MURAJ
Minnesota Undergraduate Research & Academic Journal
z.umn.edu/MURAJ

Effects of pH Changes on Zebrafish Microbiome

By Aishwarya Belhe[1], Grace Ha[1], Dana Kang[1], Jayzryn Thompson[1], Philip Truong[1]

[1]College of Biological Sciences, University of Minnesota, Twin Cities

Abstract: The pH values of the different regions of the human gastrointestinal tract are known, though their effects on the prevalent microbiota in the respective regions are unknown. This is important to note because digestion in warm-blooded animals, like humans, can only occur within a restricted range of pH and temperature. Here we tested the effects of varying pH values on the microbiome of our model organisms: zebrafish embryos. A 16S rRNA sequencing analysis was performed to compare the relative abundance and the alpha diversity of the different bacterial families present in the zebrafish embryo gut. The derived microbial isolates of the two main bacterial genera under observation, Aeromonas and Vibrio, from the zebrafish embryo water were then assessed for growth by performing various bioassays to see the effects of pH level changes on growth related factors. It was hypothesized that Aeromonas, being an acidophile, would better grow and survive in acidic conditions and that Vibrio, being a basophile, would better grow and survive in basic conditions. The results obtained from the various bioassays indicated a higher relative fitness of Aeromonas compared to Vibrio. They also showed the tendency of Aeromonas to grow better in an acidic medium and that of Vibrio to grow better in a basic medium. Overall, the diversity of the microbiota increased due to changes in the pH value of the surroundings. This increase was observed to be greater with an acidic treatment (i.e., lowering of pH) compared to a basic treatment (i.e., increasing the pH).

Introduction

Gut microbiota has been observed to be a key mediator of gut homeostasis and to have a significant impact on host metabolism (Rastelli et al., 2018). This has led to extensive studies related to the gut microbiota and the factors that influence their growth and sustenance. One such factor is the pH of the gut, which greatly affects the microbiota. In humans, different regions of the gut have different pH values: the pH of the mouth is close to neutral, and the saliva contains enzymes that inhibit bacterial growth. On the other hand, the stomach is extremely acidic (with a pH value of 2) and the pH gradually increases in the small intestine and the colon (with values around 4-5 and 7 respectively) (Evans et al., 1988). The microbial species prevalent in different regions of the gut are influenced by their

respective pH conditions. These species play a crucial role in the host's health, as they contribute to the metabolism of the host. Turnbaugh et al. (2006) showed that when the gut microbiota of obese mice was transferred into the gut of germ-free mice (i.e., mice that are devoid of any microbes), those mice had an increase in body weight and fat mass. Gut microbes have also been found to regulate physiological processes in humans, including the nervous, immune, and endocrine routes (Rastelli et al., 2018).

Bacterial species are one of the most abundant microbes that inhabit the gut microbiota (Thursby and Juge, 2017). The number of microorganisms inhabiting the GI tract has been estimated to exceed 1014, which comprises about 10 times more bacterial cells than human cells and over 100 times the amount of genomic content of the human genome (Gill et al., 2006). Thus, the important roles that the gut microbes, especially bacteria, play in the overall development and sustenance of the human body make it necessary for us to understand the factors that affect the gut microbiota. This would help to maintain a healthy gut environment for these microbes to thrive in. The effects of pH changes have been extensively studied in fecal samples from humans (Ilhan et al., 2017) but the interactions between acidophilic (acid loving) or basophilic (base/alkali loving) bacterial isolates when subjected to pH changes have not been extensively studied.

Zebrafish are model organisms for studying early development in vertebrates, genetics, and human biology and disease due to their transparency during the larval and juvenile stages and the similarities they share with humans with respect to their genetic composition and microbiome (Rawls et al., 2004). Hence, in the following study, the microbiome of zebrafish embryos was used as a model to study the effects of changes in the pH of the environment on the abundance of the bacterial species found in the zebrafish microbiome. The main species under consideration belong to the genera Shewanella, Aeromonas and Vibrio, as these are three of the main genera which constitute the culturable gut microbiota diversity of the zebrafish (Cantas et al., 2012). A varied spectrum would be observed with respect to the survival rate of these species in acidic and alkaline pH values. For example, certain Vibrio spp., being an alkaliphiles, are expected to survive in an alkaline pH medium (i.e., potassium hydroxide solution) and Aeromonas sp., being acidophiles, are expected to survive in an acidic pH medium (i.e., sulfuric acid solution). All the species are expected to grow efficiently in the control pH medium (i.e., embryo water) (Keenleyside et al., 2012). Thus, by this approach, the survival rate of the specific bacterial species and that of the zebrafish embryos at a particular pH can be correlated. This information can be expanded to more complex systems in the future to improve our understanding of human-associated bacterial communities and how they change in response to environmental conditions.

Materials and Methods

Preparation of stock solutions for treatment groups

100ml of 0.1M HEPES buffer was made by adding 2.38g of HEPES to 80ml of water. The pH value of the buffer was

brought up to 7.4 from 5.3 using two pellets of sodium hydroxide. The acidic treatment solution of pH 5.5 was prepared by mixing 10.89ml of freshly prepared HEPES buffer, 0.082mL of sulfuric acid and 40.96ml of embryo water. The basic treatment solution of pH 7.7 was prepared using 75ml of freshly prepared HEPES buffer, 0.15ml of potassium hydroxide and 75ml of embryo water.

Animals and pH exposure

All the zebrafish experiments were conducted in accordance with the standard protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota, Twin Cities. 120 zebrafish embryos 0dpf were obtained from the University of Minnesota Zebrafish Facility and divided into four treatment groups: control without buffer (embryo water, pH range = 6.4-7.2), control with buffer (embryo water with HEPES buffer, pH range = 6.4-7.2), acidic group (sulfuric acid solution buffered to a pH of 5.5 using HEPES buffer) and basic group (potassium hydroxide solution buffered to a pH of 7.7 using HEPES buffer). The pH values were selected in conformity with the approved values (Zahingir et al. 2015). 30 zebrafish per petri dish were allowed to grow for 7 days in their respective treatment solutions at room temperature. The experiment was staggered at day 2 of the previous set. Another set of 120 embryos were obtained and exposed to various pH levels in the same manner and with the same treatment groups as the previous set for 5 days.

DNA Isolation and 16S rRNA Sequencing

Kit based DNA isolation was performed with the second set of embryos using the Qiagen DNeasy® Blood & Tissue Kit (cat. Nos. 69504 & 69506). The extracted DNA samples were submitted to the University of Minnesota Genomics Center for 16S rRNA Nanopore sequencing to determine the relative abundance and the alpha diversity of the bacterial strains present in the guts of the embryos of each treatment group.

Statistical Analysis

The 16S rRNA sequencing data was analyzed for relative abundance and alpha diversity using the mothur software package (Schloss et al., 2009). A relative abundance graph and rarefaction curve were prepared using Microsoft Excel. The rarefaction curve was obtained using the analysis data of the alpha diversity of the zebrafish microbiome.

Obtaining single colonies of strains from frozen stocks

LB Agar Media was prepared following the standard protocol (CSHL 2009b). Archival frozen stocks for Aeromonas veronii, Vibrio sp., Shewanella WT and Shewanella JG1543 were obtained from the communal stocks and confirmed via PCR and gel electrophoresis.

Carbon Source Assay

The M9 media and solutions of glucose, glycerol, acetate, and lactate were prepared using the IACUC approved standard protocol (CSHL, 2010; Sambrook 1989). A carbon source assay was performed using two replicates of each carbon source for

Aeromonas, Vibrio, Shewanella JG 1543, and Shewanella WT (the standard protocol in 16mm liquid culture tubes (Sambrook, 1989). The tubes were then tested for turbidity as a measure of growth in each carbon source (- for no/low growth, +/- for moderate growth, and + for high growth).

Minimum Inhibitory Concentration (MIC) Assay

A minimum inhibitory concentration (MIC) assay was set up in a 96-well plate with three different acid and base concentrations for each of the strains (0.2uM, 0.4uM and 0.6uM of acid and base respectively) and a control (embryo water, pH range 6.4-7.2). The pH of each of the concentration groups was determined and recorded (pH values of acid solutions = 5.5, 5.4, 5.6, respectively, and those of basic solutions = 7.7, 7.7, 7.8, respectively). The plate was then stored in a 30°C incubator for 48hours. The plate was then scored for the growth of each strain in each of the concentration groups based on the turbidity observed (- for no/low growth, +/- for moderate growth, and + for high growth).

Ferrozine Assay

A ferrozine assay was performed using a protocol adapted from Coursolle et al. (2010). Lactate was used as the carbon source for two replicates of Aeromonas, Vibrio, Shewanella JG 1543 and Shewanella WT. The presence or absence of a purple color was recorded (as yes/no color change).

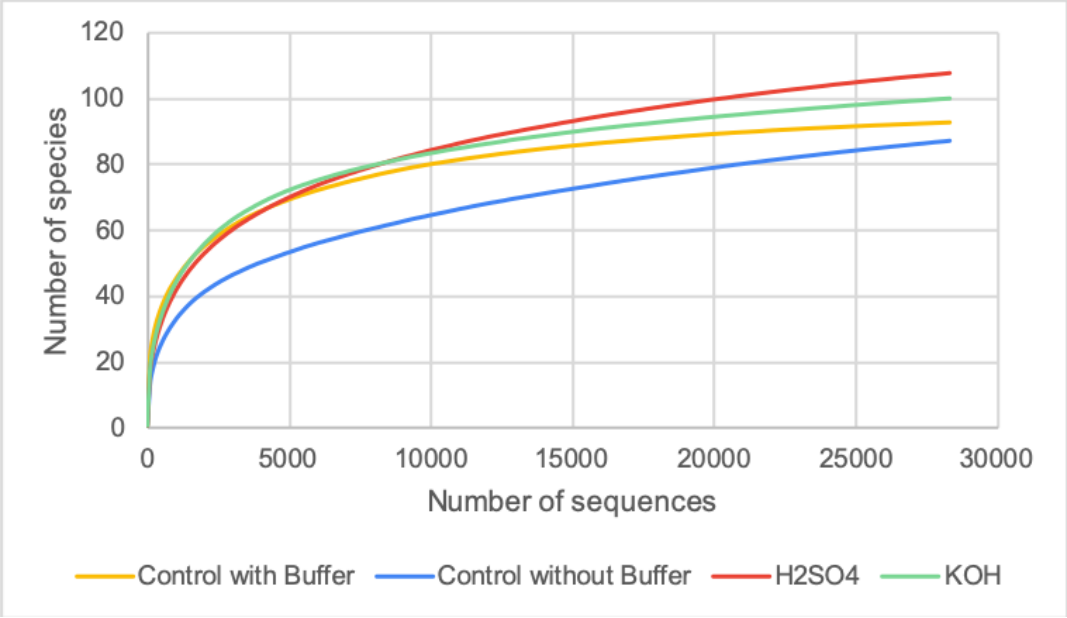

Figure 1. *Rarefaction curve showing the alpha diversity of the Operational Taxonomic Units (OTUs) found in the microbiome of the treated zebrafish embryos.* 16SrRNA sequencing using Nanopore technology was performed, and their alpha diversity was obtained using the mothur software

Competition Assay

A competition assay was set up using Aeromonas and Vibrio spp. (Lenski et al. 1991). Four 1.5ml microcentrifuge tubes were set up with 400ul of the liquid culture of each strain and 200ul of each treatment solution (control with buffer, pH range 6.4-

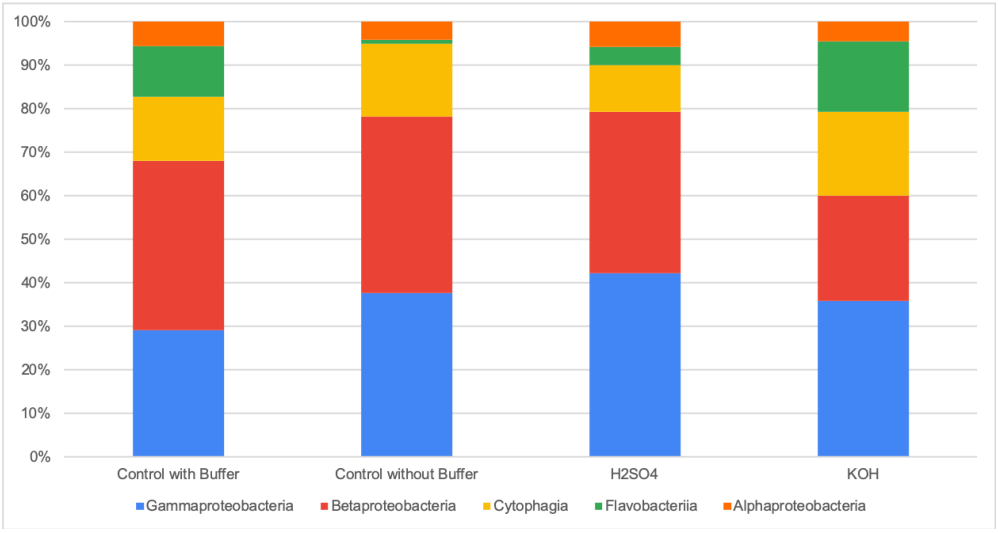

Figure 2. *Relative abundance of the Operational Taxonomic Units (OTUs) belonging to the five most populous classes of bacteria present in the microbiome of the treated zebrafish embryos.* Relative abundances using the mothur software package were obtained through 16SrRNA Nanopore sequencing. The treatment groups included control with buffer (6.8-7.2 pH), control without buffer (6.8-7.2 pH), sulfuric acid (5.5 pH) and KOH (7.7 pH).

7.2; acidic, pH 5.5; and basic, pH 7.7). 10-1, 10-2, 10-3, 10-4 and 10-5 serial dilutions were prepared and plated for day 0. The tubes were then incubated in a 30°C incubator for 5 days. 10-1, 10-2, 10-3, 10-4 and 10-5 dilutions were then plated on day 5. The relative fitness of each strain was calculated using the "best" dilution plates (determined by comparing the OD values to standards) for each culture on days 0 and 5 and the CFU/ml values of each strain at each dilution. This was done using the number of times a strain's population doubled its size over the competition experiment (D value).

Results

Alpha diversity and relative abundance analysis using the 16S DNA Sequencing method and mothur software

The analysis of the alpha diversity of the zebrafish microbiome showed more diversity at the acidic and basic pH values of 5.5 and 7.7, respectively, compared to the controls with a pH range of 6.8-7.2 (Fig. 1). The acidic group showed more diversity than the basic groups. The relative abundance of the Operational Taxonomic Units (OTUs) belonging to the five most populous classes of bacteria (i.e., Gammaproteobacteria, Betaprotobacteria, Cytophagia, Flavobacteriia and Alphaproteobacteria) varied across the different treatment groups (Fig. 2). The Gammaproteobacteria had a higher relative abundance in the acidic and the basic group (42% and 36%, respectively) than in the control group (28%). Aeromonas and Vibrio, which are the focus of this paper, belong to the class of Gammaproteobacteria. They also showed variation in relative abundances across treatment groups (Fig. 3), with Aeromonas being dominant in all of them. Aeromonas had a higher relative abundance in the control without buffer group (98.0%), the acid group (99.8%) and the basic groups (100%), than Vibrio (2%, 0.5% and 0%, respectively) (Fig. 3).

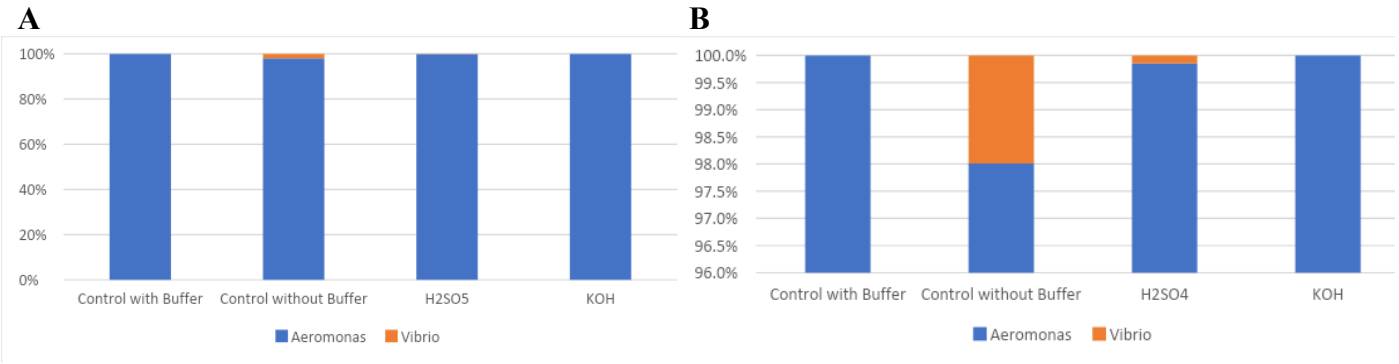

Figure 3. *Relative abundance of the Operational Taxonomic Units (OTUs) belonging to the Aeromonas and Vibrio genera present in the microbiome of the zebrafish embryos under consideration*. The treatment groups included control with buffer (6.8-7.2 pH), control without buffer (6.8-7.2 pH), sulfuric acid (5.5 pH) and KOH (7.7 pH). A) Graph with the Y-axis range from 0-100%; B) Graph with Y-axis range of 96-100%

Bioassays to test the growth and MICs of Aeromonas and Vibrio strains

The growth of two strains of Aeromonas and one strain of Vibrio was tested in the presence of glucose, lactate, acetate and glycerol, with embryo water as the control by determining the turbidity of the media and recording the Optical Density (OD) readings for each of the strains (Table 1). Strains of these two genera were found to grow best in the media with lactate as the carbon source. Shewanella strains tested were used as positive and negative controls in another experiment in the same lab.

The MIC assay showed that the MIC for the Aeromonas strain is 2% solution of potassium hydroxide (KOH) and that of the Vibrio strain is 0% solution of sulfuric acid (S.

	Glucose	Lactate	Acetate	Glycerol	Control
***Aeromonas* Elena**	clear; 0.003	clear; 0.006	clear; 0.016	clear; -0.007	clear; 0.006
***Aeromonas* Lab**	clear; -0.003	clear; 0.006	small specks; -0.011	medium-sized specks; 0.003	clear; -0.011
***Shewanella* JG 1543**	clear; -0.001	faint cloudy swirl; -0.002	faint cloudy; 1.148	clear; -0.014	cloudy specks; 0.018
***Shewanella* WT**	cloudy swirl; -0.001	cloudy swirl; 0.054	cloudy specks; 0.037	cloudy swirl; 0.023	faint cloudy specks; 0.020
***Vibrio* Luke L.**	cloudy; 0.103	cloudy; 0.129	small white specks; -0.002	cloudy; 0.122	clear; 0.005

Table 1. *Turbidity observations and optical density (OD) readings at 600nm for various strains when grown in the different carbon sources.* Each of the strains of bacteria were grown in different carbon sources and OD readings were taken.

	Shewanella WT - 1	2	*Shewanella* JG1543 - 1	2	*Aeromonas* Elena - 1	2	*Aeromonas* Lab - 1	2	Vibrio Luke L - 1	2
EW(4ul) S. Acid(6ul) [pH 5.6]	+	+	+	+	+	+	+	+	-	-
EW(6ul) S. Acid(4ul) [pH 5.4]	+	+	+	+	+	+	+	+	-	-
EW(8ul) S. Acid(2ul) [pH 5.5]	+	+	+	+	+	+	+	+	-	-
EW(10) [pH 6.6]	+	+	+	+	+	+	+	+	+/-	+/-
EW(8ul) KOH(2ul) [pH 7.7]	+	+	+	+	+/-	+/-	+/-	+/-	+	+
EW(6ul) KOH(4ul) [pH 7.7]	+/-	+/-	+/-	+/-	-	-	-	-	+	+
EW(4ul) KOH(6ul) [pH 7.8]	+/-	+/-	+/-	+/-	-	-	-	-	+	+

Table 2. *Scores for the MIC Assay.* The assay was performed for each strain having replicates, indicated above by 1 and 2, with different amounts of embryo water (EW) containing either the specified amount of sulfuric acid (S. acid) or potassium hydroxide (KOH). The presence or absence of growth was scored as + and – respectively. +/- indicates intermediate growth.

acid) (Table 2). The Aeromonas strain grows better in solutions with a greater concentration of S. acid than KOH, while the Vibrio strain grows better in solutions with a greater concentration of KOH than S. acid (Table 2).

Competition experiments show a higher relative fitness of the Aeromonas strain with respect to the Vibrio strain in all treatment groups

The relative fitness (W value) for each of the strains in each treatment group was calculated using the number of times the population doubles its size over the competition experiment (D value). The Aeromonas strain showed a higher relative fitness in all the treatment solutions when in di-association with the Vibrio strain (Table 3). The relative fitness of the Aeromonas strain was higher in the acidic solution (W = 270.37) than that in the basic solution (W = 8.25).

Treatment Group	Strains	Population Doubling (D) Value	Relative Fitness (W) Value for *Aeromonas* strain
Control with buffer	*Aeromonas* *Vibrio*	0.794 0.009	88.22 0.011
Acidic Solution (pH 5.5)	*Aeromonas* *Vibrio*	0.198 0.024	270.37 0.121
Basic Solution (pH 7.7)	*Aeromonas* *Vibrio*	14.60 0.054	8.25 0.004

Table 3. *Results for the competition experiments for Aeromonas and Vibrio strains in three of the treatment groups.* The D value is the number of times a strain's population has doubled in size during growth of the competition culture and the relative fitness value (W) is the ratio of the population di-associations under consideration.

Discussion

One of the long-term goals of the Human Microbiome Project has been to understand the associations between changes in the microbiome and health/disease by studying several different medical conditions (Peterson et al., 2009). One of these changes is the change in pH values of the different regions of the gastrointestinal (GI) tract. The pH of the gut has a significant effect on the composition of its microbiota. Wolf et al. (2014) showed that an acidic pH altered the gut microbiome and decreased the risk of diabetes in non-obese diabetic (NOD) mice. Though the significance of the pH of the gut is known, little is known about how changes can affect the gut microbiota, especially during development. To fill this gap in knowledge, we first studied the gut microbiome of the treated zebrafish embryos at the genomic level in order to determine the bacterial families present in their microbiota. Two control groups were set up: one with HEPES buffer and one without, as this buffer regulates the pH values of acidic and basic solutions. The results of the relative abundance and alpha diversity analyses showed that the treatments under study led to the diversification of the microbiota of the embryos over the span of their development (Fig. 1 and 2). These results contribute to our understanding of how the use of various pharmaceutical agents can shape the development of the microbiome of an infant, as many drugs are known to alter the pH of the gut (Neill et al., 1997). These drugs should be designed to combat disease-causing bacteria by counteracting their normal living conditions, like causing a change in the pH of the gut. This is the reason why Aeromonas and Vibrio, which belong to the class of Gammaproteobacteria, were studied under the same conditions, because not only these genera contain some disease causing strains but are also distinct in the way they interact with different pH values. Their growth under the different treatments was assessed by performing various bioassays. Aeromonas, being an acidophile, favored an acidic pH, showing more growth in the acidic solution than the basic one. In contrast, Vibrio favored the basic solution, consistent with its basophilic nature (Table 2). This suggests that a drug treatment that

makes the gut microbiota more basic could be used to combat diseases caused by acidophiles, like Aeromonas and an acidic drug could do the same for diseases caused by basophiles, like Vibrio.

The gray area would be when both acidophilic and basophilic pathogens attack a host. Here, it would be interesting to know which strain would dominate, which would help to determine the drug that would be most effective. To better understand this, a competition assay was performed with Aeromonas and Vibrio in a di-association (Table 3), with only the control with buffer, as the buffer was present in the other two treatment solutions and hence, seemed more relevant to the experiments. The results of this assay suggested that Aeromonas had outcompeted Vibrio in all treatment groups, as it had a higher relative fitness in each. The higher relative fitness of Aeromonas in the acidic solution (270.37), compared to basic solution (8.25), also confirmed its acidophilic tendency (Table 3). This contradicts previous studies with these strains under normal conditions, where Vibrio outcompeted Aeromonas (Rolig et al., 2015). How this strain of Aeromonas outcompetes this strain of Vibrio under altered pH conditions could be studied further.

In the future, this study could be performed on mice or rats, as they are extensively used as models for human health and are genetically closer to humans than zebrafish. This would help to predict the effects of changes in pH in humans and would contribute to the development of more effective drug treatments.

References

Cantas, L., Sørby, J. R., Aleström, P., & Sørum, H. (2012). Culturable gut microbiota diversity in zebrafish. Zebrafish, 9(1), 26–37. https://doi.org/10.1089/zeb.2011.0712

CSHL. (2009b). LB agar. Cold Spring Harb. Protoc. 2009, doi:10.1101/pdb.rec11683

CSHL. (2010). M9 minimal medium (standard). Cold Spring Harb. Protoc. 2010, doi:10.1101/pdb.rec12295.

Coursolle, D., Baron, D. B., Bond, D. R., & Gralnick, J. A. (2010). The Mtr respiratory pathway is essential for reducing flavins and electrodes in Shewanella oneidensis. Journal of bacteriology, 192(2), 467–474. https://doi.org/10.1128/JB.00925-09

Evans, D. F., Pye, G., Bramley, R., Clark, A. G., Dyson, T. J., & Hardcastle, J. D. (1988). Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut, 29(8), 1035–1041. https://doi.org/10.1136/gut.29.8.1035

Faith D.P. (1992). Conservation evaluation and phylogenetic diversity. Biol Conserv 61: 1–10.

Gill, S. R., Pop, M., Deboy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M., & Nelson, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. Science (New York, N.Y.), 312(5778), 1355–1359. https://doi.org/10.1126/science.1124234

Ilhan, Z. E., Marcus, A. K., Kang, D. W., Rittmann, B. E., & Krajmalnik-Brown, R. (2017). pH-Mediated Microbial and Metabolic Interactions in Fecal Enrichment Cultures. mSphere, 2(3), e00047-17. doi:10.1128/mSphere.00047-17

Jian Ye, Scott McGinnis, Thomas L. Madden, BLAST: improvements for better sequence analysis, Nucleic Acids Research, Volume 34, Issue suppl_2, 1 July 2006, Pages W6 W9, https://doi.org/10.1093/nar/gkl164

Jin, Cuiyuan & Luo, Ting & Zhu, Zhihong & Pan, Zihong & Yang, Jiajing & Wang, Wenchao & Fu, Zhengwei. (2017). Imazalil exposure induces gut microbiota dysbiosis and hepatic metabolism disorder in zebrafish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 202. 10.1016/j.cbpc.2017.08.007.

Keenleyside W. et al. (2012). The Effects of pH on Microbial Growth. In Microbiology Canadian Edition (Pressbooks Publishing), pp. 100-120.

Lane, D.J., Weisburg, W.G., Barns, S.M., Pellertier, D.A. (1991). 16S ribosomal DNA amplification for phylogenetic study, 173(2): 697-703.

Lenski et al (1991). Long-term experimental evolution in Escherichia coli. I. Adaptation and divergence during 2,000 generations. American Naturalist, 138(6): 1315-1341.

Nalbant, P., Boehmer, C., Dehmelt, L., Wehner, F., & Werner, A. (1999). Functional characterization of a Na+-phosphate cotransporter (NaPi-II) from zebrafish and identification of related transcripts. The Journal of physiology, 520 Pt 1(Pt 1), 79–89. doi:10.1111/j.1469-7793.1999.00079.

Neill J., Hickman R.J. (1997). Influence of pH on drug absorption from the gastrointestinal tract. J. Chem. Educ. 1997; 74:855–856.

Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA et al. (2009). The NIH Human Microbiome Project. Genome Res 19: 2317–2323.

Rastelli, M. et al. (2018). Gut Microbes and Health: A Focus on the Mechanisms Linking Microbes, Obesity, and Related Disorders. Obesity (Silver Spring). 26(5): 792-800.

Rawls, J.F. et al. (2004). Gnotobiotic zebrafish reveal evolutionary conserved responses to the gut microbiota. PNAS. 101(13): 4596-4601.

Rolig, A. S., Parthasarathy, R., Burns, A. R., Bohannan, B. J., & Guillemin, K. (2015). Individual Members of the Microbiota Disproportionately Modulate Host Innate Immune Responses. Cell host & microbe, 18(5), 613–620. doi:10.1016/j.chom.2015.10.009

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd edition ed. Cold Spring Harbour laboratory, Cold Spring Harbour, NY.

Schloss et al. (2009). Introducing mothur: Open source, platform-independent, community supported software for describing and comparing microbial communities. Apply. Environ. Microbiol., 75(23): 7357-41.

Thursby, E. and Juge, N. (2017). Introduction to the human gut microbiota. Biochem J. 474(11): 1823-1836.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. Nature, 444(7122), 1027–1031. https://doi.org/10.1038/nature05414

Turner, S., Pryer, K.M., Miao, V.P., Palmer, J.D. (1999). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis, 46(4): 327-38.

von Canstein H, Ogawa J, Shimizu S, Lloyd JR. (2008). Secretion of flavins by Shewanella species and their role in extracellular electron transfer. Appl Environ Microbiol 74:615–623. doi:10.1128/AEM.01387-07.

Wolf, K. J., Daft, J. G., Tanner, S. M., Hartmann, R., Khafipour, E., & Lorenz, R. G. (2014). Consumption of Acidic Water Alters the Gut Microbiome and Decreases the Risk of Diabetes in NOD Mice. Journal of Histochemistry & Cytochemistry, 62(4), 237–250. https://doi.org/10.1369/0022155413519650

Zahangir, M., Haque, F., Mostakim, G.M., Islam, M.S., (2015). Secondary Stress Responses of Zebrafish to Different pH: Evaluation in a Seasonal Manner. Aquaculture Reports, 2: 91-96.