Short-chain fatty acids are produced by zebrafish microbiota

and influence glucose homeostasis

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Abstract

Increasingly, attention has been drawn to the association between gut microbiomes and host health, particularly to the production short-chain fatty acids (SCFAs) from indigestible carbohydrates by colonic microbiota. It is known that the main SCFAs produced by mammalian intestinal microbiota are acetate, propionate, and butyrate. These SCFAs are a significant source of nutrition, providing 10% of a human’s caloric intake, 30% for many herbivores, and up to 70% in ruminants. Additionally, they play a variety of roles in human health, influencing metabolism, treating ulcerative colitis, and conferring both anti-cancer and anti-inflammatory benefits to the colon. However, relatively little is known about production and function of SCFA in non-mammalian vertebrates. One model for studying gut physiology, metabolism, and development is the zebrafish (*Danio rerio*). The ease of access to transgenic tools and gnotobiotic manipulation, coupled with its establishment as a model system for studying many SCFA-associated physiological outcomes make zebrafish an attractive model system for studying SCFA. However, no studies have tested whether SCFA synthesis occurs in zebrafish intestines. We demonstrate that bacterial communities from adult zebrafish intestines synthesize all three main SCFAs *in vitro*, though no SCFA was detected in zebrafish intestines *in vivo*. Importantly, we find that treating zebrafish larvae with propionate reduces liver phosphoenolpyruvate carboxykinase 1 expression and overall glucose level, suggesting SCFA production in the intestine may play an important role in regulating glucose homeostasis. These results suggest that zebrafish may serve an important model to understand the physiological role of SCFAs in the context of host-microbe interactions.
Introduction

Goal:

Test the suitability of zebrafish as a model system for studying short-chain fatty acid (SCFA) production and function in the context of host-microbe interactions.

Hypotheses:

1. SCFA are produced by the zebrafish gut microbiota.
2. SCFA influence zebrafish glucose metabolism.

The human gastrointestinal tract contains roughly as many bacteria as there are cells in the rest of the body (Sender et al., 2016). These microbes play key roles in numerous aspects of host health and have been implicated in a variety of conditions, from immune disorders such as inflammatory bowel disease to metabolic diseases like type 2 diabetes (Nyangale et al., 2012). The research on the mechanisms by which microbes modulate host health is still ongoing, but it is conceivable that they do so by producing metabolites which are responsible for modulating host physiology.

One such class of metabolites produced by the gut microbiota is SCFA. SCFA are the main end products produced when recalcitrant dietary fibers that avoid digestion in the stomach and small intestine are fermented by bacteria in the large intestine (Roy et al., 2006). Acetate, propionate, and butyrate are the primary SCFA produced, with acetate composing the majority of measured SCFA content in feces (Louis et al., 2007). These three compounds have been implicated in metabolism and gastrointestinal disorders. SCFA contribute greatly to human nutrition, providing up to 10% of a human’s daily caloric intake and up to 70% of a colonocyte’s caloric needs (Bergman, 1990; Roediger, 1995).
immunity, SCFA inhibit growth of pathogenic microorganisms and increase absorption of select nutrients by reducing the pH of the gut lumen (Macfarlane & Macfarlane, 2012). For example, uptake of folate is mediated by an acidic pH dependent transporter in the colon (Said, 2011). Furthermore, butyrate improves structural defenses against pathogens by increasing mucin production and improving the integrity of tight-junctions between intestinal epithelia (Jung et al., 2015; Peng et al., 2009). Previous literature also establishes a link between SCFA and both fatty acid and glucose metabolism, with SCFA being linked to the prevention of dietary-induced obesity and reduction of glycemia in diabetic mice (den Besten et al., 2013). Thus, SCFA are critical compounds through which the gut microbiome modulates host physiology.

Given that SCFA are a known nutrition source and influence both metabolic pathways and gut physiology, studying the influence of microbially derived SCFA on host physiology therefore requires an established model that is suitable for studying the aforementioned biological processes. As such, mice are a popular model for studying SCFA in the context of host-microbe interactions (Kostic et al., 2013). However, we propose that zebrafish (Danio rerio) are a suitable model system for studying SCFA in host-microbe interactions, and offer certain unique benefits absent from other model systems.

Zebrafish have recently been used as a model for studying the gut microbiome and host-microbe interactions (Borrelli et al., 2016; Davis et al., 2016). For example, a review of zebrafish host-microbiota interaction experiments reported various studies in which phenotypes observed in germ free mice paralleled those found in germ free zebrafish, such as reduced cell proliferation, reduced immune cell presence, alteration of metabolism-related genes, and reduced expression of innate immunity-associated genes among others (Milligan-Mhyre et al., 2011). Furthermore, experiments demonstrated that the zebrafish gut sculpts its microbial communities,
paving the way for using zebrafish to reveal which host factors exert these selective forces and how they do so (Rawls et al., 2006). Additionally, microbiota regulate the absorption and metabolism of fatty acids in zebrafish much as they do in mammals (Semova et al. 2012; Martinez-Guryn et al., 2018). And though zebrafish gut microbiomes are dominated by Proteobacteria and Fusobacteria, whereas human and mouse guts are dominated by Bacteroidetes and Firmicutes (Rawls et al., 2006), studies have demonstrated that vertebrate responses to the presence of gut microbiota are highly conserved (Rawls et al., 2004). As such, zebrafish offer a translatable model system for probing host-microbiota interactions.

Furthermore, zebrafish have been used extensively to study metabolism and metabolic disease. For example, Gut et al. (2013) demonstrated that expression of phosphoenolpyruvate carboxykinase 1 (pck1), an enzyme which is a central regulatory point for gluconeogenesis, is similar in zebrafish and mammals, as evidenced by its response to starvation and various pharmacological compounds. A review by Seth et al. (2013) highlights the similarities between fat storage in zebrafish and humans, neural regulation of food intake, and expression of adipocyte related genes, as reasons for the zebrafish’s suitability for studying fat metabolism and obesity (2013). With regards to metabolic disease, Hill et al. (2016) found a conserved bacterial protein which was vital for β-cell neogenesis in zebrafish, suggesting a potential link between low childhood fecal microbial diversity and risk for diabetes.

Finally, zebrafish gut function and physiology resemble that of mammalian guts. Zebrafish intestines can be divided into three morphologically distinct sections: intestinal bulb, mid-intestine, and caudal intestine, which, based on gene expression, have functional differences much like mammalian intestines (Wang et al., 2010). Zebrafish gut epithelium show four cell types: absorptive enterocytes, goblet cells, enteroendocrine cells, and proliferative
stem/progenitor cells, which are found in mammalian guts as well. However, it should be noted that no Paneth cells have been identified, nor do zebrafish have intestinal crypts or submucosal glands (Yang et al., 2014). Thus, while differences between zebrafish and mammalian guts certainly exist, there are still numerous similarities that make zebrafish an appropriate model for the study of human gut physiology.

However, to our knowledge, no literature regarding the production of SCFA and the effects of SCFA in zebrafish with regards to host-microbe interactions, metabolism, or gut function and physiology have been published. The lack of knowledge regarding the presence and or influence of microbially-produced SCFA in this system therefore constitute a significant gap in the literature.

Previous research regarding SCFA in teleost (bony, ray-finned fish) guts in general is relatively limited. Of the published articles, both Romano et al. (2016) and Ebrahimi et al. (2017) found small amounts of acetate, propionate, and butyrate in the guts of red hybrid tilapia 
(Oreochromis sp.), while Hao et al. (2017) found similarly small amounts of the same SCFA in hindguts of grass carp (Ctenopharyngodon idellus). Though marine herbivorous fish feed more on algae than vascular plant tissue, Mountfort et al. (1994) also found the same SCFA in the hindguts of three different marine herbivorous fish: butterfish (Odax pullus), marblefish (Aplodactylus arcticidens), and silver drummer (Kyphosus sydneyanus). Thus, a reasonable assumption may be that zebrafish guts may also contain SCFA, especially because zebrafish consume both vascular plant tissue and algae, potential sources of recalcitrant carbohydrates which could be fermented into SCFA, as parts of their natural diets (Spence et al., 2007). We therefore hypothesize that there are detectable amounts of SCFA produced by gut microbiota present in the adult zebrafish gut.
With regards to the influence of SCFA on teleost physiology, the literature is similarly scarce, but studies have demonstrated the ability of tilapia (*Oreochromis mossambicus*) to absorb SCFA through gut tissue and that such absorption is primarily driven by osmotic differences between the intestinal lumen and the bloodstream (Titus & Ahearn, 1998). Additionally, other marine herbivorous fish: herring cale (*Odax cyanomelas*), butterfish, and sea carp (*Crinodus lophodont*) can metabolize SCFA via the enzyme acetyl-CoA synthetase (Clements *et al*., 1994). There also exists a positive correlation between the distribution of SCFA and the distribution of microbes in the intestines of those marine herbivorous fish (Clements *et al*., 1994). Kihara and Sakata (1997) found that nile tilapia (*Oreochromis niloticus*) fed a starch rich diet had both elevated intestinal SCFA concentrations and thicker tunica muscularis, the muscle layer around the gut. Finally, given the evidence that SCFA influences teleost physiology and a potential link between SCFA and glucose metabolism in mammals (den Besten *et al*., 2013), we hypothesize that SCFA may play a similar regulatory role in zebrafish glucose metabolism.

**Specific Aims:**

1. Determine whether SCFA can be found in detectable concentrations in adult zebrafish intestines.

   We will do so by homogenizing dissected and pooled gastrointestinal tracts from adult zebrafish, then running the samples through Gas Chromatography with Flame Ionization Detector (GC-FID). We opt for this method because GC-FID has lower limits of detection and tends to be more accurate for quantification than GC-Mass Spectroscopy (GC-MS) (Primec *et al*., 2017). This is particularly important because we expect relatively low levels of SCFA in zebrafish gut tissue, given the low levels of SCFA found in other teleost gut tissues. Additionally, we will test whether the amount of
SCFA in adult zebrafish intestines is influenced by dietary carbohydrate and fiber content.

2. Determine whether SCFA can be synthesized by the zebrafish gut microbiota \textit{in vitro}.

   The purpose of doing so is because host processes and metabolism may interfere with SCFA detection \textit{in vivo}. We do so by homogenizing adult zebrafish guts and culturing the microbial communities found in the guts in nutrient rich media under both aerobic and anaerobic conditions. We then use GC-FID to detect SCFA in the supernatant media.

3. Determine whether SCFA has an influence on glucose metabolism in larval zebrafish.

   We examine the expression of \textit{pck1}, a protein which is a central regulatory point for the process of gluconeogenesis in response to supplemented SCFA in larval zebrafish. We do so by using a \textit{pck1} reporter transgenic line (Gut \textit{et al.}, 2013), in which expression of the fluorescence protein \textit{Venus} is driven by the zebrafish \textit{pck1} promoter.

   Ultimately, demonstrating that SCFA are present and play physiological roles in zebrafish would be a significant step in demonstrating the suitability of zebrafish as a model organism for studying the biology of SCFA. Establishing zebrafish as a model to study SCFA stands to add the advantages of using zebrafish as a model organism to the toolkit for researchers studying the role of SCFA in host-microbial interactions: amenability to high-quality imaging, manipulation of microbiomes via gnotobiotic experiments in which we may control the presence or absence of microbes, ease of genetic manipulation, access to transgene lines, and high capacity for experimental replication among others.
Methods and Materials

Husbandry

Zebrafish diet and feeding schedules are described in Lickwar et al. (2017). For other relevant details of zebrafish care, please refer to Westerfield (2000). Zebrafish studies were approved by the Institutional Animal Care and Use Committees of Duke University.

Dietary manipulation for varied carbohydrate content diets

58 adult zebrafish (6 months – 2 years old) were sorted into three size classes (small, medium, and large) based on a visual estimate of length to ensure that smaller fish had an equal opportunity to feed on the high or low carb diets. Final average weight, standard length, and height at anterior of anal fin (HAA) are provided for each size class (Table 1). The fish in each size class were split evenly and randomly into two experimental groups, one which received the high carb diet and one which received a low carb diet. Dividing fish by both diet and size resulted in 6 experimental groups, each of which were placed in a new clean tank on a recirculating aquaculture facility. Fish were given a one-week adjustment period, during which their feeding regimen remained unchanged from the husbandry methods above. Size classes, genotypes, and number of fish in each diet group are listed below (Table 1).

Following the one-week adjustment period, fish were fed either a high carb or low carb diet based on the following regimen. Fish were fed roughly equal amounts of artemia (brine shrimp) at 10 AM. Designated groups were then fed with 100 mg of either the high carb or low carb diet around noon and 5 PM. This dietary manipulation lasted for 3 weeks. The composition of the high and low carb diets can be found in Table 2 (Ziegler Brothers Inc.). The methods of manufacture and preparation for the diets can be found in the methods of Wong et al. (2015).
<table>
<thead>
<tr>
<th>Size*</th>
<th>Low Carb</th>
<th>High Carb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>0.38 ± 0.04</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>Medium</td>
<td>0.40 ± 0.02</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>Large</td>
<td>0.55 ± 0.03</td>
<td>0.36 ± 0.03</td>
</tr>
</tbody>
</table>

**EK WT stands for Ekkwill wild-type, a typical laboratory strain of zebrafish.**

**Generally, there were 10 fish in this group, but one died during the course of the experiment.**

**Originally, there were 10 fish in this group, but one died during the course of the experiment.**

<table>
<thead>
<tr>
<th>Zebrafish Line</th>
<th># of Fish</th>
<th>Mean ± SE Standard Length (cm)</th>
<th>Weight (g) Mean ± SE</th>
<th>HAA (cm) Mean ± SE</th>
<th>Diet</th>
<th>Zebrafish Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Carb</td>
<td>9</td>
<td>2.72 ± 0.09</td>
<td>0.33 ± 0.3</td>
<td>0.58 ± 0.04</td>
<td>EK WT</td>
<td></td>
</tr>
<tr>
<td>High Carb</td>
<td>13</td>
<td>2.62 ± 0.07</td>
<td>0.30 ± 0.2</td>
<td>0.49 ± 0.02</td>
<td>EK WT, 13x EK with neurod:lifActin-EGFP transgenic background</td>
<td></td>
</tr>
<tr>
<td>Low Carb</td>
<td>10</td>
<td>2.36 ± 0.03</td>
<td>0.30 ± 0.3</td>
<td>0.47 ± 0.03</td>
<td>EK WT</td>
<td></td>
</tr>
<tr>
<td>High Carb</td>
<td>13</td>
<td>2.73 ± 0.10</td>
<td>0.36 ± 0.03</td>
<td>0.49 ± 0.03</td>
<td>EK WT</td>
<td></td>
</tr>
<tr>
<td>Low Carb</td>
<td>10</td>
<td>0.36 ± 0.03</td>
<td>0.36 ± 0.03</td>
<td>0.47 ± 0.03</td>
<td>EK WT</td>
<td></td>
</tr>
<tr>
<td>High Carb</td>
<td>13</td>
<td>2.36 ± 0.07</td>
<td>0.30 ± 0.2</td>
<td>0.49 ± 0.02</td>
<td>EK WT, 13x EK with neurod:lifActin-EGFP/NBT:dsRed transgenic background</td>
<td></td>
</tr>
</tbody>
</table>

Some fish with transgenic backgrounds were used to improve sample size, but the transgenic backgrounds are not relevant to

Table 1. A total of six tanks of fish were kept for this experiment, divided into three size classes of fish and two diet regimens.
Table 2. Composition of high and low carbohydrate diets used in this experiment.

<table>
<thead>
<tr>
<th>Ingredient (g/100g wet weight)</th>
<th>High carbohydrate diet</th>
<th>Low carbohydrate diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>38.13</td>
<td>45.08</td>
</tr>
<tr>
<td>Fat</td>
<td>8.02</td>
<td>15.08</td>
</tr>
<tr>
<td>Carbohydrate (not including fiber)</td>
<td>38.67</td>
<td>7.32</td>
</tr>
<tr>
<td>Vitamin supplement</td>
<td>1x</td>
<td>1x</td>
</tr>
<tr>
<td>Ash</td>
<td>5.08</td>
<td>25.15</td>
</tr>
<tr>
<td>Fiber</td>
<td>1.4153</td>
<td>0.7629</td>
</tr>
<tr>
<td>Gross energy (kcal/100g)</td>
<td>457.15681</td>
<td>430.03448</td>
</tr>
</tbody>
</table>

Sample preparation for SCFA quantification

Zebrafish were euthanized with 200 – 300 mg/L of tricaine methanesulfonate prior to dissection. For each sample, intestines dissected from five adult (90+ dpf) EK WT zebrafish males (roughly 0.2 g total) were pooled in a Precellys lysis kit tube containing 1 mL of ice cold phosphate buffered saline and kept on ice during dissection. Samples were homogenized using a Precellys 24 High-Powered Bead Homogenizer at 5500 rpm for 3 cycles at 20 seconds per cycle with a 10 second delay between cycles. 30 µL of 1 M HCl was then added to each tube to acidify samples. Samples were centrifuged at 14,000x rcf for ten minutes at room temperature. The supernatant was filtered through a 0.22 µL syringe filter. Filtrate was stored at -80°C until quantification via GC-FID.
Gas Chromatography

Samples were transferred to glass autosampler vials and loaded on the Agilent 7890B GC FID, with an HP-FFAP capillary column (25m length, ID 0.2mm, film thickness 0.33um). Settings are as follows. The front inlet was set to 220°C at 25.75psi with a total flow of 44mL/min and a split ratio of 40:1 using Helium as the carrier gas. The oven initial setpoint was 120°C and held for 1 minute after injection. Subsequently, the temperature increased at a rate of 10°C/min up to 170°C. 170°C is held for 1 minute. The average column velocity is 34.991 cm/sec (still at 25.75psi). Concentrations were determined using a linear model fit of a standard curve that encompasses the sample concentration range. Standardized concentrations used were as follows: 0.2mM, 0.5mM, 1mM, 2mM, 4mM, and 8mM. For an exhaustive list of settings used, please refer to Appendix I.

In vitro synthesis of SCFA by zebrafish gut commensals

Isolation of microbiome complex from adult zebrafish intestine and mouse feces

Intestines from four adult (6-month-old) EK WT zebrafish males were each placed in separate Precellys lysis kit tubes filled with 0.5 mL of phosphate-buffered saline reduced with 0.1% cysteine to preserve the anaerobic environment in the zebrafish intestines. Mouse feces pellets were collected into a sterile Eppendorf tube and frozen at -80°C until preparation. Four fecal pellets were then similarly prepared as the zebrafish intestines. Samples were moved to an anaerobic chamber and vortexed for three minutes to break up tissue and fecal pellets. 30 µL of each sample were used to inoculate three tubes containing 3 mL of brain-heart infusion media (Thermo Scientific, OXOID) or three tubes containing 3mL of GIFU media (Sigma), both supplemented with hemin and vitamin K to a final concentration of 12.5 mg/L of hemin and 2.5
mg/L of vitamin K which were deoxygenated for 24 hours in the anaerobic chamber prior to inoculation.

*Anaerobic and aerobic bacterial culture*

Tubes were placed in a sealed anaerobic chamber with a Gas-Pak (Becton and Dickinson) to maintain anaerobic conditions and placed in an incubator at 28°C, the typical incubation temperature for zebrafish, for 24 hours. After anaerobic cultures were prepared, the tissue and fecal homogenate was removed from the anaerobic chamber. 30 µL of each sample was used to inoculate three tubes containing 3 mL of brain-heart infusion media or three tubes containing 3mL of GIFU media supplemented with hemin and vitamin K. Tubes were placed in an incubator at 28°C for 24 hours.

*Preparation of samples for gas chromatography*

1 mL of broth was removed from each culture and 10 µL of 10M hydrochloric acid was added. Each sample was then centrifuged at 14,000x rcf for five minutes at room temperature. The supernatant was filtered through a 0.22 µm syringe filter and stored at -80°C until quantification with GC-FID. Quantification methods are identical to those listed above in the initial SCFA quantification section.

*Quantifying effect of SCFA on PCK1 expression*

*SCFA treatment of zebrafish larvae*

Transgenic zebrafish from the *Tg(pck1:Venus,cryaa:mcherry)s953* (referred to hereafter as *pck1:Venus*) line created and described by Gut *et al.* (2013) were outcrossed with EK WT fish. Eggs and larvae were kept in egg water (formulation in Lickwar *et al.*., 2017) until 3 dpf. At 3
dpf, larvae were placed in a 0.03M solution of acetate, propionate, butyrate, or a control solution. Each solution of SCFA was prepared by adding acetic acid, propionic acid, or butyric acid to egg water. Then, pH was adjusted to 7 with 1M sodium hydroxide. Given that this resulted in the solution being at the equivalence point, meaning any small changes in pH caused by the larvae would significantly alter pH, a buffer was required, so a commercially available aquarium buffer, Liquid Neutral Regulator (SeaChem), was added to a final concentration of 1.5 mL buffer/L egg water. Control solution prepared by adding Liquid Neutral Regulator to the same final concentration in egg water. Every 24 hours until the larvae were 6 days old, larvae were placed in freshly made solutions to avoid bacterial overgrowth, which was observed both when larvae were kept in the same solution for 3 days and when the solutions were not prepared anew each day.

**Imaging**

At 6 dpf, larvae were mounted on a 1% methylcellulose solution and imaged under using the Leica AF6000 system on a Leica M205 FA fluorescence stereo microscope. Images were captured using a Leica DFC 365 FX camera, at 67x magnification and an exposure time of 500 ms using the 488 nm GFP filter.

**Quantification**

Quantification was performed using FIJI version 2.0.0-rc-61/1.51s (Schindelin *et al.*, 2012). The mean fluorescence of a box, roughly 9,600 pixels² was drawn across the same location, centered on a portion of the liver with no overlap with the intestine of each larvae as pictured below, was calculated with FIJI (Figure 1).
Figure 1. An example of the area for which the fluorescence of pck1::Venus was quantified in zebrafish larvae livers. The larva pictured is one which was treated with acetate.

Quantification of effect of SCFA on glucose concentration

SCFA treatment was identical to the one described above. At 6 dpf, ten larvae from each treatment group were placed in a tube containing 100 µL of Glucose Assay Buffer from the Biovision Colorimetric/Fluorimetric Assay Kit. Larvae were then anaesthetized with 200 – 300 mg/L of tricaine methanesulfonate and homogenized via sonication (QSonica q700) for 3 minutes with an amplitude of 90, pulse on time of 2 seconds, and pulse off time of 1 second. The rest of the procedure followed the protocol specified in the Biovision kit.

Data Analysis

Data analysis was performed using RStudio, version 1.0.143 (RStudio Team, 2015). For code and raw data, please see Appendix I. For SCFA synthesis by zebrafish gut microbiota in
vitro, final concentrations were found by averaging the SCFA content across each sample for an experimental condition (GIFU + anaerobic, GIFU + aerobic, BHI + anaerobic, BHI + aerobic), then subtracting the average SCFA content in negative control broths. Mean liver fluorescence was calculating by averaging the mean fluorescence value of the drawn section across the larvae for each treatment. An ANOVA and Tukey-post hoc analysis was performed to determine whether any significant differences in mean liver fluorescence could be detected across SCAF treatment groups. An ANOVA and Tukey-post hoc analysis was also performed on glucose quantification readings to determine whether any significant differences in glucose concentration in fish could be detected across different SCFA treatments.
Results

Presence of SCFA in adult zebrafish intestines

No detectable amounts of SCFA were found in adult zebrafish intestines in the initial measurement experiment performed on WT adults without dietary modifications.

In vitro synthesis of SCFA by zebrafish gut commensals

Communities of microbes cultured from WT adult male zebrafish intestines were able to synthesize acetate under both aerobic and anaerobic conditions (Figure 2). However, only under anaerobic conditions were concentrations of propionate within the range of standards used to calculate concentrations. Butyrate was detected within standard ranges only in BHI broth under anaerobic conditions. The highest concentrations of SCFA were detected under anaerobic conditions in BHI, and acetate, propionate, and butyrate, the three most biologically relevant SCFA in mammals are present in a roughly 90:5:5 ratio. Valerate, isobutyrate, and isovalerate are SCFA which are less well-studied and less relevant to gut physiology, but were quantified nonetheless. No valerate was detected in any sample. Isobutyrate and isovalerate were only detectable in trace amounts in GIFU media, but given that the media already contained trace amounts prior to incubation, actual synthesis of isobutyrate and isovalerate is dubious. At least under certain conditions, zebrafish microbiota demonstrate the capacity to synthesize the three primary SCFA found in mammalian intestines: acetate, propionate, and butyrate.
Figure 2. Amounts of short-chain fatty acids synthesized by microbial communities from mouse feces and adult zebrafish intestines grouped by experimental conditions. SCFA content of nutrient media used to culture microbes is provided under “Negative”. Asterisks indicate measurements within the range of standards. Bars without the asterisk should be interpreted with caution; the concentrations are ordinal but cannot be accurately determined (e.g., butyrate concentration in Zebrafish BHI Anaerobic is greater than in Zebrafish GIFU Anaerobic, though it is uncertain by how much).
Toxicity of SCFA to zebrafish

The maximum acceptable concentration of SCFA in egg water for which all zebrafish larvae survived was found in order to determine the maximum dosage that could be given in future experiments. The maximum dosage of acetate for which all zebrafish survived was 0.05M, while the maximum dosage of butyrate and propionate for which all zebrafish survived was 0.03M (Figure 3). Notably, 24 hours after treatment with butyrate at a concentration higher than 0.05M, larvae exhibited hyperactivity, characterized by constant, erratic swimming behavior. At or below the maximum dosage for which all zebrafish survived, no abnormal phenotypes or behaviors were observed, nor were any obvious toxic effects observed.

Figure 3. Each graph plots the average number of surviving EK WT zebrafish larvae each day following their initial exposure to the listed concentrations of short-chain fatty acid.
SCFA influences expression of phosphoenolpyruvate carboxykinase 1

Fluorescence of the livers of zebrafish larvae expressing pck1:Venus was determined following 3 days of treatment with different SCFA. The fluorescence under a 488 nm GFP filter is a proxy for the expression of pck1. Treatment of larvae with 0.03M acetate and 0.03M butyrate did not significantly alter pck1:Venus fluorescence relative to a control solution, but treatment with 0.03M propionate significantly reduced expression relative to acetate, butyrate, and control (Fig. 4). Representative images of larvae treated with SCFA are pictured in Figure 5.

Figure 4. Box plots are drawn for the fluorescence at 488 nm of pck1:Venus zebrafish larvae livers following 72 hours of exposure to 0.03M acetate, butyrate, propionate, or an egg water control. Asterisks indicate the groups between which statistically significant differences in fluorescence were detected (* = p < 0.001, ** = p < 0.01, *** = p < 0.05).
Figure 5. Representative images taken at 488 nm of pck1:Venus zebrafish larvae livers following 72 hours of exposure to A, A') 0.03M acetate; B, B') propionate; C, C') butyrate or D, D') an egg water control. Selected images had fluorescence values nearest the mean fluorescence value. In panels D and H, fluorescence along the intestinal tract was observed, but was determined to be autofluorescence.
SCFA influences glucose levels in larval zebrafish

It was observed that larval EK WT zebrafish treated with either 0.03M acetate or 0.03M propionate for 72 hours had less glucose present in their bodies than those not treated with SCFA (Fig. 6).

Figure 6. The concentration of glucose in EK WT zebrafish following 72 hours of treatment with 0.03M acetate, 0.03M propionate, or a control solution. Asterisks indicate significant differences at p < 0.05. Both acetate and propionate-treated fish had significantly less glucose than control fish.
Discussion

The suitability and value of zebrafish as a model organism for studying host-microbe interactions by way of SCFA depends on whether microbes in the zebrafish gut are capable of synthesizing SCFA, whether they produce SCFA under conditions found in zebrafish intestines, and whether SCFA influences host physiology in a similar way as in humans. In our study, we find that 1) zebrafish gut commensals are capable of synthesizing SCFA in vitro, however, 2) with our current methods, we are unable to detect SCFA in zebrafish intestines and 3) SCFA influence glucose homeostasis.

Zebrafish gut commensals produce SCFA in vitro

Data indicate that zebrafish gut commensals possess metabolic pathways required for synthesis of the three most relevant SCFA in mammalian systems: acetate, propionate, and butyrate. However, the ratio of acetate, propionate, and butyrate produced under anaerobic conditions in BHI media (90:5:5) differed from the ratio typically observed in mammalian colons (60:20:20) (den Besten et al., 2013). One explanation for this discrepancy may be the differing bacterial communities found in zebrafish and mammalian intestines. While zebrafish intestinal commensals are primarily composed of Proteobacteria and Fusobacteria, mouse and human intestines are dominated by Bacteroidetes and Firmicutes (Rawls et al., 2006). Morrison and Preston (2016) note that studies have indicated that though acetate production is a widely distributed trait across a broad range of bacterial taxa, the pathways for propionate and butyrate synthesis are present in only a handful of bacterial genera. The relatively lower amounts of propionate and butyrate produced by zebrafish gut commensals may simply be because zebrafish have fewer bacterial taxa capable of synthesizing these SCFA.
Alternatively, because propionate and butyrate pathways are highly substrate specific, the BHI and GIFU media used in this experiment may not have contained the proper substrates in sufficient quantities for propionate or butyrate synthesis (Morrison & Peterson, 2016). This possibility is supported by our observation that bacterial communities from mouse feces synthesized similar ratios of acetate to propionate to butyrate as the zebrafish communities, despite previous studies finding different ratios of these SCFA in mouse feces, suggesting that propionate and butyrate synthesis may be limited by substrate availability (den Besten et al., 2013). As GIFU and BHI are undefined media, data about the chemical composition of the media is not readily available, but experiments with defined media containing known substrates for propionate and butyrate such as cellulose may reveal whether substrate concentrations or lack of synthesis pathways in zebrafish gut commensals is responsible for the shift in SCFA ratios relative to SCFA ratios found in mammalian intestines. Additionally, RNA-seq could reveal whether differences in quantity of mRNAs from acetate synthesis pathways as opposed to propionate and butyrate synthesis pathways could provide a similar explanation.

**SCFA in zebrafish intestines**

Though it was demonstrated that communities of zebrafish gut commensals produced SCFA in vitro, no SCFA were detected in homogenized adult zebrafish intestines. This was initially somewhat surprising, as SCFA have been found in a variety of other teleosts as mentioned in the introduction (Titus & Ahearn, 1998; Mountford et al., 2002; Romano et al., 2016). It is unlikely that the bacteria in the zebrafish lack the synthesis pathways for SCFA, as it was demonstrated above that zebrafish gut commensals are capable of SCFA synthesis. Instead, this discrepancy may be due to dietary differences between the zebrafish tested in this experiment and the fish tested in the other studies. Zebrafish are omnivores, and in the Duke
aquaculture facility, fish are fed protein rich diets, whereas Titus and Ahearn caught wild Mozambique tilapia, which are vegetarians, and whose diets consist mostly of diatoms and vegetation (Dyer et al., 2013). Fiber and complex carbohydrates found in plant material, the primary substrates for SCFA production by enteric bacteria, are therefore enriched in the tilapia diets, and depleted in the Duke zebrafish diets (Roy et al., 2006). To rule out the possibility that no SCFA was detected in zebrafish due to this discrepancy, we are currently feeding adult zebrafish a modified diet containing roughly three times as much carbohydrate content and 50% more fiber content than is the typical laboratory feed, thereby providing zebrafish with a diet containing more SCFA substrates.

An alternate explanation for the lack of detectable SCFA may be that the intestinal epithelia are removing SCFA from the lumen and metabolizing it too quickly for detection. It is known that the zebrafish genome encodes an acetyl-CoA synthetase, which converts acetate into acetyl-CoA for its subsequent metabolism in the TCA cycle or incorporation into fatty-acid chains (Lopes-Marques et al., 2013). Additionally, experiments have shown that teleost epithelia contain active transporters which exchange SCFA from the lumen with intracellular bicarbonate (Titus & Ahearn, 1998). Furthermore, the zebrafish genome contains putative protein-coding genes \textit{slc5a8l} and \textit{slc16a3}, which are homologs for sodium-coupled monocarboxylate transporter 1 (SMCT1) and monocarboxylate transporter 4 (MCT4) respectively (Howe et al., 2013). The monocarboxylate transporter family is known to transport various SCFA across intestinal epithelia in mammalian intestines, which may suggest that zebrafish intestines are capable of similar transport (Kirat & Kato, 2006; Halestrap & Wilson, 2012). The presence of both active transporters of SCFA and enzymes which metabolize SCFA in the zebrafish genome may mean that SCFA does not accumulate in appreciable quantities in the zebrafish intestine. However, this
is speculative at best, and verification of the function of both acetyl-CoA synthetase and the zebrafish homologs for the MCT family in zebrafish intestinal epithelia will be required for a more definitive answer.

**Propionate alters glucose homeostasis**

Though treatment with 0.03M acetate and 0.03M butyrate did not alter fluorescence intensity of larval zebrafish livers, treatment with 0.03M propionate for reduced fluorescence, indicating decreased expression of *PCK1* in response to propionate. Previously, Zhang et al. had shown that bovine calf *PCK1* mRNA levels increased in response to supplementation with propionate (2015). Additionally, in a later paper, Zhang et al. showed that propionate activates the cytosolic promoter for *PCK1* in bovine hepatocytes (2016). These findings are seemingly contrary to our findings that propionate reduces *PCK1* expression, but there are a handful of factors which may explain the difference. The most notable difference in experimental conditions between our study and the two Zhang et al. studies are that their studies looked at *PCK1* expression under a fed state, while in our study, the zebrafish larvae are in a fasting state by the time imaging occurs. The two states differ immensely in terms of the predominant metabolic reactions taking place; gluconeogenesis, which depends on *PCK1*, typically only occurs during a fasting state and is inactive during a fed state. To our knowledge, there is no literature on the action of propionate during fasting versus fed states, but speculatively, it may be that propionate only promotes *PCK1* expression in a fed state. However, a reduction in *PCK1* activity is still interesting, as propionate is the primary organic acid substrate for gluconeogenesis in bovines (Aschenbach et al., 2010), so we had expected that supplementation of propionate would increase *PCK1* expression. We must be careful to note that *PCK1* expression levels may not correlate with the actual rate of gluconeogenesis in zebrafish larvae,
and that hepatic fluorescence in \textit{pck1:Venus} zebrafish is merely a proxy for gluconeogenic activity. However, data from our experiments suggest that treatment of zebrafish larvae with propionate reduces the bodily glucose concentrations, suggesting that gluconeogenesis may indeed be suppressed.

We propose that the observed decrease in hepatic PCK1 expression is due to intestinal gluconeogenesis. Cherbuy \textit{et al.}, (2017) demonstrated that in bovine calves, intestinal gluconeogenesis can account for nearly 10\% of the entire body’s glucogenic output. Additionally, Mithieux (2007) notes that hepatoporal glucose sensors can detect intestinally derived glucose. We propose that propionate is being synthesized into glucose in enterocytes, and that small amount of glucose is detected by the liver, reducing hepatic gluconeogenesis because the liver no longer needs to contribute the entirety of the body’s glucose needs (Fig. 7). The \textit{pck1:Venus} reporter is active in the liver but not in other gluconeogenic tissues such as intestine, therefore effects of SCFA on intestinal gluconeogenesis were not measured in our experiments. In future experiments, we will measure intestinal gluconeogenesis markers to determine whether propionate indeed increases the rate of gluconeogenesis in enterocytes.

\textbf{Figure 7.} A diagram of our proposed model for how propionate influences glucose homeostasis in zebrafish larvae.
Ideally, we would also like to perform an isotopic tracing study to determine whether propionate that is being supplemented is being used as a glucogenic substrate in enterocytes, or if it has some other regulatory role in gluconeogenesis. Additionally, there is a possibility that microbes in the lumen of the zebrafish intestines may be influencing metabolism by producing other metabolites or by metabolizing the supplemented SCFA. As such, we would like to conduct an experiment in which germ-free pck1:Venus zebrafish larvae are supplemented with SCFA to control for microbial activity in determining what effect SCFA have on PCK1 expression. As mentioned in the methods, bacterial overgrowth was previously observed in the media when treating zebrafish larvae with SCFA. It may be that the bacteria such as Escherichia coli, which are known to grow on acetate if other sources of organic carbon are depleted, were consuming the SCFA as a source of organic carbon, which would reduce the effective dosage of SCFA administered to the zebrafish (Cozzone, 1998). Additionally, bacteria may produce metabolites with unknown or uncharacterized functions which alter the physiology of the larvae. Granted, our refined methods eliminated the bacterial overgrowth problem, but the same consumption of SCFA and potential metabolite production likely still occur at a smaller scale. A germ-free experiment would allow us to eliminate these confounding sources of error.

Conclusions

We demonstrate that though zebrafish gut commensals are capable of synthesizing SCFA in vitro, there is no detectable SCFA in zebrafish intestines in vivo. Moreover, the SCFA propionate seemingly plays an opposing role in gluconeogenesis in zebrafish as it does in mammals, by reducing pck1 expression rather than increasing it. Thus, the suitability of zebrafish as a model organism for studying host-microbe interactions mediated by SCFA is dubious. However, the fact alone that SCFA influences glucose homeostasis may provide a potential link
between microbial activity in intestines and diabetes outcomes. Thus, while numerous confounding factors in this study must be ruled out before we can make a claim with any certainty about the suitability of zebrafish as a model organism in this field of study, the potential for unveiling new findings regarding relevant health concerns such as diabetes warrants continued research into the role of SCFA in zebrafish biology.
Acknowledgements

I would first like to thank my PI, Dr. John Rawls, and the post-doc who assisted me with this project, Dr. Lihua Ye, who were invaluable in their expertise and guidance. I greatly appreciate the time and effort they dedicated to helping me push this project forward. Additionally, this project would not have been possible without the help of Zachary Holmes from the lab of Dr. Lawrence David, who helped me develop protocols and ran my samples and quantified SCFA content. Furthermore, Dr. Jessica McCann helped me design the protocol for the in vitro SCFA synthesis experiments as well. I would also like to thank Dr. Brian Coggins for dedicating his time and energy to reading drafts of my thesis. Finally, I would like to thank the Rawls lab and Duke University’s MGM department at large for providing the resources and opportunities to conduct research as an undergraduate.
References


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and host energy metabolism. *Journal of Lipid Research, 54*(9), 2325–2340. 
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Sender, R., Fuchs, S., & Milo, R. (2016). Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLOS Biology, 14*(8), e1002533. https://doi.org/10.1371/journal.pbio.1002533


Appendix I

Settings used for the GC-FID are as follows:

Pre-Run Cmd/Macro: off
Data Acquisition: on
Standard Data Analysis: on
Customized Data Analysis: off
Save GLP Data: off
Post-Run Cmd/Macro: off
Save Method with Data: off

Injection Source and Location
Injection Source: GC Injector
Injection Location: Front

=====================================================================  
Agilent 7890B
=====================================================================  
GC
GC Summary
Run Time 7 min
Post Run Time 1 min

Oven
Equilibration Time 0.25 min
Max Temperature 240 °C
Maximum Temperature Override Disabled
Slow Fan Disabled

Temperature
Setpoint On
(Initial) 120 °C
Hold Time 1 min
Post Run 220 °C

Program
#1 Rate 10 °C/min
#1 Value 170 °C
#1 Hold Time 1 min

ALS
Front Injector
Syringe Size 10 μL
Injection Volume 0.5 μL
Solvent A Washes (PreInj) 1
Solvent A Washes (PostInj) 2
Solvent A Volume 8 μL
Solvent B Washes (PreInj) 1
Solvent B Washes (PostInj) 2
Solvent B Volume 8 μL
Sample Washes 1
Sample Wash Volume 8 μL
Sample Pumps 3
Dwell Time (PreInj) 0 min
Dwell Time (PostInj) 0 min
Solvent Wash Draw Speed 300 μL/min
Solvent Wash Dispense Speed 3000 μL/min
Sample Wash Draw Speed 300 μL/min
Sample Wash Dispense Speed 3000 μL/min
Injection Dispense Speed 300 μL/min
Viscosity Delay 0 sec
Sample Depth Disabled
Injection Type Standard
L1 Airgap 0.2 μL
Solvent Wash Mode A-A2,B-B2

Sample Overlap Mode Sample overlap is not enabled

Front SS Inlet He Mode Split
Heater On 220 °C
Pressure On 25.75 psi
Total Flow On 44 mL/min
Septum Purge Flow On 3 mL/min
Gas Saver On 20 After 2 min mL/min
Split Ratio 40 : 1
Split Flow 40 mL/min
Liner Agilent 5190-2295: 870 μL (Universal, low pressure drop, ultra i)

Column Column Outlet Pressure 0 psi
Column #1 Column Information Agilent 19091F-102
HP-FFAP Temperature Range 60 °C—240 °C (250 °C)
Dimensions 25 m x 200 μm x 0.33 μm
Column lock Unlocked
In Front SS Inlet He
<table>
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<th>Setting</th>
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</thead>
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<td>Front Detector FID</td>
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<tr>
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<tr>
<td>Flow Setpoint</td>
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<tr>
<td>Post Run</td>
<td>1 mL/min</td>
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<tr>
<td>Front Detector FID</td>
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<tr>
<td>Makeup</td>
<td>N2</td>
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<tr>
<td>Heater</td>
<td>On 250 °C</td>
</tr>
<tr>
<td>H2 Flow</td>
<td>On 30 mL/min</td>
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<tr>
<td>Air Flow</td>
<td>On 400 mL/min</td>
</tr>
<tr>
<td>Makeup Flow</td>
<td>On 25 mL/min</td>
</tr>
<tr>
<td>Carrier Gas Flow Correction</td>
<td>Does not affect Makeup or Fuel Flow</td>
</tr>
<tr>
<td>Flame</td>
<td>On</td>
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<td>Signals</td>
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<tr>
<td>Signal #1: Front Signal</td>
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</tr>
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<td>Description</td>
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<tr>
<td>Details</td>
<td>Front Signal (FID)</td>
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<td>Save</td>
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</tr>
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<td>Data Rate</td>
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</tr>
</tbody>
</table>
Code for the R programs used to analyze the data can be found here:

**SCFA Survival Curves**

Alvin Han

December 8, 2017

data <- read.csv("C:/Users/bobmh/Documents/SCFA Project/Survival Curves_Updated.csv")

colnames(data) <- c("SCFA","Day","Well1","Well2","Well3","Avg Survivors","STD ev")

data<- data[-c(73,74,75,76,77,78,79,80), ]
data$SE <- data$STDev/sqrt(20)
data$SCFA <- as.factor(data$SCFA)
data

library("plyr")
library("ggplot2")
ggplot(data,aes(x=Day, y=Avg Survivors)) + geom_point() + geom_line() +    facet_wrap("SCFA", nrow=3) +    theme(strip.text = element_text(size=8), legend.position="none",panel.background = element_blank(), panel.grid = element_blank(), axis.line = element_line(color = "black",size=0.2)) +    geom_errorbar(aes(ymin=Avg Survivors-SE,ymax=Avg Survivors+SE),width=0.2, p    osition=position_dodge(0.9))+    ylab("Average Survivors") + xlab("Days after initial treatment")
#import data

data <- read.csv("C:/Users/bobmh/Documents/SCFA Project/Pck1 expt 3 1_9_18/pck1 venus experiment 3_long exposure_cols.csv")

#set column names

colnames(data) <- c("Sample_Type","Well","Individual","Area","Mean", "Min", "Max","col_order")

#set sample type as a factor

data$Sample_Type = as.factor(data$Sample_Type)

library("plyr")

#summarize data and plot it

sumdata <- ddply(data, c("Sample_Type"), summarise, N = length(Mean), mean = mean(Mean), sd = sd(Mean), se = sd/sqrt(N))

sumdata$col_order <- c(1,3,4,2)
sumdata <- sumdata[order(sumdata$col_order),]
sumdata

##         Sample_Type   N     mean        sd       se col_order
## 1    Acetate 0.03 M 15 46.77573 22.982620 5.934087         1
## 4  Propionate 0.03 M 15 14.05080  5.164677 1.333514         2
## 2    Butyrate 0.03M 15 41.13887 29.440051 7.601388         3
## 3           Control 15 37.49207 23.759723 6.134734         4

library("ggplot2")

ggplot(sumdata,aes(x=reorder(Sample_Type,col_order), y=mean, fill=Sample_Type)) +
  geom_bar(position=position_dodge(), stat="identity") +
  geom_errorbar(aes(ymin=mean-se,ymax=mean+se),width=0.2, position=position_dodge(0.9)) +
  xlab("Treatment") + ylab("Average of mean pck1:venus Fluorescence") +
  theme_classic()
ggplot(data, aes(x=reorder(Sample_Type, col_order), y=Mean, color=Sample_Type)) + geom_point() + xlab("Treatment") + ylab("Mean pck1:venus Fluorescence")
#png("Plot.png", width = 8, height = 4, units = 'in', res = 600)
```r
ggplot(data, aes(x=reorder(Sample_Type, col_order), y=Mean, color=Sample_Type)) +
  geom_boxplot() + xlab("Treatment") + ylab("Mean pck1:venus Fluorescence") +
  theme_classic() +
  theme(axis.text=element_text(size=11, color = "black"),
        panel.background = element_blank(),
        panel.grid = element_blank(),
        axis.line = element_line(color = "black", size=0.2),
        axis.title = element_text(size = 12, face="bold"),
        strip.text.x =element_text(size = 12, face="bold"))

#dev.off()
```

#run an anova and tukey post hoc
```r
anova <- aov(Mean ~ Sample_Type, data)
summary(anova)
##             Df Sum Sq Mean Sq F value Pr(>F)
## Sample_Type  3   9320  3106.8   6.257  0.001 ***
## Residuals   56  27806   496.5
## ---
## Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
TukeyHSD(anova)
```
In Vitro SCFA Synthesis by Zebrafish Gut Microbes

Alvin Han

March 21, 2018

sumdata <- read.csv("C:/Users/bobmh/Documents/SCFA Project/In Vitro Experiment 12_9_17/sumdata.csv")
sumdata$scfa <- factor(sumdata$scfa, levels = c("acetate", "propionate", "butyrate", "isobutyrate", "valerate", "isovalerate"))
sumdata$Organism = factor(sumdata$Organism, levels = c('Zebrafish', 'Mouse', 'Negative'))
colnames(sumdata)[3] <- c("SCFA")
library("ggplot2")

ggplot(sumdata,aes(x = Treatments, y = mean, fill = SCFA)) +
geom_bar(position=position_dodge(), stat="identity") +
geom_errorbar(aes(ymin=mean-se,ymax=mean+se),width=0.2, position=position_dodge(0.9)) +
xlab("Conditions") +
ylab("SCFA Concentration (mM)") +
facet_grid(. ~ Organism, scales = "free_x") +
theme(axis.text.x=element_text(angle=40, hjust=1, size=12, color = "black"),
    panel.background = element_blank(),
    panel.grid = element_blank(),
    axis.line = element_line(color = "black",size=0.2),
    axis.title = element_text(size = 12, face="bold"),
    strip.text.x =element_text(size = 12, face="bold"))

#dev.off()
Response of Glucose to SCFA

Alvin Han

April 11, 2018

# import data
data <- read.csv("C:/Users/bobmh/Documents/SCFA Project/SCFA Glucose 4_7_18/glucose_data.csv")
# set column names
colnames(data) <- c("Treatment","Group","Glucose_Concentration")

library("plyr")
# summarize data and plot it
sumdata <- ddply(data, c("Treatment"), summarise, N = length(Treatment), mean = mean(Glucose_Concentration)/10, sd = sd(Glucose_Concentration)/10, se = sd/sqrt(N))
sumdata$Treatment <- factor(sumdata$Treatment, levels = c("Acetate", "Propionate", "Control"))

#png("Plot.png", width = 8, height = 4, units = 'in', res = 600)
library("ggplot2")
ggplot(sumdata,aes(x=Treatment, y=mean, fill=Treatment)) + geom_bar(position=position_dodge(), width = 0.5, stat="identity") + geom_errorbar(aes(ymin=mean-se,ymax=mean+se),width=0.2, position=position_dodge(0.9)) + xlab("Treatment") + ylab("Concentration of Glucose per Larva (nM)") + theme_classic() + theme(axis.text=element_text(size=11, color = "black"), panel.background = element_blank(), panel.grid = element_blank(), axis.line = element_line(color = "black",size=0.2), axis.title = element_text(size = 12, face="bold"), strip.text.x =element_text(size = 12, face="bold"))
# dev.off()

# Run an anova and tukey post hoc
anova <- aov(Glucose_Concentration ~ Treatment, data)
summary(anova)

##             Df    Sum Sq   Mean Sq F value Pr(>F)
## Treatment    2 0.0002647 1.323e-04   6.544 0.0311 *
## Residuals    6 0.0001213 2.022e-05
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

TukeyHSD(anova)

##    Tukey multiple comparisons of means
## 95% family-wise confidence level
##
## Fit: aov(formula = Glucose_Concentration ~ Treatment, data = data)
##
## $Treatment
##             diff     lwr     upr     p adj
## Control-Acetate 0.011666667 0.0004008414 2.293249e-02 0.0437398
## Propionate-Acetate 0.0003333333 -0.0109324919 1.159916e-02 0.9954699
## Propionate-Control -0.0113333333 -0.0225991586 -6.750808e-05 0.0488818

45