Alternative Data Analysis Approach and Ecological Perspectives on the Age-Related Changes in the Gut Microbiota of the Chinese Giant Salamanders (Andrias Davidianus)

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Abstract: The gut microbiome of an organism plays a pivotal role in host fitness through contributions to host metabolism and behavior. In a previous study, Zhang et al (2018) discovered The Chinese Giant Salamander's (CGS) (*Andrias davidianus*) gut microbiome reflects age development and growth, indicating both extrinsic (dietary) and intrinsic (host traits) forces influence the establishment and subsistence of microbes in the gut. Oftentimes, the effects of host-mediated selection on associated assemblages are more subtle than dietary-derived colonization for gut microbes, potentially drowning out important host effects on symbiotic associations. That said, recent advances in high thruput sequencing (HTS)have enabled researchers to uncover sequence-level differences through the usage of amplicon sequence variants (ASVs), uncovering cryptic variations at the lowest taxonomic levels. In this study, we reassess the gut microbiome of the CGS over the course of development using ASVs as opposed to 97% clustering to understand the effects of host-mediated selection on dietary derived microbes in larval and juvenile salamanders[1]. Our results will provide much-needed clarity concerning the definition of a species in HTS studies as well as an understanding of how gut microbiomes are established and maintained over development in a highly endemic, critically endangered salamander.

Keywords: Environmental Science; Technical Research; ASV

Introduction

Microbiome is a community constituted by micro-organisms in a particular habitat or host (Microbiology society,2022). Although not all species have microbiome, organisms such as plants, animals and human have peculiar structure of microbiome. New findings on studies on host-associated microbiomes of individual organisms and free-living microbiota shows patterns related to immune system. Host-associated microbiomes also influence evolutionary, immunological, and ecological processes (Woodhams,2020). The host and its microbes is another distinct biological system which plays an important role in metabolism, immunity, and development (Margulis and Fester,1991; Rosenberg and Zilber-Rosenberg 2018). In the paper, we are focusing on the host-associated microbiome, more specifically, the microbiome of the salamanders.

The study of microbiome covers difference species and classes of animals. Recently, there are few studies amphibian microbiota which largely contributed to the study of salamanders and frogs. As amphibians are facing rapid population decline and extinctions, an understanding of the effects of microbiome symbiosis and mitigation is a prerequisite for amphibian protection (Jiménez,2017)[2]. The microbiota of amphibian species such as frogs or salamanders are highly unique to species, which means the individuality and uniqueness of the microbiota could help scientists to navigate different genes and contribute to the study of gene shift. Also, there is a balance exists between respiratory, skin, and gastrointestinal microbiota and the host (Ludek,2021). Study of the single microbiota or the relation between can positively effect the realm of amphibian and amphibian microbiome. In particular, the gut microbiota can positively and negatively affect host health[13]. There are trillions of micro-organisms living in the gastrointestinal tract (Zhang,2018)[14]. Environment factors such as diet,

host environment, maternal effect, stochasticity, and so on interact to shape the gut microbiota, as well as the interaction with host genetics, influencing the composition of the microbiota (Spor A,2020)[15]. A detailed understanding of how an organism's gut microbiome community is constructed and utilized across an organism's lifespan is essential to understand how anthropogenic and natural factors influence amphibian species (Zhang,2018)[16]. The composition of an organism's gut microbiome include dietary preference and prey availability (David et al., 2014; Knutie, Shea, et al. 2017; Ley, Lozupone, et al. 2008; Zhang et al. 2010), metamorphic transition from the larval stage (tadpole) to the adult (frog) stage in Anura (Kohl et al. 2013; Vences et al. 2016), endocrine disruptors (Vences et al., 2016), and internal regulation facilitating hibernation (Weng et al, 2016)[17].

Salamanders are examples of host-associated microbiomes since the microorganisms occurs inside and on host surface. The understanding of interactions between host and microbiome which could affect metabolism and contribute to host adaptation to changing environments is crucial to protect endangered salamander species (Prado-Irwin,2017). The Chinese Giant Salamander (Andrias davidianus), one of the largest salamanders and one of the largest amphibians in the world, is endemic to China. Being a part of the family Cryptobranchidae which dates back 170 million years ago, there are fewer than 50,000 in the wild found in rocky mountain streams and lakes in the Yangtze river basin of central China (Mongabay,2015). The Chinese Giant Salamanders are critically endangered due to over-harvesting for human consumption, habitat loss, water pollution, and being used in traditional Chinese medicine (San Diego Zoo,2022). The Chinese giant salamander is often called a living fossil and is considered a valuable model species for phylogenetic and evolutionary studies (Geng et al. 2017).Thus, the study of the Chinese Giant Salamander became important. A previous paper (Zhang,2018), the team utilizes the changes in gut microbiota relative to age of the Chinese Giant Salamanders with skills such as bioinformatics, alpha diversity and beta diversity[8].

In this paper, we offer an alternative approach to analyze and evaluate the data to provide new perspectives and insights on the gut microbiome of the Chinese Giant Salamander and compare the results between the methods.

1. Material and Methods

1.1 Sequence acquisition and Bioinformatics

To generate sequence tables to assess within and between sample differences, we utilized bioinformatic approaches to produce OTU (97% clustering) and ASV tables (amplicon sequence variants). We aim to discover precise and subtle sequence-level difference through recent advances in high thruput sequencing (HTS), and understand the effect of host-mediated selection on dietary derived microbes in larval and juvenile salamanders. Raw .fasta sequences were downloaded from Figshare (https://doi.org/10.6084/m9.figshare.7243463.v1) and split into separate files, one file for each sample. We will analyze the data with bioinformatics and computation to interpret biological data. Bioinformatics incorporates math, physics, computer science, information engineering, statistics, and biology under a big umbrella to help produce the highest quality of scientific research and papers. Since Bioinformatics utilizes software tools, it is usually used for interpreting or decoding sizeable complex data sets. More importantly, bioinformatics can determine genes and establish evolutionary relationships.We generated 30813 taxa and 53 samples with 3 sample variables and 7 taxonomic ranks.

To do this, we made a sample-specific barcode and primer and then wrote a .fasta file for each sample. Once organized, we then recursively removed the primers and barcodes, resulting in one folder with a file for each sample. Next, we dereplicated each sequence to get how many counts were in each sample of each sequence. This resulted in a different ASV table for each sample. These ASV tables were then merged along shared sequences to produce a single ASV with all samples and sequences. Finally, relevant metadata was incorporated, taxonomy was assigned using the IDtaxa algorithm (cite), and all information was constructed into a phyloseq object (cite). The 97% clustered OTU table was constructed by clustering ASV sequences using DECIPHER (cite) and formulated into an OTU table.

1.2 Statistics

To assess the richness and evenness of the alpha diversity (the within sample component of diversity), we utilize Hill Number'sto estimate the data sets. Hill numbers are a mathematically unified family of diversity indices that incorporate relative abundance and species richness and overcome many shortcomings of microbiome research, chiefly that diversity is not evenly distributed, with a large proportion of species being relatively 'rare' and not contributing much relative abundance. The estimation varies by an exponent q, which represents diversity and relative abundance measurement. In addition, if two completely distinct assemblages (i.e., no species in common) have identical relative abundance distributions, then the Hill number doubles if the assemblages are combined with equal weights.

We estimated alpha diversity at three orders of q. When $q=0$, the effective number of species is just the richness of a sample, or the number of distinct 'species' in each sample. When $q=1$, the species are weighed in proportion to their frequencies which are the effective number of common or "typical" species. When $q = 2$, abundant species are favored and rare species are discounted, which counts as the effective number of dominant or very abundant species in the sample. The metrics generated can conclude an ecological community's structure with respect to its richness (number of taxonomic groups), evenness (distribution of abundances of the groups), or both. Differences in alpha diversity for each order of q between age and sample type will be assessed using a tukeyHSD test. Additionally, we will assess the decrease in evenness by comparing diversity estimates for each sample type over q.

Beta diversity as the method to compare the microbiome between the stomach, duodenum, ileum, and rectum groups from the Chinese Giant Salamanders of different ages. Beta diversity describes the between sample component of diversity and multivariate analyses of beta diversity rely on estimates of community dissimilarity, a transformation of a multiplicative beta. Beta diversity, in short, is the ratio between regional and local species diversity. In this paper, maximum beta diversity is 2 (no shared species) and the minimum is 1 (completely shared Conveniently, Jost (2007) used mathematical proofs to establish the relationship between dissimilarity indices and Hill Numbers. The Sørensen index, the proportion of species in one sample that is not detected in another, corresponds to a $q=0$ beta diversity metric, since it is only reliant on the detection of species (eg: binary). The Horn dissimilarity (1-Horn index of overlap) takes into account the relative abundance of taxa, while the Morisita-Horn dissimilarity index is most influenced by the dominant community members, corresponding to $q=1$ and $q=2$ Hill Numbers, respectively. We estimate the beta diversity through Hill numbers and compare the similarity and differences between the samples.

Aside from the diversity measurement, we will find changes of microorganisms with age and compare the microbial community across gastrointestinal tract sections using different methods and analysis approach.
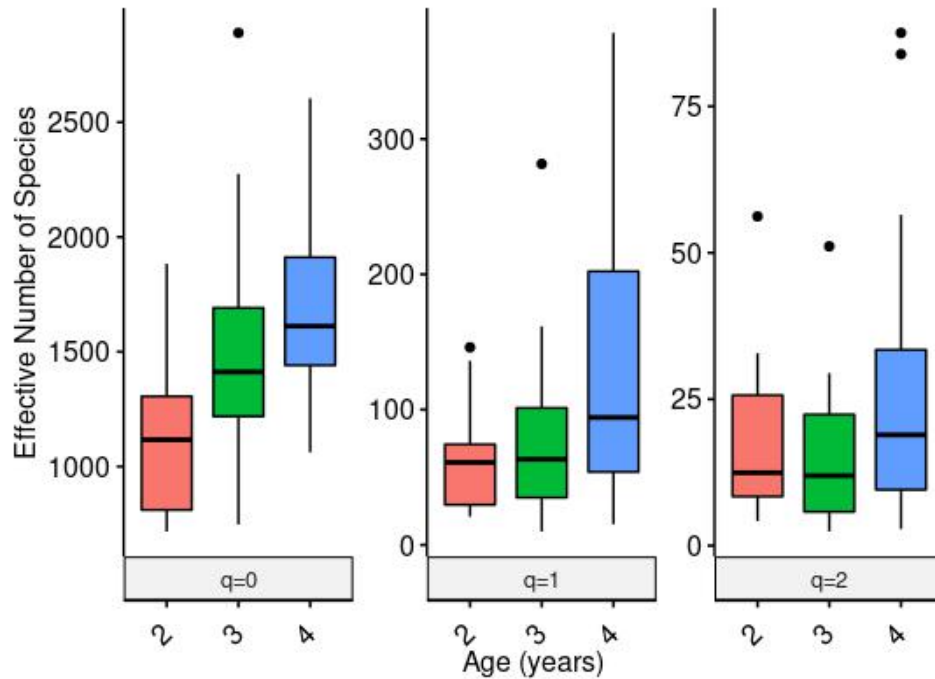
2. Results

2.1 97% clustering Alpha diversity

In Zhang's paper, they collect a total of 135 individual Chinese giant salamanders ranging from age 1 to 4 (Appendix 1) from a farm located in Lueyang

County in Shanxi Province in December 2016. For each of the 53 gastrointestinal samples, Zhang's team run the Shannon, Chao 1 and Ace indexes to calculate and estimate diversity and richness. We use R to reproduce the 97% clustering graph to compare with the ASV graph. When $q=0$, the richness increases as age develop. When $q=1$, the Shannon diversity which is effective number of common species remains the relatively same from age 2 to 3, but changes significantly when the salamanders reach to age 4. When $q=2$, the Simpson diversity which counts as the effective number of dominant or very abundant species in the sample varies differently from age 2 to age 4. The diversity and richness index in gastrointestinal samples tended to increase from age 1 to 4, and minimum and climax diversities were almost always observed in samples from age 2 and 4 individuals(Zhang et al. 2018). In general, the difference observed in the index of gastrointestinal samples

from age 2 to 4 individuals was statistically significant, and samples of age 2 individuals had the lowest index (Figure 1) (Zhang et al. 2018).

Figure 1 97% clustering index sample from age 2 to 4

2. ASV Alpha diversity

In the data set composed with 30813 taxa and 53 samples with 3 sample variables and 7 taxonomic ranks. For each of the 53 samples, we run with ASV to produce the statistic results and compare with the 97% clustering. Similar to the 97% clustering, ASV shows richness increases as age develop when $q=0$. In addition, when $q=0$, most of the taxa in the gut microbiome that causes differences are not relatively rare or very abundant. However, there is a significant difference when $q=1$. The ASV graph shows the Shannon diversity remains relatively consistent throughout age 2 to 4, which means the abundance of the bacterial composition has no influential changes. When $q=2$, the Simpson diversity varies as the 97% clustering estimation. The diversity index in gastrointestinal samples tended to increase from age 1 to 4, and minimum and climax diversities were almost always observed in samples from age 2 and 4 individuals(Figure 2). However, the richness or abundance has no significant changes through age 2 to 4. In general, the difference observed in the index of gastrointestinal samples from age 2 to 4 individuals was statistically significant when $q=0$ and $q=2$, but not when $q=1$, and samples of age 2 individuals had the lowest index compares to age 3 and 4 (Figure 2).

Figure 2 ASV index sample from age 2 to 4
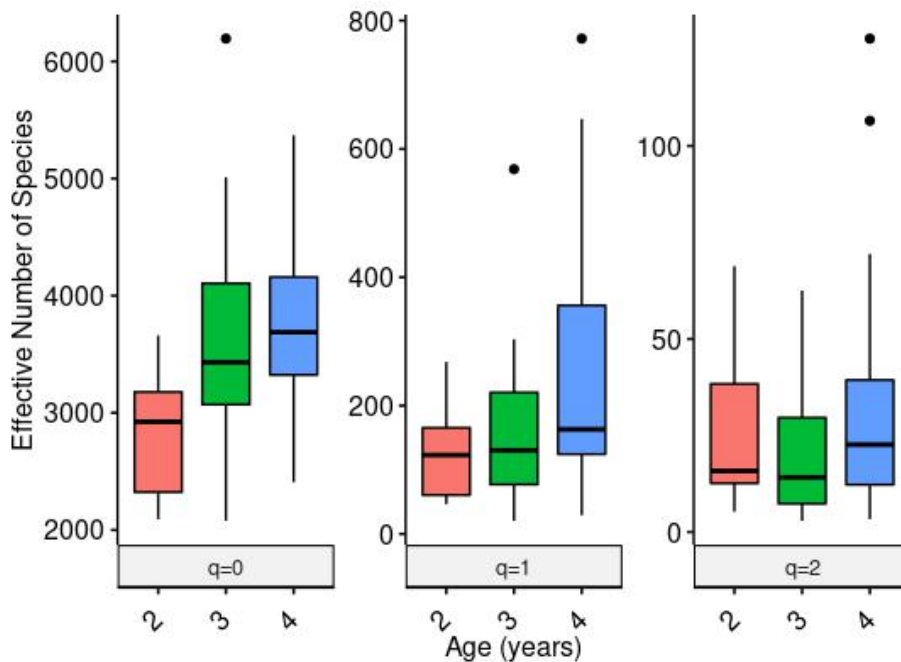
3. Conclusion

Using the Hill numbers and ASV, we found out that the Chinese Giant salamanders gut microbiome shift in diversity as they grow and change diet, however, the evenness and relative abundance remains statistically consistent with no relative changes.

4. Discussion

4.1 The shift of nutrition source related to the gastrointestinal community

In this study, we found out that although the gut microbiome tend to increase in complexity when counting the number of species, there is no significant changes through age two to four related to abundance or richness. As Zhang's team concluded, the diversity increases as the Chinese Giant Salamanders grow base on the observation on dietary preference. During their first year of life, Chinese giant salamanders are entirely aquatic and rely solely on the yolk sac for nutrition(Zhang et al. 2018). After age 2, Chinese giant salamanders continue to depend on the yolk sac for nutrition but begin feeding on redworms supplied by the aquaculture facility(Zhang et al. 2018). After age 3, they rely solely on external food sources, mainly shrimp and crab(Zhang et al. 2018). In a lot of studies, researchers have found a high-fat diet leads to an increase in Firmicutes and a high‐fiber diet leads to an increase in Bacteroidetes (Clarke et al., 2012; Turnbaugh et al., 2006). As Zhang estimated, we speculated that these changes in the gut microbiome might be related to the transition between endogenous and exogenous nutrition sources across their development (from age 1–4 years.). The shift of dietary preference also lead to changes in microorganisms.

4.2 Ecological perspectives and different focus

Zhang's team yield the conclusion from Chao 1, Shannon, and Ace indexes, which put emphasis rare tax along with producing richness and evenness. In this study, we focus on the ecological perspectives and yield results focusing on the broader concept. Using the Hill numbers and ASV, R produced the relative abundance of the sample which refers to the

evenness of distribution within the taxa. Nevertheless, both Zhang's research and our research aim to study the gastrointestinal (gut) microbiome since both extrinsic (dietary) and intrinsic (host traits) forces influence the establishment and subsistence of microbes in the gut, which help researchers to understand the Chinese Giant Salamanders better and improve the diet to build healthier microbiome. Furthermore, both gastrointestinal and skin microbiome of the amphibian organisms are important for researchers to study. Both skin and gut microbiomes constitute a critical component of the amphibian immune system, and provide a model for understanding the processes shaping the forces of colonization and competition. The bacterial community dynamics of amphibian is an understudied host group in the field, but influence the host in various and significant ways. In addition, amphibians play a pivotal role in ecosystem as secondary consumers in many food chains. Amphibians also play important roles in ecosystem nutrient cycling and energy flow. They are important predators of insects, worms, and other invertebrates. However, up to one third of all amphibian species are at risk of extinction because of human actions, such as habitat destruction, climate change, and pollution. Amphibians are also important to biodiversity and evolution. They consume insects and help control pest populations to prevent plant damage. For example, frog tadpoles feed on algae, which helps keep waters clean. From evolutionary perspectives, amphibians have showed adaptation which allow them to stay out of water for longer period with their evolved lungs and heavier skeleton. If the amphibian are distinct, the ecosystem will collapses and influence the nature world fiercely. Researchers should spend more time on the amphibian species.

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