The role of gut microbiota and diet in lipid metabolism

Ivana Semova

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Approved by:

John Rawls, PhD

Kay Lund, PhD

Manzoor Bhat, PhD

Michael Goy, PhD

Matthew Wolfgang, PhD

ABSTRACT

IVANA SEMOVA: The role of microbiota and diet in lipid metabolism
(Under the direction of Dr. John Rawls)

The large community of microorganisms in the intestine (microbiota) has been identified as an additional metabolic organ in our body. However, the microbial role in dietary lipid absorption in the intestine is unclear. Improved understanding of the trialogue between dietary nutrients, microbiota and host lipid metabolism can help develop strategies for decreased or increased absorptive capacity as respective treatments for obesity or malnutrition.

Here, we use *in vivo* imaging of fluorescent fatty acid analogues to investigate the effect of microbiota and nutrients on dietary fatty acid absorption in a zebrafish host. Our results demonstrate that the microbiota stimulates fatty acid uptake and lipid droplet accumulation in the intestinal epithelium. The microbiota promotes an increase in enterocyte lipid droplet number, but not size in a diet-dependent manner. We show that the presence of microbial community also results in enhanced dietary fatty acid absorption into the liver and non-gastrointestinal tissues. These findings show that the microbiota stimulates intestinal and extra-intestinal fatty acid absorption in a diet-dependent manner.

To determine whether diet affects the zebrafish microbial community composition, we performed sequencing of the bacterial 16S ribosomal RNA gene in gut and water samples from colonized zebrafish under different dietary conditions. Our analysis of bacterial community composition is the first one to show that dietary nutrients promote intestinal Firmicutes abundance in the zebrafish host. Even in the absence of bacterial competition,

the Firmicutes primary isolate *Exiguobacterium* sp. can't survive and colonize the intestine under starved conditions. These findings indicate that Firmicutes survival and growth may require a nutrient-rich environment.

Finally, we tested whether the diet-induced increase in intestinal Firmicutes abundance could be partially responsible for the observed increase in lipid droplet number in fed zebrafish colonized with the microbiota. Colonization of germ-free zebrafish with single bacterial strains revealed that lipid droplet number is increased by Firmicutes and lipid droplet size is increased by other bacterial types. These results indicate that the microbiota stimulates intestinal and extra-intestinal fatty acid absorption and that different microbial members mediate intestinal fatty acid absorption via distinct mechanisms. This work demonstrates novel interactions between nutrients and microbiota that enhance dietary fat absorption.

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LIST OF ABBREVIATIONS

16S rRNA	16 Svedberg unit of ribosomal ribonucleic acid
AMPK	Adenosine monophosphate-activated protein kinase
Angpt4	Angiopoietin-like protein 4 (also called Fiaf)
ANOVA	Analysis of variance
apoB	ApolipoproteinB
ATP	Adenosine triphosphate
BODIPY-FL	Boron-dipyrromethene fluorescent dye
C diet	Control diet
CFU	Colony forming units
CM	Chylomicron
CoA	Coenzyme A
CONVD	Conventionalized
DGAT	Diacylglycerol acyltransferase
dpf	days post fertilization
EnzChek	Boron-dipyrromethene TexasRed casein
ER	Endoplasmic reticulum
FA	Fatty acid
FABP	Fatty acid binding protein
FABPpm	Fatty acid binding protein at plasma membrane
FAT/CD36	Fatty acid translocase
FATP	Fatty acid transport protein
FFA	Free fatty acid
Fiaf	Fasting-induced adipose factor (also called Angptl4)

GF	Germ-free
GFP	Green fluorescent protein
GI	Gastrointestinal
GZM	Gnotobiotic zebrafish media
HDL	High density lipoprotein
hrs	hours
HSL	Homoserine lactone
IAP	Intestinal alkaline phosphatase
IDL	Intermediate density lipoprotein
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC diet	Low-calorie diet
LCFA	Long-chain fatty acid
LD	Lipid droplet
LDL	Low density lipoprotein
LPL	Lipopolysaccharide
MCFA	Medium-chain fatty acid
MG	Monoacylglycerol
mL	Mililiter
μm	Micrometer
μM	Micromolar
mpo	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MTP	Microsomal triglyceride transfer protein
NAFLD	Non-alcoholic fatty liver disease

nm	Nanometer
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCoA	Principal coordinates analysis
PED6	N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-1-palmitoyl-2-BODIPY-FL-pentanoyl-sn-glycero-3-phosphoethanolamine
PFA	Paraformaldehyde
PLA_2	Phospholipase A_2
PPAR	Peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid
QS	Quorum sensing
RT-PCR	Reverse transcriptase polymerase chain reaction
SCFA	Short-chain fatty acid
SD	Standard deviation
SEM	Standard error of the mean
sp	species
TG	Triglycerides
TMAO	trimethylamine-N-oxide
VLDL	Very low density lipoprotein

CHAPTER ONE

INTRODUCTION

The trialogue between microbiota, dietary nutrients and metabolic function

Beginning at birth, we are colonized with a complex microbial community that changes in density, composition and function throughout our lives. The trillions of microorganisms residing in our intestines, collectively called the gut microbiota, impact numerous biological processes in our bodies. The dynamic interactions with these microbial members influence physiological processes involved in nutrient metabolism. For example, the gut microbiota is an important environmental factor in energy storage (Bäckhed et al., 2004), and diseases of energy imbalance such as obesity (Bäckhed et al., 2007; Ley et al., 2006) and diabetes (Wen et al., 2008). The host-microbe interactions that affect host energy homeostasis and pathogenicity are mediated by dietary nutrients (Musso et al., 2011). Dietary nutrients can contribute to microbial regulation of metabolic function in three major ways: 1) dietary nutrients affect the microbial community composition and structure, 2) the microbiota in turn affects the metabolic fates of dietary nutrients in the intestine and whole body or 3) diet modifies host physiology that impacts the composition or activity of the microbiota.

Dietary effect on microbial community composition

Numerous studies in hosts from worm to human showed that different nutrients (fats, proteins, carbohydrates) affect bacterial abundance of certain microbial species (Ley et al., 2008; Wu et al., 2011; Muegge et al., 2011). In zebrafish (Figure 3.4), pythons (Secor et al., 2008) and mammals (Ley et al., 2005; Turnbaugh et al., 2009) we and others have shown that an increase in caloric input promotes the abundance of a major bacterial phylum called Firmicutes in the intestine. In rodents fed a "Western diet" (high-fat/high-carbohydrate diet), increase in Firmicutes levels has been associated with increased energy harvest from polysaccharides and fat storage in the body (Turnbaugh et al., 2006). Our zebrafish studies suggest similar effects of Firmicutes on intestinal lipid absorption, as a single Firmicutes strain induced fatty acid uptake in the intestinal epithelium (Figure 3.6). Increased Firmicutes abundance has been associated with both diet and genetic mouse models of obesity (Turnbaugh et al., 2008; Ley et al., 2005). Collectively, these studies suggest that Firmicutes may affect the development of obesity; however, more direct evidence is needed to determine the contribution of this bacterial phylum to energy harvest from different types of nutrients.

While the presence of diet appears to be sufficient to induce changes in the microbial community structure, the specific nutrient content of the diet is also a factor. Most studies investigating the development and pathobiology of obesity and related metabolic diseases have used high-fat/high-carbohydrate diets. However, other nutrients have also been evaluated for their ability to alter microbial composition and gene expression. A simpler community of 10 bacterial species was monitored in gnotobiotic mice to determine changes in species abundance as a result of perturbations of four different major nutrients (casein, starch, sucrose and oil) (Faith et al., 2011). The authors showed that changes in casein levels were highly correlated with total community abundance and abundance of each

species. High levels of dietary protein also had an impact on the microbiota of domesticated cats (Lubbs et al., 2009). Surprisingly, reduced dietary protein levels did not have a significant impact on the zebrafish gut or water microbiota (Figure 3.4). In addition, studies have shown that dietary fiber can alter the microbial community of mice (Neyrinck et al., 2010), dogs (Middelbos et al., 2010), and humans (De Filippo et al., 2010). Together, these findings suggest that both caloric value and nutrient content of diet affect the microbial community composition in the intestine.

Microbiota impacts intestinal metabolic processes

The microbiota can impact nutrient metabolism along the intestinal length. In humans, the microbiota has been shown to modify bile acids and stimulate lipid emulsification in the small intestine (Ridlon et al., 2006; Martin et al., 2007). Members of the microbial community in the distal intestine, on the other hand, possess genes that encode enzymes involved in digestion of complex carbohydrates (Xu et al., 2003; Xu et al., 2007). Therefore, the distal gut microbiota may complement some of the human digestive functions by degrading carbohydrates that cannot be digested otherwise due to lack of human hydrolytic enzymes (Flint et al., 2008; Louis et al., 2007). In mice, the microbiota affects gut permeability which is important in fatty acid diffusion and lipopolysaccharide-mediated inflammatory response (Cani et al., 2009). Another study in germ-free (GF; animals without any microbes) mice suggested that increased excretion of fat in the intestine could contribute to the observed obesity-resistance phenotype of these animals (Rabot et al., 2010). Altogether, these findings clearly demonstrate the microbial impact on nutrient digestive and absorptive processes in the intestinal lumen and epithelium.

Microbial factors also regulate transcript levels of metabolic genes, such as fatty acid binding proteins and transporters, as demonstrated in the intestine and other organs of

zebrafish and mice (Rawls et al., 2004; Rawls et al., 2006; Larsson et al., 2012). One of these microbially-regulated genes, called *Angiopoietin-like protein 4*/*Fasting-induced adipose factor* (*Angptl4/Fiaf*), is an important mediator of host lipid metabolism (Yoshida et al., 2002). The microbiota regulates energy storage in adipose tissue via suppression of intestinal *Fiaf* transcript levels in a mouse host (Bäckhed et al., 2004). Therefore, understanding the mechanisms involved in microbial regulation of Fiaf expression in the intestine is of great importance for obesity prevention and treatment strategies. **My work presented in the appendix shows preliminary results of bacterial factors that regulate transcript levels of *fiaf* as well as another biomarker of lipid metabolism (*fatty acid binding protein 2*) and innate immune function (*myeloperoxidase*) in a zebrafish host.**

Microbiota impacts metabolism in non-intestinal tissues

The microbiota also regulates peripheral organ metabolism as determined by quantification of transcript, protein and metabolite levels in non-intestinal tissues. Since this is a broad research topic, my focus will be on microbial regulation of lipid metabolites. A study using mass spectrometry suggested increased uptake of circulating triglycerides in adipose tissue of conventionally-raised (CONVR; raised in the presence of microbes) mice in comparison to GF mice potentially due to increased activity of lipoprotein lipase (LPL) which hydrolyzes triglyceride-rich lipoproteins in circulation (Velagapudi et al., 2010; Bäckhed et al., 2004). Colonization of GF mice increased glucose and short-chain fatty acids in serum, which are thought to stimulate hepatic *de novo* synthesis of fatty acids (Bäckhed et al., 2004). In addition to the metabolic effects of the microbiota, individual microbial community member *Bifidobacterium breve* alters the fatty acid composition profile by increasing levels of the bioactive conjugated linoleic acid in mouse and pig livers and omega-3 fatty acid levels in adipose tissue (Wall et al., 2009). These results demonstrate

microbial roles in regulation of lipid metabolite levels systemically; however, it is not always clear whether the studied metabolites are derived from exogenous (dietary) or endogenous lipid sources. In addition, there is lack of *in vivo* evidence for microbial stimulation of dietary lipid absorption in the intestine. **In order to distinguish between the microbial effects on exogenous versus endogenous lipids, I summarize our current understanding of the metabolic fates of these two types of lipid in the body as well as the microbial contribution to some of these processes in chapter 2 of my dissertation. In chapter 3, I elucidate the role of the microbiota and individual bacterial species on dietary fatty acid absorption in zebrafish intestinal and extra-intestinal tissues.**

Zebrafish as a model organism to study host-microbe interactions and lipid metabolism

Model organisms are valuable surrogate hosts for understanding host-microbe interactions that occur in humans and other mammals. Zebrafish have proven to be an excellent model organism for *in vivo* studies and high-throughput genetic and chemical screens for several reasons. Studies show extensive similarities between mammalian and zebrafish intestinal development, organization and function (Ng et al., 2005; Wallace et al., 2005; Hama et al., 2009). Similar to mammals, zebrafish early development occurs in a sterile environment and includes differentiation of intestinal epithelial cells into absorptive enterocytes, secretory goblet cells and enteroendocrine cells (Bates et al., 2006). The digestive tract consists of intestine, liver, endocrine and exocrine pancreas and gallbladder. The zebrafish intestine has specialized regions (segments) that perform similar functions to the small and large intestines in mammals. Segment 1 is responsible for lipid absorption, segment 2 for protein and macromolecule pinocytosis and segment 3 may play a role in water and ion absorption (Bates et al., 2006). Zebrafish development is rapid and the

intestine is fully functional by 5 days post fertilization (dpf) when lipid and protein digestion and absorption commence (Farber et al., 2001; Hama et al., 2009). Dietary lipid composition, digestive physiology and lipid metabolic processes are generally conserved among zebrafish and humans (Carten and Farber, 2009). As a result, the zebrafish is an attractive model organism for human diseases including cardiovascular disease, fatty liver disease and obesity (Carten and Farber, 2009; Hölttä-Vuori et al., 2010). Zebrafish have a large advantage over mammalian models for *in vivo* studies of the host's metabolic processes since the optical transparency of the larvae allows for observation of digestive and absorptive function using fluorescent protein and lipid probes (Farber et al., 2001; Hama et al., 2009; Carten et al., 2011; Chapter 3). We took advantage of available fluorescent lipid and protein substrates to show that the microbiota can regulate some of these digestive and absorptive processes in the intestine (Chapter 3).

In addition, the zebrafish rapid growth and body transparency allow for high throughput screening for bacterial and host factors involved in metabolic function. **In the appendix, I show that *Pseudomonas aeruginosa* products regulate transcript levels of several fatty acid metabolic and innate immune genes.** In our future studies, we intend to utilize a *P. aeruginosa* PA14 strain mutant library (Liberati et al., 2006) to screen for bacterial genes and mechanisms required for regulation of metabolic activity using quantitative RT-PCR and *in vivo* assays of digestive and absorptive function.

In summary, the work presented in my dissertation identifies several novel roles for the gut microbiota in dietary lipid metabolism. First, we demonstrate microbial stimulation of dietary fatty acid absorption in intestinal and extra-intestinal tissues of a zebrafish host. Second, we also provide important insights into the effects of dietary nutrient levels on zebrafish microbial community composition. Third, we present novel findings that individual microbial members regulate distinct aspects of dietary fatty acid absorption in the intestine.

These results have broad implications for the field of host-microbe interactions because they provide the foundation for future characterization of bacterial and host metabolic mechanisms involved in microbial regulation of dietary lipid absorption. Identification of these molecular mechanisms could contribute to the development of treatment strategies for human metabolic diseases of energy imbalance such as obesity and malnutrition.

CHAPTER TWO

INTESTINAL MICROBIOTA IN LIPID METABOLISM

SUMMARY

The metabolic fates of exogenous (dietary) and endogenous (*de novo* synthesized) lipids are affected by environmental challenges such as dietary nutrients and the large intestinal community of microorganisms (gut microbiota). Despite previous research efforts, our understanding of the trialogue between the microbiota, dietary lipids and host lipid metabolism is incomplete. This review summarizes our current knowledge of the molecular mechanisms involved in dietary lipid processing in the small intestine as well as the generation of lipoprotein particles from exogenous and endogenous lipid substrates in peripheral tissues. Later in the review, we discuss the impact of the microbiota on host lipid metabolism and the effect of dietary nutrient composition on microbial community structure and metabolic function.

INTRODUCTION

Fat is a major macronutrient in animal diet. Dietary fat metabolism is affected by host digestive physiology and anatomy, as well as by environmental factors such as ingested dietary nutrients and the gut microbiota. In mammals, dietary lipid digestion occurs primarily in the stomach and the anterior region of the small intestine (duodenum) by gastric and pancreatic lipases, respectively. Emulsification of fat by bile acids is important for efficient lipase hydrolysis and uptake of fatty acids by enterocytes. Once taken up, dietary fatty acids

get activated as acyl-CoA derivatives to be used as energy sources, get reassembled into triglycerides and stored in cytosolic lipid droplets or packaged in lipoprotein particles (chylomicrons) for exocytosis and extra-intestinal metabolism. Once transported out of the intestine, chylomicrons (CMs) circulate through the lymphatics and enter the vasculature via the portal vein. In circulation, CMs interact with lipoprotein lipase (LPL) that hydrolyzes triglycerides and releases free fatty acids (FFAs) for uptake into peripheral tissues (Borensztajn, 1979). After LPL hydrolysis, the smaller, cholesteryl ester-rich chylomicron remnant circulates to the liver where it enters the pool of endogenous lipid to be used for hepatic very low density lipoprotein (VLDL) formation (see Figure 2.1). Secreted VLDLs circulate and undergo hydrolysis by LPL resulting in generation of intermediate density lipoprotein (IDL) and low density lipoprotein (LDL) particles. Another lipoprotein (high density lipoprotein, HDL) is responsible for cholesterol transport from peripheral tissues to the liver for excretion (Fava et al., 2006). These metabolic processes are summarized in Figure 2.1 and described in more depth throughout this review, with emphasis on their regulation by environmental factors such as dietary nutrients and the gut microbiota.

The microbiota can contribute to lipid metabolism by affecting both intestinal and extra-intestinal metabolic processes. In the intestinal lumen, the microbiota is thought to contribute to increased lipid digestion (Kosa and Ragauskas, 2011) and bile salt modification that improves fatty acid emulsification and absorption (Martin et al., 2008). In the intestinal epithelium, the microbiota affects the absorptive capacity via changes in intestinal permeability (Cani et al., 2008). The microbiota also provides bacterial or nutrient-derived ligands that can regulate the host's transcriptional machinery by interaction and activation of host transcription factors in intestinal and extra-intestinal tissues (Sanderson et al., 2009). Finally, on a systemic level, the presence of microbiota regulates circulating levels of lipid metabolites, fat storage in adipose tissue and liver (Velagapudi et al., 2010), and immune

system development and response (Sharma et al., 2010). However, currently there is very little distinction between the microbial impact on exogenous versus endogenous lipid sources. Improving our understanding of how the microbiota mediates dietary lipid absorption in the intestine can provide us with new preventative and treatment strategies for diet-induced obesity and malnutrition. The purpose of this review is to summarize the most current findings on lipid metabolism with a more in-depth coverage of dietary lipids and the role of gut microbiota in mediating these metabolic processes on a systemic level.

Fat metabolism in various animals

Lipid is a major macronutrient for humans that provides 40-55% of the caloric value in Western diet (Binder and Reuben, 2009). At birth, human infants switch from glucose-rich to lipid-rich food source as almost half of the energy content in human milk comes from fat (Lindquist and Hernell, 2010). It has been hypothesized that the high fat content in our diet is partially due to our high level of encephalization (large brain:body mass). In comparison to other primates and mammals of similar size, we allocate a larger portion of our daily calories as energy for the brain. As a result, we have increased our demands for energy-dense food which is typically rich in fats. This dietary change may have also altered our gastrointestinal tract, resulting in expansion of our small intestine and reduction of the colon as well as enhanced capacity to digest and metabolize higher fat diets in comparison to chimpanzees and gorillas (Leonard et al., 2010).

Despite these differences in GI anatomy and dietary fat preferences between us and our closest relatives, most of our knowledge of the lipid metabolic fates in humans is based on discoveries made in cell culture and animal studies. It is important to note that each model has similarities and differences with human physiology and metabolism. Intestinal cell

culture studies have allowed for an important initial characterization of cellular metabolic processes; however, most cell lines do not express many of the metabolic genes found *in vivo* (Shulzhenko et al., 2011) and fail to recapitulate the effect of the surrounding luminal environment that includes bile, mucus and microbiota. Research in worms, flies, fish, mice, pigs and many other animals has generated a more complete picture of the mechanisms involved in lipid metabolism at a systemic level. It is important to note that interspecies differences in gastrointestinal (GI) physiology and diet preferences affect lipid metabolic processes, which is the reason why animal hosts used in the summarized studies are highlighted throughout this review. Despite innate differences in GI physiology and nutrition, animal models have provided us with important discoveries of evolutionarily conserved molecular factors involved in lipid metabolism such as fatty acid binding proteins and transporters. These findings emphasize the importance of lipid metabolic processes for the survival of different species.

Metabolic routes of exogenous (dietary) lipids in the intestine

Types of dietary lipids: Dietary lipid composition factors (such as saturation and length of the FA acyl chain) are important for determining physiological outcomes and disease development in humans. Cholesterol, saturated and trans-fatty acid levels in diet have been linked to coronary heart disease (Austin, 1991) and atherosclerosis (Katan, 2000). On the other hand, (ω-3) polyunsaturated fatty acids eicosapentaenoic and docosahexaenoic acid protect against macrophage-induced inflammation and stimulate systemic insulin sensitivity (Oh et al., 2010). Dietary fat content consists primarily of triglycerides (TGs), phospholipids and cholesterol. In energetic terms, the typical Western diet consists of approximately 14% of saturated fatty acids (mostly palmitic acid), 14% of

monounsaturated fatty acids and 6% of polyunsaturated fatty acids (Nassir and Abumrad, 2009).

In addition to saturation, the acyl chain length also has an impact on fatty acid metabolic fate. Short-chain fatty acids (SCFA; C2-C5) get absorbed via sodium-coupled transporter SLC5A8 in the ileum and colon to mediate many beneficial functions in epithelial biology and metabolism (Cresci et al., 2010). Medium-chain fatty acids (MCFA; C6-C16), the predominant fat form in infant diet, are thought to enter enterocytes via diffusion where they get used primarily as energy source (Papamandjaris et al., 1998). Long-chain fatty acids (LCFA; C17-C22), representing the majority of dietary fatty acids found in human diet, are thought to be actively transported by fatty acid translocases and binding proteins discussed below. MCFA, which are saturated, are rarely found in human food; however, in the past decade there has been an effort to synthesize structured triglycerides with lower fat-producing value by chemical interesterification of medium- and long-chain FAs onto the same glycerol backbone (Phan and Tso, 2001). Previous studies in rodents and humans have reported reduced body weight, fat storage and increase in plasma triglyceride levels on a medium-chain vs long-chain triglyceride diet which may be a result from a shift towards increased use of medium-chain fatty acids for energy and *de novo* lipogenesis in the liver rather than storage in the body (Geliebter et al., 1983; Hill et al., 1989). It has been argued that this increase in lipogenesis on medium-chain triglyceride diet is due to faster intestinal absorption, hepatic portal transport, carnitine-independent mitochondrial metabolism (peroxisomal and omega-oxidation) and a low affinity for esterification, all of which lead to a faster and greater oxidation of MCFA in comparison to LCFA (Papamandjaris et al., 1998). Together, these findings indicate that differences in fatty acyl chain length and saturation impact the metabolic fates of FAs in the body, and that MCFA could be useful as agents for obesity treatment.

Dietary fat digestion: In humans, fat digestion begins in the mouth with lingual lipase, produced by salivary glands in the tongue, and continues in the stomach with the activity of both lingual and gastric lipase produced by chief cells. However, the majority of fat digestion occurs in the small intestine, as only 15% of digestion occurs prior gastric emptying (Binder and Reuben, 2009). Triglyceride digestion in the stomach plays an important role especially for neonates. Human milk fat contains a considerable amount of medium-chain triglycerides and acid lipases (lingual and gastric) are more efficient at digestion of medium- vs long-chain triglycerides. In addition, the lingual and gastric lipases are the primary enzymes involved in neonatal fat digestion as the pancreatic lipase system is still not fully functional (Hamosh, 1996). The stomach is also the location where most of the fat emulsification aided by dietary phospholipids occurs, which is required for efficient digestion by the pancreatic lipase.

Upon entry into the duodenum, pancreatic lipase hydrolyzes the fatty acids on the sn-1 and sn-3 positions of a triglyceride and produces two free fatty acids (FFA) and a 2-monoacylglycerol (2-MG). Further hydrolysis results in a release of another FFA and a glycerol backbone. Phospholipid and cholesteryl ester digestion also occurs in the small intestine by the activity of pancreatic phospholipase A_2 (PLA_2) and cholesterol esterase, respectively. Released FFA and mostly 2-MG get taken up by enterocytes, a process which is enhanced by micellar solubilization by bile salts (Westergaard and Dietschy, 1976). In order for single molecules to reach the epithelial brush border, they need to diffuse across an unstirred water layer, which is a challenge for hydrophobic FA and MG molecules. Mixed micelles, which result from bile salt emulsification of FFA, 2-MG, cholesterol and phospholipids, are important vesicles that get sensed by enterocytes via scavenger receptor SR-BI/CLA-1 (Béaslas et al., 2009). A study in pig intestinal explants incubated with lipid mixture of corn oil, cholesterol, bile and pancreatin showed that 3 hrs after incubation, SR-

BI/CLA-1 was endocytosed from the brush border and accumulated in cytosolic lipid droplets but not secreted lipoproteins (Hansen et al., 2003). The role of these lipid droplet structures is discussed below.

Lipid uptake in enterocytes: Intestinal intracellular trafficking and processing of fatty acids is not completely understood and controversial. Once fatty acids in mixed micelles reach the brush border, they become protonated and leave the micelle to be taken up via passive diffusion or active transport by binding proteins. The exact fatty acid uptake mechanisms are unresolved. Studies in rats suggest that active transport is the major route for linoleate (derived from ω-6 linoleic acid) uptake at low concentrations, while passive diffusion predominates at high concentrations (Chow and Hollander, 1979). Furthermore, the length of the acyl chain affects FA solubility and transport across the epithelium. MCFA are less hydrophobic than LCFA and are therefore, thought to diffuse more freely across the epithelium and enter the portal venous blood that transports it directly to the liver. On the other hand, longer FA need active transport by proteins such as fatty acid translocase (FAT/CD36) and fatty acid binding protein at the plasma membrane (FABPpm). Fatty acid transport proteins (FATP2-FATP4) were originally thought to contribute to FA transport, however FATP4 was later shown to have a CoA acylase function that serves to activate FA prior storage or use as energy via FA oxidation (Jia et al., 2009; Milger et al., 2006). Cholesterol uptake is mediated by adenosine-triphosphate binding cassettes A1, G5 and G8 and Niemann-Pick C1 like 1 transporter (Plösch et al., 2005).

Regulation of transport proteins is dependent on dietary fat levels. As a consequence, one of the frequent problems in obesity is intestinal adaptation to high-fat diet and increased absorptive capacity (Lynes and Widmaier, 2011). However, the mechanisms that mediate epithelial transport capacity in response to dietary fat levels are only partially understood. A mouse study showed that a diet rich in linoleic acid induced colonic expression of the

transcription factor peroxisome proliferator-activated receptor γ (PPARγ) and CD36 (Bassaganya-Riera et al., 2004). In humans, however, CD36 mRNA levels did not correlate with protein levels along the length of the intestine since the ileum showed the highest protein expression, with lower mRNA levels than in the duodenum, jejunum and colon (Masson et al., 2010). Therefore, transcript levels of fatty acid transporters do not necessarily correlate with protein expression at least in human subjects.

Intestinal fatty acid fates: Free fatty acids that get taken up have several metabolic fates, which are partially dependent on their site of entry (apical or basolateral side of the enterocyte). FAs bind the intestinal FA binding protein (FABP2) and liver FA binding protein (FABP1). The two proteins are expressed in the intestine, and are thought to be transcriptionally regulated by C/EBP (Cohn et al., 1992), although their expression might be regulated by genetic and not dietary factors. These proteins belong to a larger family of intracellular binding proteins that evolved after the animal kingdom separated from plants and fungi and is present in both invertebrates and vertebrates (Haunerland and Spener, 2004). Their known roles include protecting cells from cellular damage induced by excess FA and increasing the FA concentration gradient by binding FFAs in the cell (Haunerland and Spener, 2004). However, FABP2 overexpression and knock-out in both cells and mice have lead to inconclusive results about the function of this binding protein in fat absorption, suggesting that there might be compensatory mechanisms involved in such a critical process. On the other hand, FABP1 KO mice on a western diet were resistant to obesity (Newberry et al., 2006), suggesting that this protein influences fat storage in the body.

An additional intestinal enzyme has been implicated in nutrient absorption in the small intestine. Intestinal alkaline phosphatases (IAP) are metalloenzymes that hydrolyze a spectrum of phosphomonoesters such as the 5' terminal phosphate group of DNA and RNA, the terminal phosphate of ATP and phosphate groups on serine and threonine residues

(Millán, 2006). IAP is expressed at the brush border (Xie et al., 1997; Bates et al., 2007), the membranes of particles secreted by enterocytes basolaterally and at the apical membrane after their passage through tight junctions (Mahmood et al., 2003), and around lipid droplets in enterocytes (Moss, 1982; Narisawa et al., 2007; Warnes, 1972). Furthermore, IAP expression increases in the duodenum and jejunum of rats (Kaur et al., 2007), piglets (Dudley et al., 1994) and mice (Millán, 2006; Nakano et al., 2007) in response to increased dietary fat levels. IAP dephosphorylates the fatty acid translocase CD36 in murine 3T3 fibroblasts, which suggests functional interaction between CD36 and IAP (Ho et al., 2005). CD36 phosphorylation is thought to be important for its function, since Luiken and colleagues (2002) showed that cAMP phosphodiesterase inhibitors increase cAMP levels and the CD36 activity potentially via increased phosphorylation in skeletal muscle cells. Based on these findings, it is predicted that CD36 physically interacts with IAP to potentially regulate LCFA uptake in the small intestine. This interaction between CD36 and IAP is thought to be responsible for the adaptation of the distal intestine and increased absorptive capacity in mammals in response to high-fat diet (Lynes and Widmaier, 2011).

Bound fatty acids get trafficked to the endoplasmic reticulum (ER), which is the site of triglyceride re-esterification and prechylomicron formation. The transfer mechanism is unresolved. At the ER, FFA and MG are used as substrates to re-synthesize TG primarily via the MG pathway and with the activity of MG acyltransferases (MGAT1, 2 and 3) and DG acyltransferases (DGAT1 and 2) (Buhman et al., 2002). A DGAT1 knockout mouse model showed that there might be compensatory mechanisms that involve DGAT2 and DG transacylase activity in FA uptake and TG formation in enterocytes (Buhman et al., 2002). The FAs used in TG formation usually reflect the ones found in the diet (Redgrave and Dunne, 1975; Parks et al., 1981), and TG synthesized from dietary FA is preferentially transported into chylomicrons (CM) due to different route of delivery by different acyl-CoA

synthetases (Mansbach and Nevin, 1998). The exception was observed during fasting when very low density lipoprotein (VLDL)-size particles are formed in enterocytes consisting of lipids derived from bile, sloughed enterocytes and plasma FAs (Hussain, 2000).

Another metabolic fate of fatty acyl-CoA is its use as an energy source via fatty acid oxidation. Even though only a small portion of the dietary FA pool is oxidized in enterocytes, it becomes an important energy source during fasting. This process is compartmentalized to the mitochondrion during β-oxidation and requires a membrane transport system facilitated by carnitine palmityl transferase-1α (CPT-1α) for LCFA substrates. Medium-chain FA on the other hand, can cross the mitochondrial membrane freely to be oxidized (Friedman et al., 1990).

FA metabolic fate in the enterocyte depends on the uptake route from the lumen. Studies in humans and rodents have shown that the site of cellular entry can impact the intracellular metabolic fate of FA and MG (Gangl and Renner, 1978; Storch et al., 2008). Both palmitate and oleate substrates undergo oxidation at higher rates when taken up via the basolateral than apical surface of enterocytes, which the authors speculate is due to the subcellular localization of mitochondria (even distribution at the apical and basolateral side) and ER (primarily at the apical side). Furthermore, FAs taken up on the apical side get incorporated into TG, DG or MG more often than into phospholipids in comparison to FAs that get taken up basolaterally (Storch et al., 2008).

Intestinal lipid droplet formation: Lipid droplets (LDs) have been recognized as important organelles in the body that serve as temporary TG stores that can get depleted or replenished depending on dietary fat levels. In addition to their storage function, LDs also play a role as building elements for organelles by providing phospholipids and sterols (Farese and Walther, 2009). During feeding, progressively larger TG-rich lipid droplets form

in the ER lumen independent of synthesis of CM lipoprotein apolipoproteinB (apoB) (Hamilton et al., 1998). In fact, lack of apoB synthesis resulted in larger LD formation in the ER (Mak and Trier, 1975). A study in suckling mice identified cytosolic LDs that lacked cell membrane (Young et al., 1995), which suggests of two separate lipid pools – an ER-independent TG stored in cytosolic LD and an ER-dependent TG used in CM formation. Both lipid droplet pools (cytosolic LD and CM) have various size distribution, which is thought to increase with fat feeding and independent of apoB levels (Davidson et al., 1987; Davidson et al., 1988).

Cytosolic LDs get coated with proteins of the PAT (perilipin, adipophilin, TIP47) family that play various functions in LD biogenesis (Bickel et al., 2009). For example, TIP47 mediates the incorporation of newly-synthesized TG into LDs, while perilipin and adipophilin associate only with pre-existing LDs so they get degraded through a proteosome-depedent pathway when LDs are absent from cells (Masuda et al., 2006). Furthermore, LD-associated PAT protein levels are dependent on the duration of the dietary fat challenge in mice, as TIP47 expression is higher after an acute than chronic challenge, while adipophilin expression follows the opposite trend. Localization was also affected, with TIP47 but not adipophilin coating LDs after an acute high-fat feeding, while adipophilin but not TIP47 is observed on LDs after a chronic exposure to high-fat diet (Lee et al., 2009). Therefore, these proteins are unique to cytosolic LDs and get regulated by the amount and the duration of dietary fat feedings.

Chylomicron assembly: The process of CM formation begins at the ER where re-synthesized TG binds the microsomal triglyceride transfer protein (MTP) and gets transferred to newly-synthesized ApoB protein and cholesterol esters to form a primordial chylomicron. MTP is a protein complex localized to the ER that consists of protein disulfide isomerase (PDI) and a unique 97 kDa subunit with lipid transfer activity. Mutations in the

MTP large subunit are the cause of the metabolic disease abetalipoproteinemia in humans (Wetterau et al., 1992), mice (Raabe et al., 1998) and zebrafish (Schlegel et al., 2006). These studies demonstrate that MTP is an essential protein in the initial assembly of intestinal lipoproteins in several different vertebrate species.

ApoB is another important protein involved in CM formation. It exists in two forms, ApoB100 and ApoB48. The larger protein, ApoB100 is enriched in the liver and the prenatal small intestine and has a low density lipoprotein-binding domain (Black, 2007). The other form, ApoB48 is enriched in the small intestine and lacks the low density-binding domain. Both forms are produced by the same gene with ApoB48 being generated by posttranscriptional mRNA editing (Davidson and Shelness, 2000). While ApoB48 is required for primordial CM assembly in the ER, ApoB100 is required for VLDL assembly in hepatocytes. Lipidation of ApoB48 by MTP activity is important to prevent nascent ApoB degradation and lipoprotein assembly (Hussain et al., 2012). In addition to MTP and ApoB48, another protein Apo A-IV is also added to the surface of the forming particle (van Greevenbroek and de Bruin, 1998). Additional factors that regulate CM formation include rate of TG synthesis, the size of the intracellular lipid pool and lipid trafficking and translocation in cells (van Greevenbroek and de Bruin, 1998), all of which are dependent on the amount of dietary FA taken up from the lumen.

The primordial CM gets transported from the ER to the *cis*-Golgi in a prechylomicron transport vesicle, which is the rate-limiting step in lipid absorption in a rat model (Siddiqi et al., 2006). This process is mediated by FABP2 that assists the budding off of the ER, while the trafficking and fusion with the Golgi is mediated by the coating protein II (COPII) family members Sar1 and Sec23/24 and the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) fusion complex which consists of vesicle-associated membrane protein 7 (VAMP7), syntaxin 5, Bet1 and vti1a (van Greevenbroek and de Bruin, 1998). This

process is well-conserved even in human newborns that adapt to large dietary lipid loads by adjusting gene expression levels.

The final step of mature CM formation in the Golgi is followed by basolateral secretion of these lipoprotein particles into the lymphatic lacteals of humans and suckling pigs whose GI tracts are closely related to ours. In contrast, in suckling rodents the portal vein is the primary lipid transport route, since suckling rats exhibit inefficient dietary lipid transport in lymphatic chylomicrons (Black, 2007). Once chylomicrons exocytose into the lymphatics, they enter circulation through the thoracic duct, circulate in the vasculature and interact with lipases such as lipoprotein lipase (LPL) for TG hydrolysis and uptake of FA into tissues such as the muscle and adipose tissue.

Endogenous lipid synthesis (de novo lipogenesis) in the liver

The liver supplies both exogenous- and endogenous-derived lipid substrates to peripheral tissues via uptake of FFA from chylomicron remnants or de novo fatty acid synthesis, respectively. Both lipid substrates get packaged into VLDL particles and released into the circulation. Hepatic lipid levels are affected by metabolic imbalances in insulin resistance, type 2 diabetes (Adiels et al., 2007; Adiels et al., 2008), obesity and fatty liver diseases (Dumas et al., 2006). In hepatocytes, like in enterocytes, there are several different lipid pools: cytosolic LDs, ER-luminal apoB-free LDs and apoB-containing VLDL precursors (Olofsson et al., 2000; Shelness and Sellers, 2001). These lipid pools serve as substrates during lipolysis and TG generation for VLDL particles.

Hepatic enzymes involved in lipolysis: The well-studied lipases such as hormone-sensitive lipase and adipose triglyceride lipase are absent (Holm et al., 1987) or expressed at very low levels in the mammalian liver, respectively (Zimmermann et al., 2004). One ER-

associated lipase that is expressed at high levels in the liver is the triacylglycerol hydrolase (TGH). Knockout of this enzyme in mice resulted in smaller cytosolic LDs in primary hepatocytes. Furthermore, absence of TGH had no effect on nascent LD formation which colocalized with TIP47, while ADPR colocalized with the surface of pre-formed LDs that were larger in size. When the authors looked at the LD maturation process, they observed that colocalization and lipid transfer from nascent into preformed LDs was delayed but not absent in TGH KO hepatocytes. Therefore, these findings suggest that TGH is important in determining the dynamics of lipid transfer from newly synthesized to preformed LDs in hepatocytes (Wang et al., 2010). In addition to its role in lipid transfer, lack of TGH also resulted in a shift in the lipid metabolite pool with increased DG levels in the ER, which is thought to recruit CTP:phosphocholine cytidyltransferase to the active membrane-associated lipid pool and increase phosphotidylcholine (PC) synthesis (Jamil et al., 1993). The increase in PC levels is correlated with increased surface area and reduced size of LDs, which may be due to failure to form new LDs or defective fusion process as suggested by a study in Drosophila S2 cells (Guo et al., 2008).

Hepatic VLDL production: The process of VLDL formation is similar to CM production in the intestine, and occurs in the ER where lipids are transferred from cytosolic LD pools in an MTP-dependent manner. The lipid substrates used in VLDL formation are dependent on the nutritional status and hormonal factors. Under fed conditions, VLDL lipids are derived from TG hydrolysis in LDs or CM remnants. Under fasted conditions, the substrates include circulating FFA from adipose tissue lipolysis, *de novo* TG synthesis and phospholipids hydrolysis (Xiao et al., 2011). These conditions also regulate the intracellular FA fates, with elevated FFA flux to the liver and elevated de novo lipogenesis priming the liver to store and secrete lipids as VLDL particles. This phenotype is observed in insulin-resistant states. Hormonal regulation of exogenous and endogenous lipids is beyond the

scope of this review (for further reading, please refer to Xiao et al, 2011). Circulating FFA are taken up in hepatocytes and re-esterified by DGAT1 to contribute to the cytosolic LD pool. Lipid transfer from LDs to the site of VLDL formation is mediated by TGH.

Apolipoproteins involved in VLDL formation: Like in the intestine, ApoB is involved in VLDL in its longer form, ApoB100. This protein is exclusively expressed in the liver of humans, while it is found in both the liver and intestine of rodents. Unlike intestinal apoB formation, hepatic apoB molecule quality is regulated by several mechanisms that involve ER-associated degradation (ERAD) and post-ER proteolysis pathway (PERPP) mechanisms (Xiao et al., 2011). Additional apolipoproteins associated with VLDL particles include apoC-III which helps recruit TG to nascent apoB and promotes larger VLDL formation (Sundaram et al., 2010).

Upon generation of a nascent particle in the ER, VLDL exits in an ER-derived vesicle, which fuses with the Golgi in a SNARE complex-dependent manner (Siddiqi et al., 2010). After lipidation is completed in the Golgi, VLDL is secreted into the vasculature and circulates to provide lipid substrates to peripheral tissues via LPL-mediated lipolysis. Since both CM and VLDL particles require the same removal machinery from circulation and therefore compete for LPL activity in humans (Brunzell et al., 1973) and rats (Karpe and Hultin, 1995).

Lipid metabolism in peripheral tissues

Lipid is an energy-rich nutrient whose metabolism is dependent on the overall energy status and homeostasis of the organism. Secreted exogenous lipids from the intestine (chylomicrons) and endogenous lipids from the liver (VLDL) circulate in the vasculature where they interact with lipoprotein lipase (LPL) and TG get hydrolyzed to FFA that enter

peripheral tissues based on energy demands. For example, intestinal chylomicron production is stimulated not only by an increase in intestinal luminal fat content but also by circulating FFA levels (Xiao et al., 2011). Similarly, circulating FFA can also stimulate VLDL production in the liver, while prolonged exposure to FFA in the plasma leads to hepatic insulin resistance which causes increased VLDL production (Lewis et al., 2002).

Fed vs fasted states: Lipid metabolism is sensitive not only to dietary lipid but also carbohydrate and protein levels. During overnight fasting, circulating FFA released from adipose tissue fat hydrolysis lead to increased VLDL production, while prolonged fasting results in an increase in adipose tissue hydrolysis that provides more than 90% of FFA in VLDL particles (Barrows and Parks, 2006). Circulating VLDL particles get hydrolyzed to provide FFA as energy source in peripheral tissues resulting in the formation of intermediate density lipoproteins (IDL) and low density lipoproteins (LDL). After feeding, chylomicrons are the predominant lipoprotein particles secreted from the intestine into the lymphatics (Tso et al., 1984). VLDL production is decreased in comparison to fasted state, while circulating lipoproteins get hydrolyzed and FFA get taken up for storage primarily in adipose tissue. Therefore, dietary nutrient availability mediates the metabolic fates of exogenous and endogenous lipids in the body.

The intestinal microbiota as a metabolic partner

Our microbial self: Our mammalian ancestors evolved ~160 million years ago in a microbe-dominated world. In order to ensure our survival, we learned to co-exist with this large microbial community that outnumbers us. In particular, we form a very intimate bond with the group of microorganisms that reside on and within our body, including the large and complex community that colonizes our intestine (gut microbiota). The gut microbiota

represents the largest microbial community on our body, and outnumbers the total number of human cells in an adult human. The biological impact of this microbial community has been demonstrated in biological events such as intestinal cell proliferation (Cheesman et al., 2011), vascular remodeling (Reinhardt et al., 2012), nutrient metabolism (Claus et al., 2011), and immune function (Round et al., 2011) in numerous hosts.

The role of intestinal microbiota in human health and disease

The role of the intestinal community of microorganisms in metabolism has been established to the point where the gut microbiota is now accepted as an additional metabolic organ in our body. Studies in vertebrates and invertebrates have identified the importance of gut microbiota in host physiology (Sekirov et al., 2010) as well as pathologies related to metabolism such as the ones presented in obesity (DiBaise et al., 2008), diabetes (Musso et al., 2010), metabolic syndrome (Tilg, 2010), fatty liver disease (Abu-Shanab and Quigley, 2010). Part of the culprit for the recent high prevalence of these metabolic diseases in humans is the increased consumption of high-calorie diets such as the Western diet (30-40% of its caloric value comes from fat) that has high levels of saturated and *trans* fatty acids and low levels of the anti-inflammatory n-3 PUFAs (Kris-Etherton et al., 2002; Simopoulos, 2002). As a result of these recent dietary changes, diseases of energy imbalance have become a major health concern in developed and a growing concern in developing countries. The effects of diet and microbiota on the development and progression of these metabolic diseases is discussed in further detail below.

The effect of diet on microbiota

Microbial ecology: Evolutionary studies show that changes in environmental conditions (particularly dietary habits) caused our microbial partners to co-evolve with us. Ley and colleagues used bacterial 16S ribosomal RNA gene analysis of 60 mammalian species from several different locations around the globe to show that the host diet and phylogeny both influence the gut microbial community composition and diversity (2008). Herbivores showed the highest genus-level richness in comparison to omnivores and carnivores. The authors suggested that this dietary effect on microbial diversity is due to functional and anatomical adjustments in the intestine of these dietary groups. In order to digest dietary nutrients from plants, herbivores adapted by extending their gut retention time to allow for microbial fermentation in the foregut or the hindgut. Microbial communities in omnivores on the other hand, cluster separately into hindgut fermenters and ones with simple guts (Ley et al., 2008).

Another study sampled fecal DNA from 33 mammalian species and used shotgun and targeted sequencing of bacterial 16S ribosomal RNA genes to compare the gut microbial communities. Principal coordinates analysis plots of microbial communities showed that bacterial communities associated with herbivores clustered separately from those in omnivores and carnivores, further emphasizing the importance of plant-based versus meat-based diet in mediating microbial community composition (Muegge et al., 2011). However, the lack of correlation between mammalian phylogeny and overall distribution of microbial species and function suggests that there may not be a significant co-evolution between mammals and their gut microbiota and microbiome. The authors argue that this discrepancy between microbial composition and function may be due to differences in abundance of shared enzymes. For example, the microbiomes of herbivores are enriched in

enzymes involved in amino acid biosynthesis, while the carnivore samples have increased levels of enzymes involved in amino acid degradation.

Microbial community composition and function has also been extensively studied in lean versus obese hosts in order to identify which bacterial phyla are enriched under excess nutrient availability. Both diet (high-fat, high-carbohydrate) and genetic (*ob/ob*) models of obesity show increased abundance of Firmicutes vs Bacteroidetes, which represent the two major phyla in human and mouse gut microbiota (Ley et al., 2005; Ley et al., 2006; Turnbaugh et al., 2008). Microbiota transplantation from obese mouse donors into ex-GF mice showed that the obese microbiota increases adiposity in a lean host, which suggested that the microbiota from obese hosts has increased energy harvesting capacity relative to the microbiota from lean mouse hosts (Turnbaugh et al., 2008). Another study showed that the distal microbiome of obese twins is enriched in pathways involved in fatty acid biosynthesis and phosphotransferase system (Turnbaugh et al., 2009), which could alter the levels and composition of carbohydrate and fatty acid metabolites that become available to the host. Therefore, host dietary habits can modify nutrient metabolism via direct transfer of calories from nutrients and indirect effects on host energy storage via alterations of microbial community structure and function.

The effect of microbiota on metabolism of exogenous lipids

The microbial role in complex carbohydrate metabolism in the colon has been studied extensively (Turnbaugh et al., 2008; Turnbaugh et al., 2009; Flint et al., 2012) and will not be covered in detail here. Briefly, bacterial genes, enzymes and metabolites are thought to complement the function of host digestive enzymes that cannot digest complex plant carbohydrates. This microbial enhancement of digestion in the lumen results in release

of short-chain fatty acids (acetate, propionate and butyrate) that serve as an energy source in the intestine and other organs. The microbial enhancement of polysaccharide digestion and increase in nutrient availability is thought to be partially responsible for larger fat storage observed in mouse and human hosts (Bäckhed et al., 2004; Turnbaugh et al., 2008). However, a comparative study in children on a modern Western diet or on a rural African diet showed that the children on African diet had higher levels of SCFA than the children on Western diet (De Filipo et al., 2010). Furthermore, SCFA have beneficial effects on intestinal health as these nutrients contribute to differentiation of epithelial cells, provide fuel for colonocytes, modulate ion and water transport and decrease colorectal cancer incidence (Topping and Clifton, 2001; Wong et al., 2006; Sellin, 1999). Propionate specifically has been shown to have beneficial effects on a systemic level by lowering liver and plasma fatty acid levels and inflammation and increasing satiety, therefore potentially contributing to improved insulin sensitivity and weight reduction (Al-Lahham et al., 2010). However, it is acetate and butyrate levels that are increased in the ceca of obese versus lean conventionalized (CONVD; ex-GF mice colonized with microbiota) mice, while propionate levels are low at least in this host organism (Turnbaugh et al., 2006).

The microbial role in exogenous lipid metabolism is not well understood, since the majority of lipid digestion and absorption is completed in the small intestine, which is a difficult anatomical region to study *in vivo* especially in rodents and humans. However, as mentioned earlier, under high fat feedings, the distal intestine also becomes involved in lipid absorption (Lynes and Widmaier, 2011) to enhance energy harvest from diet despite an efficient lipid uptake in the small intestine (>90% uptake in vertebrates) (Labonté et al., 2008; Karasov and Hume, 1997). As summarized below, our current understanding of the role of gut microbiota in lipid metabolism comes primarily from genomic, transcriptomic and metabolomic findings that indicate of microbial regulation of host lipid metabolites.

Microbial role in lipid metabolism: The microbiota can potentially regulate lipid metabolism by providing microbial genes encoding proteins involved in lipid metabolism (Turnbaugh et al., 2006), which could contribute to lipid digestion and increased lipid bioavailability to the host. Another possibility is that the microbiota regulates the expression of host genes involved in lipid metabolism. Larsson and colleagues (2011) showed that the gut microbiota regulates transcript levels of genes along the length of the mouse intestine and liver, which include genes involved in fatty acid activation (Fatp4), TG reesterification (Dgat1 and 2), fatty acid and phospholipid binding (Fabp2, phospholipid transfer protein - Pltp) and chylomicron formation (Apob precursor, Mtp). Analysis of enriched groups among the 500 most significantly regulated genes by gene ontology shows lipid, fatty acid and cholesterol biosynthetic and metabolic processes amongst the microbially-regulated metabolic mechanisms. The majority of genes involved in lipid metabolism are downregulated in the presence of microbiota, while many of the adaptive immunity genes are upregulated.

Another recent study showed similar trend of microbial transcriptional regulation of host genes involved in intestinal steroid, lipid, cholesterol metabolic processes and lipid transport in immunodeficient mice (Shulzhenko et al., 2011). The authors showed that in the absence of B cells or IgA, the intestinal epithelium upregulates interferon-inducible immune responses as a protective mechanism against the microbiota. This leads to decreases in metabolic function and lipid malabsorption as observed in immunodeficient humans. However, subjects with a normal function of their immune system and consuming a high-fat diet do not present with malabsorption, but rather hyperlipidemia, hypercholesterolemia and increase in fat storage in the presence of microbiota, suggesting that the microbial suppression of transcript levels of lipid metabolic genes does not correlate with decreased lipid availability to the host.

Metabolomic studies have also shown differences in the lipidomes of serum, adipose tissue and liver of GF and CONVR mice (Velagapudi et al., 2010). Specifically, serum cholesterol, TG and FFA were reduced while liver and adipose tissue TG levels were increased in CONVR versus GF mice, which is indicative of increased lipid clearance from the circulation and stimulated tissue storage. Furthermore in the presence of microbiota, there were detectable increases in serum cholesteryl esters, sphingomyelins, and phosphatidylcholines. Serum lipoprotein profiles showed reduced serum chylomicron levels in CONVR mice after a 4 hr fast, which are thought to be the result of increased clearance of serum TG and not decreased absorption from the intestine of CONVR animals. On the other hand, serum VLDL and HDL levels were similar between GF and CONVR mice despite increased VLDL production in the liver, which also suggests increased clearance rates of the endogenous lipid supplies from the circulation of colonized mice. However, these altered lipid profiles could be due to effects of microbiota on intestinal absorption or metabolism of exogenous dietary lipids or on hepatic production or metabolism of endogenous lipids. Therefore, the relative contribution of the microbiota to exogenous and endogenous lipid metabolism in not fully understood.

The microbiota has also been shown to modulate luminal lipid metabolism via modification of bile acids released into the intestinal lumen from the gallbladder. Bile acids are derivatives of cholesterol metabolism and facilitate absorption of dietary lipids and lipid-soluble vitamins in the intestine (Martin et al., 2007). Bile acids also maintain the integrity of the intestinal epithelial barrier to prevent entry of enteric bacteria and inflammatory response. The ability of microbiota to process bile acids has been examined in numerous studies (Martin et al., 2007; Martin et al., 2008; Blaut et al., 2007). Martin and colleagues performed microbial and metabolic profiling using H^1-NMR and bile acid profiling of liver, plasma, urine and ileum using ultra performance liquid chromatography – mass spectroscopy (UPLC-MS)

(2007). The results from this study show a variety of bile acid modifications (tauro- and glyco-conjugation as well as unconjugation) in response to microbial processing. The tauro-conjugation reduces the hydrophobic/hydrophilic ratio and increases their ability to emulsify lipids. Lipid emulsification results in increased uptake of lipid by the intestinal epithelial cells. In addition, tauro-conjugation also increases the amount of bile acids that re-enter enterohepatic circulation and therefore decrease the synthesis of bile acids and cholesterol by the liver (Martin et al., 2007).

Microbial processing of another dietary lipid (phosphatidylcholine) has been associated with an animal model of non-alcoholic fatty liver disease (NAFLD). Choline is the primary source for hepatic phosphotidylcholine production. Dietary choline gets processed by the gut microbiota (al-Waiz et al., 1992), which releases methylamines (dimethylamine, trimethylamine and trimethylamine-N-oxide or TMAO). Dumas and colleagues (2006) tested the effects of high-fat diet on plasma and urine metabolic ^{1}H NMR profiles in BALB/c (resistant to NAFLD and insulin resistance) and 129S6 (susceptible to NAFLD and insulin resistance) mice and showed that NAFLD is associated with low plasma phosphotidylcholine and high urinary methylamine levels, which is the microbiota-mediated phenotype. The authors interpreted the low plasma levels of phosphotidylcholine to be a consequence of microbial degradation of choline into methylamines. These findings suggest that microbiota of NAFLD- and insulin resistance-susceptible hosts are partially responsible for the disease progression by mimicking choline-deficient conditions.

The host's response to microbially-regulated nutrient metabolism

The evidence for the host's response to microbial regulation of nutrient metabolism comes from gnotobiotic rodent studies that investigate regulation of metabolic processes in

the host upon microbial colonization. Studies by Bäckhed et al. showed that in GF mice there is increased activity of AMP-activated protein kinase (AMPK) compared to CONVD animals (2007). This enzyme senses metabolic energy levels via AMP/ATP ratios and is activated during a nutrient deprivation and energy depletion state. The authors showed that GF animals are protected against diet-induced obesity in two major, but independent ways: (i) increase in AMPK activity and (ii) induction in peroxisomal proliferator-activated receptor coactivator (Pgc-1α) and enzymes involved in fatty acid oxidation, such as Carnitine palmitoyltransferase1a (Cpt1a) and Medium chain acyl-CoA dehydrogenase (Mcad) in an Fiaf-dependent manner (Bäckhed et al., 2004; Bäckhed et al., 2007). Fiaf is a circulating peptide that inhibits the LPL activity in vascular endothelial cells. As described earlier, this lipase hydrolyzes serum TG, releasing free fatty acids and glycerol into heart, adipose or muscle tissue. The FFA enter lipogenesis and get stored in the form of fat or get oxidized in tissues like muscle or heart. Therefore, the microbial role in intestine-specific suppression of Fiaf results in alleviated suppression of lipoprotein lipase (LPL) in the vasculature, and increase in TG hydrolysis from chylomicrons and FA uptake in peripheral tissues (Bäckhed et al., 2004). A study of transcriptional regulation of *fiaf* shows that different cis-intronic modules are responsible for tissue-specific expression in a zebrafish host (Camp et al., 2012). Interestingly, the gut microbiota regulates the cis-intronic modules that regulate intestinal *fiaf* expression, suggesting that the microbiota may suppress the intestine-specific transcriptional enhancer.

The microbiota and diet also contribute to transcriptional activity and lipid metabolic gene expression. The microbiota stimulates polysaccharide digestion and absorption, which initiates hepatic de novo lipogenesis (Bäckhed et al., 2004). Conventionalization of mice also increased hepatic mRNA expression of sterol-responsive element-binding protein (SREBP-1) and carbohydrate-responsive element-binding protein (ChREBP), which are two

transcription factors that regulate lipogenic enzymes (Bäckhed et al., 2004). Microbial colonization has been shown to induce intestinal alkaline phosphatase expression in a zebrafish host (Bates et al., 2007), which as mentioned earlier activates CD36 to stimulate LCFA uptake from the lumen. Bates and colleagues showed that zebrafish IAP protects against LPS toxicity and neutrophil infiltration into the intestinal epithelium as a response to microbial colonization (2007). These findings suggest that the gut microbiota regulate metabolic processes involved in exogenous and endogenous lipid fates, which is closely associated with immune function and inflammation induced by high-fat diet. However, direct evidence of the impact of microbial induction of IAP on lipid metabolism has not been explored.

Molecular mechanisms involved in gut microbiota- and diet-mediated inflammation

Metabolic endotoxemia: The gastrointestinal tract is under constant low-grade inflammation that worsens with excess of caloric intake and is thought to precede and contribute to obesity and insulin resistance in CONVD but not GF mice (Ding et al., 2010). One proposed method for HFD-mediated endotoxemia is by LPS absorption and incorporation into chylomicrons (Ghoshal, 2009; Laugerette, 2011), indicating that the gut microbiota and dietary lipids can mediate intestinal lipid absorption and host responses to elevated lipid and LPS uptake. LPS is the breakdown product of Gram-negative bacteria that activates the toll-like receptor-4 (TLR4) to initiate secretion of proinflammatory cytokines (Sweet and Hume, 1996). Obese Sprague-Dawley rats on a high-fat diet showed increased ileal TLR4 expression, which mediates the intestinal inflammatory response (de La Serre et al., 2010).

High fat diets have also been shown to affect epithelial integrity and permeability, which leads to systemic inflammation also known as metabolic endotoxemia (Cani et al., 2007; Cani et al., 2008). The mechanisms mediated by high-fat diet involve changes in gut microbiota and reduced expression of the tight junction proteins ZO-1 and occludin in the intestine (Cani et al., 2008). Furthermore, prebiotic treatment with fermentable dietary fiber oligofructose ameliorated the effects of endotoxemia in *ob/ob* mice and improved intestinal permeability via induction of intestinotrophic proglucagon-derived peptide (GLP-2) (Cani et al., 2009). These and other studies establish the interactions between gut microbiota, high-fat diet and inflammation that mediate diet-induced obesity and insulin resistance.

Therefore, understanding the microbial and dietary signals that regulate FA uptake, LD and chylomicron formation and secretion into the lymphatics could potentially lead to new approaches for limiting HFD-associated endotoxemia and associated disorders. Similarly, microbial pathways could be targeted to limit the intestinal absorption of potentially harmful products of microbial lipid metabolism, such as TMAO, the atherogenic microbial metabolite of dietary choline (Wang, 2011).

FUTURE DIRECTIONS

The role of microbiota in exogenous lipid metabolism has only recently been examined, despite significant independent evidence of dietary fat and microbiota as two environmental factors involved in numerous metabolic processes and diseases in humans. The importance in studying microbial regulation of lipid metabolism lies in the fact that the microbiota stimulates excess energy harvest and storage in the host, and lipid is the only

macronutrient that can be stored at large capacities in the body (Papamandjaris et al., 1998). Therefore, in order to improve our knowledge of the role of microbiota in lipid metabolism we need to better understand microbial regulation of different metabolic fates of exogenous and endogenous lipids under various dietary conditions.

The role of gut microbiota in the development and progression of obesity and other metabolic diseases has also been established; however, it is important to note that additional environmental (high-fat, high-carbohydrate diet) or host genetic factors are important mediators of this microbial role. Metabolic diseases are complex pathologies that affect multiple tissues and involve numerous biological pathways (Ashley et al., 2010). Our current evidence for the microbial regulation of disease development and progression is mostly based on metabolomic studies and tissue metabolic profiles, which provide us with snapshots of the dynamic and complex interactions between the gut microbiota, dietary nutrients and host physiology.

In order to better understand the microbial contribution to human metabolism, we need to take a more direct and systemic approach for studying the physiological response to changing dietary nutrients and microbial community composition. *In vivo* studies of the metabolic fates of exogenous lipids provide a more complete picture of the microbial impact on dietary lipid metabolism at a systemic level. Zebrafish have proven to be important model organisms for such investigations due to their transparency prior adulthood, and same lipid metabolic mechanisms (Carten and Farber, 2009) and microbial community members (at the phyla level) as humans (Rawls et al., 2006; Chapter 3). We have utilized fluorescent lipid analogues to show that the gut microbiota stimulates fatty acid uptake from the lumen into the epithelium in live zebrafish (Semova et al., submitted). Furthermore, our studies also indicate that Firmicutes enrichment under nutrient-rich conditions is partially responsible for the epithelial lipid absorption phenotype observed in fish. One of the important implications

of this report is that the diet-dependent selective pressures governing Firmicute abundance in the gut might be conserved across diverse vertebrate lineages, and that probiotic/antibiotic/prebiotic strategies for manipulating Firmicute abundance and associated community function might be applicable to a diversity of vertebrate hosts. Since Firmicute-enriched communities arising from genetic or diet-induced obesity have been shown to promote positive energy balance (Turnbaugh et al., 2006; Turnbaugh et al., 2008), such strategies might be effective at controlling energy balance in humans and other animals.

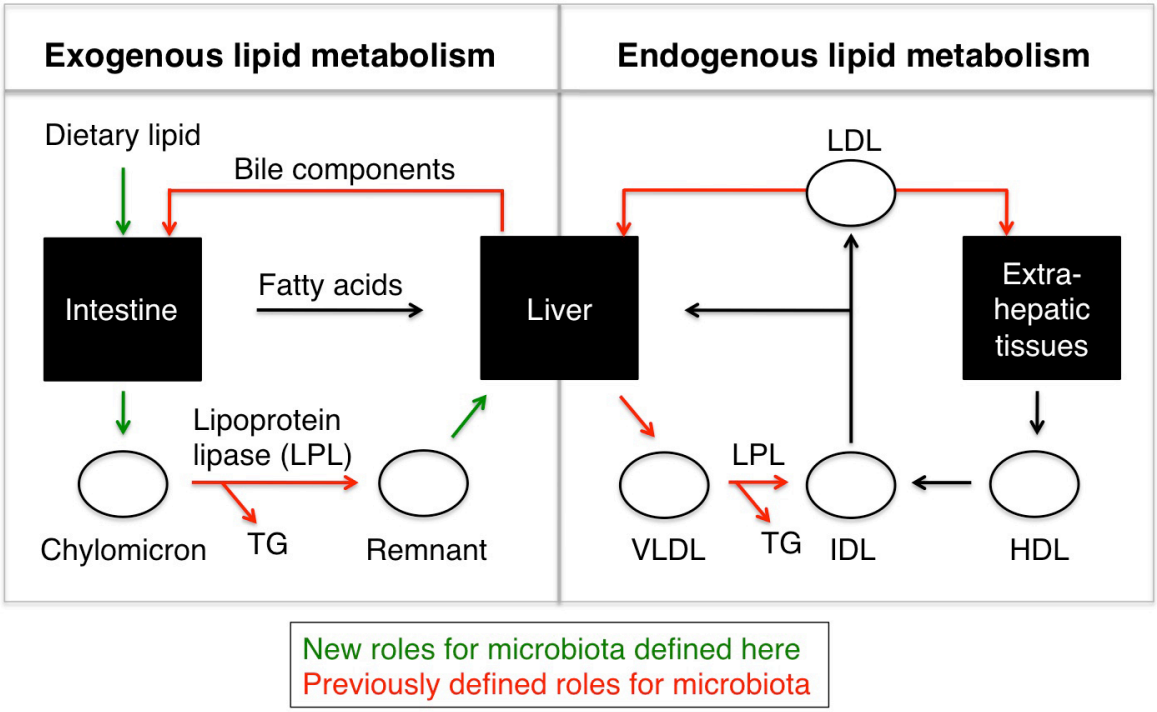

Figure 2.1. Novel and known roles of microbiota on lipid metabolism. Roles for the microbiota have been previously defined in multiple steps in exogenous and endogenous lipid metabolism (red arrows). Our data reveal new roles for microbiota in absorption of dietary lipid into the intestine, and export to the liver perhaps via chylomicrons (green arrows). Triglyceride, TG; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LPL, lipoprotein lipase. Adapted from Sheridan, M.A. (1988). Comp. Biochem. Physiol. *90B (4). 679-690.*

CHAPTER THREE

MICROBIOTA AND DIET REGULATE FATTY ACID ABSORPTION IN THE ZEBRAFISH INTESTINE[1]

SUMMARY

The intestinal microbiota is known to impact host nutrition and energy balance. However, its role in dietary fat absorption in the intestine is unclear. Here, we use *in vivo* imaging of fluorescent fatty acid (FA) analogs delivered into gnotobiotic zebrafish to reveal that the microbiota stimulates FA uptake and lipid droplet (LD) formation in the intestinal epithelium. Comparison of animals that were starved or fed a sterile diet reveals that the microbiota promotes epithelial LD number, but not size, in a diet-dependent manner. The presence of food results in Firmicutes enrichment in the microbiota of the zebrafish intestine, but not in the surrounding water. Monoassociation studies reveal that LD number is increased by diet-enriched Firmicutes and LD size is increased by other bacterial types. These results indicate that members of the gut microbiota regulate intestinal FA absorption via distinct mechanisms and that diet-induced alterations in gut microbiota composition might indirectly influence FA absorption.

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[1] Co-authors: Carten, J.D., Stombaugh, J., Mackey, L.C., Knight, R., Farber, S.A., Rawls, J.F.

INTRODUCTION

Dietary fat absorption in the intestine is a key determinant of energy balance. Dietary lipids supply 45-55% of the energy requirements in breastfed human infants (Boudry et al., 2010) and 40-55% of the calories in Western diet (Binder and Reuben, 2009; Meek et al., 2010). In vertebrates, dietary fats in the form of triglycerides are digested by lipases within the intestinal lumen and the released free fatty acids (FAs) and monoglycerides are absorbed with a high efficiency (>90%) by enterocytes in the intestinal epithelium (Labonté et al., 2008; Karasov and Hume, 1997). FA absorption at the brush border of enterocytes is enhanced by solubilization in bile salt micelles or liposomes (Kindel et al., 2010). Once absorbed by enterocytes, FAs are either oxidized to generate energy or reesterified into triglycerides temporary storage as cytoplasmic lipid droplets (LDs), or incorporation into chylomicrons for secretion into the lymph. Lipid droplets provide cells with the ability to store potentially toxic FAs during energy excess and use the stored lipids as an energy source during starvation (Farese and Walther, 2009). The mechanisms underlying FA uptake into enterocytes and their subsequent assembly into LDs and chylomicrons are unresolved due in part to the limitations of the mammalian animal and cell culture models often used to study these dynamic physiologic processes (Carten and Farber, 2009). An improved understanding of factors controlling dietary FA absorption and LD formation could lead to new approaches for decreasing the efficiency of dietary energy harvest in the context of obesity and increasing efficiency in the context of malnutrition.

Environmental factors such as intestinal microorganisms and diet represent attractive targets for controlling dietary lipid absorption and energy balance. The digestive tract is colonized beginning at birth by complex assemblages of microorganisms (gut microbiota)

that profoundly influence intestinal and extra-intestinal physiology (Sekirov et al., 2010). The ability of the gut microbiota to modify dietary nutrient metabolism has emerged as a key feature of host-microbe relationships in the intestine (Musso et al., 2011). This capability has been most extensively described in the ruminant foregut and in the human and rodent hindgut (colon), where microbial fermentation of otherwise indigestible plant polysaccharides produces monosaccharides and short chain FAs that can then be absorbed by the host (Flint et al., 2008). In contrast, the potential impact of the microbiota on intestinal absorption of dietary lipids remains relatively unexplored. The presence of a gut microbiota in mice increases fat storage in adipose tissue (Bäckhed et al., 2004) and causes significant alterations in secondary lipid metabolites in serum, liver, and adipose tissue (Martin et al., 2009; Velagapudi et al., 2010). However, it remains unknown if the microbiota regulates intestinal absorption of dietary lipid.

Although the microbiota can influence dietary nutrient harvest, the diet can also strongly impact microbial community composition and function. Gut microbial community membership is strongly correlated with diet composition in humans and other mammalian hosts (Ley et al., 2008; Wu et al., 2011; Muegge et al., 2011; Neyrinck et al., 2011; Sonnenburg et al., 2010). Although not all studies have reported similar associations, an emerging pattern is that caloric intake can influence the relative abundance of the Firmicutes and Bacteroidetes phyla that dominate the intestines of mammals (Ley et al., 2008). For example, mice starved for 24 hours show a reduction of Firmicutes and increase of Bacteroidetes in their gut microbiota (Crawford et al., 2009). Conversely, obese humans (Ley et al., 2006), humans consuming a high-calorie diet (Jumpertz et al., 2011), hyperphagic obese *ob*/*ob* mice (Ley et al., 2005), mice fed high-calorie Western diets (Turnbaugh et al., 2008; Hildebrandt et al., 2009; Murphy et al., 2010), and postprandial pythons (Costello et al., 2010) assemble gut microbial communities with an enrichment of

Firmicutes at the expense of Bacteroidetes and other major phyla. Diet-induced enrichment of Firmicutes in the gut microbiota has been associated with a positive energy balance in gnotobiotic mouse hosts (Turnbaugh et al., 2006; Turnbaugh et al., 2008) and with alterations in the relative abundance of bacterial genes involved in a wide range of metabolic processes (Hildebrandt et al., 2009; Turnbaugh et al., 2009; Turnbaugh et al., 2006). However, the mechanisms by which certain bacterial taxa such as Firmicutes become enriched in the gut as a function of dietary caloric intake remain unresolved. Furthermore, the potential impact of diet-induced alterations in microbiota composition on dietary lipid absorption in the intestine is completely unknown.

In this study, we use the zebrafish model to investigate how the microbiota and diet interact to regulate lipid absorption in the intestinal epithelium. Digestive tract anatomy and physiology as well as lipid metabolism pathways in the zebrafish are similar to mammals and other vertebrates (Babin and Vernier, 1989; Carten and Farber, 2009; Hölttä-Vuori et al., 2010). Moreover, the optical transparency of the zebrafish permits visualization of fluorescent lipid analogs and lipophilic probes in the intact physiologic context of a living vertebrate (Farber et al., 2001; Schlegel and Stainier, 2006; Flynn et al., 2009; Hama et al., 2009; Fang et al., 2011; Carten et al., 2011). Previous work comparing zebrafish raised germ free (GF) to those colonized with a normal zebrafish microbiota (conventionalized, CONVD) revealed roles for the microbiota on diverse aspects of host physiology (Rawls et al., 2004; Bates et al., 2006; Rawls et al., 2006; Kanther et al., 2011; Cheesman et al., 2011). The zebrafish gut microbiota, like that of humans and other mammals, is dominated by the bacterial phyla Proteobacteria, Firmicutes, and Bacteroidetes (Rawls et al., 2004; Rawls et al., 2006; Roeselers et al., 2011). However, the impact of diet on the zebrafish gut microbiota, and its relationship to the microbiota in the surrounding aqueous environment, is unknown. We recently developed a new method to monitor FA absorption into the intestinal

epithelium by incubating zebrafish in liposomes containing FA analogs fluorescently labeled with BODIPY (BODIPY-FL) (Carten et al., 2011). Here, we used this method to investigate the impact of microbial colonization and diet on intestinal FA absorption. Our work reveals a novel diet-dependent role for the microbiota in stimulating dietary FA absorption and LD formation in the intestinal epithelium. We find that the composition of the zebrafish gut microbiota varies in a diet-dependent manner, with feeding inducing a gut-specific enrichment of Firmicutes. Colonization of GF zebrafish with individual bacterial species uncovers two distinct pathways by which the microbiota stimulates dietary lipid absorption: a Firmicutes-specific induction of LD number, and a non-Firmicutes bacterial induction of LD size. These results identify the gut microbiota as a new target for controlling dietary fat absorption and provide an ecological explanation for the diet-dependent nature of this host-microbe interaction.

MATERIALS AND METHODS

Gnotobiotic zebrafish husbandry

All experiments using zebrafish 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. All zebrafish were TL wild-type strain unless otherwise noted. Zebrafish embryos were derived germ-free and maintained in sterile conditions or colonized according to published protocols (Pham et al., 2008). Zebrafish were maintained in gnotobiotic zebrafish media (GZM) at 28.5°C in 50 mL and 250 mL sterile tissue culture flasks (VWR International, LLC) on a 14 hr light cycle with daily 80% media changes. Colonization with microbiota and individual bacterial strains was achieved by inoculating the GZM at approximately 10^4 colony

forming units (CFU)/mL at 3 dpf. For the monoassociation studies, we used primary bacterial isolates from zebrafish adults (*Exiguobacterium* sp. ZWU0009 and *Pseudomonas* sp. ZWU0006) (Rawls et al., 2006) and 3 dpf embryos (*Chryseobacterium* sp. ZOR0023; a generous gift from E. Mittge and K. Guillemin) (see Table 3.S5). Overnight cultures grown in Brain Heart Infusion broth (aerobic; 30°C) were used to inoculate the GZM at a final density of 10^4 CFU/mL. Sterility of GF zebrafish flasks was assessed using culture-based methods as previously described (Pham et al., 2008). All zebrafish were treated with 0.015 mg/L 1-phenyl-2-thiourea (PTU)(Lancaster Synthesis, Inc.) to reduce melanin synthesis. Control (C) and Low-Calorie (LC) diets (Table 3.S1) were custom formulated and ground to a pellet size of 50-100 μm (Ziegler Brothers Inc.) and were then sterilized by irradiation (absorbed dose range 106.5-135.2 kGy; Neutron Products Inc.). Zebrafish were either fed once per day beginning 3 dpf with approximately 2.5 and 4 mg sterilized C or LC diet (for the 50 and 250 mL flasks, respectively) or not fed for the duration of the experiment (starved). At 6 dpf, intestines of colonized animals were dissected and homogenized for 1 min using a Tissue-Tearor (Biospec Products). Homogenized intestinal and water samples were serially diluted in phosphate buffered saline (PBS) and plated on Tryptic Soy (for the CONVD condition) or Brain Heart Infusion (for the monoassociated conditions) agar, grown overnight at 28.5°C, and CFU density was determined.

Liposome delivery assay

Preparation of fluorescent liposome solution was performed as described (Carten et al., 2011). Briefly, liposomes were generated by sonicating 5% chicken egg yolk solution in sterile GZM and BODIPY-FL C_5 (Invitrogen, D-3834) or C_{16} (Invitrogen, D-3821) at a final concentration of 6.4 μM. Fluorescent liposome formation was confirmed by confocal

microscopy (data not shown). Zebrafish were first washed 3 times in GZM, and then the medium was replaced with freshly-prepared fluorescent liposome solution. For live imaging experiments, the labeled fish were then washed 3 times with GZM after liposome incubation, anaesthetized with 0.4% tricaine (w/v) (Argent Chemical Laboratories) and mounted in 3% methylcellulose (Sigma-Aldrich) on a coverslip (Surgipath Medical Industries INC.) for imaging. For the lipid droplet measurements, zebrafish were fixed in freshly-made 4% paraformaldehyde (PFA) (Acros) in PBS for 1 hr at room temperature to preserve LD structure (DiDonato and Brasaemle, 2003) and arrest peristalsis, washed 3 times in PBS and stored in a sterile 12-well plate at 4°C in the dark. Fixed zebrafish were mounted on Superfrost Plus microscope slides (Fisher Scientific) in 1% low melting temperature agarose (Fisher Scientific) and sealed with high vacuum grease (Dow Corning). For confocal imaging methods and lipid droplet quantification, see Supplemental Information.

Statistical methods

The significance of diet and microbial status on lipid absorption, digestive enzyme activity, growth, and feeding behavior was determined by two-way ANOVA (p values reported in Results section). Statistical significance between individual conditions was based on individual Student's t test adjusted for unequal variances (determined by the Bartlett's test) and corrected by Bonferroni's method for multiple comparisons (p values reported in Figures). We used ANOVA corrected for false discovery rate (Benjamini and Hochberg, 1995) to identify significant differences in relative abundance of bacterial taxa based on the Ribosomal Database Project (RDP) classifier version 2.2 (Wang et al., 2007) and in normalized alpha diversity distances (p values reported in Results). Any p values less than 0.05 with correction were considered significant.

Supplemental Information

Supplemental Information accompanying this manuscript includes Supplemental Results, Supplemental Experimental Procedures, Supplemental References, six Figures, and five Tables.

RESULTS

The microbiota promotes intestinal fatty acid accumulation in a diet-dependent manner

To determine the impact of the microbiota on dietary lipid absorption in the intestine, we used our recently developed BODIPY-FL delivery assay to study the metabolic fates of dietary FA analogs in zebrafish larvae (Carten et al., 2011). We used BODIPY-labeled palmitic acid (BODIPY-FL C_{16}), which represents the most common saturated long-chain FA found in triglycerides, and medium chain pentanoic acid (BODIPY-FL C_5; the BODIPY fluorophore effectively adds 2-3 carbons in length to the C5:0 FA backbone), which is more rapidly absorbed in the intestine (Carten et al., 2011). We incubated 6 dpf GF and CONVD animals with BODIPY-FL C_5 or BODIPY-FL C_{16} emulsified in egg yolk liposomes for 6 hrs (Figure 3.1A). To test whether the prior nutritional status of the animal can impact dietary lipid absorption, we performed this assay on GF and CONVD zebrafish that were either fed a control diet since 3 dpf (C-fed) or never fed (starved). Confocal imaging of the proximal intestine in live zebrafish revealed luminal and epithelial fluorescence under all microbial and dietary conditions, indicating FA analog ingestion and absorption respectively (Figure 3.1B,C). Quantification of intestinal epithelial fluorescence revealed higher levels of BODIPY-FL C_5 and C_{16} fluorescence in CONVD compared to GF zebrafish ($p<0.001$ for both

C_5 and C_{16}), which was enhanced by the presence of diet (Figure 3.1D,F). In contrast, we observed a consistent trend of higher luminal fluorescence in GF than CONVD animals ($p<0.05$ for C_5, n.s. for C_{16})(Figure 3.1E,G). These results suggest that the microbiota promotes dietary FA accumulation in the intestinal epithelium in a diet-dependent manner.

The microbiota increases lipid droplet number and size in the intestinal epithelium

We next sought to determine if the observed BODIPY-FL accumulation in the intestinal epithelium was due to alterations in LD formation in the enterocytes. Because microbial colonization and feeding appeared to have similar effects on epithelial accumulation of BODIPY-FL medium- and long-chain FAs (Figure 3.1), we used the medium-chain FA analog BODIPY-FL C_5 to develop a quantification assay for LD formation in the intestinal epithelium (see Supplemental Experimental Procedures and Figure 3.S1). After a 3 hr incubation with BODIPY-FL C_5, LDs accumulated in enterocytes of GF and CONVD fish (Figure 3.S2A) and were detected in circulation (indicated by arrows in Figure 3.S2A). Longer, 6 hr incubation resulted in increased LD numbers only in animals that were both colonized with a microbiota and fed control diet (Figure 3.2A,B), confirming that interactions between microbial colonization and diet stimulate FA absorption ($p<0.01$)(Figure 3.2B). By analyzing the relative frequency of LD sizes, we observed a high percentage of small LDs after the 3 hr incubation in all conditions (Figure 3.S2C). The percentage of very large LDs was increased in all CONVD conditions after the 6 hr incubation (i.e., 5.6-11 μm^2 area, $p<0.01$), whereas the percentage of small (i.e., <0.55 μm^2 area, $p<0.001$) and medium LDs (i.e., 0.55-1.64 μm^2 area, $p<0.001$) was strongly altered by diet (Figure 3.2C). These results suggest that the presence of microbiota promotes two distinct phenotypes of epithelial LD formation: increased LD size regardless of dietary status and increased LD number only in fed animals. Intriguingly, the microbiota also increased LD number and size

in zebrafish fed a low calorie diet (LC-fed) that has 31.4% fewer calories than the control diet due to a 55.6% reduction in protein content (Table 3.S1 and data not shown), suggesting that diet-dependent microbial stimulation of FA absorption is not determined by the caloric value or protein content of the diet. Furthermore, the increase in epithelial LD accumulation in fed CONVD animals was not associated with significant differences in ingestion rates, digestive organ size, or in the *in vivo* activity of digestive enzymes phospholipase and protease in the intestinal lumen (Figures 3.S3 and 3.S4).

Microbial stimulation of intestinal epithelial lipid droplet accumulation is associated with increased lipid absorption into extra-intestinal tissues

We postulated that the increased LD number and size in CONVD fed zebrafish could be due to either delayed lipid clearance via exocytosis of chylomicrons into the circulation or increased absorption of FAs from the lumen. Indeed, previous studies in mice have shown that accumulation of large LDs can be associated with reduced chylomicron exocytosis (Buhman et al., 2002). To test the possibility that chylomicron exocytosis is impaired in CONVD fed zebrafish, we performed a washout experiment to compare the ability of GF and CONVD zebrafish to clear LDs from their epithelium after BODIPY-FL C_5 incubation. We incubated 6 dpf GF and CONVD C-fed zebrafish with BODIPY-FL C_5 liposomes for 3 hrs, and subsequently washed a subset of animals from each condition in sterile GZM for an additional 5 hrs (Figure 3.3A). Consistent with our prior observations (Figure 3.2), the 3 hr labeling resulted in similar LD numbers in the intestines of both GF and CONVD animals (Figure 3.3B-D). The 5 hr washout reduced the number of LDs in the epithelium of both GF and CONVD zebrafish; however, this reduction was more prominent in CONVD fish ($p<0.001$)(Figure 3.3B-D). This indicates that the rate of chylomicron export in CONVD animals is similar to, and perhaps even more robust than, GF zebrafish. Analysis of LD size

frequency following the 5 hr washout in GF and CONVD animals revealed similar reductions in small LDs (i.e., <0.55 μm^2, $p<0.01$) and increases in medium (i.e., 1.65-5.5 μm^2, $p<0.01$) and large LDs (i.e., 5.6-11 μm^2, $p<0.0001$; 11-27.4 μm^2, $p<0.05$) (Figure 3.3D). This indicates that the significant reduction in LD number in CONVD animals (Figure 3.3C) is not due to enhanced LD fusion in the presence of microbiota. These data suggest that the observed increase in LD number and size in CONVD fish observed in Figure 3.2 is not due to impaired chylomicron exocytosis.

We next asked whether the increases in intestinal epithelial LD accumulation elicited by the microbiota are associated with increased FA export from the intestine to other tissues. After BODIPY-FL C5 molecules are absorbed and exported from the zebrafish intestine, a primary site of accumulation is in LDs within the liver (Carten et al., 2011). We found that the presence of a microbiota resulted in increased BODIPY fluorescence in liver LDs after a 6 hr incubation in BODIPY-FL C_5 liposomes (Figure 3.2D,E). This was observed both starved and C-fed zebrafish larvae (Figure 3.2E), suggesting that the microbiota promotes intestinal absorption and export of FA to liver irrespective of diet history and diet-dependent effects of the microbiota on intestinal LD number (Figure 3.2B). Additionally, CONVD animals displayed a significant increase in accumulation of BODIPY C_5 fluorescence in non-GI tissues compared to GF controls (82 ±16% increase, $p<0.02$) (Figure 3.2F). Based on these results, we conclude that the ability of the microbiota to induce accumulation of LDs in the intestinal epithelium is associated with increased absorption of dietary FAs into extra-intestinal tissues.

Diet determines bacterial community composition in the zebrafish gut

Our results show that the microbiota promotes intestinal LD formation in fed but not in starved zebrafish. We hypothesized that diet-dependent microbial stimulation of intestinal

LD number may be due to diet-induced alterations in gut microbial community composition. Diet-dependent differences in zebrafish gut microbiota composition could be due to direct effects of diet on the selective pressures present in the host gut habitat (e.g., alterations in host digestive tract physiology) or to indirect effects of diet on the selective pressures in the surrounding aqueous environment (e.g., altered water chemistry and nutrient availability) that would modify the microbial community in the water that is available to colonize the zebrafish gut. We therefore analyzed the impact of diet on the bacterial communities that form in the zebrafish gut and in the surrounding water (Figure 3.S5). GF zebrafish embryos were colonized at 3 dpf with a common conventional zebrafish microbiota (inoculum, Figure 3.S5) and subsequently either starved, or fed C or LC diet until 6 dpf. Microbial genomic DNA was extracted from zebrafish guts and the housing water from each flask (3 biological replicates flasks/diet condition), and the respective bacterial communities were analyzed using 16S rRNA gene pyrosequence-based surveys (61,429 sequences in total; Figure 3.S5 and Table 3.S3). UniFrac principal coordinates analysis (PCoA) plots derived from both unweighted (an evaluation of community composition) and weighted algorithms (an evaluation of community structure) (Lozupone et al., 2007) provided several insights. First, biological replicates from each of the sample groups consistently clustered together (Figure 3.4A,B), suggesting that the principles governing bacterial community composition are reproducible. Second, we observed a striking separation of gut and water samples, revealing that the zebrafish gut selects or enriches a distinctive subset of bacteria from the surrounding water. Notably, the inoculum sample appeared central to all other samples, reflecting the fact that the majority of operational taxonomic units (OTUs) observed in the inoculum sample were also detected in other samples (108/111 or 97.3%; Figure 3.S6). Third, fed samples clustered together and were separate from the starved samples for both the gut and water environment, suggesting that feeding markedly alters the gut and water

bacterial communities in distinctive ways. Furthermore, the clustering of LC-fed and C-fed samples indicates that the effect of diet on those communities is not strongly determined by the caloric and protein content of the diet.

The distinct separation of gut and water samples observed in the PCoA analysis was accompanied by significant differences in the relative abundances of several bacterial taxa (Figure 3.4C,D and Table 3.S3). Although all samples were dominated by Proteobacteria phylum sequences, sequences from the β-Proteobacteria class were enriched in water (mean 49.7% vs. 6.4% of all water and gut samples, respectively; $p<0.0001$). Bacteroidetes phylum sequences were also enriched in water (mean 19% vs. 3.3% of all water and gut samples, respectively; $p<0.01$), through increases in classes Flavobacteria and Sphingobacteria. In contrast, Firmicutes phylum sequences were enriched in gut samples (mean 30.2% vs. 0.6% of all gut and water samples, respectively; $p<0.05$) through increases in classes Bacilli and Clostridia. Strikingly, gut-specific enrichment of Firmicutes occurred only in animals that had been fed, and not in starved animals (Figure 3.4C,D), which indicates that feeding results in gut-specific enrichment of Firmicutes in zebrafish hosts.

In addition to affecting the relative abundance of specific bacterial taxa, the overall diversity of these bacterial communities also varied as a function of diet and environment. In the gut, bacterial diversity and richness were markedly lower in starved animals compared to those fed C or LC diet (e.g., mean phylogenetic distances of 5.41 vs. 1.87 in starved vs. C/LC-fed gut samples, respectively, $p<0.005$; Figure 3.4E,F and Table 3.S4). In contrast, diversity and richness in water communities were not significantly different between fed and starved conditions. These diet-induced increases in gut microbiota diversity were not accompanied by significant alterations in the density of culturable microorganisms in the gut (Figure 3.5A). These results reveal that the presence of diet promotes diversity of bacterial

communities in the zebrafish gut, without inducing similar alterations in the surrounding water.

Although the trends among biological replicates were largely consistent, there were two instances in which one replicate deviated significantly from the others (C-Fed gut 1 and Starved gut 3; Figure 3.4A-C; Table 3.S5). The sequences in these samples grouped into OTUs that were also present in other samples (data not shown), suggesting that these deviations are not due to contamination from external sources but due to differences in abundance. These two deviant samples may be the result of technical variation or may reflect unappreciated stochasticity in gut microbial community assembly (Robinson et al., 2010).

A Firmicutes strain isolated from the zebrafish intestine is sensitive to exogenous nutrient levels in the absence of microbial competition

Although diet-dependent enrichment of Firmicutes has been observed in the intestines of multiple vertebrate hosts, the ecological processes underlying this enrichment remain unknown. We speculated that the Firmicutes enrichment is due in part to their improved ability to initiate or maintain colonization of the intestine in the presence of dietary nutrients. To test this possibility, we inoculated GF zebrafish at 3 dpf with individual, culturable members of the zebrafish microbiota (a process called monoassociation; Table 3.S5) and fed them a C or LC diet, or starved them, until 6 dpf. Then, we measured colonization efficiency in the zebrafish gut and surrounding water in the absence of competition from other microbes. Notably, colony forming unit (CFU) densities of a representative Firmicutes *Exiguobacterium* sp. ZWU0009 (class Bacilli) were below the level of detection in both gut and water under starved conditions but established robust colonization under fed conditions (Figure 3.5B). In contrast, the strains *Chryseobacterium*

sp. ZOR0023 (Bacteroidetes class Flavobacteria; Figure 3.5C) and *Pseudomonas* sp. ZWU0006 (γ-Proteobacteria; Figure 3.5D) were able to colonize the gut and water under starved conditions, with only modest increases in CFU density under fed conditions. This finding reveals that even in a simple community without microbial competition, the Firmicutes species we tested (*Exiguobacterium* sp.) requires nutrient-rich conditions to survive and colonize the intestine, whereas other species (*Chryseobacterium* sp. and *Pseudomonas* sp.) can successfully colonize even in the absence of dietary nutrients. These results suggest that diet-dependent Firmicutes enrichment in the intestines of CONVD animals might occur due to increased colonization efficiency of Firmicutes, independent of competition from other microbes.

Monoassociations reveal two distinct pathways for bacterial stimulation of intestinal fatty acid absorption

Our BODIPY-FL C_5 labeling experiments revealed that the microbiota stimulated two phenotypes in intestinal FA absorption: a diet-dependent increase in LD number and a diet-independent increase in LD size. This result raised the possibility that bacterial taxa that were enriched in a diet-dependent manner, such as Firmicutes, might promote LD number. To test this hypothesis, we performed monoassociation experiments on C-fed GF zebrafish using the same bacterial strains as above and performed the BODIPY-FL C_5 delivery assay (Figure 3.6A-D). Intriguingly, monoassociation with the Firmicutes strain *Exiguobacterium* sp. ZWU0009 (Figure 3.6B), but not with the other two tested bacterial strains (Figure 3.6C,D), resulted in a significant increase in LD number (Figure 3.6E) compared to GF controls (Figure 3.6A). In contrast, monoassociation with *Exiguobacterium* sp. had no effect on LD size frequency, whereas the other two tested bacterial strains induced increases in the relative frequency of large LDs in the intestinal epithelium compared to GF controls

(Figure 3.6F). These results suggest two novel mechanisms for bacterial induction of intestinal FA absorption: a Firmicutes-specific induction of LD number and a non-Firmicutes bacterial induction of LD size (Figure 3.7).

DISCUSSION

Studies identifying the gut microbiota as a key environmental factor regulating energy balance have suggested that an improved understanding of the microbial contributions to host metabolism could lead to novel therapies for disorders such as obesity and malnutrition (Musso et al., 2011). Although microbial contributions to degradation of complex dietary carbohydrates have been studied extensively (Flint et al., 2008), the impact of the microbiota on dietary lipid metabolism has received relatively little attention. Previous investigations of dietary lipid metabolism in gnotobiotic mammals suggested that the presence of a microbiota results in reduced or unaltered dietary lipid harvest. However, these studies performed evaluations of serum lipid metabolites (Bäckhed et al., 2004; Bäckhed et al., 2007), serum chylomicrons (Velagapudi et al., 2010) or fecal crude fat (Yoshida et al., 1968), which do not distinguish between exogenous and endogenous lipid sources or between dietary and microbe-produced lipids. Here, we use an *in vivo* imaging strategy in transparent zebrafish larvae to uncover a novel role for the microbiota in stimulating dietary FA absorption in the intestinal epithelium.

Fatty acid absorption, intracellular LD assembly in enterocytes, and subsequent secretion as chylomicrons have been extensively studied. However, our mechanistic understanding of these *in vivo* physiologic processes remains incomplete (Phan and Tso,

2001), which poses challenges for understanding how they are regulated by environmental factors such as the microbiota. Our data reveals that colonization with a microbiota promotes epithelial absorption of FAs, resulting in increased number and size of LDs in enterocytes and increased accumulation of dietary FAs in extra-intestinal tissues. Our washout experiments indicate that the accumulation of LDs in the intestines of CONVD animals is not due to impaired export of chylomicrons, consistent with previous studies in mice (Bäckhed et al., 2007; Velagapudi et al., 2010). We propose three nonexclusive mechanisms by which microbes might stimulate FA absorption into enterocytes. First, microbes might increase bioavailability of FAs by modifying the production or composition of bile salts. Gut microbes can convert primary bile acids to a variety of secondary bile acids with different characteristics (Ridlon et al., 2006; Martin et al., 2007); however, the effect of secondary bile acids on enterocyte FA absorption has not been explored. Second, microbes could directly contribute to luminal lipolytic activity that promotes release of FAs from dietary triglycerides for potential absorption by the intestinal epithelium (Ringø et al., 1995). Finally, microbes might enhance FA absorption indirectly by stimulating pancreatic enzyme activity or the inherent absorptive capabilities of the intestinal epithelium. We anticipate that *in vivo* imaging and genetic analysis in the gnotobiotic zebrafish system will provide useful means of distinguishing between these possibilities.

Our results show that the presence of a microbiota promotes two distinct phenotypes of LD formation within the enterocyte: increased LD number and increased LD size. That these increases in LD size and number are detected 6 hrs after liposome exposure and not at the 3 hr timepoint suggests that the microbial colonization may increase enterocyte capacity for LD accumulation instead of accelerating LD formation. Enterocyte LD size and number are used here as distinct quantifiable phenotypes of FA absorption, but it is likely that the mechanisms regulating LD size and number are connected. Previous genetic

analyses of LD formation have established that these dynamic organelles are under complex regulatory control and functionally linked to other organelles and pathways (Guo et al., 2008; Beller et al., 2008). This is supported by our observation that LD size and number can be induced by distinct bacterial species and have distinct sensitivity to dietary status (see below), suggesting that these phenotypes are regulated at least in part by distinct mechanisms. Investigation of the mechanisms underlying the microbial and dietary regulation of these phenotypes could provide new insights into the complex process of enterocyte lipid metabolism.

In addition to revealing a novel role for the microbiota in stimulating FA absorption, our data establish a major role for diet in mediating that interaction. Although enterocyte LD size and hepatic LD accumulation was increased by the microbiota regardless of dietary status, increases in enterocyte LD number were only observed in fed, but not starved, animals. This finding raised the intriguing possibility that diet-induced alterations in gut microbiota composition might lead to enrichment of microbial species that promote increased LD number. A frequently observed pattern in humans, mice, and pythons is that the relative abundance of Firmicutes in the intestine is positively correlated with dietary caloric intake (see Introduction). Our evaluation of intestinal bacterial communities in fed and starved zebrafish revealed that this ecological principle also applies to bony fishes. Strikingly, diet-dependent enrichment of Firmicutes bacteria in the zebrafish intestine was not accompanied by a parallel enrichment in the surrounding water, which provides strong evidence that the presence of diet exerts different selective pressures on microbial communities in the zebrafish gut versus the surrounding water. In the future, longitudinal analyses of the zebrafish gut and surrounding water could help resolve temporal relationships between the respective diet-induced alterations in microbial community composition in these different habitats. Because Firmicutes-enriched communities arising

from genetic or diet-induced obesity have been shown to promote positive energy balance, the mechanisms underlying diet-dependent enrichment of Firmicutes could be targeted to control energy balance in humans and other animals.

The mechanisms that promote Firmicutes abundance in nutrient-rich environments remain unknown. Our monoassociation experiments suggest that the diet-dependent enrichment of Fimicutes may be due, at least in part, to an autonomous bacterial requirement for diet-derived nutrients to allow colonization. In contrast, the non-Firmicutes bacteria tested here colonized in both starved and fed conditions, suggesting that nutritional niches provided by the host are sufficient for these organisms to colonize the zebrafish intestine. It will be interesting to determine if diet-dependent enrichment of Firmicutes in the context of a more complex microbial community is also mediated in part by inter-microbe competitions. Furthermore, it will be important to determine whether the impact of diet on bacterial colonization of the gut is mediated by permitting initial gut colonization and/or maintenance of colonization over time.

Strikingly, the diet-dependent effects on FA absorption, microbial community composition and diversity, and bacterial colonization efficiency were observed in animals fed a control or low-calorie diet. The low-calorie diet was generated by specifically depleting protein, which is one of the major energy sources in the zebrafish diet. Our results indicate that these diet-dependent events are not mediated by gross caloric value or protein content in the diet and that they may instead be mediated by other dietary nutrients. Identification of the specific nutrients that regulate these aspects of gut microbial ecology and host FA absorption could provide new dietary approaches for controlling these processes to promote health in a diversity of vertebrate hosts.

Our results also reveal that different bacterial species are capable of eliciting distinct FA absorption phenotypes in fed zebrafish: a Firmicutes strain induced increased LD

number, and the other two tested non-Firmicutes strains induced increased LD size. Although it remains unknown if these findings are generalizable to other members of their respective phyla, these data are consistent with our observations that fed CONVD animals enriched with gut Firmicutes display increased LD number and all CONVD animals display increased LD size. Based on these results, we propose two distinct mechanisms to explain the observed diet-dependent interactions between gut microbial ecology and host FA absorption. First, Firmicutes are enriched in the intestines of fed animals, where they enhance the ability of host enterocytes to create or retain LDs from absorbed FAs. Second, other non-Firmicutes bacteria that colonize the intestine regardless of dietary status induce an increased accumulation of large LDs within host enterocytes. In animals that are fed, these two bacterial signals combine to stimulate FA absorption through increases in both LD size and number (Figure 3.7). Because the enterocyte LDs evaluated here are likely to include both the temporary storage organelles under conditions of high-fat feedings (Glatz et al., 2010) and chylomicrons, the relative impact of microbial status on these two types of enterocyte LDs will require investigation. Future work will need to define the specific bacterial factors or activities that elicit these distinct phenotypes and to reveal how the host perceives and responds to these bacterial cues to modify distinct aspects of dietary lipid absorption.

FIGURES

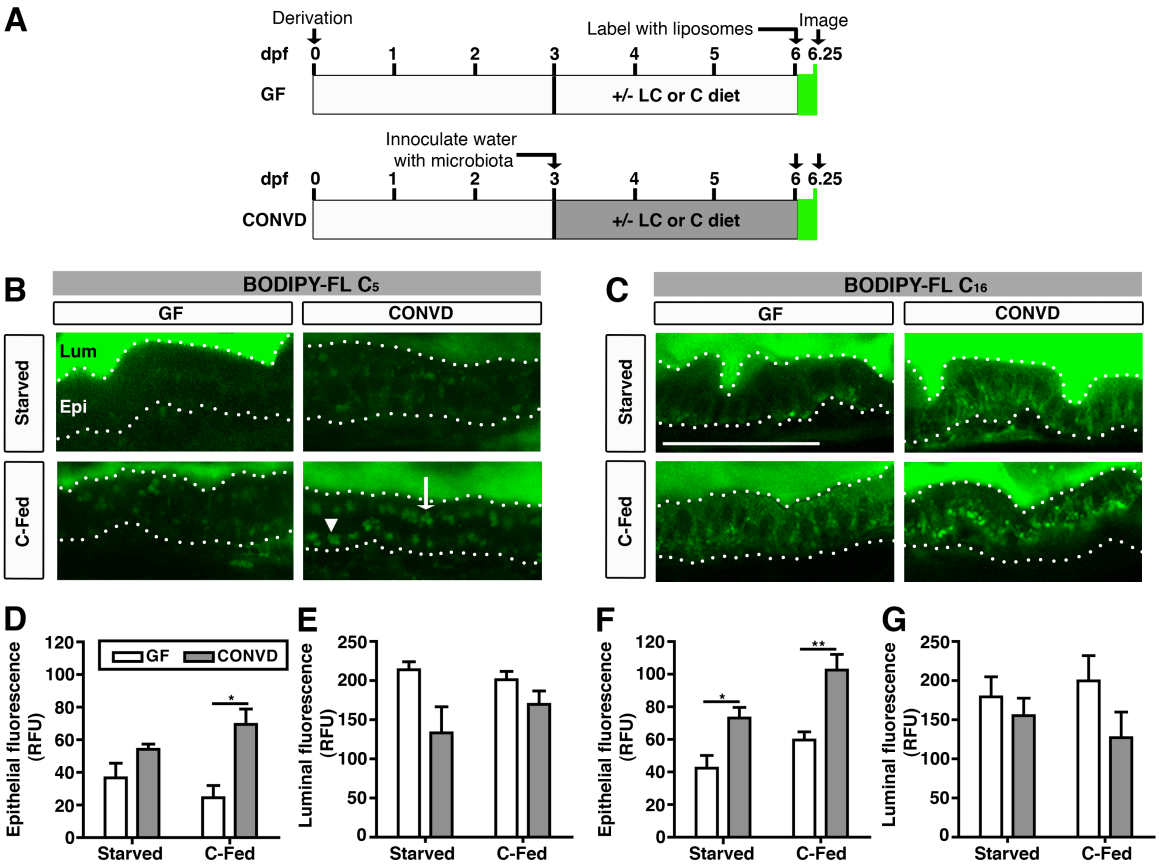


Figure 3.1. Liposome delivery assay shows that fatty acids accumulate in the intestinal epithelium in the presence of microbiota and diet.
(A) Schematic of BODIPY-FL delivery assay in gnotobiotic zebrafish. We derive zebrafish embryos germ-free (GF) at 0 days post-fertilization (dpf) and either rear them GF (top) or inoculate their water at 3 dpf with a normal microbiota (conventionalized, CONVD; bottom). From 3-6 dpf, fish are either starved (all figures except 3.3 and 3.6), or are fed a control (C) (all figures) or low calorie (LC) (Figures 3.4,3.6,3.S1-6) diet. At 6 dpf, we incubate zebrafish with BODIPY-FL liposomes for 6 hrs and image or fix them for later imaging.
(B,C) Representative confocal images of the intestines of live 6 dpf GF and CONVD zebrafish incubated with BODIPY-FL liposomes. Scale bar, 50 μm.
(B) The intestinal lumen (Lum) and epithelium (Epi; dotted line) of GF and CONVD zebrafish incubated with BODIPY-FL C_5 liposomes. The epithelium shows apical (arrow) and basolateral accumulation of lipid droplets (arrowhead).
(C) Incubation with BODIPY-FL C_{16} liposomes shows fluorescent signal accumulation in the intestinal epithelium that is similar to, but more diffuse than, that observed with BODIPY-FL C_5.
(D-G) Quantification of total epithelial (D,F) and luminal (E,G) fluorescence expressed in relative fluorescence units (RFU). Values represent the means ± SEM from 3 independent

experiments, with significance by Student's t test using Bonferroni's correction for multiple comparisons: *, $p<0.05$; **, $p<0.01$.

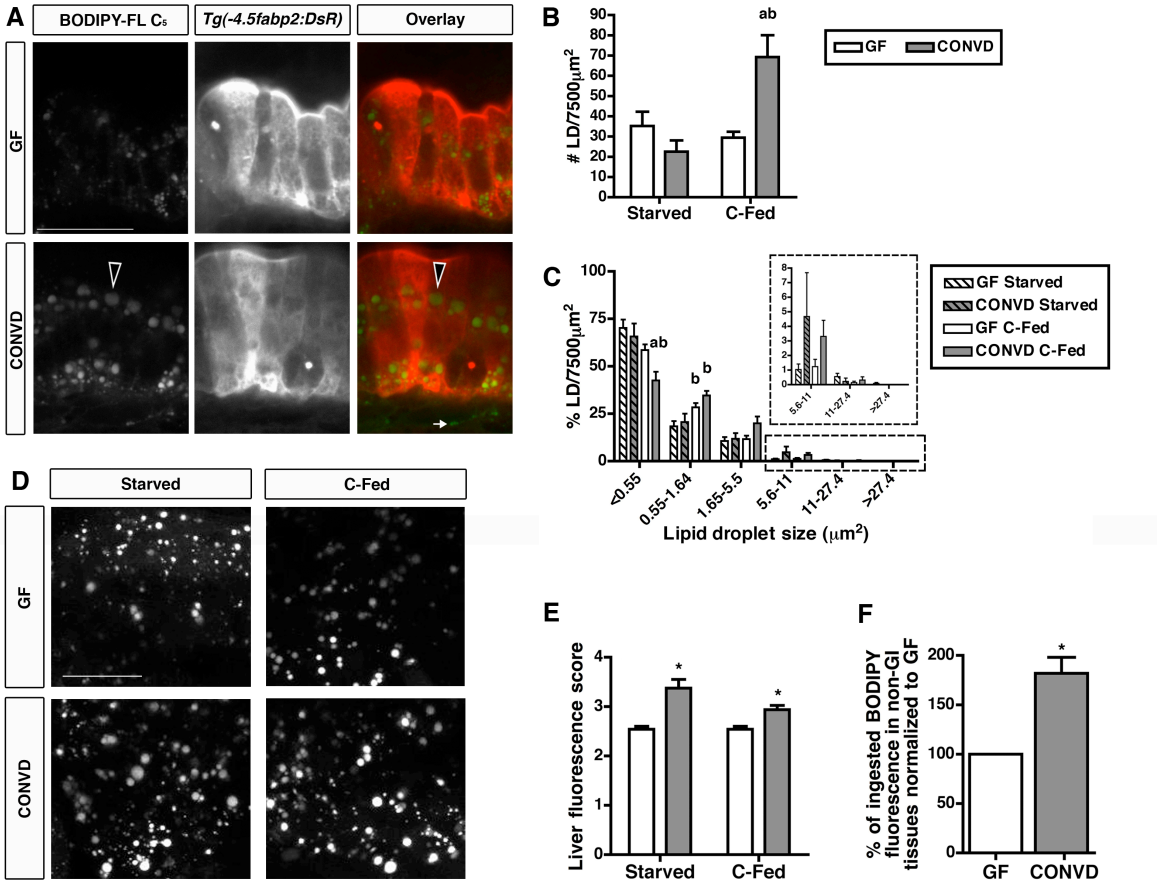


Figure 3.2. The microbiota stimulates lipid absorption into intestinal epithelial lipid droplets and extra-intestinal tissues.

(A) Representative confocal images of fixed 6 dpf *Tg(-4.5fabp2:DsRed)* GF and CONVD zebrafish fed a control diet and incubated with BODIPY-FL C_5 liposomes for 6 hrs. Scale bar, 20 μm. Intestinal epithelial cells identified by DsRed expression show BODIPY-FL accumulation as lipid droplets (LDs) in the epithelium (arrowheads) and as secretion in chylomicrons (arrow; A). Large LDs are detected in the epithelium of CONVD zebrafish (arrowhead; A).

(B,C) Lipid droplet quantification assay was developed using Volocity software (see Figure 3.S1) to determine LD number (B) and size frequency (C) in an epithelial region of interest (7500 μm^2). The graphs depict the mean ± SEM of at least two independent experiments (3-15 fish/condition/experiment). Results of Student's t test with the Bonferroni's correction for multiple comparisons: a, significant vs. GF fed same diet; b, significant vs. starved in same microbial condition. See Figure 3.S2 for data from the 3 hr timepoint.

(D) Representative confocal images from the livers of 6 dpf GF and CONVD zebrafish that were starved or fed a control diet and incubated with BODIPY-FL C_5 FA liposomes for 6 hrs. The images are maximum fluorescence projections from 10 Z-stack slices (total Z-depth = 12.5 μm). Scale bar, 20 μm.

(E) Fluorescence quantification in livers of 6 dpf GF and CONVD zebrafish that were starved or fed control diet and incubated with BODIPY-FL C_5 FA liposomes for 6 hrs. The graph

depicts the mean ± SD of two independent experiments (3-5 fish/condition/experiment) that were scored blindly (score scale 0-5; based on LD number, size, and fluorescence intensity). (F) Non-GI BODIPY-FL C_5 FA fluorescence in GF and CONVD C-fed zebrafish. The data represents mean ± SD of two experiments (20-30 carcasses or 9-10 whole larvae/condition/experiment). Significance was determined by Student's t test: *, $p<0.05$ (E,F).

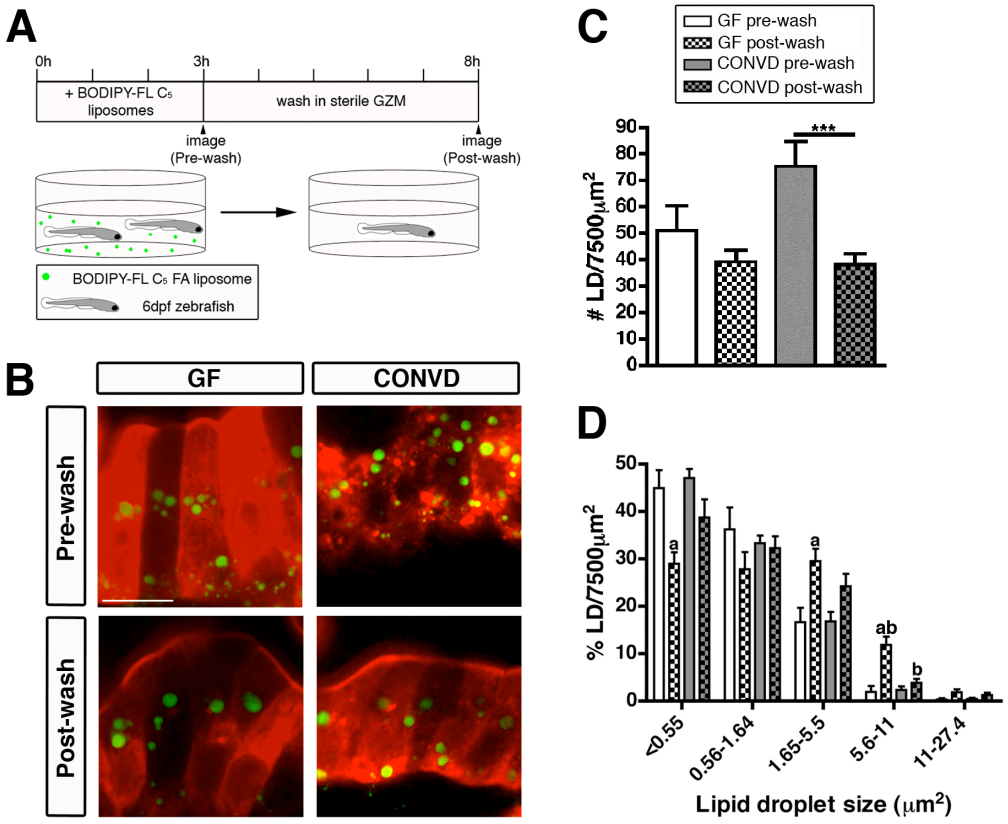


Figure 3.3. Lipid droplet clearance is more efficient in the presence of microbiota.
(A) Schematic representation of the BODIPY-FL C_5 washout experiment. We incubate 6 dpf zebrafish with BODIPY-FL C_5 for 3 hrs. A subgroup is briefly washed and fixed for confocal imaging (Pre-wash), and the remainder are transferred to sterile GZM for additional 5 hrs prior to fixation and imaging (Post-wash).
(B) Representative confocal images of control-fed GF and CONVD zebrafish pre- and post-wash. Scale bar, 10 μm.
(C,D) Quantification of lipid droplet (LD) number (C) and relative size frequency (D), shown as the mean ± SEM from two independent experiments (4-14 fish/condition/experiment). Results of Student's t test using Bonferroni's correction for multiple comparisons: ***, $p<0.001$; a, significant vs. pre-wash in same microbial condition; b, significant vs. same wash in other microbial condition.

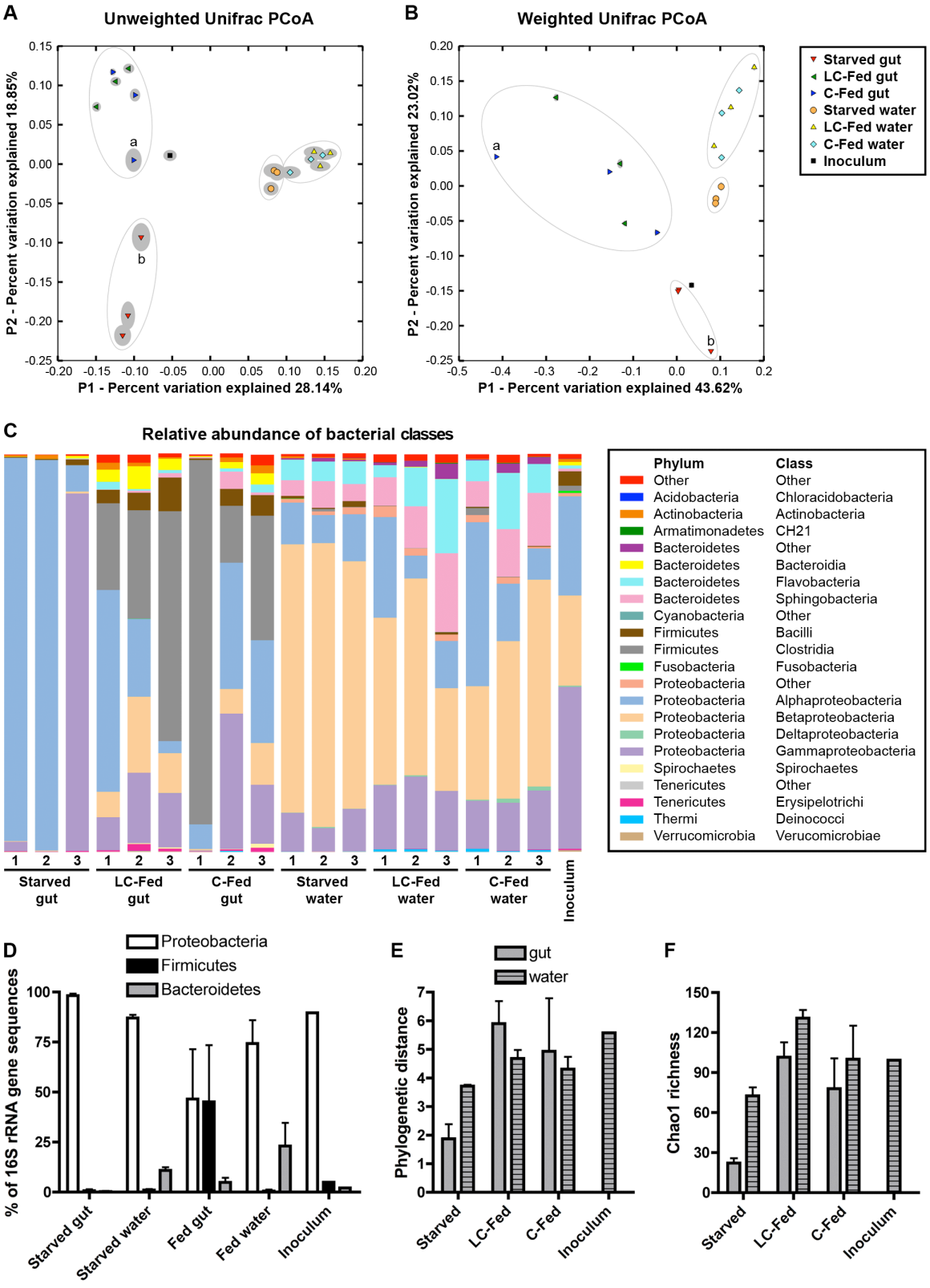

A
Unweighted Unifrac PCoA
P2 - Percent variation explained 18.85%
P1 - Percent variation explained 28.14%
a
b
B
Weighted Unifrac PCoA
P2 - Percent variation explained 23.02%
P1 - Percent variation explained 43.62%
a
b
Starved gut
LC-Fed gut
C-Fed gut
Starved water
LC-Fed water
C-Fed water
Inoculum
C
Relative abundance of bacterial classes
Starved gut
LC-Fed gut
C-Fed gut
Starved water
LC-Fed water
C-Fed water
Inoculum
Phylum
Class
Other
Other
Acidobacteria
Chloracidobacteria
Actinobacteria
Actinobacteria
Armatimonadetes
CH21
Bacteroidetes
Other
Bacteroidetes
Bacteroidia
Bacteroidetes
Flavobacteria
Bacteroidetes
Sphingobacteria
Cyanobacteria
Other
Firmicutes
Bacilli
Firmicutes
Clostridia
Fusobacteria
Fusobacteria
Proteobacteria
Other
Proteobacteria
Alphaproteobacteria
Proteobacteria
Betaproteobacteria
Proteobacteria
Deltaproteobacteria
Proteobacteria
Gammaproteobacteria
Spirochaetes
Spirochaetes
Tenericutes
Other
Tenericutes
Erysipelotrichi
Thermi
Deinococci
Verrucomicrobia
Verucomicrobiae
D
Proteobacteria
Firmicutes
Bacteroidetes
% of 16S rRNA gene sequences
Starved gut
Starved water
Fed gut
Fed water
Inoculum
E
gut
water
Phylogenetic distance
Starved
LC-Fed
C-Fed
Inoculum
F
Chao1 richness
Starved
LC-Fed
C-Fed
Inoculum

Figure 3.4. 16S rRNA gene sequencing reveals distinct bacterial communities in the zebrafish gut and water that are strongly influenced by dietary status.
(A,B) UniFrac principal coordinates analysis (PCoA) plots using unweighted (A; community composition) and weighted (B; community structure) algorithms. Each replicate sample is represented by a single shape, as shown in the legend at right, with the solid grey ellipsoid around each shape indicating the confidence interval from 100 jackknife replicates of 500 sequences per sample. Apparent clusters of samples are indicated with open ovals. Samples C-Fed gut 1 (a) and Starved gut 3 (b) are labeled.
(C) Stacked bar graph showing relative abundance (Y-axis) of 16S rRNA gene sequences from different bacterial classes (legend at right) observed in different samples (X-axis).
(D) Percentage of 16S rRNA gene sequences classified as Proteobacteria, Firmicutes, and Bacteroidetes, shown as the mean ± SD across different replicate sample groups and the inoculum sample. See also Table 3.S3. Alpha diversity measures of (E) Phylogenetic distance and (F) Chao1 richness are shown as the mean ± SD across different replicate sample groups and the inoculum sample. See also Figure 3.S4 and Table 3.S4.

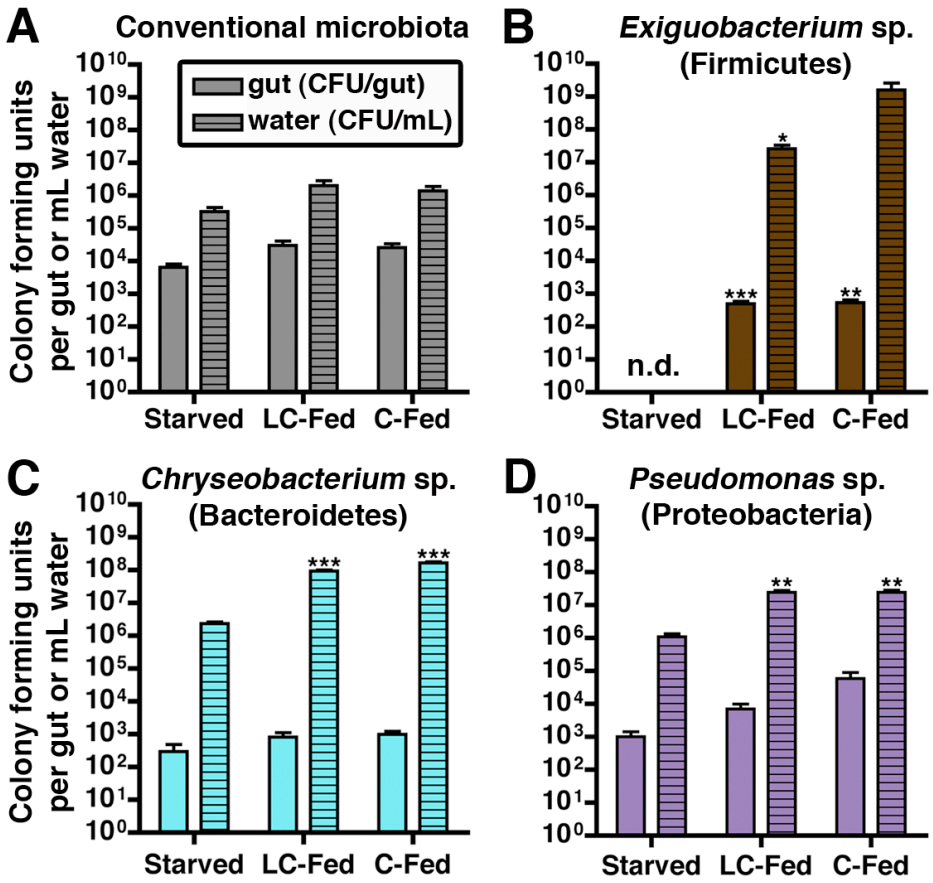


Figure 3.5. Monoassociation with individual community members reveals diet-dependent colonization of a representative Firmicutes species.
(A-D) Colony forming units (CFU) in the intestine (per dissected gut; n=4-5 per condition) or surrounding water (per mL; n=3 per condition) of 6 dpf zebrafish. The results represent the mean ± SEM of at least two independent experiments (n.d., not detected). Results of Student's t test using Bonferroni's correction for multiple comparisons: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.
(A) Density of the conventional microbiota in CONVD zebrafish.
(B-D) Bacterial densities in GF zebrafish monoassociated with (B) *Exiguobacterium* sp. ZWU0009 (Firmicutes), (C) *Chryseobacterium* sp. ZOR0023 (Bacteroidetes), or (D) *Pseudomonas* sp. ZWU0006 (γ-Proteobacteria).

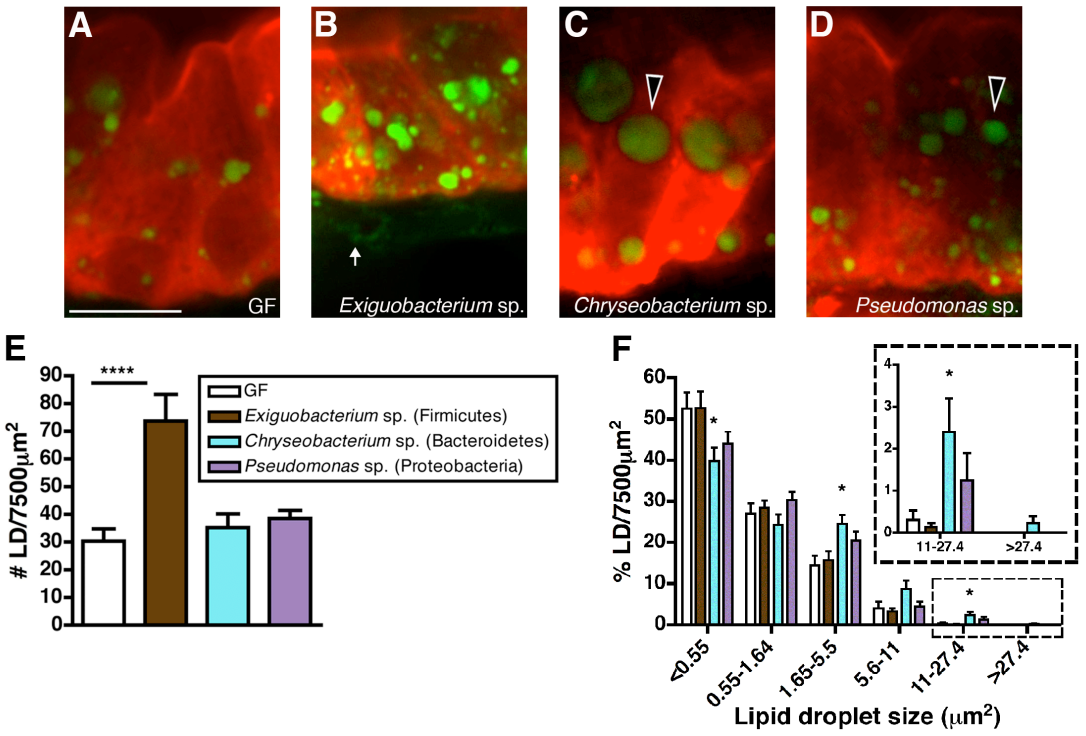


Figure 3.6. Monoassociations reveal distinct bacterial mechanisms for inducing fatty acid absorption in the intestinal epithelium.

(A-D) Representative confocal images of the intestinal epithelium of 6 dpf C-fed zebrafish raised GF (A) or monoassociated with *Exiguobacterium* sp. ZWU0009 (Firmicutes) (B), *Chryseobacterium* sp. ZOR0023 (Bacteroidetes) (C), or *Pseudomonas* sp. ZWU0006 (γ-Proteobacteria) (D). Prior to imaging, zebrafish are incubated with BODIPY-FL C_5 liposomes for 6 hrs and then treated as described in Figure 3.2A.

(E) Lipid droplet quantification shows increased epithelial LD number in zebrafish monoassociated with *Exiguobacterium* sp. but not with other bacterial species compared to GF controls.

(F) Analysis of the relative frequency of LD sizes shows an increased frequency of medium and large lipid droplets in the zebrafish monoassociated with *Chryseobacterium* sp. or *Pseudomonas* sp. but not with *Exiguobacterium* sp.. The data represent the mean ± SEM of at least two independent experiments (5-16 fish/condition/experiment). Significant differences to GF controls were identified by Student's t test using Bonferroni's correction for multiple comparisons: *, $p<0.05$; ***, $p<0.001$.

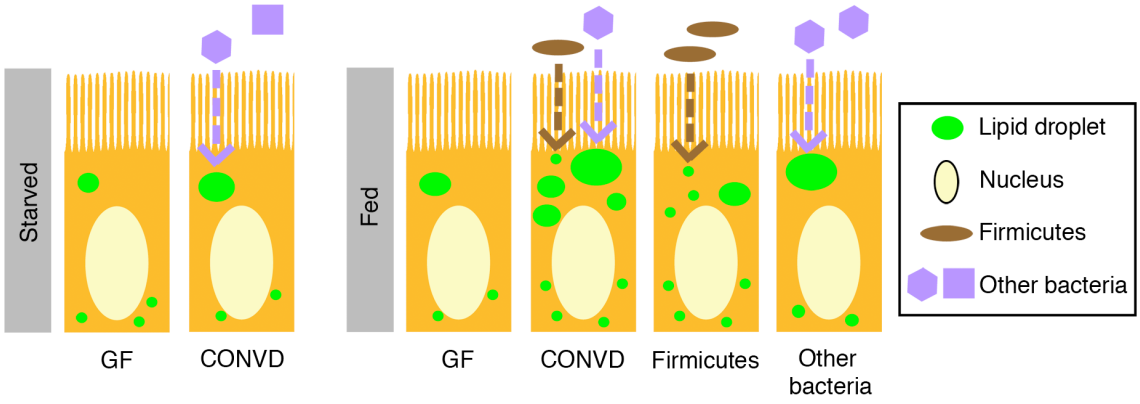


Figure 3.7. Model for diet-dependent microbial regulation of intestinal fatty acid absorption.
The microbiota promotes epithelial LD size independent of diet (dashed violet arrow). The presence of diet promotes LD number in CONVD and in zebrafish monoassociated with a Firmicutes strain (i.e., *Exiguobacterium* sp. ZWU0009; dashed brown arrow). Monoassociation with other bacterial strains from Bacteroidetes (i.e., *Chryseobacterium* sp. ZOR0023) or γ-Proteobacteria (i.e., *Pseudomonas* sp. ZWU0006) promotes LD size. Although the extent to which these findings are generalizable to their respective phyla remains unclear, these data suggest two bacterial mechanisms that promote distinct LD phenotypes: a Firmicutes-induced increase in LD number and a non-Firmicutes bacterial induction in LD size.

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL RESULTS

The microbiota and diet do not affect feeding behavior or digestive organ size in 6 dpf zebrafish

To explain the observed diet-dependent microbial regulation of dietary lipid accumulation in the zebrafish intestine (Figures 3.1, 3.2), we tested several working hypotheses. We first tested the hypothesis that C-fed CONVD fish are hyperphagic and ingest more liposome substrate than GF controls, leading to increased levels of fatty acids available for uptake in the intestine. Using a fluorescent bead-eating assay to measure feeding behavior, we found a small effect of diet on ingestion rates between all tested microbial conditions ($p<0.05$), suggesting that feeding increases ingestion behavior independent of microbial colonization (Figure 3.S3A). We next tested the hypothesis that microbial colonization and feeding promote dietary lipid absorption in 6 dpf zebrafish larvae by altering the overall body size or the size of digestive organs. Although the presence of microbiota affected body length (measured as standard length) ($p<0.05$) (Figure 3.S3B), liver, pancreas and pancreatic islet size revealed no significant effect of microbial or dietary status through 6 dpf (Figure 3.S3C-H). Therefore, diet-dependent microbial stimulation of fatty acid accumulation in the intestinal epithelium (Figures 3.1, 3.2) is not due to effects of microbiota on feeding behavior or digestive organ size.

The microbiota and exogenous food do not stimulate phospholipase or protease activity in the 6 dpf zebrafish intestine

Because digestive organ size and ingestion rates were similar between GF and CONVD zebrafish, we next tested the hypothesis that the microbiota stimulates digestive

function. We recently showed that BODIPY-FL fatty acid analog absorption into the intestinal epithelium requires emulsification in phosphatidylcholine liposomes, suggesting that digestive enzymes acting on liposomes might promote epithelial absorption of fatty acid analogs (Carten et al., 2011). We previously developed a method for measuring the activity of digestive enzymes phospholipase and protease in the intestinal lumen by feeding zebrafish caged fluorescent substrates PED6 and EnzChek, respectively (Farber et al., 2001; Hama et al., 2009). We fed PED6 and EnzChek reporters to 6 dpf GF and CONVD zebrafish to investigate how the microbiota and diet affect digestive function. Fluorescent PED6 and EnzChek products were observed in both the intestinal lumen as well as the gallbladder (Figure 3.S4A-F). Quantification of luminal fluorescence revealed that feeding increased PED6 fluorescence in GF but not CONVD zebrafish (effect of microbiota and diet $p<0.0001$) (Figure 3.S4G). Feeding also increased luminal EnzChek fluorescence in GF and in CONVD animals, although CONVD levels were consistently lower than GF controls (effect of microbiota and diet $p<0.0001$)(Figure 3.S4H). This result suggests that phospholipase and protease activity levels in the intestine are reduced in CONVD compared to GF zebrafish, which is consistent with previous studies in other gnotobiotic vertebrate hosts (Reddy et al., 1969; Lepkovsky et al., 1964; Pollak and Montgomery, 1994). Our observations that phospholipase activity is reduced by microbes only in fed but not in starved zebrafish is consistent with a previous report (Bates et al., 2006). These results are inconsistent with the hypothesis that the microbiota increases digestive function in fed zebrafish and suggest that diet-dependent microbial induction of lipid accumulation in the epithelium occurs through other mechanisms.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Confocal imaging of live BODIPY-FL labeled zebrafish

Live imaging of BODIPY-FL labeled zebrafish was performed on the Leica TCS SP2 at the Carnegie Institution of Embryology. Anaesthetized zebrafish larvae were mounted on a coverslip in 3% methylcellulose as previously described (Carten et al., 2011). Another coverslip was gently added, and the larvae were imaged. Images were taken using HCX APO L W 40x (3.3 WD, 0.8 NA) and 63x (2.2 WD, 0.9 NA) oil-immersion objectives. Fluorescence quantification was performed in ImageJ by box analysis and was normalized to controls that were not incubated with liposomes. Quantified results are expressed as the mean ± SEM ($n \geq 5$ fish per condition; at least two independent experiments).

Confocal imaging of fixed BODIPY-FL labeled zebrafish

Imaging of fixed BODIPY-FL labeled zebrafish was performed on the Olympus FV1000 laser scanning microscope at the UNC-Olympus Research Imaging Center at UNC-Chapel Hill. For these experiments, we used the *Tg(-4.5fabp2:DsRed)*^{pd1000} zebrafish line that expresses DsRed specifically in intestinal enterocytes (Kanther et al., 2011), facilitating confident identification of BODIPY-FL LDs that accumulate in the epithelial monolayer. Imaging was performed with a 60x water-immersion objective (0.28 WD, 0.9 NA). We took Z-stacks through the same region of intestinal segment 1 in every fish using the multi Ar laser (488 nm excitation) and LD laser (559 nm excitation) at laser capacity of 3% and 6.9%, respectively. Z-stacks were collected using unidirectional laser scanning at 1.0–1.5 μm per slice. The image size was 640x640 pixels in the XY direction for quantified Z-stacks and 1600x1600 pixels for the images shown in Figures 3.2, 3.3 and 3.6.

Lipid droplet quantification protocol

Three independent epithelial regions of interest in each fish were selected randomly from sagittal Z slices near the middle of the intestine that contained a single layer of DsRed-positive epithelial cells below a clear luminal space. Quantification of BODIPY-FL fluorescence in the intestinal epithelium was performed in three individual slices from the overlay Z-stacks using the Volocity Visualization+Quantification module (Improvision) (see Figure 3.S1). For each slice, we identified a region of interest (ROI, 2500 μm^2) and applied a measurement protocol (Figure 3.S1F) to identify individual objects (lipid droplets) and their size (represented by area). The LD identification and measurement protocol consisted of the following steps, which are presented in order: find objects using % intensity (lower: 50%; upper: 100%); clip objects to ROIs; separate touching objects (object size guide: 5 μm^2); and filter measurements (ID > 3). Collected measurement values were exported to Microsoft Excel to determine total LD number and the number of LDs for each size category (as defined in Figures 3.2, 3.3 and 3.6). The total LD number is presented as the mean ± SEM of at least two independent experiments. The relative frequency of LD size categories was determined as a percentage of the total LD number from each individual fish and is presented as the mean ± SEM of at least two independent experiments.

Quantification of BODIPY fluorescence in liver

The Olympus FV1000 confocal microscope (60x objective) was used to create liver Z-stacks (Z-depth = 1.25 μm). The stacks were opened in the FluoView10-ASW viewer (version2.0) and exported as RAW data (16 bit; TIFF file). The same number of slices (10) were imported in ImageJ and Z-projections were created based on maximum fluorescence

intensity. All projections were clipped to the same area, randomized and blinded. Four subjects scored the images based on lipid droplet number and size. The individual scores were averaged per condition and then per experiment. The results are mean ± SEM from two experiments (3-5 animals per condition per experiment).

Quantification of ingested BODIPY in extra-intestinal tissues

GF and CONVD 6 dpf zebrafish were incubated with BODIPY-FL C_5 FA liposomes for 6 hrs. After incubation, the animals were washed three times with GZM. While kept on ice, the GI tracts (including intestine, liver, pancreas, gall bladder, and swim bladder) were microdissected and the rest of the animal (the carcass) was collected in Tris-EDTA extraction solution (20 mM Tris and 1 mM EDTA, pH 8.0; 100 μL of solution per 10 animals pooled, 20-30 animals per condition). In parallel, whole larvae were collected from each condition to calculate total ingested BODIPY and control for inter-experimental variation in BODIPY-FL C_5 loading. The carcasses were disrupted with a microtip sonicator on ice (5 second cycle; 1 sec ON/1 sec OFF) and lipid was extracted in a chemical hood at room temperature following a modified Bligh and Dyer method (1959). The following specified volumes are based on 10 carcasses per condition, except for the final resuspension volume. Briefly, the organic phase was extracted after mixing with 375 μL of Chloroform:Methanol (1:2, v/v). The samples were vortexed for 30-60 sec and spun at low speed to remove solvent from the cap. The mixture was left for 10 min at room temperature to permit complete lipid dissociation and interrupt enzyme activity. Chloroform and Tris-EDTA solution were added (125 μL each) subsequently to the mixture, and the samples were vortexed for 30 sec and spun at low speed to remove liquid from the cap. The samples were centrifuged at 2000 x g for 5 min to separate the aqueous (upper) from the organic (lower) phase. Upon

discarding the aqueous phase, the organic phase containing lipids was collected in a new microcentrifuge tube with a pipette tip pre-calibrated in chloroform. The middle phase (larval debris) was avoided. The total volume of the organic phase was measured and the samples were stored at -80°C overnight. Same volume from each condition was completely dried in a speed vacuum and the samples were resuspended in a final volume of 50 μL of Chloroform:Methanol (2:1, v/v). Ten microliters were immediately transferred to a new microcentrifuge tube, dried and resuspended in ethanol. BODIPY-FL C_5 FA standards were prepared in ethanol, and fluorescence from samples and standards was determined using FluoroMax-4 compact spectrofluorometer (HORIBA Scientific). Total lipid amount per carcass was determined based on a standard curve and the leftover volume in the organic phase. The percentage of non-GI BODIPY fluorescence was determined based on the ratio of fluorescence in the carcass preparation and the fluorescence in the whole animal preparation from a given experiment, which was then normalized to the respective GF sample.

Gross developmental measurements

Feeding behavior was determined using a bead-eating assay as previously described (Farber et al., 2001). Briefly, zebrafish were washed 3 times in GZM and transferred to 15 mL conical tubes, where they were incubated with a 0.5% solution of Fluoresbrite™ YG 2.0 μm microspheres (18338, Polysciences, Inc.). The fish were washed in GZM after a 45 min incubation, fixed in 4% PFA and 1% dimethyl sulfoxide (DMSO) for 1 hr, and washed prior to storage (4°C, O/N). Bead counting and imaging was performed on a Leica MZ 16F fluorescence stereomicroscope. Standard length was measured from the snout to the end of the notochord (Parichy et al., 2009). For digestive organ size

measurements, *Tg*(*lfabp:dsRed*; *elaA:EGFP*)^{gz15} zebrafish (Korzh et al., 2008) were used to calculate the area of the liver and pancreas. The transgenic line *Tg(in3.3-Mmu.Fos:GFP)* (Camp et al., 2012) was used to determine the area of the endocrine pancreas in 6 dpf zebrafish. The area of each digestive organ was measured using ImageJ.

Preparation of fluorescent substrates

PED6 [N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-1-palmitoyl-2-BODIPY-FL-pentanoyl-sn-glycero-3-phosphoethanolamine, D23739], EnzChek [BODIPY-TxR casein, E6639] and BODIPY FL C_5 [4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid, D3834] were purchased from Invitrogen, CA. PED6 was dissolved in chloroform and was TLC-purified as previously described (Hama et al., 2009). Purified PED6 was dissolved in chloroform (1 mg/mL) and stored long term at -80°C. EnzChek was dissolved in 0.1 M sodium bicarbonate (pH 8.3) at 1 mg/mL and stored at -20°C. BODIPY-FL C_5 was dissolved in chloroform at 1 mg/mL and stored at -80°C. Immediately prior to treatment, PED6 aliquots were dried and resuspended in 0.5% EtOH in sterile GZM (v/v). BODIPY-FL C_5 aliquots were also dried and resuspended in 0.1% EtOH in sterile GZM (v/v), prior to emulsification with egg yolk.

In vivo assay of phospholipase and protease activity

6 dpf zebrafish were washed with GZM and transferred to a 6-well plate at 40 fish/mL GZM. Both PED6 and EnzChek were added to the media at a final concentration of 2 μg/mL and 5 μg/mL, respectively. The fish were incubated with the reporters for 3 hrs at room temperature on a rocker. After incubation, the fish were washed 3 times with pre-chilled GZM that served as anaesthetic because it was previously shown that tricaine treatment

reduces the intestinal fluorescent signal of both reporters (Hama et al., 2009). Fish were mounted in 3% methylcellulose and imaged at identical short and long exposure times with Leica MZ 16F fluorescence stereomicroscope using GFP and DsRed filter sets. The short exposure time images were used to quantify the intestinal fluorescence in ImageJ, to limit pixel saturation. Briefly, a rectangular ROI was placed over the whole intestinal region, and a pixel intensity threshold was set to include all intestinal pixels but minimize the number of background pixels. Any fish that displayed hallmarks of developmental delay, including the presence of yolk or lack of inflated swim bladder, were excluded from the analysis. Normalized integrated density was generated against the average integrated density of the respective GF starved control. The data presented in box-and-whiskers plot represent the combination of three independent experiments (8-20 fish per condition were analyzed in each experiment).

Analysis of gut and water microbiota by pyrosequencing of 16S rRNA gene sequences

A large, single clutch of embryos was derived GF, split into sterile vented flasks, and raised until 3 dpf in sterile GZM. At 3 dpf, a sample of housing water was collected from our conventional aquaculture facility and was used to inoculate all sterile flasks using our standard protocols (Pham et al., 2008). A 2 mL aliquot of this inoculum was snap frozen for subsequent analysis. From 3-6 dpf, each flask was also fed with sterilized Zeigler Control or Low-Calorie diet or was starved (3 biological replicate flasks/diet, 25 larvae/flask). On 6 dpf, one 1 mL sample of housing water and one pool of 10 dissected digestive tracts from each flask were collected and snap frozen, and additional gut and water samples were taken for CFU analysis. Genomic DNA was extracted from the inoculum, gut, and water samples using established protocols (Ley et al., 2008). To evaluate the composition of the gut and water microbiota, the V1-V3 region of the 16S rRNA gene was amplified from genomic DNA

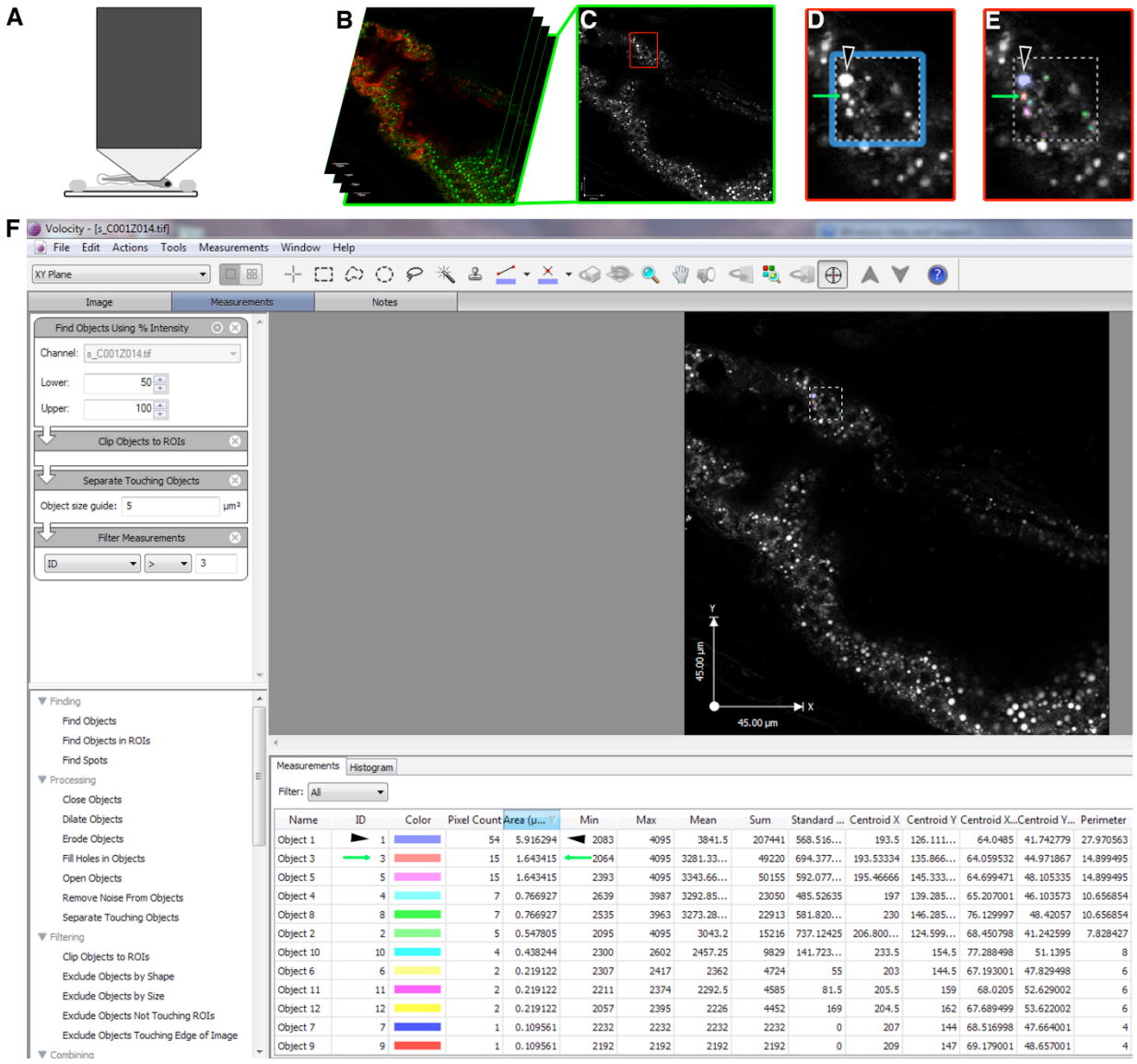
extracts using bar coded universal PCR primers 8F and 518R containing the A and B sequencing adaptors (454 Life Sciences). The forward primer (A-8FM) was 5'-gcctccctcgcgccatcag-AGAGTTTGATCMTGGCTCAG-3', where the lowercase is the A sequencing primer. The reverse primer (B-518) was 5'-gccttgccagcccgctcag-NNNNNNATTACCGCGGCTGCTGG-3', where the lowercase is the B sequencing primer and N represents a 6-base bar code that is unique for each sample. Prior to sequencing, amplicons from the individual PCR reactions samples were gel purified and quantified using the Quant-IT Pico-green dsDNA assay (Invitrogen). Final sample quality was assessed on an Agilent 2100 Bioanalyzer. For sequencing, amplicons from each reaction were mixed in equal amounts, based on concentration and subjected to emulsion PCR as recommended by 454 Life Sciences. Using the 454/Roche B sequencing primer kit, sequencing was performed from the "B" end on the Roche Genome Sequencer GS-FLX. Samples were combined in a single region of the pico-titre plate, and approximately 1000-2000 sequences were obtained from each sample. The data analysis pipeline removed low quality sequences (*i*) that do not perfectly match the PCR primer at the beginning of a read, (*ii*) that are shorter than 150 bp in length, (*iii*) that contain ambiguous nucleotides, or (*iv*) that do not match a barcode (allowing for 1.5 errors). Sequences were quality controlled and then binned according to bar codes.

Sequence analysis

Sequences were denoised and analyzed with the software package Quantitative Insights into Microbial Ecology (QIIME) version 1.3.0 using default parameters (Caporaso et al., 2010), except for allowing a minimum sequence length of 150 bp. Sequences were assigned to samples according to their barcodes (Table 4.S2), and similar sequences with a minimum pairwise identity of 97% were binned into OTUs using UCLUST

(http://www.drive5.com/usearch/) (Edgar, 2010). The most abundant sequence in each OTU was chosen to represent its OTU. Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier version 2.2 (Wang et al., 2007), which was retrained on the February 4, 2011 Greengenes dataset (http://greengenes.lbl.gov/Download/Sequence_Data/Fasta_data_files/Caporaso_Reference_OTUs/gg_otus_4feb2011.tgz) (McDonald et al., 2011). Sequences belonging to singleton OTUs or OTUs classified as plant chloroplasts were considered contaminants and were removed from the analysis.

SUPPLEMENTAL FIGURES



Name	ID	Color	Pixel Count	Area (µ...	Min	Max	Mean	Sum	Standard ...	Centroid X	Centroid Y	Centroid X...	Centroid Y...	Perimeter
Object 1	1		54	5.916294	2083	4095	3841.5	207441	568.516...	193.5	126.111...	64.0485	41.742779	27.970563
Object 3	3		15	1.643415	2064	4095	3281.33...	49220	694.377...	193.53334	135.866...	64.059532	44.971867	14.899495
Object 5	5		15	1.643415	2393	4095	3343.66...	50155	592.077...	195.46666	145.333...	64.699471	48.105335	14.899495
Object 4	4		7	0.766927	2639	3987	3292.85...	23050	485.52635	197	139.285...	65.207001	46.103573	10.656854
Object 8	8		7	0.766927	2535	3963	3273.28...	22913	581.820...	230	146.285...	76.129997	48.42057	10.656854
Object 2	2		5	0.547805	2095	4095	3043.2	15216	737.12425	206.800...	124.599...	68.450798	41.242599	7.828427
Object 10	10		4	0.438244	2300	2602	2457.25	9829	141.723...	233.5	154.5	77.288498	51.1395	8
Object 6	6		2	0.219122	2307	2417	2362	4724	55	203	144.5	67.193001	47.829498	6
Object 11	11		2	0.219122	2211	2374	2292.5	4585	81.5	205.5	159	68.0205	52.629002	6
Object 12	12		2	0.219122	2057	2395	2226	4452	169	204.5	162	67.689499	53.622002	6
Object 7	7		1	0.109561	2232	2232	2232	2232	0	207	144	68.516998	47.664001	4
Object 9	9		1	0.109561	2192	2192	2192	2192	0	209	147	69.179001	48.657001	4

Figure 3.S1. Development of a lipid droplet quantification assay for the zebrafish intestine.

(A) Z-stack movies collected on the Olympus FV1000 laser scanning microscope were scanned using the FV1000 FLUOVIEW software. To determine single epithelial layers, we overlayed the GFP signal from the BODIPY-FL C_5 fatty acid and the DsRed signal from the *Tg(-4.5fabp2:DsRed)* line, which expresses DsRed in intestinal epithelial cells. We selected three slices with independent regions of interest per fish and extracted the GFP signal only in Volocity 5.5.1.

(B-F) Lipid droplet number and size quantification in Volocity. We imported the Z-stacks from individual fish in Volocity and opened the selected slices (B) to quantify the fluorescent signal. (C) Scale arrow, 20 µm. The red box shows the magnified region in D and E. After

the region of interest was selected (purple box in D), we applied our quantification protocol (F) to identify individual lipid droplets as objects (E,F) with displayed size (Area, μm^2). (F) Scale arrow, 45 μm. The selected slice shows a medium (green arrow) and a large LD (black arrowhead, E,F).

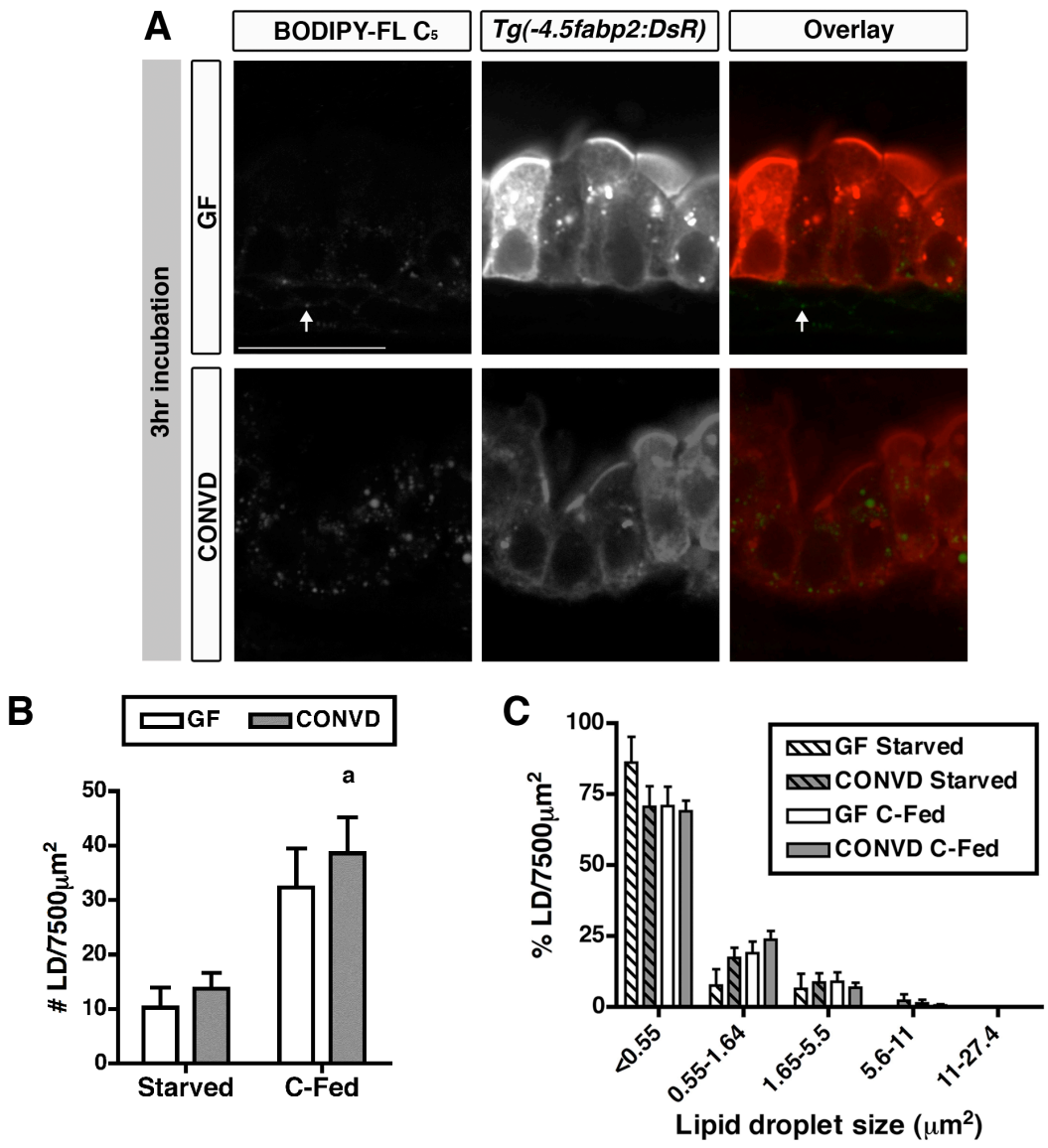


Figure 3.S2. A short (3 hr) incubation with BODIPY-FL C_5 liposomes results in lipid droplet formation in 6 dpf GF and CONVD zebrafish.
(A) Representative confocal images of fixed 6 dpf *Tg(-4.5fabp2:DsRed)* GF and CONVD zebrafish fed a control diet and incubated with BODIPY-FL C_5 liposomes for 3 hrs. The lumen is at the top and epithelium at the bottom of all images. Scale bar, 20 μm. Intestinal epithelial cells are identified by DsRed expression, and BODIPY-FL is detected in the epithelium as small lipid droplets (LDs) and secreted as chylomicrons (arrow; A).
(B and C) Lipid droplet quantification assay was developed using Volocity software (see Supplemental Experimental Procedures and Figure 3.S1) to determine LD number (B) and relative size frequency (C) in an epithelial region of interest (7500 μm^2). The graphs represent the mean ± SEM in starved and control-fed (C-Fed) zebrafish (at least two independent experiments; 4-10 fish/condition/experiment). Results of Student's t test corrected by Bonferroni's method for multiple comparisons: a, significant vs. same microbial

condition on different diets. The LD number (B) and size frequency (C; area) are similar between GF and CONVD zebrafish after the 3 hr incubation.
(C) Lipid droplet size (represented as %LD from total) shows high abundance of small LDs (<0.55 μm^2) in all microbial and dietary conditions. Similar data from longer 6 hr incubations with BODIPY-FL C_5 liposomes are shown in Figure 3.2.

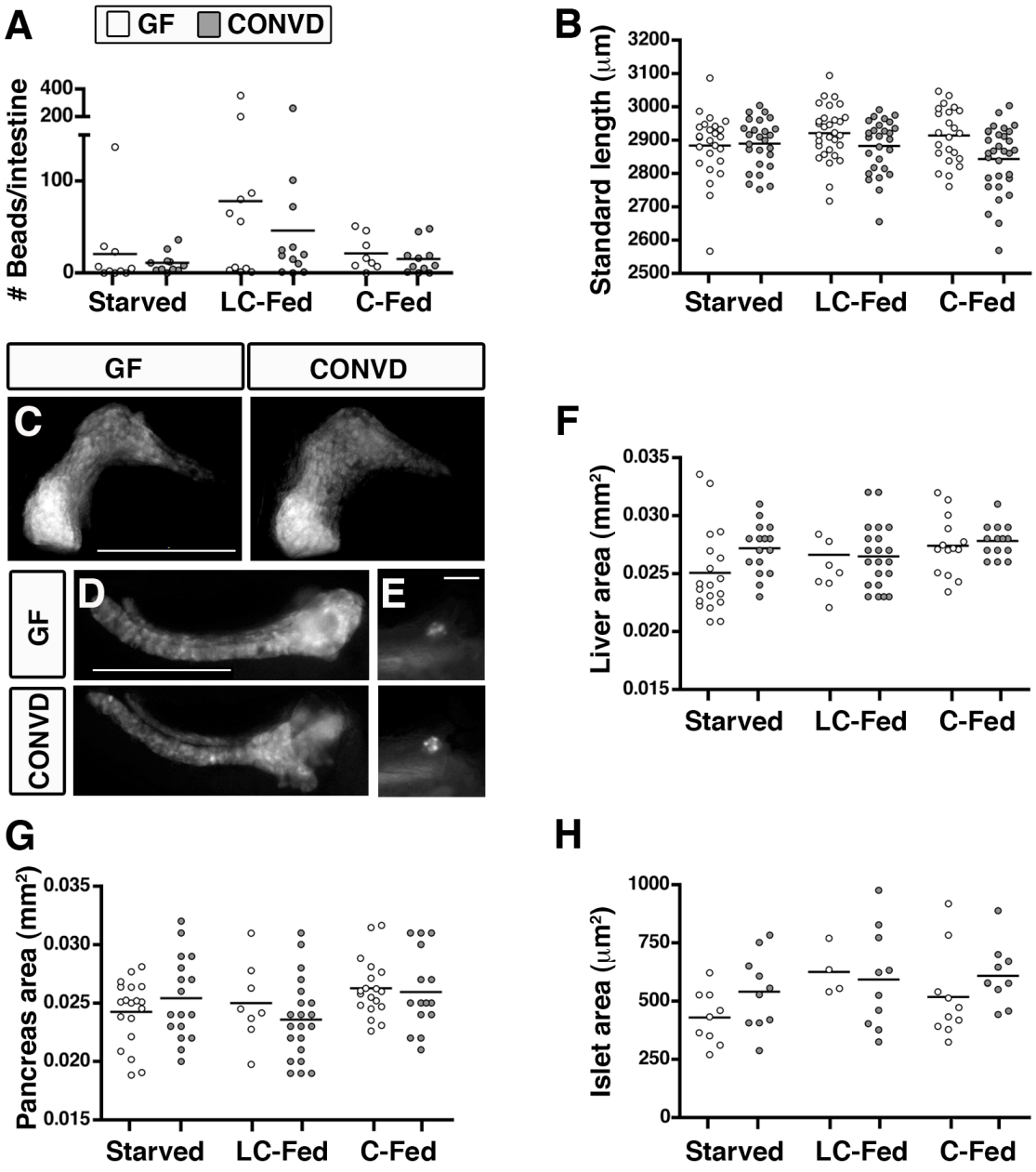


Figure 3.S3. GF and CONVD zebrafish have similar feeding behavior, growth rates and development of the GI tract.

(A) A bead-eating assay was used to determine the feeding behavior of 6 dpf GF and CONVD zebrafish reared under different dietary conditions. We counted the number of fluorescent microspheres in the intestines of individual fixed zebrafish. Zebrafish under the different microbial and dietary stimuli show similar number of ingested beads. The results show individual samples and the means from two independent experiments.

(B) Body size was determined by measuring the length from the snout to the end of the notochord (standard length). The graph represents means from at least two independent experiments.

(C-E) Transgenic zebrafish lines were used to measure the area of GI tract organs – liver, pancreas and pancreatic islet. We used the *Tg(lfabp:DsRed; elaA:GFP)* line (C,D) to determine the size of the liver and pancreas. Scale bar, 200 μm. We used *Tg(in3.3-Mmu.Fos:GFP)* fish to measure the area of the single pancreatic islet (E). Scale bar, 50 μm. (F-H) Quantification of area size for liver, pancreas and islet. Measurements were collected from two independent experiments. The data analysis was performed in ImageJ and shows no significant effects of the microbiota and exogenous food on the development of liver (F), pancreas (G) and the islet (H) in 6 dpf zebrafish. Each circle represents results from a single animal. Student's t tests adjusted with Bonferroni's correction for multiple comparisons revealed no significant differences.

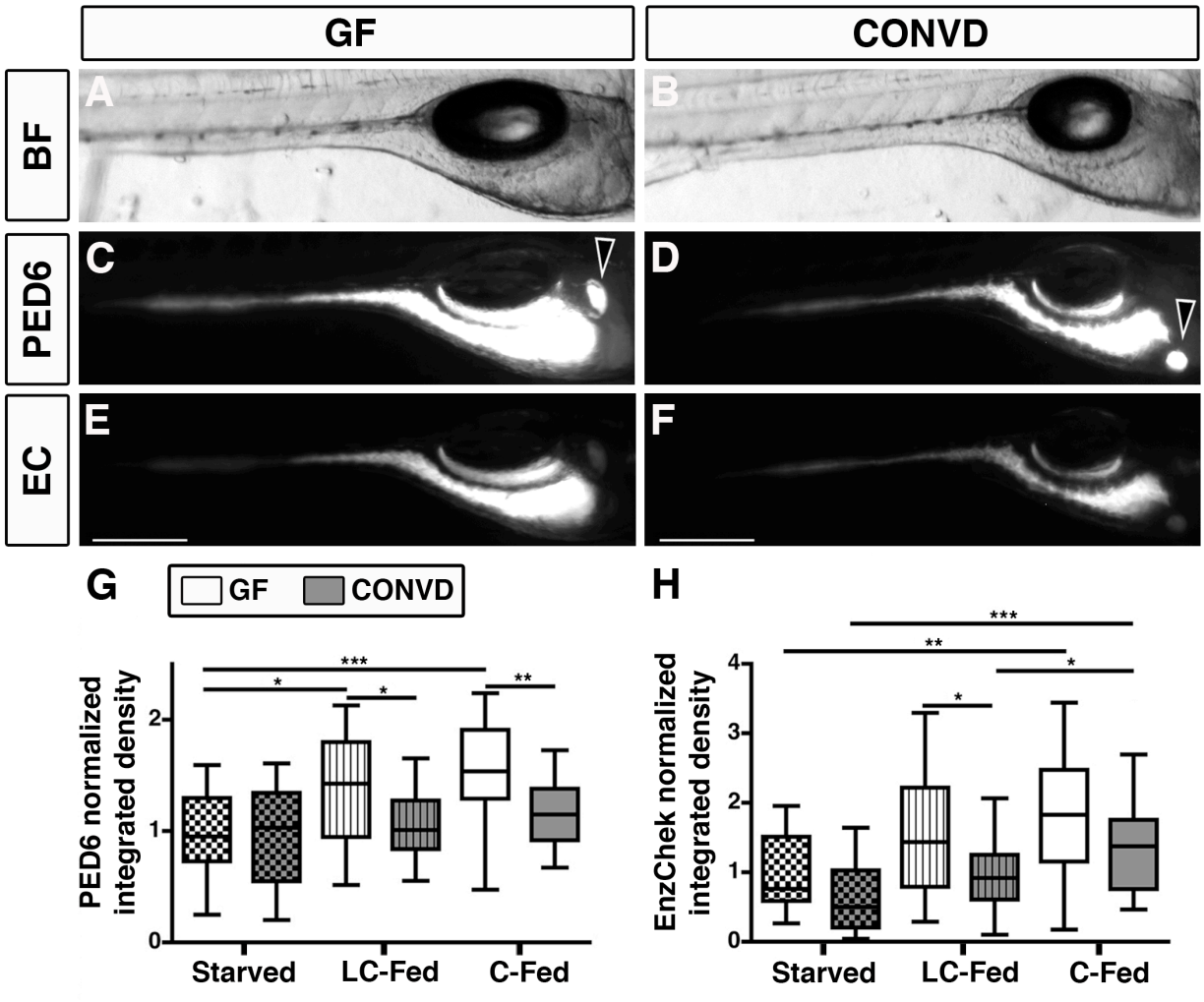


Figure 3.S4. The presence of microbiota and host diet does not stimulate digestive function in 6 dpf zebrafish.

(A-F) Representative images of live 6 dpf GF and CONVD zebrafish fed a control diet that were simultaneously soaked in quenched phospholipid PED6 and casein EnzChek (EC) for 3 hrs. Brightfield images (A,B) show the intestinal region of GF and CONVD zebrafish. (C,D) Overexposed images of unquenched PED6 show fluorescence in the intestinal lumen and gallbladder of GF (C) and CONVD (D) zebrafish (arrowhead).

(E,F) Overexposed images of unquenched EnzChek shows that TxRed signal is primarily detected in the intestinal lumen. Scale bar, 200 μm.

(G,H) Box-and-whiskers plots of total intestinal fluorescence in GF and CONVD zebrafish under three dietary conditions: starved, fed low-calorie (LC-Fed) or control diet (C-Fed). The fluorescent signal was quantified using underexposed images in ImageJ software as integrated density (area x mean fluorescence). The data were normalized to the mean of the GF starved condition for each substrate. PED6- (G) and EnzChek- (H) normalized integrated densities show reduced fluorescent signal in CONVD zebrafish fed LC or C diet vs. their GF controls. Results of Student's t test corrected with Bonferroni's method for multiple comparisons: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

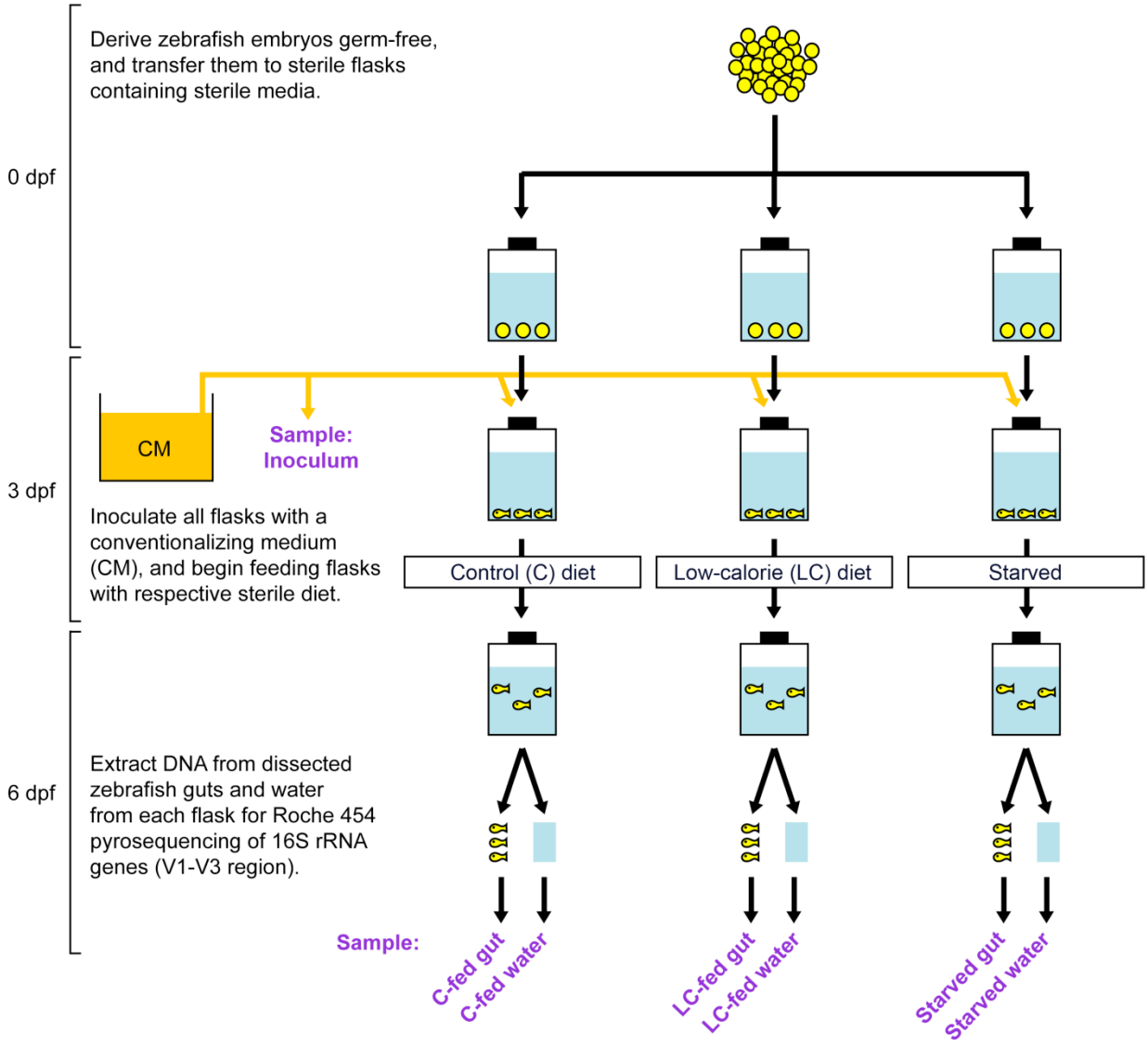


Figure 3.S5. Schematic depiction of experimental design for 16S rRNA gene sequence-based evaluations of gut and water bacterial communities under different diet conditions.
A single cohort of GF zebrafish embryos were split into multiple sterile flasks and colonized at 3 dpf by inoculating their housing water with a common conventionalizing medium containing a zebrafish microbiota (inoculum). From 3 to 6 dpf, flasks containing CONVD zebrafish were either starved or fed a C or LC diet. At 6 dpf, we extracted genomic DNA from dissected zebrafish guts and housing water from each flask and analyzed the respective bacterial communities using culture-independent 16S rRNA gene pyrosequence-based surveys (61,429 sequences in total; Table 3.S3). A single inoculum sample was collected, and biological triplicate samples were collected for all other sample types.

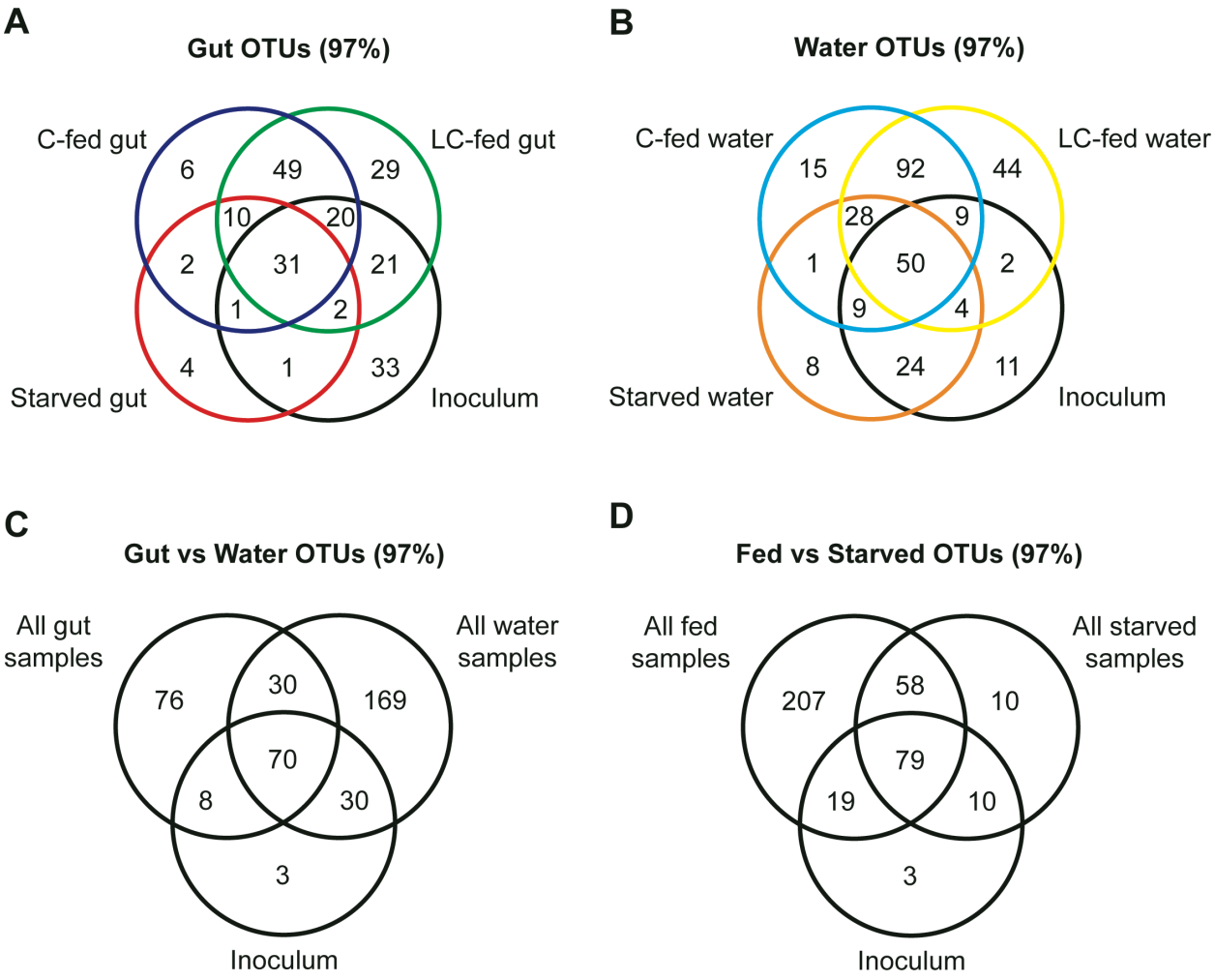


Figure 3.S6. Distribution of bacterial operational taxonomic units (OTUs) across samples.
We defined bacterial OTUs as 16S rRNA gene sequences that share a minimum pairwise identity of 97%. An OTU was called present in a sample type if it was observed in at least one of the respective samples.
(A) OTUs within different gut sample types referenced against the inoculum sample.
(B) OTUs within different water sample types referenced against the inoculum sample.
(C) OTUs within the inoculum sample referenced against those in all gut samples and all water samples.
(D) OTUs within the inoculum sample referenced against those in all fed samples and all starved samples.

SUPPLEMENTAL TABLES

Ingredient	Control Diet	Low Calorie Diet
Protein	45.14	20.03
Fat	15.09	15.08
Carbohydrate (not including fiber)	12.11	12.45
Fiber	0.93	0.62
Ash	19.92	45.08
Vitamin supplement	1x	1x
Gross Energy kcal/100g	450.96	309.13

Table 3.S1. Calculated proximate composition of custom Zeigler zebrafish larval diets

Sample Name	Flask	Diet	Environment	Barcode Sequence	Sample ID	Description of Sample	No. Seqs/Sample
Starved gut 1	10	Unfed	Gut	ATTACGCG	10g	Pooled digestive tracts from ten 6dpf ex-germ-free zebrafish larvae that were conventionalized but unfed since 3dpf	3013
Starved gut 2	11	Unfed	Gut	CAAGTGGA	11g	Pooled digestive tracts from ten 6dpf ex-germ-free zebrafish larvae that were conventionalized but unfed since 3dpf	2946
Starved gut 3	12	Unfed	Gut	CAGTTGAC	12g	Pooled digestive tracts from ten 6dpf ex-germ-free zebrafish larvae that were conventionalized but unfed since 3dpf	2886
LC-fed gut 1	4	Zeigler Low-Calorie Diet	Gut	ATCGGCTA	4g	Pooled digestive tracts from ten 6dpf ex-germ-free zebrafish larvae that were conventionalized and fed sterilized Zeigler Low-Calorie diet since 3dpf	1141
LC-fed gut 2	5	Zeigler Low-Calorie Diet	Gut	ACCAGTTG	5g	Pooled digestive tracts from ten 6dpf ex-germ-free zebrafish larvae that were conventionalized and fed sterilized Zeigler Low-Calorie diet since 3dpf	1927
LC-fed gut 3	6	Zeigler Low-Calorie Diet	Gut	ACGTCAAC	6g	Pooled digestive tracts from ten 6dpf ex-germ-free zebrafish larvae that were conventionalized and fed sterilized Zeigler Low-Calorie diet since 3dpf	2168
C-fed gut 1	1	Zeigler Control Diet	Gut	AACCAACC	1g	Pooled digestive tracts from ten 6dpf ex-germ-free zebrafish larvae that were conventionalized and fed sterilized Zeigler Control diet since 3dpf	2180
C-fed gut 2	2	Zeigler Control Diet	Gut	ATTAGGCC	2g	Pooled digestive tracts from ten 6dpf ex-germ-free zebrafish larvae that were conventionalized and fed sterilized Zeigler Control diet since 3dpf	871
C-fed gut 3	3	Zeigler Control Diet	Gut	ACACACTG	3g	Pooled digestive tracts from ten 6dpf ex-germ-free zebrafish larvae that were conventionalized and fed sterilized Zeigler Control diet since 3dpf	599
Starved water 1	10	Unfed	Water	ATTAGGCC	10M	Water from a flask containing 6dpf ex-germ-free zebrafish larvae that were conventionalized but unfed since 3dpf	5675
Starved water 2	11	Unfed	Water	ACACACTG	11M	Water from a flask containing 6dpf ex-germ-free zebrafish larvae that were conventionalized but unfed since 3dpf	3473
Starved water 3	12	Unfed	Water	ATCGGCTA	12M	Water from a flask containing 6dpf ex-germ-free zebrafish larvae that were conventionalized but unfed since 3dpf	4513
LC-fed water 1	4	Zeigler Low-Calorie Diet	Water	CTCTCTCT	4M	Water from a flask containing 6dpf ex-germ-free zebrafish larvae that were conventionalized and fed sterilized Zeigler Low-Calorie diet since 3dpf	5250
LC-fed water 2	5	Zeigler Low-Calorie Diet	Water	CTTCACCA	5M	Water from a flask containing 6dpf ex-germ-free zebrafish larvae that were conventionalized and fed sterilized Zeigler Low-Calorie diet since 3dpf	4692
LC-fed water 3	6	Zeigler Low-Calorie Diet	Water	GAACACCT	6M	Water from a flask containing 6dpf ex-germ-free zebrafish larvae that were conventionalized and fed sterilized Zeigler Low-Calorie diet since 3dpf	4564
C-fed water 1	1	Zeigler Control Diet	Water	CATCCATC	1M	Water from a flask containing 6dpf ex-germ-free zebrafish larvae that were conventionalized and fed sterilized Zeigler Control diet since 3dpf	4051
C-fed water 2	2	Zeigler Control Diet	Water	CCAATTGG	2M	Water from a flask containing 6dpf ex-germ-free zebrafish larvae that were conventionalized and fed sterilized Zeigler Control diet since 3dpf	3433
C-fed water 3	3	Zeigler Control Diet	Water	CGAACCAT	3M	Water from a flask containing 6dpf ex-germ-free zebrafish larvae that were conventionalized and fed sterilized Zeigler Control diet since 3dpf	3659
Inoculum	n/a	n/a	n/a	ACCAGTTG	CM	Conventionalizing media collected from a recirculating zebrafish aquaculture facility used to inoculate all germ-free fish flasks at 3dpf	4388

Table 3.S2. Description of samples subjected to 16S rRNA gene pyrosequencing

	Starved gut			LC-fed gut			C-fed gut			Starved water			LC-fed water			C-fed water			
Taxon	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**Inoculum**
Root;k__Bacteria;Other	0.10%	0.07%	0.24%	2.02%	2.02%	0.65%	0.23%	0.69%	2.67%	0.42%	0.60%	0.86%	1.90%	1.49%	1.99%	1.01%	1.98%	0.46%	1.07%
Root;k__Bacteria;Other;Other	0.10%	0.07%	0.24%	2.02%	2.02%	0.65%	0.23%	0.69%	2.67%	0.42%	0.60%	0.86%	1.90%	1.49%	1.99%	1.01%	1.98%	0.46%	1.07%
Root;k__Bacteria;Other;Other;Other	0.10%	0.07%	0.24%	2.02%	2.02%	0.65%	0.23%	0.69%	2.67%	0.42%	0.60%	0.86%	1.90%	1.49%	1.99%	1.01%	1.98%	0.46%	1.07%
Root;k__Bacteria;Other;Other;Other;Other	0.10%	0.07%	0.24%	2.02%	2.02%	0.65%	0.23%	0.69%	2.67%	0.42%	0.60%	0.86%	1.90%	1.49%	1.99%	1.01%	1.98%	0.46%	1.07%
Root;k__Bacteria;p__Acidobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%
Root;k__Bacteria;p__Acidobacteria;c__Chloracidobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%
Root;k__Bacteria;p__Acidobacteria;c__Chloracidobacteria;o__	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%
Root;k__Bacteria;p__Acidobacteria;c__Chloracidobacteria;o__;f__	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%
Root;k__Bacteria;p__Actinobacteria	0.37%	0.95%	0.24%	1.75%	0.88%	0.37%	0.18%	1.15%	2.00%	0.48%	0.20%	0.35%	0.00%	0.04%	0.09%	0.12%	0.09%	0.08%	0.96%
Root;k__Bacteria;p__Actinobacteria;c__Actinobacteria	0.37%	0.95%	0.24%	1.75%	0.88%	0.37%	0.18%	1.15%	2.00%	0.48%	0.20%	0.35%	0.00%	0.04%	0.09%	0.12%	0.09%	0.08%	0.96%
Root;k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales	0.37%	0.95%	0.17%	1.75%	0.88%	0.37%	0.18%	1.15%	2.00%	0.48%	0.12%	0.35%	0.00%	0.02%	0.09%	0.12%	0.09%	0.08%	0.89%
Root;k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae	0.00%	0.00%	0.00%	0.09%	0.10%	0.00%	0.00%	0.23%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.36%
Root;k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Gordoniaceae	0.00%	0.00%	0.00%	0.61%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae	0.17%	0.48%	0.03%	0.18%	0.47%	0.32%	0.05%	0.34%	0.67%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.14%
Root;k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	0.03%	0.10%	0.03%	0.70%	0.16%	0.00%	0.09%	0.23%	0.33%	0.18%	0.03%	0.07%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.18%
Root;k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Pseudonocardiaceae	0.17%	0.14%	0.00%	0.00%	0.10%	0.05%	0.00%	0.00%	0.00%	0.16%	0.06%	0.24%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%
Root;k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;Other	0.00%	0.24%	0.10%	0.18%	0.05%	0.00%	0.05%	0.34%	1.00%	0.09%	0.03%	0.00%	0.00%	0.02%	0.07%	0.02%	0.03%	0.08%	0.11%
Root;k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%
Root;k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%
Root;k__Bacteria;p__Actinobacteria;c__Actinobacteria;Other	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Actinobacteria;c__Actinobacteria;Other;Other	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Armatimonadetes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.18%	0.00%	0.15%	0.05%	0.00%
Root;k__Bacteria;p__Armatimonadetes;c__CH21	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.18%	0.00%	0.15%	0.05%	0.00%
Root;k__Bacteria;p__Armatimonadetes;c__CH21;o__	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.18%	0.00%	0.15%	0.05%	0.00%
Root;k__Bacteria;p__Armatimonadetes;c__CH21;o__;f__	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.18%	0.00%	0.15%	0.05%	0.00%
Root;k__Bacteria;p__Bacteroidetes	0.07%	0.03%	0.69%	5.08%	6.69%	4.75%	0.50%	6.77%	5.51%	9.50%	12.58%	10.44%	10.90%	21.99%	42.40%	11.95%	28.55%	22.36%	2.16%
Root;k__Bacteria;p__Bacteroidetes;c__Bacteroidia	0.07%	0.03%	0.45%	3.07%	5.76%	2.86%	0.18%	1.61%	2.84%	0.02%	0.00%	0.04%	0.00%	0.11%	0.04%	0.00%	0.03%	0.00%	0.66%
Root;k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales	0.07%	0.03%	0.45%	3.07%	5.76%	2.86%	0.18%	1.61%	2.84%	0.02%	0.00%	0.04%	0.00%	0.11%	0.04%	0.00%	0.03%	0.00%	0.66%
Root;k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__	0.00%	0.00%	0.00%	0.00%	0.36%	0.14%	0.00%	0.00%	0.17%	0.02%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.25%
Root;k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae	0.07%	0.03%	0.45%	0.18%	0.47%	0.18%	0.00%	0.23%	1.67%	0.00%	0.00%	0.04%	0.00%	0.09%	0.04%	0.00%	0.03%	0.00%	0.41%
Root;k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	0.00%	0.00%	0.00%	0.00%	0.05%	0.09%	0.00%	0.23%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;Other	0.00%	0.00%	0.00%	2.89%	4.88%	2.40%	0.18%	1.15%	1.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Bacteroidetes;c__Flavobacteria	0.00%	0.00%	0.14%	1.93%	0.42%	0.78%	0.05%	0.80%	2.00%	5.16%	4.95%	5.69%	3.05%	9.83%	18.67%	5.26%	14.13%	7.30%	0.80%
Root;k__Bacteria;p__Bacteroidetes;c__Flavobacteria;o__Flavobacteriales	0.00%	0.00%	0.14%	1.93%	0.42%	0.78%	0.05%	0.80%	2.00%	5.16%	4.95%	5.69%	3.05%	9.83%	18.67%	5.26%	14.13%	7.30%	0.80%
Root;k__Bacteria;p__Bacteroidetes;c__Flavobacteria;o__Flavobacteriales;f__Cryomorphaceae	0.00%	0.00%	0.03%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.56%	0.52%	0.42%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.02%
Root;k__Bacteria;p__Bacteroidetes;c__Flavobacteria;o__Flavobacteriales;f__Flavobacteriaceae	0.00%	0.00%	0.10%	1.93%	0.42%	0.69%	0.05%	0.80%	2.00%	4.60%	4.43%	5.27%	3.05%	9.83%	18.67%	5.21%	14.13%	7.30%	0.77%
Root;k__Bacteria;p__Bacteroidetes;c__Sphingobacteria	0.00%	0.00%	0.10%	0.09%	0.52%	1.11%	0.28%	4.36%	0.67%	3.96%	6.71%	4.30%	7.16%	10.53%	19.83%	6.39%	12.03%	13.34%	0.68%
Root;k__Bacteria;p__Bacteroidetes;c__Sphingobacteria;o__Sphingobacteriales	0.00%	0.00%	0.10%	0.09%	0.52%	1.11%	0.28%	4.36%	0.67%	3.96%	6.71%	4.30%	7.16%	10.53%	19.83%	6.39%	12.03%	13.34%	0.68%
Root;k__Bacteria;p__Bacteroidetes;c__Sphingobacteria;o__Sphingobacteriales;f__	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.23%	0.00%	1.11%	1.58%	0.93%	3.92%	3.18%	10.41%	3.09%	4.28%	5.99%	0.16%
Root;k__Bacteria;p__Bacteroidetes;c__Sphingobacteria;o__Sphingobacteriales;f__Flexibacteraceae	0.00%	0.00%	0.10%	0.00%	0.42%	1.11%	0.28%	4.13%	0.67%	2.10%	2.74%	2.46%	3.12%	7.16%	9.01%	3.21%	7.31%	6.83%	0.05%
Root;k__Bacteria;p__Bacteroidetes;c__Sphingobacteria;o__Sphingobacteriales;f__Sphingobacteriaceae	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.76%	2.39%	0.86%	0.08%	0.15%	0.24%	0.07%	0.35%	0.52%	0.48%
Root;k__Bacteria;p__Bacteroidetes;c__Sphingobacteria;o__Sphingobacteriales;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.04%	0.04%	0.18%	0.02%	0.09%	0.00%	0.00%
Root;k__Bacteria;p__Bacteroidetes;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.35%	0.92%	0.40%	0.69%	1.53%	3.86%	0.30%	2.36%	1.72%	0.02%
Root;k__Bacteria;p__Bacteroidetes;Other;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.35%	0.92%	0.40%	0.69%	1.53%	3.86%	0.30%	2.36%	1.72%	0.02%
Root;k__Bacteria;p__Bacteroidetes;Other;Other;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.35%	0.92%	0.40%	0.69%	1.53%	3.86%	0.30%	2.36%	1.72%	0.02%
Root;k__Bacteria;p__Cyanobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.04%	0.07%	0.05%	0.03%	0.00%	0.00%
Root;k__Bacteria;p__Cyanobacteria;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.04%	0.07%	0.05%	0.03%	0.00%	0.00%
Root;k__Bacteria;p__Cyanobacteria;Other;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.04%	0.07%	0.05%	0.03%	0.00%	0.00%
Root;k__Bacteria;p__Cyanobacteria;Other;Other;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.04%	0.07%	0.05%	0.03%	0.00%	0.00%
Root;k__Bacteria;p__Firmicutes	0.30%	0.34%	1.56%	25.24%	31.71%	66.37%	92.20%	18.60%	36.56%	0.65%	0.89%	1.57%	0.10%	0.00%	0.59%	2.07%	0.09%	0.22%	4.95%
Root;k__Bacteria;p__Firmicutes;c__Bacilli	0.20%	0.24%	1.39%	3.42%	4.41%	8.49%	0.41%	4.25%	5.18%	0.63%	0.29%	1.46%	0.06%	0.00%	0.59%	0.32%	0.06%	0.22%	3.65%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales	0.00%	0.07%	0.80%	0.26%	0.10%	0.05%	0.00%	0.11%	2.84%	0.12%	0.14%	0.82%	0.02%	0.00%	0.00%	0.00%	0.00%	0.08%	1.53%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae	0.00%	0.03%	0.80%	0.09%	0.05%	0.00%	0.00%	0.00%	2.50%	0.12%	0.14%	0.80%	0.02%	0.00%	0.00%	0.00%	0.00%	0.08%	1.28%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae	0.00%	0.03%	0.00%	0.18%	0.00%	0.05%	0.00%	0.11%	0.33%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.16%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales	0.20%	0.17%	0.55%	2.37%	1.71%	5.03%	0.37%	3.44%	1.00%	0.49%	0.14%	0.64%	0.04%	0.00%	0.22%	0.22%	0.03%	0.14%	1.98%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae	0.00%	0.00%	0.00%	0.61%	0.00%	0.28%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae	0.00%	0.07%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae	0.03%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.11%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.02%	0.00%	0.00%	0.02%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae	0.00%	0.03%	0.21%	0.70%	0.52%	4.61%	0.18%	1.84%	0.17%	0.41%	0.14%	0.47%	0.04%	0.00%	0.00%	0.20%	0.00%	0.14%	1.21%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae	0.17%	0.07%	0.35%	0.96%	1.14%	0.14%	0.18%	1.49%	0.83%	0.09%	0.00%	0.18%	0.00%	0.00%	0.18%	0.00%	0.03%	0.00%	0.75%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;o__Turicibacterales	0.00%	0.00%	0.03%	0.70%	2.23%	2.72%	0.05%	0.46%	1.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae	0.00%	0.00%	0.03%	0.70%	2.23%	2.72%	0.05%	0.46%	1.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;Other	0.00%	0.00%	0.00%	0.09%	0.36%	0.69%	0.00%	0.23%	0.33%	0.02%	0.00%	0.00%	0.00%	0.00%	0.37%	0.10%	0.03%	0.00%	0.14%
Root;k__Bacteria;p__Firmicutes;c__Bacilli;Other;Other	0.00%	0.00%	0.00%	0.09%	0.36%	0.69%	0.00%	0.23%	0.33%	0.02%	0.00%	0.00%	0.00%	0.00%	0.37%	0.10%	0.03%	0.00%	0.14%
Root;k__Bacteria;p__Firmicutes;c__Clostridia	0.10%	0.10%	0.17%	21.82%	27.30%	57.89%	91.79%	14.35%	31.39%	0.02%	0.60%	0.11%	0.04%	0.00%	0.00%	1.75%	0.03%	0.00%	1.30%
Root;k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales	0.07%	0.10%	0.17%	21.65%	27.19%	57.80%	91.74%	14.35%	31.05%	0.02%	0.58%	0.09%	0.04%	0.00%	0.00%	1.75%	0.03%	0.00%	1.05%
Root;k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__	0.00%	0.00%	0.00%	0.26%	0.31%	0.69%	0.00%	0.00%	3.67%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae	0.00%	0.00%	0.00%	0.53%	0.42%	1.75%	0.05%	0.00%	0.50%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	1.73%	0.00%	0.00%	0.55%
Root;k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiales Family XIII. Incertae Sedis	0.00%	0.00%	0.00%	0.79%	0.16%	0.23%	0.00%	0.11%	0.67%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae	0.03%	0.07%	0.00%	0.00%	0.26%	0.46%	0.05%	0.11%	1.84%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.09%

	Starved gut			LC-fed gut			C-fed gut			Starved water			LC-fed water			C-fed water			
Taxon	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**Inoculum**
Root;k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	0.00%	0.03%	0.14%	1.84%	2.08%	4.01%	90.50%	0.92%	7.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	0.03%	0.00%	0.03%	4.73%	4.98%	5.58%	0.28%	2.18%	3.67%	0.02%	0.03%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.18%
Root;k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other	0.00%	0.00%	0.00%	13.50%	18.99%	45.06%	0.87%	11.02%	13.69%	0.00%	0.55%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.23%
Root;k__Bacteria;p__Firmicutes;c__Clostridia;Other	0.03%	0.00%	0.00%	0.18%	0.10%	0.09%	0.05%	0.00%	0.33%	0.00%	0.03%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.25%
Root;k__Bacteria;p__Firmicutes;c__Clostridia;Other;Other	0.03%	0.00%	0.00%	0.18%	0.10%	0.09%	0.05%	0.00%	0.33%	0.00%	0.03%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.25%
Root;k__Bacteria;p__Fusobacteria	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.52%
Root;k__Bacteria;p__Fusobacteria;c__Fusobacteria	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.52%
Root;k__Bacteria;p__Fusobacteria;c__Fusobacteria;o__Fusobacteriales	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.52%
Root;k__Bacteria;p__Fusobacteria;c__Fusobacteria;o__Fusobacteriales;f__Fusobacteriaceae	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.52%
Root;k__Bacteria;p__Proteobacteria	99.00%	98.57%	97.05%	65.56%	56.51%	26.80%	6.79%	72.10%	51.25%	88.74%	85.69%	86.64%	86.48%	75.70%	54.36%	84.08%	68.89%	76.25%	89.63%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria	96.48%	98.30%	6.55%	50.83%	19.62%	3.04%	6.19%	31.80%	25.88%	10.48%	7.08%	11.85%	25.35%	5.75%	11.88%	41.30%	14.48%	7.87%	24.98%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.02%	0.00%	0.03%	0.03%	0.00%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.02%	0.00%	0.03%	0.03%	0.00%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales	38.77%	36.93%	1.63%	14.46%	7.06%	1.43%	2.02%	12.51%	12.85%	3.12%	2.79%	4.50%	0.61%	0.77%	0.59%	0.35%	1.05%	1.34%	7.59%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae	38.77%	36.90%	1.63%	14.46%	7.06%	1.38%	2.02%	12.06%	12.85%	1.43%	1.01%	1.79%	0.32%	0.30%	0.24%	0.12%	0.47%	0.85%	5.95%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.46%	0.00%	1.69%	1.70%	2.68%	0.06%	0.15%	0.11%	0.00%	0.29%	0.25%	0.80%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.09%	0.02%	0.23%	0.32%	0.24%	0.22%	0.29%	0.25%	0.84%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales	0.00%	0.00%	0.00%	0.00%	0.05%	0.09%	0.00%	0.00%	0.00%	0.09%	0.14%	0.16%	0.08%	0.26%	0.28%	0.42%	0.44%	0.08%	0.25%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae	0.00%	0.00%	0.00%	0.00%	0.05%	0.09%	0.00%	0.00%	0.00%	0.09%	0.14%	0.16%	0.08%	0.26%	0.28%	0.42%	0.44%	0.08%	0.25%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.32%	0.26%	0.20%	0.02%	0.02%	0.09%	0.02%	0.20%	0.14%	0.05%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.02%	0.00%	0.04%	0.00%	0.03%	0.05%	0.00%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.28%	0.26%	0.20%	0.00%	0.02%	0.04%	0.02%	0.17%	0.08%	0.05%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.71%	0.17%	0.55%	0.44%	0.17%	0.42%	0.22%	0.12%	0.22%	0.16%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.59%	0.17%	0.49%	0.36%	0.17%	0.42%	0.22%	0.12%	0.22%	0.16%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__Rickettsiaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.12%	0.00%	0.07%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales	57.72%	61.37%	4.82%	36.37%	12.20%	1.06%	4.13%	18.48%	12.69%	4.18%	2.42%	5.58%	0.32%	0.19%	0.20%	0.05%	0.61%	0.90%	16.11%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae	57.72%	61.37%	4.82%	36.37%	12.20%	1.06%	4.13%	18.48%	12.69%	4.18%	2.42%	5.58%	0.32%	0.19%	0.20%	0.05%	0.61%	0.90%	16.11%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;Other	0.00%	0.00%	0.10%	0.00%	0.31%	0.42%	0.05%	0.80%	0.33%	1.07%	1.30%	0.82%	23.89%	4.35%	10.28%	40.24%	12.03%	5.17%	0.82%
Root;k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;Other;Other	0.00%	0.00%	0.10%	0.00%	0.31%	0.42%	0.05%	0.80%	0.33%	1.07%	1.30%	0.82%	23.89%	4.35%	10.28%	40.24%	12.03%	5.17%	0.82%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria	0.17%	0.20%	0.49%	6.40%	19.15%	10.01%	0.37%	6.20%	10.52%	67.59%	71.49%	62.31%	42.06%	49.57%	25.88%	28.59%	39.67%	52.09%	22.70%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales	0.17%	0.17%	0.10%	5.17%	17.07%	7.43%	0.37%	6.08%	9.18%	25.66%	27.15%	23.24%	35.45%	41.16%	20.07%	21.60%	34.23%	44.66%	11.83%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__	0.03%	0.14%	0.10%	2.80%	8.15%	2.68%	0.18%	3.21%	3.34%	1.48%	1.15%	1.91%	6.90%	4.04%	0.72%	4.62%	8.74%	6.34%	2.46%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.04%	0.10%	0.00%	0.00%	0.00%	0.11%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae	0.00%	0.00%	0.00%	0.00%	0.16%	0.09%	0.00%	0.00%	0.00%	0.00%	0.06%	0.04%	0.02%	0.02%	0.00%	0.00%	0.00%	0.00%	0.25%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae	0.13%	0.03%	0.00%	2.37%	5.66%	2.31%	0.18%	1.72%	4.67%	5.27%	5.10%	7.07%	16.67%	18.29%	11.74%	10.47%	13.25%	14.59%	3.28%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae	0.00%	0.00%	0.00%	0.00%	3.06%	2.35%	0.00%	1.15%	1.17%	18.91%	20.79%	14.18%	11.66%	17.84%	7.38%	6.27%	11.91%	23.64%	5.72%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.04%	0.21%	0.28%	0.18%	0.15%	0.32%	0.08%	0.00%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Hydrogenophilales	0.00%	0.03%	0.00%	0.35%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Hydrogenophilales;f__Hydrogenophilaceae	0.00%	0.03%	0.00%	0.35%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales	0.00%	0.00%	0.00%	0.00%	0.67%	1.75%	0.00%	0.00%	0.50%	0.21%	0.14%	0.33%	2.13%	1.00%	1.10%	1.38%	0.44%	0.57%	1.44%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae	0.00%	0.00%	0.00%	0.00%	0.67%	1.75%	0.00%	0.00%	0.50%	0.21%	0.14%	0.33%	2.13%	1.00%	1.10%	1.38%	0.44%	0.57%	1.44%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Rhodocyclales	0.00%	0.00%	0.17%	0.09%	0.93%	0.14%	0.00%	0.00%	0.17%	12.67%	13.25%	12.79%	0.08%	1.09%	0.13%	0.20%	0.55%	0.55%	9.39%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Rhodocyclales;f__Rhodocyclaceae	0.00%	0.00%	0.00%	0.09%	0.16%	0.14%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Rhodocyclales;Other	0.00%	0.00%	0.17%	0.00%	0.78%	0.00%	0.00%	0.00%	0.17%	12.67%	13.25%	12.79%	0.08%	1.09%	0.13%	0.20%	0.55%	0.55%	9.39%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;Other	0.00%	0.00%	0.21%	0.79%	0.47%	0.69%	0.00%	0.11%	0.67%	29.06%	30.95%	25.95%	4.40%	6.33%	4.58%	5.41%	4.46%	6.31%	0.05%
Root;k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;Other;Other	0.00%	0.00%	0.21%	0.79%	0.47%	0.69%	0.00%	0.11%	0.67%	29.06%	30.95%	25.95%	4.40%	6.33%	4.58%	5.41%	4.46%	6.31%	0.05%
Root;k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.07%	0.29%	0.07%	0.13%	0.34%	0.02%	0.27%	1.05%	1.01%	0.32%	
Root;k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.07%	0.29%	0.07%	0.13%	0.34%	0.02%	0.27%	1.05%	1.01%	0.32%	
Root;k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales;f__Bacteriovoracaceae	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.07%	0.06%	0.04%	0.00%	0.00%	0.02%	0.15%	0.09%	0.05%	0.21%	
Root;k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales;f__Bdellovibrionaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.23%	0.02%	0.13%	0.34%	0.00%	0.12%	0.96%	0.96%	0.11%	
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria	2.36%	0.07%	90.02%	8.33%	17.75%	13.70%	0.23%	34.10%	14.86%	9.64%	5.90%	10.68%	16.19%	18.18%	14.96%	12.12%	12.09%	14.84%	40.79%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales	0.37%	0.00%	7.97%	0.00%	0.36%	0.28%	0.00%	7.00%	0.17%	0.42%	0.60%	1.57%	0.53%	2.20%	1.56%	1.83%	0.61%	0.52%	0.46%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Aeromonadaceae	0.37%	0.00%	7.97%	0.00%	0.36%	0.28%	0.00%	7.00%	0.17%	0.42%	0.60%	1.57%	0.53%	2.20%	1.56%	1.83%	0.61%	0.52%	0.46%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales	0.00%	0.00%	2.60%	0.09%	0.16%	0.14%	0.00%	0.11%	0.00%	0.09%	0.03%	0.20%	0.42%	0.17%	0.28%	0.05%	0.26%	0.60%	0.11%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Chromatiaceae	0.00%	0.00%	0.00%	0.00%	0.10%	0.05%	0.00%	0.11%	0.00%	0.05%	0.03%	0.20%	0.42%	0.04%	0.04%	0.02%	0.23%	0.11%	0.07%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Shewanellaceae	0.00%	0.00%	2.60%	0.09%	0.05%	0.09%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.13%	0.24%	0.02%	0.03%	0.49%	0.05%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales	1.99%	0.00%	79.38%	5.61%	10.28%	3.78%	0.14%	19.29%	6.18%	8.88%	4.84%	8.04%	0.86%	0.94%	0.74%	0.44%	0.55%	0.98%	38.61%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae	1.99%	0.00%	79.38%	5.61%	10.28%	3.78%	0.14%	19.29%	6.18%	8.88%	4.84%	8.04%	0.86%	0.94%	0.74%	0.44%	0.55%	0.98%	38.61%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Legionellaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales	0.00%	0.00%	0.00%	0.00%	0.31%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.08%	0.34%	0.00%	0.05%	0.00%	0.00%	0.21%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Halomonadaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.34%	0.00%	0.02%	0.00%	0.00%	0.14%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;Other	0.00%	0.00%	0.00%	0.00%	0.31%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.07%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales	0.00%	0.07%	0.07%	2.37%	5.40%	9.13%	0.09%	5.51%	4.84%	0.07%	0.17%	0.07%	13.18%	11.59%	9.90%	9.18%	7.95%	11.81%	1.03%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.04%	0.00%	0.02%	0.00%	0.00%	0.03%	0.00%	0.34%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae	0.00%	0.00%	0.00%	0.00%	0.16%	0.37%	0.00%	1.26%	0.17%	0.04%	0.06%	0.00%	2.15%	3.45%	2.70%	3.43%	1.92%	1.20%	0.00%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae	0.00%	0.07%	0.07%	2.37%	5.24%	8.76%	0.09%	4.25%	4.67%	0.04%	0.09%	0.02%	11.03%	8.12%	7.21%	5.75%	5.50%	10.60%	0.68%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales	0.00%	0.00%	0.00%	0.09%	1.09%	0.32%	0.00%	2.18%	0.50%	0.18%	0.23%	0.75%	0.82%	2.69%	2.17%	0.54%	2.39%	0.85%	0.25%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__	0.00%	0.00%	0.00%	0.00%	0.67%	0.32%	0.00%	1.38%	0.33%	0.16%	0.20%	0.60%	0.44%	1.26%	0.39%	0.22%	1.86%	0.57%	0.05%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.03%	0.16%	0.02%	0.04%	0.00%	0.00%	0.03%	0.03%	0.00%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae	0.00%	0.00%	0.00%	0.09%	0.42%	0.00%	0.00%	0.80%	0.17%	0.00%	0.00%	0.00%	0.36%	1.39%	1.77%	0.32%	0.50%	0.25%	0.21%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;Other	0.00%	0.00%	0.00%	0.18%	0.16%	0.05%	0.00%	0.00%	3.17%	0.00%	0.00%	0.02%	0.30%	0.26%	0.31%	0.02%	0.32%	0.08%	0.00%
Root;k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;Other;Other	0.00%	0.00%	0.00%	0.18%	0.16%	0.05%	0.00%	0.00%	3.17%	0.00%	0.00%	0.02%	0.30%	0.26%	0.31%	0.02%	0.32%	0.08%	0.00%
Root;k__Bacteria;p__Proteobacteria;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.95%	0.92%	1.73%	2.74%	1.85%	1.62%	1.80%	1.60%	0.44%	0.84%
Root;k__Bacteria;p__Proteobacteria;Other;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.95%	0.92%	1.73%	2.74%	1.85%	1.62%	1.80%	1.60%	0.44%	0.84%

	Starved gut			LC-fed gut			C-fed gut			Starved water			LC-fed water			C-fed water			
Taxon	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**Inoculum**
Root;k__Bacteria;p__Proteobacteria;Other;Other;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.95%	0.92%	1.73%	2.74%	1.85%	1.62%	1.80%	1.60%	0.44%	0.84%
Root;k__Bacteria;p__Spirochaetes	0.00%	0.00%	0.00%	0.00%	0.21%	0.28%	0.00%	0.11%	1.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Spirochaetes;c__Spirochaetes	0.00%	0.00%	0.00%	0.00%	0.21%	0.28%	0.00%	0.11%	1.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales	0.00%	0.00%	0.00%	0.00%	0.21%	0.28%	0.00%	0.11%	1.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae	0.00%	0.00%	0.00%	0.00%	0.21%	0.28%	0.00%	0.11%	1.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Tenericutes	0.13%	0.03%	0.21%	0.35%	1.71%	0.69%	0.05%	0.34%	1.00%	0.04%	0.03%	0.00%	0.00%	0.06%	0.04%	0.00%	0.06%	0.14%	0.32%
Root;k__Bacteria;p__Tenericutes;c__Erysipelotrichi	0.13%	0.03%	0.21%	0.35%	1.71%	0.65%	0.05%	0.34%	1.00%	0.04%	0.03%	0.00%	0.00%	0.06%	0.04%	0.00%	0.06%	0.14%	0.32%
Root;k__Bacteria;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales	0.13%	0.03%	0.21%	0.35%	1.71%	0.65%	0.05%	0.34%	1.00%	0.04%	0.03%	0.00%	0.00%	0.06%	0.04%	0.00%	0.06%	0.14%	0.32%
Root;k__Bacteria;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	0.13%	0.03%	0.21%	0.35%	1.71%	0.65%	0.05%	0.34%	1.00%	0.04%	0.03%	0.00%	0.00%	0.06%	0.04%	0.00%	0.06%	0.14%	0.32%
Root;k__Bacteria;p__Tenericutes;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Tenericutes;Other;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Tenericutes;Other;Other;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Root;k__Bacteria;p__Thermi	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.23%	0.00%	0.09%	0.00%	0.07%	0.55%	0.66%	0.28%	0.72%	0.17%	0.44%	0.02%
Root;k__Bacteria;p__Thermi;c__Deinococci	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.23%	0.00%	0.09%	0.00%	0.07%	0.55%	0.66%	0.28%	0.72%	0.17%	0.44%	0.02%
Root;k__Bacteria;p__Thermi;c__Deinococci;o__Deinococcales	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.23%	0.00%	0.09%	0.00%	0.07%	0.55%	0.66%	0.28%	0.72%	0.17%	0.44%	0.02%
Root;k__Bacteria;p__Thermi;c__Deinococci;o__Deinococcales;f__Deinococcaceae	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.23%	0.00%	0.09%	0.00%	0.07%	0.55%	0.66%	0.28%	0.72%	0.17%	0.44%	0.02%
Root;k__Bacteria;p__Verrucomicrobia	0.03%	0.00%	0.00%	0.00%	0.16%	0.09%	0.05%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.32%
Root;k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae	0.03%	0.00%	0.00%	0.00%	0.16%	0.09%	0.05%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.32%
Root;k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales	0.03%	0.00%	0.00%	0.00%	0.16%	0.09%	0.05%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.32%
Root;k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae	0.03%	0.00%	0.00%	0.00%	0.16%	0.09%	0.05%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.32%

Table 3.S3. Bacteria taxon abundance as defined by 16S rRNA gene sequences

		PD whole tree			Chao1			Observed species			Shannon			Simpson		
Sample	**Replicate no.**	**Replicate[a]**	**Mean[b]**	**Error[c]**	**Replicate[a]**	**Mean[b]**	**Error[c]**	**Replicate[a]**	**Mean[b]**	**Error[c]**	**Replicate[a]**	**Mean[b]**	**Error[c]**	**Replicate[a]**	**Mean[b]**	**Error[c]**
Starved gut	1	1.63	1.87	0.51	19.22	22.21	3.65	10.10	12.30	3.40	1.23	1.22	0.07	0.51	0.46	0.07
	2	1.40			20.08			9.70			1.12			0.49		
	3	2.58			27.34			17.10			1.30			0.36		
LC-fed gut	1	5.03	5.90	0.79	86.72	101.61	11.13	61.30	71.80	8.16	3.83	4.43	0.45	0.83	0.89	0.05
	2	6.93			113.46			81.20			4.92			0.94		
	3	5.73			104.66			72.90			4.53			0.90		
C-fed gut	1	2.46	4.93	1.86	46.04	77.88	22.81	17.40	54.93	27.72	0.75	3.44	1.94	0.19	0.68	0.35
	2	5.40			89.33			63.90			4.31			0.91		
	3	6.94			98.28			83.50			5.27			0.95		
Starved water	1	3.65	3.71	0.05	81.16	72.55	6.42	43.00	43.97	1.44	3.64	3.69	0.12	0.86	0.86	0.02
	2	3.75			65.73			42.90			3.57			0.85		
	3	3.74			70.76			46.00			3.85			0.88		
LC-fed water	1	4.41	4.68	0.30	122.12	130.80	6.18	66.00	70.07	3.48	4.18	4.43	0.18	0.90	0.92	0.02
	2	4.53			134.23			69.70			4.50			0.92		
	3	5.09			136.04			74.50			4.61			0.93		
C-fed water	1	4.11	4.31	0.43	89.96	100.05	25.13	58.30	58.73	7.48	3.97	4.24	0.24	0.83	0.89	0.04
	2	4.91			134.61			68.10			4.55			0.93		
	3	3.93			75.58			49.80			4.21			0.91		
Inoculum	n/a	5.58	n/a	n/a	99.30	n/a	n/a	60.20	n/a	n/a	3.52	n/a	n/a	0.80	n/a	n/a

a Mean values from 10 iterations of 550 sequences per replicate sample.

b Mean values from the respective 3 biological replicate samples.

c 90% confidence intervals surrounding the respective mean.

Table 3.S4. Alpha diversity of 6 dpf zebrafish gut and water samples

Strain name	Strain number	Phylum	Class	Order	Old names	References
Exiguobacterium sp.	ZWU0009	Firmicutes	Bacilli	Bacillales	ZF1EB02	Rawls et al., 2006
Chryseobacterium sp.	ZOR0023	Bacteroidetes	Flavobacteria	Flavobacteriales	KG3dpf.1	gift from E. Mittge and K. Guillemin
Pseudomonas sp.	ZWU0006	Proteobacteria	γ-Proteobacteria	Pseudomonadales	ZF1EE07	Rawls et al., 2006

Table 3.S5. Bacterial strains used in this study

CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

My dissertation research focuses on how microbial colonization impacts dietary lipid metabolism in a zebrafish host. In Chapter 2, I reviewed our current understanding of dietary and *de novo* synthesized lipid metabolism in various hosts. I emphasized the dietary and microbial challenges that affect lipid metabolism along the gastrointestinal tract and elsewhere in the body. I also summarized important gaps in our knowledge that I addressed in my dissertation research. In Chapter 3, I presented my main research study that explored the effects of microbes and diet on fatty acid digestion and absorption in intestinal and extra-intestinal tissues in zebrafish. The work presented in the Appendix shows some preliminary but highly intriguing findings on bacterial factors that regulate transcript levels of two fatty acid metabolism genes, *fiaf* and *fabp2*, and the innate immune gene myeloperoxidase (*mpo*). Altogether, my work identifies novel roles for the microbiota in stimulating intestinal fatty acid uptake and accumulation in lipid droplets, as well as promoting absorption in extra-intestinal tissues. In addition, our work is the first to report diet-induced alterations in zebrafish gut microbiota composition, including diet-dependent increases in Firmicutes abundance. This finding complements previous studies in humans and mice, which highlights the utility of zebrafish as a model organism to study dietary impact on microbial community composition and function. We also showed that when deprived of dietary nutrients, one tested Firmicutes strain could not survive and colonize the intestine at

detectable levels using culture-based methods. This finding is the first in any host to show that Firmicutes fitness in the intestine might be reduced in nutrient-poor conditions independent of microbial competition. Finally, we dissected the bacterial effects on intestinal fatty acid uptake and lipid droplet accumulation. Monoassociation with a Firmicutes strain recapitulated the increase in lipid droplet number seen with a complex microbiota. Conversely, colonization with two non-Firmicutes bacterial strains recapitulated the lipid droplet size phenotypes that were observed with the conventional microbiota (Figure 3.6).

Future directions

There are several important implications of my work that can be tested to further elucidate the factors involved in regulating dietary lipid metabolism. The lipid metabolism field has acknowledged the paucity of *in vivo* methods and models to study physiological responses to dietary and microbial challenges. Our analysis confirms the importance of *in vivo* approaches to study complex and dynamic host-microbe interactions that impact the metabolic fates of lipids in the intestine. In my dissertation, I present a novel experimental approach that can be utilized to dissect both microbial and host factors involved in mediating these metabolic processes. Using this approach, we demonstrate a novel role of the microbiota in dietary lipid absorption in intestinal and extra-intestinal tissues in a zebrafish host (Semova et al., submitted).

Microbe-centric approaches to study diet-mediated lipid metabolism

The zebrafish is an excellent model for high-throughput screens to identify primary bacterial isolates and lab strains that replicate the changes triggered by larger microbial communities on intestinal digestive and absorptive function. In our lab, we found several *Pseudomonas aeruginosa* lab strains (PAK, PA14, PAO1) that modulated transcript levels of

lipid metabolism and immune genes including *fiaf*, *fabp2* and *mpo* in a manner comparable to the complex microbiota (Figure A.2A,B). *P. aeruginosa* is one of the best studied Gram-negative bacteria due to its pathogenic role in opportunistic infections of immunocompromised patients (Wood, 1976; Bodey, 1983). In zebrafish, however, *P. aeruginosa* does not present as pathogen when administered orally (Rawls et al., 2007; Clatworthy et al., 2009). One advantage of *P. aeruginosa* is that there are widely available libraries of non-essential gene mutants (Jacobs et al., 2003; Lewenza et al., 2005; Liberati et al., 2006). These mutant collections could be utilized in genetic screens to identify the specific bacterial factors and mechanisms required for lipid metabolism changes in the zebrafish intestine. One of the candidate bacterial mechanisms is quorum sensing.

Quorum sensing (QS) is a bacterial cell communication mechanism that involves release of small diffusible signal molecules (autoinducers) to sense bacterial community density and regulate numerous functions (Keller and Surette, 2006; Williams et al., 2007). Some of these QS-regulated functions include regulation of fatty acid and phospholipid metabolism genes in strain PAO1 (Wagner et al., 2003), and release of membrane vesicles containing extracellular DNA, LPS and hydrolytic enzymes that are important for biofilm formation in *P.a.* strain PAO1 (Nakamura et al., 2008). QS-regulated biofilm formation is important in *P. aeruginosa* colonization of human hosts (Lowery et al., 2008). Biofilm-like structures have been detected in the lumen of CONVD zebrafish (Rawls et al., 2004; Rawls et al., 2007). In mammalian hosts, it has been proposed that microbes inhabiting the mucus gel layer above the epithelium form a biofilm-like community (Sonnenburg et al., 2004). Biofilms are thought to be sensitive to nutrient levels in the environment, since nutrient starvation leads to release of *P. aeruginosa* strain PAO1 from biofilms *in vitro* (Hunt et al., 2004). However there is no evidence on the effect of *in vivo* nutrient abundance on QS signaling and gut microbial density. In our work, colonization density was lower under

starved conditions for both conventional microbiota and a monoassociated primary *Pseudomonas* isolate sp. ZWU0006 (Figure 3.5D and Figure A.5B). Therefore, it would be interesting to determine whether dietary nutrient availability affects QS signaling in *P. aeruginosa*. Our lab has obtained a QS reporter strain (PAO1 with lasR lasB::gfp(ASV) and Pmty::rfp) designed by Dr. Thomas Bjarnsholt (Hentzer et al., 2003; Wu et al., 2000) that could be utilized to evaluate QS signaling of *P. aeruginosa* in the zebrafish intestine under starved and fed conditions. These studies might provide more insight into the effects of diet on QS-regulated bacterial mechanisms in the intestine. Our preliminary studies suggest that QS mutants in the PAK strain regulate *fiaf*, *fabp2* and *mpo* transcript levels in a similar manner as the wild-type PAK strain, raising the possibility that QS-mediated mechanisms are not required for transcriptional regulation of these metabolism and immune-related genes (Figure A.2A,B). However, we did not yet test the effect of colonization with PAK or QS mutants on fatty acid uptake and absorption using our novel *in vivo* approach described in chapter 3. My studies have shown that the primary *Pseudomonas* zebrafish isolate (*Pseudomonas* sp. ZWU0006) can affect digestive function (Figure A.5A) and lipid droplet formation in the intestine (Figure 3.6). Therefore, some of the future efforts in the lab will focus on the used Pseudomonas lab strains as well as QS and other mutants that might impact the dietary lipid absorption phenotype observed with the microbiota (Figures 3.1 and 3.2).

In addition to *P. aeruginosa*, QS signaling molecules (autoinducers C4-HSL and C12-HSL) might also impact intestinal fatty acid uptake and absorption. Our preliminary results show that autoinducer treatment of GF zebrafish regulate *fiaf*, *fabp2* an *mpo* transcript levels (Figure A.3A), suggesting that these bacterial autoinducers directly interact with host factors to modify host transcript profiles. Since previous cell culture study showed *P. aeruginosa* autoinducer entry and function in monkey kidney COS-1 cells, this

mechanism may be important not only in bacterial signaling to a zebrafish but also to mammalian hosts (Williams et al., 2004). Several other cell culture studies showed effects of C4- and C12-HSL as well as other homoserine lactones on transcriptional activation of immune responses (IL-8, ERK1/2, NFκB) and inhibition of TNFα and IL-12 production via LPS-stimulated peritoneal macrophages in animal cells (Shiner et al., 2005), suggesting that autoinducer signaling might mediate the immune system response to microbial colonization. Autoinducer treatment can also impact lipid metabolic genes in host cells, since *P. aeruginosa* autoinducer C12-HSL functioned as a PPARβ/δ agonist and PPARγ antagonist in murine fibroblasts and human alveolar epithelial cells (Jahoor et al., 2008). PPAR activity is regulated by various FA ligands and they regulate the expression of many lipid metabolic genes, including PPARα-induced expression of FIAF/ANGPTL4 in humans (Mandard et al., 2004). Therefore, these studies suggest of interkingdom signaling via autoinducer-mediated transcriptional regulation of host lipid metabolic and immune system responses.

Host-centric approaches to study microbe-mediated lipid metabolism

One important future direction involves deciphering intestinal molecular mechanisms that mediate dietary and microbial effects on lipid metabolism. Our lipid droplet quantification protocol distinguished between number and size in the epithelium of zebrafish under different microbial and dietary conditions (Figures 3.2, 3.3 and 3.6). However, we did not provide insight on the nature of these lipid droplets (i.e., cytosolic LDs or chylomicrons) due to lack of zebrafish antibodies or transgenic lines to label these cellular structures. In order to do more proper characterization of the microbial effect on dietary FA fates in the intestinal epithelium, we performed quantitative PCR on genes involved in epithelial FA translocation (*cd36*), FA binding protein (*fabp2*), activation (*fatp4*) and trafficking through the ER (*mtp*),

chylomicron formation (*apob*) and transcription factor regulation (*pparγ*). I pooled the intestines of BODIPY-FL C_5 liposome-fed 6 dpf GF and CONVD zebrafish after a 6 hr incubation, extracted mRNA and performed quantitative RT-PCR which showed microbially-mediated suppression of all tested genes (Figure 4.1). Therefore, similar analysis could be performed for genes involved in fatty acid oxidation and lipid droplet coating proteins in order to determine whether the microbiota regulates fatty acid usage as an energy source or the temporal storage in LD form in the cytoplasm. However, it is important to note that transcript levels do not necessarily correlate with protein levels of some of these genes along the intestinal length (Masson et al., 2010) and FA absorption and chylomicron synthesis are heavily regulated at the post-transcriptional level (Siddiqi et al., 2003; Iqbal et al., 2008; Tran et al., 2011).

An additional approach that could distinguish between chylomicron- and cytosolic-derived LDs would be to determine the subcellular localization of these organelles. As summarized in Chapter 2, chylomicron formation occurs at ER and Golgi sites prior exocytosis into the lymphatics. My confocal imaging protocol allows for visualization of subcellular organelles that could be identified by recently developed transgenic line *Tg(actin:GalT-GFP; actin:ER-tdTomato)* that labels Golgi and ER (generously provided by Dr. Kirsten Sadler Edepli, Mount Sinai School of Medicine). Alternatively, we can co-inject GFP-p115 and GFP-gm130 mRNA with lyndTtomato mRNA for Golgi staining (Pouthas et al., 2008) and ER-Tracker dyes for live-cell ER labeling (E12353, Invitrogen). LDs colocalized with Golgi are prechylomicrons, while ER-associated LDs could be cytosolic LDs or prechylomicrons. To investigate microbial impact on epithelial FA oxidative processes, colocalization of BODIPY-FL C_5 FA with the mitochondrial marker DASPEI can be determined (Jonz and Nurse, 2006). These studies will help determine whether the

microbiota and dietary nutrients stimulate dietary fatty acid uptake for storage as chylomicrons or cytosolic LDs or usage as energy source.

In addition to microbial stimulation of epithelial fatty acid uptake and LD formation, I also demonstrated microbial stimulation of dietary fatty acid absorption into the liver and non-GI tissues (Figure 3.2). This is additional important *in vivo* evidence of microbial stimulation of dietary lipid absorption. However, in my study, I did not determine the nature of the lipid substrates or lipoproteins carrying the BODIPY-FL C_5 FA. Future efforts are needed to determine whether the microbiota affects the ratio of absorbed FAs in the intestine that get incorporated as TG, DG, MG or FFA in several compartments including plasma, liver and non-GI tissues. Using thin layer chromatography Carten et al. (2011) showed that the C_5 substrate can be incorporated in all the mentioned lipids; however the authors did not test the effect of the microbiota on the lipid profiles. In addition to detecting the dietary lipids, we can also detect the effect of microbiota on endogenous lipid levels by a well-established charring procedure in the presence of copper salts (Fewster et al., 1969).

The microbial stimulation of dietary fatty acid absorption in non-GI tissues could also affect the lipoprotein profile of CONVD zebrafish. To test this hypothesis, we could perform native gel electrophoresis with lipid extracts used in Figure 3.2F to determine the chylomicron, VLDL, LDL and HDL levels in non-GI tissues of GF vs CONVD animals. This method was previously pioneered in zebrafish fed a high-cholesterol diet (Fang et al., 2010). The results will provide some insights into how microbial stimulation of dietary fatty acid uptake in the intestine affects the intertwined metabolic fates of exogenous and endogenous lipid sources.

Diet-microbiota interactions are important in lipid metabolism

The zebrafish is an important model organism that can be used to study microbial community assembly and ecology as a response to dietary changes. We discovered that diet induced Firmicutes abundance in the intestine of fed but not starved zebrafish (Figure 3.4). We speculated the other microbial members might outcompete Firmicutes species in starved animals, so we performed monoassociation studies with a Firmicutes, Bacteroidetes and Proteobacteria strains to eliminate microbial competition and determine each strain's fitness in response to changing dietary environments. The fact that the Firmicutes strain, but not Bacteroidetes or Proteobacteria strains failed to colonize the zebrafish gut or water under starved conditions suggest that Firmicutes fitness and survival might be diet-dependent even in the absence of other microbes. Although these findings need to be repeated with increased number of representatives from each phyla, it would be equally interesting to perform dual and triple associations to evaluate microbial community assembly and maintenance in response to dietary challenges. Another possibility is that Firmicutes colonization is dependent on the presence of other bacteria that can survive in nutrient-poor environments and provide nutrients that facilitate Firmicutes growth and survival.

It would be also interesting to determine whether the lipid droplet phenotype changes in zebrafish colonized with this simple microbial community of two or three members. I discovered two distinct bacterial mechanisms that regulate lipid droplet formation in the epithelium – a Firmicutes-specific increase in LD number and a non-Firmicutes induced increase in LD size (Figure 3.6). Our current hypothesis based on this finding is that Firmicutes stimulate a more efficient processing and packaging of lipids from cytosolic LDs into chylomicrons, which are smaller in size. Our prediction is that dual association with the Firmicutes and one of the other two strains would result in a more similar LD phenotype to that observed in CONVD fed fish (Figure 3.2B,C). Therefore, I expect that the effects of dual

and triple associations on the lipid droplet phenotype will provide important discoveries of simple community interactions that are important in mediating dietary lipid metabolism. One can combine *in vivo* and quantitative analysis techniques discussed earlier in this chapter in order to determine bacterial and host factors that respond to certain microbial members or the triple member community.

In summary, my dissertation describes novel microbial roles in intestinal dietary fatty acid uptake and accumulation in lipid droplets, as well as absorption into non-GI tissues and liver of a zebrafish host. Future efforts focusing on the mechanisms that regulate this microbial stimulation of dietary lipid metabolism in the intestine will hopefully identify some host and bacterial factors that can be manipulated as treatment strategy for obesity and malnutrition.

Implications for the field of host-microbe interactions and lipid metabolism

The research findings summarized here set the ground for future research directions in the field of host-microbe interactions and lipid metabolism. The emerging patterns of microbial community alterations in response to dietary effectors reported in this study are suggestive of evolutionarily-conserved microbial responses to the host's nutritional status despite vast differences in anatomy and physiology between zebrafish and mammals. Furthermore, our work also indicates the importance of the microbial regulation of dietary fatty acid absorption. Many studies do not distinguish between the microbial impact on exogenous versus endogenous lipid sources, partially due to inability to monitor digestion and absorption of dietary nutrients in the small intestine of rodents and humans. These findings have great implications for the use of zebrafish as a model organism for studying the microbial impact on diet-induced diseases of energy imbalance (such as obesity). Our work also emphasizes the importance of development of tools and substrates that can be

tracked throughout the body in order to monitor the impact of microbial and dietary perturbations on specific nutrient metabolic fates.

Our *in vivo* approach showed that the microbiota stimulates dietary fatty acid accumulation in epithelial LDs (Figure 3.2A,B), despite decreased transcript levels of genes involved in fatty acid metabolism in the intestine (Figure 4.1). This finding emphasizes the importance of experimental approaches that determine the overall physiological response to dynamic microbial and dietary alterations. Expanding the extent to which we understand the microbial contribution to the host's health status will require a more integrative approach of current research techniques. One such approach was recently reported by Chen and colleagues in an attempt to develop a more complete picture of the molecular and metabolic phenotypes in a human host (Chen et al., 2012). Combining genomic, transcriptomic, proteomic and metabolomic techniques with analysis of community composition and function will allow us to obtain a more mechanistic understanding of how diet-induced microbial community changes affect the host metabolic function and contribute to the development of obesity-related pathologies.

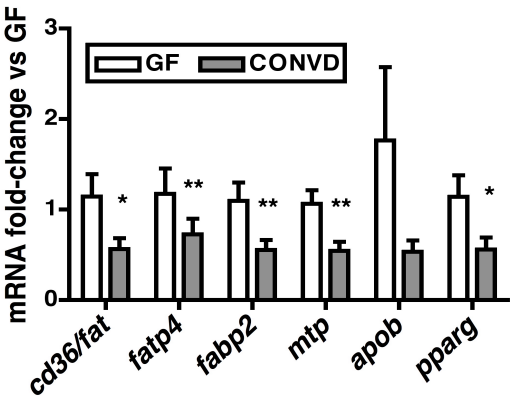


Figure 4.1. Transcript levels of FA metabolic genes are reduced in the GI tracts of 6 dpf CONVD vs GF zebrafish.
6 dpf GF and CONVD zebrafish were incubated with BODIPY-FL C_5 FA liposomes for 6 hrs. After incubation, the zebrafish were kept on ice while their GI tracts were microdissected and pooled in microcentrifuge tubes of Trizol (10-12 GI tracts/tube/condition). These samples were used to extract mRNA and perform qRT-PCR with primers against several FA metabolic genes. The results show fold-changes of two independent experiments represented as mean ± SD. Significance was determined using Student's t test (*, $p<0.05$; **, $p<0.01$).

APPENDIX

THE ROLE OF *PSEUDOMONAS AERUGINOSA* AUTOINDUCERS IN HOST METABOLIC AND IMMUNE GENE EXPRESSION

ABSTRACT

The current epidemic of obesity and overweight has dramatically increased the rates of diabetes, cardiovascular disease and metabolic syndrome. Insights from gnotobiotic animal models have demonstrated that the gut microbiota can influence fat storage via transcriptional regulation of metabolic and immune genes. More specifically, the gut microbiota suppresses intestinal expression of genes involved in lipid metabolism such as *Fasting-induced adipose factor/Angiopoietin-like4* (*Fiaf/Angptl4*) and *Intestinal fatty acid binding protein* (*Fabp2*), while it induces expression of immune genes such as *Myeloperoxidase* (*Mpo*) and *Serum amyloid A* (*Saa*). The microbial mechanisms that regulate the expression of these metabolic and immune genes in the host remain unknown, and represent potential targets for controlling host energy balance. We showed that the *Pseudomonas aeruginosa* strain PAK can recapitulate the microbial regulation of gene expression for *fiaf*, *fabp2* and *mpo*. Therefore, in this preliminary study we use *P. aeruginosa* as a model gut bacterium to investigate bacterial mechanisms and factors that regulate *fiaf, fabp2* and *mpo* expression in 6 dpf zebrafish. Here we describe our preliminary findings of novel roles for the *P. aeruginosa* QS signaling molecules (autoinducers) in transcriptional

regulation of metabolic and immune markers in a zebrafish host. Quorum sensing (QS) is a common mechanism of bacteria-bacteria communication, however potential roles for QS in regulating host gene expression are untested. Our preliminary results indicate that autoinducer treatment can regulate *fiaf*, *fabp2* and *mpo* transcript levels in both GF and zebrafish monoassociated with wild-type *P. aeruginosa* strain PAK or QS mutant, suggesting of a direct interaction of this bacterial factor with host epithelial cells. Inconsistencies with our qPCR results led us to utilize *in vivo* approaches to determine changes in immune cell recruitment and digestive function in the intestine as a response to PAK colonization and autoinducer treatments.

INTRODUCTION

The vertebrate intestine harbors a large community of microorganisms that regulates diverse aspects of energy balance (Musso et al., 2011). Comparison of germ-free (GF, animals raised in the absence of microbes), conventionalized (CONVD, GF animals colonized at selected time point with microbes) and conventionally-raised (CONVR, animals exposed to microbes from birth) mice have identified multiple ways through which the microbiota modifies energy balance. First, the microbiota can enhance processing and uptake of otherwise indigestible nutrients in the intestinal epithelium in multiple ways. The microbiota possesses enzymes that digest glycans and provide short-chain fatty acids to the host (Gill et al., 2006). The gut microbiota also plays a role in bile salt modifications that improve emulsification and absorption of dietary lipids in enterocytes (Martin et al., 2007; Martin et al., 2008; Blaut and Clavel, 2007). The microbiota can also regulate the host's production of digestive enzymes, but the effects shown in mice are contradictory (Lhoste et al., 1996; Reddy et al., 1968). In addition to mediating the digestive capacity in the lumen,

the microbiota has been suggested to affect the absorptive processes in the intestinal epithelium potentially via transcriptional regulation of fatty acid transporters and binding proteins such as *fabp2* (Larsson et al., 2011). Second, the microbiota can stimulate *de novo* hepatic lipogenesis and production of triglycerides (TG). Third, the microbiota suppresses intestinal epithelial expression of *Fasting-induced adipose factor* (*Fiaf*, also known as *angiopoietin-like 4*, *Angptl4*)(Bäckhed et al., 2004). This circulating protein inhibits lipoprotein lipase (LPL)-mediated hydrolysis of triglycerides and their storage as fat in peripheral tissues. Therefore, the microbiota promotes storage of serum TG in adipose tissues by inhibiting LPL activity through suppression of *Fiaf* levels (Figure A.1). Finally, the microbiota can regulate energy balance by evoking host pro-inflammatory signals in the intestine and whole body. High-fat diet is associated with microbiota-dependent intestinal inflammation which precedes obesity in a mouse model (Ding et al., 2010). Despite all the previous evidence of metabolic and immune changes to microbial colonization, the microbial mechanisms mediating these diverse host metabolic responses remain unclear.

In order to investigate how the microbiota impacts host metabolic and immune responses, our lab has established a protocol for rearing germ-free zebrafish through early adult stages (Rawls et al., 2007; Rawls et al., 2004; Pham et al., 2008). Our and other labs showed that the presence of gut microbiota affects intestinal motility, protein absorption and glycan expression, recruitment of immune cells to the intestine, increased mucosal barrier, but not gross anatomy in zebrafish (Rawls et al., 2004; Bates et al., 2006). Furthermore, reciprocal microbiota transplants into germ-free mice and zebrafish demonstrated a conserved host metabolic response to the zebrafish and mouse microbiota (Rawls et al., 2006). These findings validate zebrafish as an appropriate model system for studying host-microbe interactions and their role in metabolic and immune processes.

In an effort to dissect the role of the complex microbial community on metabolic

function and immune system development, we identified a single bacterial species that recapitulates many of the effects of the microbiota on nutrient and immune biomarker regulation. *Pseudomonas aeruginosa (P.a.)* is an opportunistic pathogen in susceptible humans, but acts as a commensal gut bacterium in zebrafish hosts (Rawls et al., 2007). Pseudomonads are common members of fish and some mammalian intestinal microbiotas (Rawls et al., 2004; Rawls et al., 2006; Roeselers et al., 2011). Our lab has shown that *P.a.* strains can stimulate transcript levels of innate immune biomarkers *myeloperoxidase* (*mpo*, a neutrophil marker) and *serum amyloid a* (*saa*, an acute phase protein), and suppression of transcript levels of nutrient metabolism biomarkers *fiaf* and *fabp2* in a similar manner as the normal microbial community in gnotobiotic zebrafish hosts (Rawls et al., 2006; Kanther et al., 2011; Rawls et al., 2004; Camp et al., 2012). The availability of a large number of genetic tools and reagents for *P.a.* allowed us to test the potential role of 39 virulence and metabolic genes and pathways in regulation of host *fiaf* expression (data not shown). In this study, we focus on our preliminary findings of *P.a. virulence factor regulator* (*vfr*) and quorum sensing pathway as potential regulators of *fiaf* expression in zebrafish. Vfr is a global regulator of numerous bacterial genes including those involved in quorum sensing (Wolfgang et al., 2003). Vfr is a cAMP-dependent transcriptional regulator, and is positively regulated by adenylate cyclases CyaA and CyaB and negatively regulated by phosphodiesterase CpdA (Yahr and Wolfgang, 2006). Quorum sensing is a cell-to-cell communication mechanism utilized by bacteria to control gene expression in response to bacterial cell density (Schauder and Bassler, 2001). The two major QS systems in *P.a.* consist of synthase proteins (i.e., LasI and RhlI) that produce signaling molecules called autoinducers to activate the QS receptors (i.e., LasR and RhlR) (Figure A.1B,C). QS is an attractive bacterial mechanism for regulating host gene expression in the intestine, because QS has also been shown to activate signaling cascades and gene transcription in other host

cells (Jahoor et al., 2008; Williams et al., 2004; Fujiya et al., 2007). However this role of bacterial QS in regulating gene expression in the intestinal epithelium remains largely unknown.

Here we describe our preliminary findings of novel roles for the *P. aeruginosa* QS signaling molecules in transcriptional regulation of metabolic and immune markers in a zebrafish host. Using quantitative RT-PCR methods we find that the gut microbiota, wild-type *P. aeruginosa* strain PAK and QS synthases double mutant regulate transcript levels of fatty acid metabolic genes *fiaf*, *fabp2* and innate immune gene *mpo* in a manner that doesn't require functional QS synthases. We show that treatment of GF fish with autoinducer C12-HSL elicits similar transcript levels as the ones observed in C12-HSL treated zebrafish monoassociated with PAK and QS synthase mutant. Some inconsistency in our qPCR results led us to explore and utilize *in vivo* approaches to determine the metabolic and immune system response to PAK colonization and autoinducer treatments.

MATERIALS AND METHODS

Gnotobiotic zebrafish husbandry

Experiments using zebrafish were conducted using protocols approved by the IACUC and Environmental Health and Safety (EHS) offices at the University of North Carolina – Chapel Hill. For our experiments we used Tubingen Longfin (TL) and *Tg(BACmpo:gfp)*^{i114} zebrafish lines (Renshaw et al., 2006). We followed our published protocol for gnotobiotic zebrafish husbandry (Pham et al., 2008). Briefly, to test the sterility of GF zebrafish, we aerobically culture aliquots of zebrafish media on tryptic soy agar (TSA) at 2 days post fertilization (dpf). The sterility test at end point includes culturing zebrafish

media in nutrient, brain/heart infusion (BHI) and Sabouraud Dextrose (Sab-Dex) broths under aerobic and anaerobic conditions at 28^{o}C.

We colonize 3 dpf GF animals with a normal zebrafish microbiota or the bacterial strain of choice at a final density of 1x10^{4} colony forming units (CFU) per mL. Overnight bacterial cultures of *Pseudomonas aeruginosa* PAK lab strains or *Pseudomonas* sp. ZWU0006 wild-type zebrafish isolate were grown in LB broth and used for monoassociations. To monitor bacterial density at the time of colonization and end point, we plate the inoculating solution on TSA plates. For gut CFUs we dissect intestines in biological triplicate, homogenize their respective contents, and plate technical triplicates on TSA plates. We incubate the plates overnight at 28^{o}C and count CFUs per gut. Beginning at 3 dpf, we feed daily with standard autoclaved zebrafish diet (ZM-000, ZM Ltd.), except for the experiments in Figure A.5 where we fed 0.06 mg of sterile irradiated Zeigler control diet per flask. We provide 80% water change daily using gnotobiotic zebrafish medium (GZM).

Pseudomonas aeruginosa genetic manipulation

All *P. aeruginosa* mutants were a generous donation by Dr. Matthew Wolfgang's lab at UNC (Wolfgang et al., 2003; Smith et al., 2004). The *vfr*, *cyaA, cyaB* and *cpdA* nonpolar deletion mutants were generated from PAK parent strain by removing internal fragments of coding sequence from each gene via special PCR technique (splicing by overlap extension) (Wolfgang et al., 2003). For complementation, the *vfr* gene was cloned into pMMB v1 plasmid that carries a *lacI^{q}* repressor and carbenicillin resistance cassette (Smith et al., 2004).

Autoinducer treatment of GF and monoassociated PAK strains

The *P. aeruginosa* autoinducer *N*-3-oxododecanoyl-homoserine lactone (C12-HSL) was a generous gift from Dr. Kendra Rumbaugh (Texas Tech University). *N*-Butyryl-homoserine lactone (C4-HSL) and synthetic N-dodecanoyl-homoserine lactone C12*-HSL (Williams et al., 2004) were purchased from Sigma. The autoinducers were dissolved in dimethyl sulfoxide (DMSO) and stored at -20 °C. At 2 dpf we arrayed GF fish into 6-well plates (20 fish/well) in a final volume of 4 mL of GZM per well. To reduce the amount of HSL used in each experiment, the zebrafish were fed only once at 3 dpf by adding 20 μL of ZM-000 to the media and no subsequent media changes were performed. At 5 dpf, the autoinducers were dried down, and resuspended in ethanol (final 0.2% or 0.4% for C12*-HSL v/v) at the appropriate concentrations. We treated with the appropriate autoinducer or ethanol for the negative controls for 12 hrs.

Quantitative RT-PCR analysis

Whole animals were pooled at 6 dpf in Trizol for mRNA extraction and quantitative RT-PCR. The qRT-PCR primers used were as follows – reference gene *18S*_Forward primer: 5'-CACTTGTCCCTCTAAGAAGTTGCA-3'; *18S*_Reverse primer: 5'-GGTTGATTCCGATAACGAACGA-3'; *fiaf*_Forward primer: 5'-CGAGCGCATCAAGCAACA-3'; *fiaf*_Reverse primer: 5'-TCGCTCGTTTTTCATCG-TAATCT- 3'; *fabp2*_Forward primer: 5'-TGCCCATGACAACCTGAAGA-3'; *fabp2*_Reverse primer: 5'-GTTAATTTCCAGTGTGCGGAAAG-3'; *mpo*_Forward primer: 5'-TCCAAAGCTATGTGGGATGTGA-3'; *mpo*_Reverse primer: 5'-GTCGTCCGGCAAAACTGAA-3'; *pla2g1b*_Forward primer: 5'-CCCGGTGGATGAA-CTGGAC-3'; *pla2g1b*_Reverse primer: 5'-ATTTCAGTGTAGGGGTTGTCCAAG-3';

*tryl*_Forward primer: 5'-CTGGTGTGGAGCATCTCTGA-3'; *tryl*_Reverse primer: 5'-CTTCGACAGCTACGTTGTGC-3'.

Digestive function assay

PED6 [N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-1-palmitoyl-2-BODIPY-FL-pentanoyl-sn-glycero-3-phosphoethanolamine, D23739] and EnzChek [BODIPY-TxR casein, E6639] were purchased from Invitrogen, CA. PED6 was dissolved in chloroform and was TLC-purified as previously described (Hama et al., 2009). Purified PED6 was dissolved in chloroform (1 mg/mL) and stored long term at -80°C. EnzChek was dissolved in 0.1 M sodium bicarbonate (pH 8.3) at 1 mg/mL and stored at -20°C. Immediately prior to treatment, PED6 aliquots were dried and resuspended in 0.5% EtOH in sterile GZM (v/v).

Zebrafish at 6 dpf were washed with GZM and transferred to a 6-well plate at 40 fish/mL GZM. Both PED6 and EnzChek were added to the media at a final concentration of 2 μg/mL and 5 μg/mL, respectively. The fish were incubated with the reporters for 3 hrs at room temperature on a rocker. After incubation, the fish were washed 3 times with pre-chilled GZM that served as anesthetic because it was previously shown that tricaine anesthetic treatment reduces the intestinal fluorescent signal of both reporters (Hama et al., 2009). Fish were mounted in 3% methylcellulose and imaged at identical short and long exposure times with Leica MZ 16F fluorescence stereomicroscope using GFP and DsRed filter sets. The short exposure time images were used to quantify the intestinal fluorescence in ImageJ, to limit pixel saturation. Briefly, a rectangular ROI was placed over the whole intestinal region, and a pixel intensity threshold was set to include all intestinal pixels but minimize the number of background pixels. Any fish that displayed hallmarks of developmental delay, including the presence of yolk or lack of inflated swim bladder, were excluded from the analysis. Normalized integrated density was generated against the

average integrated density of the respective GF starved control. The data presented in box-and-whiskers plot represents a single experiment (8-20 fish per condition).

Statistical analysis

Significance was determined using paired Student's t test for means with equal variances for the qPCR results (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). For the PED6 and EnzChek significance, one-way ANOVA was used with a Tukey's post-hoc test (Figure A.5A).

PRELIMINARY RESULTS

Colonization with *P. aeruginosa* regulates fatty acid metabolic and innate immune gene expression independent of *vfr* and QS synthases

We first tested the requirement for *vfr* in *P.a.* regulation of fatty acid metabolic genes *fiaf* and fatty acid binding protein (*fabp2*) and the innate immune gene myeloperoxidase (*mpo*). Colonization of zebrafish with the wild-type parent *P. a.* strain PAK results in suppression of *fiaf* and induction of *mpo* mRNA levels (Figure A.2A). Colonization with *vfr*-deficient (*Δvfr*) PAK strain resulted in similar transcript levels of *fiaf*, *fabp2* and *mpo* as the ones detected with wild-type PAK (Figure A.2A), suggesting that *vfr* may not be required for regulation of transcript levels of these metabolic and immune genes. Despite these negative results, we tested the effects of upstream effectors of *vfr* (adenylate cyclases CyaA and CyaB and the only cAMP-dependent phosphodiesterase in *P. aeruginosa*, CpdA) and attempted a complementation experiment with a wild-type copy of the *vfr* gene to validate

the lack of *vfr* phenotype. However both of these experiments failed due to lack of *fiaf* suppression by the wild-type PAK control (data not shown).

We screened for *vfr* downstream effector pathways and showed that colonization with a mutant carrying deletions in both quorum sensing (QS) synthase genes (*ΔlasIΔrhlI)* results in a reduction of *fiaf* and *fabp2* mRNA levels compared to GF fish, but showed no difference compared to the wild-type PAK strain (Figure A.2B). These results suggest that QS synthases may not be required in regulation of *fiaf* expression in zebrafish. Similarly, colonization with single synthase (*las* or *rhl*) mutants in a different *P.a.* strain (PA14) showed no significant attenuation of *fiaf* suppression. However, the PA14 wild-type strain did not cause a significant suppression of *fiaf* in these experiments, which might be due to *P.a.* strain-dependent differences in their ability to regulate *fiaf* expression (data not shown). The QS double mutant also showed induction of *mpo* mRNA levels similar to the wild-type PAK strain, suggesting that QS synthase signaling may not be involved in transcriptional regulation of this immune gene (Figure A.2B).

Autoinducer treatment regulates transcript levels of *fiaf*, *fabp2* and *mpo* in GF and monoassociated zebrafish

The QS synthases produce the signaling molecules *N*-butyryl homoserine lactone C4-HSL (produced by RhlI) and *N*-3-oxododecanoyl homoserine lactone C12-HSL (produced by LasI). These bacterial products have been previously shown to interact with transcription factors PPARβ and PPARγ and modulate expression of pro-inflammatory genes in murine fibroflast and human alveolar epithelial cell lines (Jahoor et al., 2008). Despite our negative results with the QS synthases double mutant in figure A.2B, we decided to test the effect of these QS signaling molecules on the expression of the tested

metabolic and immune genes in zebrafish. We treated GF animals with the *P. aeruginosa* autoinducers C4- and C12-HSL as well as a synthetic derivative N-dodecanoyl homoserine lactone C12*-HSL that lacks the oxygen at the third carbon in the acyl chain and can bind LasR in a similar manner at the natural substrate (Williams et al., 2004; Chhabra et al., 2003). Our preliminary results suggest that treatment with C4-HSL and C12-HSL autoinducers causes an induction rather than suppression of *fiaf* expression (Figure A.3A). On the other hand, treatment with the synthetic autoinducer C12*-HSL suppressed *fabp2* and *mpo* expression (Figure A.3A). These results raise the possibility that C4- and C12-HSL may directly interact with host transcriptional machinery to suppress *fabp2* and *mpo* expression. Furthermore, combined treatment of C4- and C12- natural or synthetic substrates did not suppress *fiaf* mRNA levels, suggesting of a lack of synergistic effect of the autoinducers on *fiaf* expression (Figure A.3B).

We also wanted to determine whether autoinducer treatment can have an impact on PAK bacterial function important in its transcriptional regulation of the metabolic and immune genes. Our preliminary results show that treatment of GF and monoassociated zebrafish with 50 μM C12-HSL induce similar expression trends as observed in Figure A.2B. The results were more dramatic with the treatment of zebrafish monoassociated with QS synthase mutants (Figure A.3C) than receptor double mutants (data not shown), suggesting that autoinducer activation of quorum sensing receptors and downstream signaling pathways might be important for regulation of *fiaf*, *fabp2* and *mpo* expression. It is important to note that monoassociation with the wild-type strain PAK does not result in a consistent suppression or induction of the metabolic and immune genes, respectively. Therefore, we decided to complement our qPCR findings with *in vivo* assays that determine the metabolic and immune response to *Pseudomonas* colonization in live zebrafish hosts.

Treatment of GF and PAK monoassociated zebrafish with C12-HSL results in recruitment of *mpo*-positive neutrophils in the zebrafish intestine

Since our preliminary studies suggest that C12-HSL suppresses *mpo* mRNA levels in both GF and zebrafish monoassociated with wild-type PAK and QS mutant strains, we hypothesized that this could be due to a decrease in the number of cells expressing *mpo*. As mentioned earlier, *mpo* expression is enriched in neutrophils. Therefore, we used transgenic zebrafish expressing GFP in neutrophils (*BACmpo:gfp*) to determine the effect of autoinducer treatment on *mpo*-positive cell number. The number of *mpo*-positive neutrophils in the intestine increased with colonization of wild-type PAK and QS receptor double mutant *ΔlasRΔrhlR*. To our surprise, treatment with 50 μM C12-HSL in both GF and monoassociated zebrafish showed further increase in neutrophil recruitment to the intestine (Figure A.4), despite the observed suppressing effect of the autoinducer on *mpo* mRNA levels (Figure A.3A). These findings suggest that even though treatment with the autoinducer molecule C12-HSL suppresses *mpo* transcript levels in the whole animal, this is not due to a decrease in total number of *mpo*-expressing cells. Further studies are needed to understand the interesting observation that C12-HSL mediates recruitment of neutrophils to the intestine.

***Pseudomonas* sp. ZWU0006 zebrafish isolate regulates zebrafish digestive physiology**

Mammalian studies demonstrated that members of the gut microbiota possess digestive enzymes that complement the host's enzymes (Gill et al., 2006). We used an *in vivo* fluorescent substrate feeding assay to study the effect of *Pseudomonas* genus members on zebrafish digestive physiology. Caged fluorescent phospholipid (PED6) and protein (EnzChek) require the respective phospholipase A_2 (PLA_2) and protease activity in

order to be cleaved and fluoresce (Hama et al., 2009). We used these reporters to assess phospholipase and protease activities since zebrafish utilize lipids and proteins as major sources of energy. Furthermore, we chose a microbiota isolate from an adult zebrafish, *Pseudomonas* sp. ZWU0006. Monoassociation of GF zebrafish with *Pseudomonas* sp. ZWU0006 resulted in a decrease in intestinal PED6 fluorescence, suggesting that *Pseudomonas* sp. ZWU0006 may mediate PLA_2 activity (Figure A.5A). This effect was also diet- and microbe-dependent, since the presence of dietary nutrients increased PED6 fluorescence in monoassociated but not GF zebrafish. On the other hand, *Pseudomonas* sp. ZWU0006 monoassociation did not have a significant effect on protease activity in the intestine. These results suggest that *Pseudomonas* sp. ZWU0006 may enhance PLA_2 activity in the intestine. The diet-dependent effect of *Pseudomonas* sp. ZWU0006 on PLA_2 activity was associated with an increase in bacterial density in the intestine but not the surrounding media, suggesting that the growth and fitness of at least this *Pseudomonas* strain was dependent on the availability of dietary nutrients (Figure A.5B). The presence of diet also mediated the *Pseudomonas* sp. regulation of transcript levels of two genes encoding the pancreas-enriched *pla2 group1b* (*pla2g1b*) and the serine protease *trypsin-like* (*tryl*) (Figure A.5C). These results suggest that *Pseudomonas* sp. ZWU0006 colonization stimulates *in vivo* digestive function of PLA_2 in zebrafish larvae in a diet-dependent manner. The increased luminal nutrient digestion is associated with increased uptake of macromolecules (Rawls et al., 2004) and dietary fatty acids into intestinal epithelial cells (Semova et al., submitted). Taken together, these findings suggest that *Pseudomonas* sp. ZWU0006 stimulate digestion and absorption of dietary lipids.

DISCUSSION AND FUTURE STUDIES

We tested two bacterial mechanisms (*vfr* and quorum sensing) for their role in regulation of *fiaf, fabp2* and *mpo* expression in the zebrafish host (Figure A.2A,B). Our preliminary observations suggest that *P. aeruginosa* regulates transcript levels of these genes independent of *vfr* and QS synthases. However, several of our preliminary findings need to be further confirmed by repeating some of the initial experiments primarily due to the inconsistency of wild-type *P. aeruginosa* strain in suppression of *fiaf*, *fabp2* or induction of *mpo* expression. First, the effects of *vfr* on the tested metabolic and immune genes need to be validated by performing complementation with a plasmid carrying wild-type *vfr* gene. Second, the effects of the upstream effector pathways (adenylate cyclases CyaA and CyaB and phosphodiesterase CpdA) need to be further examined by determining *fiaf*, *fabp2* and *mpo* transcript levels in zebrafish colonized with the *ΔcyaAΔcyaB* or the *ΔcpdA* mutant. The role of these mechanisms in transcript levels of the tested genes will indicate whether *vfr* regulates gene expression via cAMP-dependent or -independent mechanisms.

To determine bacterial genes involved in regulation of these fatty acid metabolic and immune genes, future studies need to include additional resources in our lab such as the sequenced transposon insertion library for *P.a.* strain PA14 (Liberati et al., 2006). Previous microarray analysis of *P.a.* quorum sensing regulons identified 616 genes that are regulated by quorum sensing (Wagner et al., 2003). We compared this list against 206 *vfr*-regulated genes (Wolfgang et al., 2003) and identified a small subset of 31 transcripts regulated by both mechanisms. We will obtain these mutants from the PA14 mutant library available in our lab, and screen them for aberrant host *fiaf*, *fabp2* or *mpo* expression using qRT-PCR. Since qPCR showed some inconsistent results especially from *P.a.* monoassociated conditions, the mutants can be validated for regulation of digestive and absorptive function using our *in vivo* assays. To identify the affected genes, the transposon insertion site needs

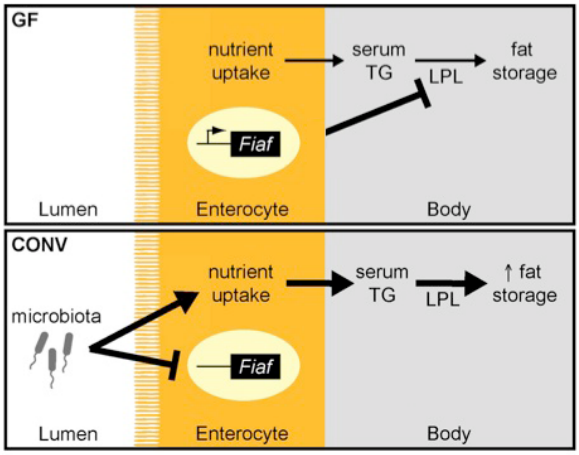
to be confirmed by inverse PCR and rescue wild-type phenotype by complementation using standard methods.

It has been shown that C12-HSL interacts with and regulates the activity of transcription factors PPARβ and PPARγ in lung epithelial cells (Jahoor et al., 2008). In the same model, the authors also showed that autoinducer treatment promotes up-regulation of pro-inflammatory cytokines, possibly via NF-κB signaling mechanisms. In our preliminary study, treatment with the synthetic derivative C12*-HSL suppressed *fabp2* in a concentration-dependent manner. This intriguing finding indicates that PPARβ or γ might transcriptionally regulate the expression of this fatty acid binding protein enriched in the intestine. A previous study in a fasting mouse model showed that hepatic PPARβ/δ can sense plasma free fatty acid levels and upregulate expression of genes such as *Lpin2* and *St3gal5* (Sanderson et al., 2009). Furthermore, the elevation of free fatty acids in plasma was due to the LPL-inhibitory activity of Fiaf that stimulates adipose tissue lipolysis and release of fatty acids that get taken up by the liver for VLDL production. Therefore, we predict that autoinducers may regulate *fabp2* and *mpo* expression by mimicking the ligand-binding properties of fatty acids to activate transcriptional factors such as PPARs. Furthermore, the C12-HSL induced recruitment of neutrophils to the intestine is also a very interesting novel finding that suggests that autoinducer signaling may be important not only as a sensing mechanism for bacterial density by *P. aeruginosa* but also by the host that can regulate bacterial growth and density via neutrophil-mediated anti-bacterial function (Nauseef, 2007).

Finally, these preliminary qPCR studies emphasized to us the need for other quantitative approaches that can test the effects of microbiota and individual bacterial strains. Our preliminary studies using the *in vivo* digestive function assay show that a

zebrafish microbiota isolate *Pseudomonas* sp. ZWU0006 can regulate zebrafish digestive physiology (Figure A.5A). This result would suggest that the digestive abilities of the intestinal bacterium might contribute to its role in regulating expression of metabolic genes such as *fiaf* and *fabp2* whose expression is mediated by dietary nutrient availability. These preliminary results led us in the direction of using fluorescent FA substrates to determine the microbial impact on digestive and absorptive function in the zebrafish intestine (work summarized in chapter 3). The BODIPY-FL FA assay is sufficiently robust and consistent to permit screening of bacterial genotypes in the future.

A



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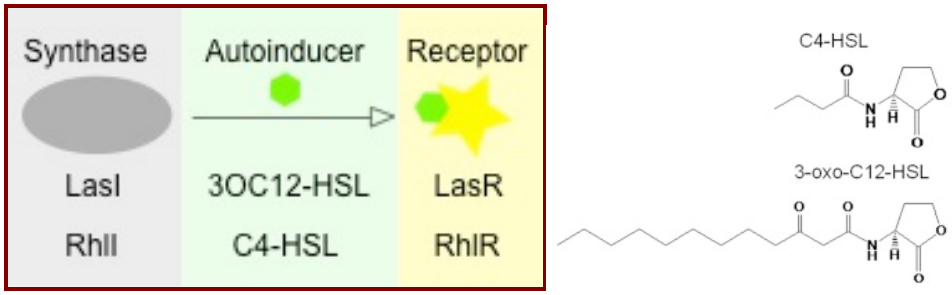

Figure A.1. (A) The role of gut microbiota in fat storage in the body via transcriptional regulation of Fasting-induced fatty acid (*Fiaf*) in the intestine.
(B) Quorum sensing (QS) signaling mechanisms in *Pseudomonas aeruginosa*. Each QS mechanism consists of a synthase molecule that produces the signaling autoinducer that gets secreted. Upon reaching a certain quorum, the autoinducer binding to the receptor causes quorum sensing signaling that regulates various bacterial mechanisms. *P. aeruginosa* has two major QS mechanisms, *Las* and *Rhl* that use homoserine lactones (HSL) as autoinducer molecules. The chemical structure of *P. aeruginosa Las* and *Rhl* homoserine lactones (C4- and C12-HSL) is shown to the right (adapted from Williams and Cámara, 2009).

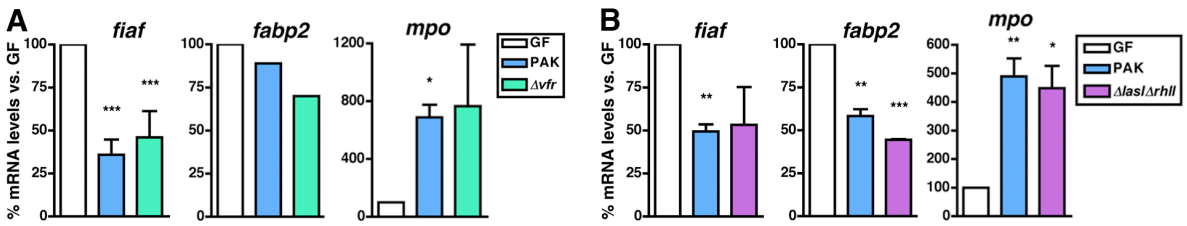

Figure A.2. *P. aeruginosa* bacterial mechanisms virulence factor regulator (*vfr*) and quorum sensing are not required for regulation of *fiaf*, *fabp2* and *mpo* transcript levels in 6 dpf zebrafish.

(A) Quantitative RT-PCR of 6 dpf GF and zebrafish colonized with zebrafish gut microbiota and *P.a.* strain PAK. Colonization with *vfr* mutant results in attenuated but non-significant *fiaf* and *cpt1a* suppression compared to wild-type PAK. Colonization with the *vfr* mutant further increases *mpo* expression in comparison to PAK.

(B) Quorum sensing synthase mutants show similar transcript levels of *fiaf*, *fabp2* and *mpo* as the ones detected in zebrafish monoassociated with wild-type *P. a.* strain PAK. Significance was against GF fish. (* $p < 0.05$; ** $p < 0.01$, ***$p<0.001$).

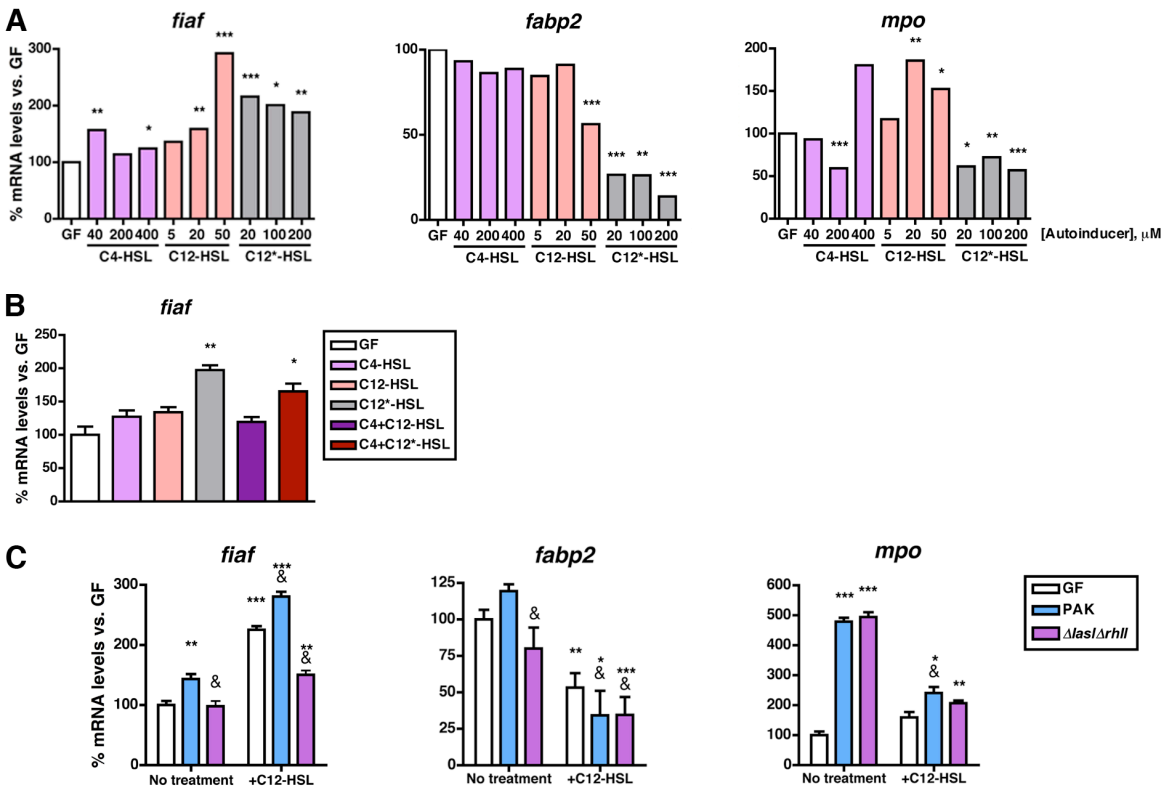

Figure A.3. Treatment with *P. aeruginosa* autoinducers C4- and C12-homoserine lactone regulate *fiaf*, *fabp2* and *mpo* expression in 6 dpf GF and zebrafish colonized with wild-type PAK and quorum sensing mutants.
(A) Expression of *fiaf*, *fabp2* and *mpo* mRNA in GF zebrafish treated with C4- and C12-HSL autoinducers as well as a synthetic C12-HSL molecule (C12*-HSL).
(B) Combined treatment of GF zebrafish with C4- and the natural or synthetic C12-HSL show no synergistic effect of the two QS signaling molecules on *fiaf* expression.
(C)Treatment with C12-HSL shows regulation of *fiaf*, *fabp2* and *mpo* in GF and zebrafish monoassociated with wild-type PAK and QS synthase double mutants. Significance is shown against GF untreated condition (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) and against PAK of the appropriate treatment (& $p<0.05$).

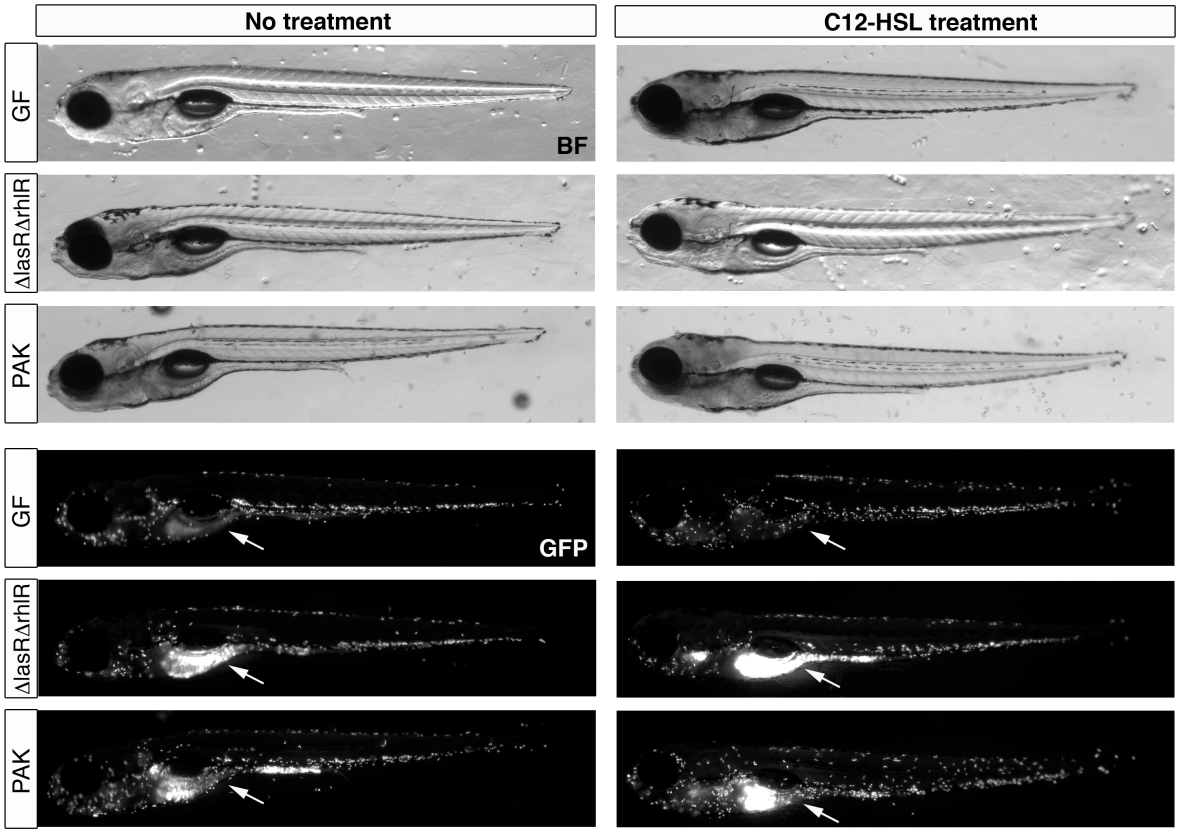

Figure A.4. Colonization with wild-type PAK and QS receptor mutant results in intestine-specific recruitment of *mpo*-positive neutrophils. The *Tg(BACmpo:gfp)* zebrafish line was used to determine neutrophil recruitment to the intestine of GF and zebrafish monossociated with wild-type *P.a*. strain PAK and the QS receptor double mutant (*ΔlasRΔrhlR*). Treatment with 50 μM C12-HSL autoinducer indicates of increased recruitment of *mpo*-positive neutrophils to the intestine (arrows) but no decrease in number of *mpo*-positive neutrophils.

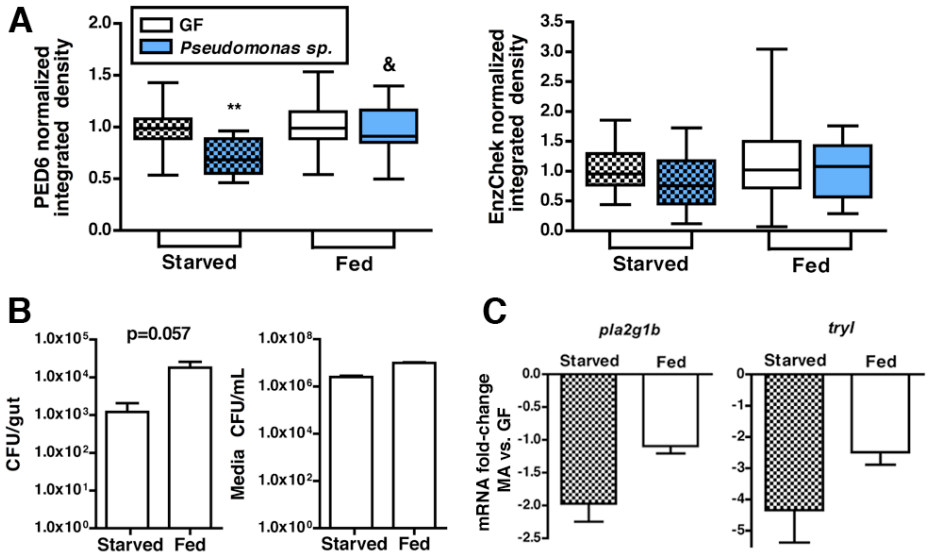

Figure A.5. Monoassociation with *Pseudomonas* zebrafish strain ZWU0006 regulates digestive enzyme expression and activity in a diet-dependent manner.
(A) Monoassociation with *Pseudomonas* sp. ZWU0006 results in a diet-dependent increase in phospholipaseA_2 activity (** $p<0.01$ against GF; & $p<0.05$ against *Pseudomonas* sp. starved).
(B) Intestinal bacterial density is increased by the presence of dietary nutrients in zebrafish monoassociated with *Pseudomonas* sp. ZWU0006.
(C) Monoassociation with *Pseudomonas* sp. ZWU0006 results in suppression of zebrafish *phospholipase a2 group 1b* (*pla2g1b*) and *trypsin-like* (*tryl*) mRNA levels in a diet-dependent manner.

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