences such as starvation or altered diet composition during specific developmental windows. To determine which host and microbiota changes persist into adulthood, host physiological parameters as well as the gut microbiota of these animals would then be assessed during and immediately after diet manipulation as well as in adulthood.

Mechanistic studies of host-microbe and microbe-microbe interactions in the gut

Improving methods to identify potential bacteria or groups of bacteria that impact host physiology

Deep sequencing surveys have identified many associations between different environmental conditions, including host diet, and increases or decreases in the relative abundance of specific gut microbiota members. However, other analytical methods may also be used to identify potentially important members of gut microbiota. For example, the identification of assemblages, which are groups of bacteria that co-vary in their occurrence and/or abundance (Fauth *et al.*, 1996), may generate hypotheses about which bacteria cooperate or possess

similar functional characteristics. The bacteria within an assemblage need not be closely phylogenetically related. Potential reasons for co-variance include the possession of similar characteristics that increase their fitness in specific niches. Alternatively, co-varying bacteria may cooperate in a functional network to make the collective group more fit in a particular niche. In Chapter Three, in addition to identifying bacterial taxa for which relative abundance correlated with age and/or diet, we also defined assemblages that differed in relative abundance in different experimental groups. Experiments in which GF animals are colonized by assemblages that differ in relative abundance between experimental groups could subsequently be performed to test the cohesiveness or cooperativity of bacteria within the assemblages. However, these analyses are limited because the use of hierarchical clustering to identify the assemblages mandates that specific microbes belong to only one assemblage. In contrast, particular microbial species may belong to multiple assemblages, with the degree of contribution to those assemblages varying with different conditions. More complex analytical methods may reveal how assemblage composition differs under different environmental conditions or in different niches. Combined with genetic information about assemblage members, such analyses may yield testable hypotheses on the bacterial mechanisms determining assemblage membership in different contexts. For example, one could identify and test specific bacterial genes hypothesized to be important for diet-dependent interactions with other bacteria.

Importantly, differences in relative abundance under different environmental conditions may be due to selective or non-selective (neutral) processes (Sloan *et al.*, 2006). The ability to distinguish between changes due to selective versus neutral processes would enhance the identification of potential bacteria that impact host physiology. In Chapter Three, I compared gut microbiota of fish fed different diets, at multiple ages, to gut microbiota that would assemble according to Sloan's Neutral Community Model for Prokaryotes (Sloan *et al.*, 2006). Use of this model identified bacteria that were likely to be under positive or negative selection in the zebrafish gut under different dietary conditions or at different ages (Table S3.6). Bacteria under

positive selection may be highly attractive candidates for species with the potential to impact host physiology. Interestingly, in accordance with the age-dependent differences in bacterial relative abundance in fish fed different diets, I also observed that age impacted which bacteria were under selection in fish fed different diets (Fig 3.3F). For example, the bacterial class Erysipelotrichi, which has previously been associated with altered adiposity and increased risk of diabetes (Woting *et al.*, 2014), contained OTUs that were under positive selection in LF but not HF guts at 10dpf and, conversely, OTUs that were under positive selection in both HF and LF guts at 35dpf (Fig 3.3F). Importantly, the results of this model suggest that the same bacteria may only impact host physiology during specific developmental stages or may exert different impacts on host physiology at different ages. Therefore, validation experiments should be mindful of physiological development status when determining how diet influences the impact of specific bacteria on host physiology.

Elucidating specific host-microbe interactions in the gut

The analytical methods I discussed above and in Chapter Three, as well as comparisons of relative abundance, have generated predictions about which specific bacteria and groups of bacteria impact host physiology under host ingestion of different levels of dietary fat. One species of interest, *Akkermansia muciniphila*, has previously been anti-correlated with adiposity and diabetes (Liou *et al.*, 2013). Similarly, in Chapter Three, we observed positive selection for a particular *A. muciniphila* OTU at 35dpf in the guts of all diet groups but at 10dpf only in LF guts. This species is an example of the few for which the hypothesized impact on host physiology has been tested (Everard *et al.*, 2013) (Lukovac *et al.*, 2014). Other gut microbes have been shown ferment carbohydrates and produce SCFAs, which can be used by colonocytes for energy (Stevens & Hume, 1998) (Clausen & Mortensen, 1995). However, most bacteria that have been implicated in altered host physiologies have yet to be tested. Moreover,

many of the mechanisms by which specific bacteria induce changes in host physiology remain unknown.

Testing the specific bacteria identified by deep sequencing studies may be challenging for several reasons, including the inability to grow pure cultures of many species. Furthermore, many culturable gut microbes remain genetically intractable, which greatly obstructs the testing of potential mechanisms by which these bacteria exert their influence on host physiology. Because bacterial species that more phylogenetically related are also more likely to possess similar characteristics (Faith, 1992), more phylogenetically-related, culturable bacteria species could be used as a proxy to test microbes of interest. For example, gut colonization has been shown to induce pro-inflammatory and repress fatty acid catabolism programs in the host, and Rawls and colleagues demonstrated that colonization by *Pseudomonas aeruginosia*, which is a member of the most abundant bacterial phylum in the larval zebrafish gut, was capable of recapitulating host responses to conventionalization (Rawls *et al.*, 2004) (Rawls *et al.*, 2007). Facilitated by the fact that *P. aeruginosa* is genetically tractable, it was further shown that induction of host responses to colonization was dependent on the ability to conduct flagellar motility and not just the result of bacterial stimulation of TLR5 by flagellin (Rawls *et al.*, 2007). Similar experiments could be performed with other culturable gut isolates to probe potential impacts and activities of other potentially influential bacteria. Furthermore, direct visualization of host-microbe interactions can be observed using simplified bacterial communities in which different species are marked with fluorescent molecules. Zebrafish present especially useful tools for such studies because their optical transparency permits imaging in live animals. For example, a recent study visualizing *Aeromonas veronii* in the guts of live zebrafish reported interesting differences in growth dynamics and location with the gut between planktonic and aggregated *Aeromonas veronii* (Jemielita *et al.*, 2014). While *A. veronii* can be genetically manipulated, genetically intractable microbes could be similarly visualized using fluorescent lipophilic membrane dyes (Yadava *et al.*). The ability to directly visualize host-microbe

interactions in specific intestinal compartments could provide unique insight into the mechanisms by which specific bacteria influence host physiology. Additionally, techniques such as RNA-seq or microarrays could generate hypotheses about particular gene products that mediate host-microbe interactions. For genetically intractable organisms, the gene of interest might then be tested by expressing the gene in other organisms such as *E. coli* and monoassociating a host organism with the transformed microbe.

Dissecting inter-species bacterial interactions in the gut

As suggested by the identification of assemblages, which I described above, different bacterial species may interact in the gut, and those interactions may be dependent on host diet (Ze *et al.*, 2012) (Falony *et al.*, 2009). In Chapter Three, I identified a number of assemblages that exhibited dynamic temporal and spatial relative abundance patterns (Fig 3.2). These included assemblages that were only enriched in gut versus environment in the presence of food and assemblages that differed in relative abundance between HF and LF guts (Fig 3.2B, Fig. 3.2E). It is therefore likely that changes in diet can impact host physiology by altering the interactions between different gut microbes or by impacting entire assemblages. However, many inter-species microbial interactions that take place within the gut remain open questions. These include questions regarding whether bacterial localization within the gut changes in the presence of other bacterial species and whether those changes change in response to different nutrient availabilities. These hypotheses could be tested through microscopy, with real-time interactions observable in live animals in which different bacterial species are labeled with different fluorescent markers. It is known that some bacteria can antagonize others through the secretion of antimicrobial compounds or through contact-dependent systems such as the type VI secretion system (T6SS), which has been shown to target both bacteria and eukaryotes (Basler *et al.*, 2013) (MacIntyre *et al.*, 2010). The impact of these bacterial activities could be tested by comparing gut microbiota of animals inoculated with strains that can or cannot

produce bacteriocins or utilize their T6SS. For example, the impact of T6SS could be tested by comparing gut microbiota of animals inoculated with wild-type *Vibrio cholerae* or a *vasH* deletion mutant (MacIntyre *et al.*, 2010). A similar experiment could be performed using *P. aeruginosa*, which our lab and others have shown does not cause pathology upon gut colonization in zebrafish (Rawls *et al.*, 2007).

Another mechanism with the potential to impact microbial interactions in the gut is quorum sensing (QS). QS has been shown to regulate bacterial virulence, including transitions to and from planktonic lifestyles and T6SS (Patriquin *et al.*, 2008) (Zheng *et al.*, 2010) (Khajanchi *et al.*, 2009). Among the molecules shown to be QS signals is autoinducer 2 (AI-2). AI-2 is sometimes referred to as a universal quorum sensing signal due to the ability of multiple bacterial species, for example *Salmonella typhimurium* and *Vibrio harveyi*, to produce and/or detect it (Bassler *et al.*, 1997) (Surette & Bassler, 1998). It is therefore possible that QS mediates gut microbiota composition by induction of bactericidal behaviors or by regulating the adoption of sessile versus planktonic lifestyles of multiple microbiota members. The impact of AI-2 could be tested by administering AI-2 to the intestine and determining whether changes in gut microbiota occur. Alternatively, one could colonize GF animals with a defined community of bacteria with different abilities to produce and/or detect AI-2. The impact of AI-2 could be tested by assessing whether or not there are differences in gut localization or population densities of the different species. Additionally, one could characterize transcriptional profiles of the different bacteria in different experimental groups.

Similarly, cobalamin (Vitamin B12), a corrinoid, is a required co-factor in enzymatic reactions in all domains of life, but many bacterial species do not possess a complete cobalamin *de novo* synthesis pathway (Degnan *et al.*, 2014) (Yi *et al.*, 2012). These bacteria must obtain cobalamin or an intermediate in the synthesis pathway, synthesized by other bacteria or provided in host diet, from their environment (Degnan *et al.*, 2014) (Butzin *et al.*, 2013) (Men *et al.*, 2014). Because some bacteria may secrete cobalamin or different cobalamin precursors or

neither (Yi *et al.*, 2012) (Degnan *et al.*, 2014), bacterial production of cobalamin or its precursors may impact microbial ecology in the gut. Microbial interactions involving corrinoids could be explored by colonizing GF animals with defined bacterial communities in which members of different communities produce different corrinoids or by administering different bacterial species that produce different corrinoids to conventionalized animals. Effects of altering the presence of corrinoid-producing microbes in the gut could be determined by assessing changes in bacterial relative abundance or gene transcription. Effects of altering the presence of corrinoid-producing microbes in the gut could be determined by assessing changes in bacterial relative abundance or gene transcription.

Implications for and future directions in the study of the tripartite relationship between gut microbiota, host physiology, and host diet

In summary, my dissertation as well as most of what I discussed above focuses on how bacteria within the gut may interact with each other as well as on the relationship between gut microbiota, host physiology, and host nutrition. Deep-sequencing has generated hypotheses about the influence of large numbers of bacteria on host physiology and how diet alters that influence. However, there remains a great gap between the number of bacteria hypothesized to impact host physiology and the number of bacteria that have been tested. While unculturability and genetic intractability are substantial obstacles to the characterization of bacteria of interest, the large number of different species, as well as combinatorial permutations, to test is also a substantial challenge (Faith *et al.*, 2011). An additional related challenge is the determination of which bacteria or groups of bacteria to test in experimental models. The assemblage and neutral model analyses I employed in Chapter Three present useful tools with which to identify bacteria for experimental characterization. Additionally, because assemblages may consist of bacteria that form a functional network, assemblage analysis has the potential to identify

culturable bacteria that, in co-culture, might promote the *in vitro* grown of an otherwise unculturable species.

While my dissertation has primarily focused on differences in macronutrients consumption, many other ingested chemical compounds, including synthetic molecules such as BPA and pharmacological drugs, have been associated with altered physiologies (Schug *et al.*, 2011) (Chamorro-Garcia *et al.*, 2012). It is possible that these effects may be mediated or inhibited in part by gut microbiota, and several groups have begun to explore the interaction between these compounds and gut microbiota. For example, *Eggerthella lenta*, which resides in the gut, has been shown to chemically inactivate the cardiac drug digoxin; furthermore, *E. lenta* inactivation of digoxin is inhibited by host protein ingestion (Haiser *et al.*, 2013) (Haiser *et al.*, 2014). The authors of this study proceeded to speculate that this and similar microbial modification of pharmaceutical compounds may be a causative factor in inter-individual differences in drug efficacy. Moreover, the liver plays a major role in metabolism of many pharmaceutical compounds. Liver physiology can also impact gut microbiota, making it likely that host physiology and gut microbiota interact in response to pharmaceutical compounds in a manner similar to the response to different diets. Future lines of research will likely provide insight into the mechanisms by which gut microbiota and hosts impact each other in their responses to pharmaceutical compounds and their breakdown intermediates. Full comprehension of how environmental factors such as diet or drugs impact host physiology will require integration of the response of gut microbiota and of multiple organ systems across multiple time scales.

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