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Dynamic Changes of Gut Microbiome in Borneo Earless Monitor Lizard Across Different Diets

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ABSTRACT

The Borneo earless monitor lizard (*Lanthanotus borneensis*) is one of the endemic animals of Indonesia. In 2019, the IUCN listed *L. borneensis* as an 'endangered' species. The declining population of the *L. borneensis* is a major concern for conservation efforts. One of the efforts to conserve endangered wild animals is to know the gut microbiome profile and the relationship between the host and the bacterial community in the body, which is associated with an influence on health. This research aims to determine the gut microbiome profile of ***L. borneensis*** animals with metagenomic analysis using Next-Generation Sequencing (NGS). This research was carried out by providing three feed types: earthworms, shrimp, and fish. Sample feces was collected by cloacal swabs and the DNA was sequenced using the Illumina NGS technique in the V4 16S rRNA region to examine the gut microbiome. The results of the metagenomic analysis showed differences in bacterial abundance in each feed treatment. The *L. borneensis* treated with earthworms and fish were dominated at the phylum level by Proteobacteria and Bacteriodota, while in shrimp, the phyla were Proteobacteria and Firmicutes. Index Shanon, Simpson, and the rarefaction curve also showed the highest bacterial diversity found in the fish treatment. This study can be used as a reference in designing suitable feed formulations for effective captive breeding for the conservation of *L. borneensis*

1. Introduction

L. borneensis is a type of Lizard belonging to the Lanthanotidae tribe and is related to other types of lizards in the Varanidae family (Ast 2001). In 2019, the IUCN (International Union for Conservation of Nature) designated *L. borneensis* as an endangered species, meaning that it faces a significant risk of extinction in part or all of its geographic range (Buechley and Sekercioglu 2013). The approach used to increase *L. borneensis* populations is conservation. Conservation efforts involve not just the preservation of natural habitats but also particular concern for animal nutrition and efficient captive breeding. Proper nutrition can impact health and performance. The effect of feed on improving performance, such as weight gain, improved health, and animal development, can be determined by understanding the diversity of the microbial community (bacteria, archaea, fungi, protozoa, and microalgae) in the gut, known as the microbiome (Mueller and Sachs 2015; West *et al.* 2019).

Symbiosis in the microbiome associated with the host affects the homeostasis of the species. In healthy homeostatic conditions, the microbes in the gut play roles in digestion, metabolism, and immune modulation. However, in a disturbed state, homeostasis can result in dysbiosis, a health problem caused by an imbalance of microbes in the gut digestion (Trevelline *et al.* 2019; Bidell *et al.* 2022). Dysbiosis is reported

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to be closely related to several diseases, such as cancer and intestinal inflammation (Baldelli *et al.* 2021). Dysbiosis is characterized by several things, such as an increase in pathobionts, namely microbiota that have the potential to become pathogens; an abundance of the Proteobacteria phylum, including Enterobacteriaceae; loss of commensal bacteria due to lack of proliferation; low and reduced bacterial diversity (alpha diversity) (Chow and Mazmanian 2010; Korem *et al.* 2015; Levy *et al.* 2017). Some of the bacteria reported to infect reptiles are *Salmonella* spp., *Clostridium*, and *Pseudomonas* (Ogunleye and Carlson 2017; Andres-Lasheras *et al.* 2018). Knowing the microbiome profile can be a basic detection and screening tool for food nutritional preferences of the species to be conserved (Behrendoff *et al.* 2016). Microbial ecology studies can determine the relationship between certain organisms and the host's health status, which can be used for early diagnosis and prevention of diseases (Xiong *et al.* 2018).

In addition, a lack of proper nutrition can hinder individuals' growth, reproduction, and survival, which, in turn, can contribute to population decline (Wu 2017). The food sources for *L. borneensis* in its natural habitat are fish, shrimp, tadpoles, small frogs, earthworms, snails, and various types of insects (Arida *et al.* 2018). The diverse diet of *L. borneensis* in their natural habitat is the basis for determining food sources for treatment in this study. This is to assess the effect of each feed on the microbiome in the gut of *L. borneensis*.

The microbiome is very complex, consisting of thousands of diverse microbial species, and their relative abundance can vary. Next-Generation Sequencing (NGS) is the newest DNA sequencing technology, capable of producing millions of DNA sequences in parallel (Vincent *et al.* 2017). NGS can comprehensively observe the diversity and track the presence of various microbial species in the microbiome. The high diversity of species' DNA in the gut of *L. borneensis* requires an approach to amplify this diversity. There has been no research regarding the microbiome profile of *L. borneensis* with different feeding methods using Next-Generation Sequencing (NGS), which is the basis for conducting this research. This research aimed to determine the microbiome profile of various types of feed so that suitable feed formulations can be found as a conservation effort for the *L. borneensis*.

2. Materials and Methods

2.1. Preparation and Sample Collection

All experimental animal procedures were approved by the Animal Care and Use Committee of the Ethical Clearance, BRIN (Number: 080/KE.02/SK/04/2023). Seven male *L. borneensis* (aged 2-3 years) were used for this experiment. This study was conducted at Animalium BRIN, Indonesia. Three types of diets (earthworms, fish meat, and shrimp meat) were used in this experiment and were fed to *L. borneensis* in fresh form. Five grams of the diets were cut into small pieces and then given to each lizard. The feed was placed in a small dish near the lizards. The amount of feed given and the remaining feed were recorded daily. The earthworms contained 43.96% protein, 11.43% fat, and 1.89% fiber; shrimp contained 74.32% protein, 12.92% fat, and 0.625% fiber; fish contained 68.58% protein, 12.72% fat, 1.01% fiber. Each experimental period lasted 14 days and consisted of 13 days for diet adaptation and 1 day for sample collection. Cloacal swabs were used for non-destructive sampling of the gut microbiome (Jiang *et al.* 2017). To perform a cloacal swab on the lizards, the outer surface of the cloaca was disinfected using alcohol-soaked cotton. We then used a sterile OneMed cotton swab (small size) and gloves to collect the swab sample, taking care to avoid contamination. After swabbing, we immediately placed the sample in a sterile 15 ml centrifuge tube containing 5 ml of 10% glycerol. The samples were stored in the freezer at -80°C.

2.2. SDNA Extraction

Total DNA from the cloacal swabs was extracted using a procedure of the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) with little modifications. The modifications were made in the lysis step. The swab samples were cleaned with RBC Lysis Buffer before it was suspended with Inhibitor EX Buffer. The lysozyme and RNAase from Genomic DNA Mini Kit (Geneaid) were then used in the middle of the procedure which was mixed with Proteinase-K.

2.3. Sequencing

DNA extracts from seven replicate samples in each treatment were pooled into one sample for sequencing using the next-generation sequencing (NGS) platform. The Amplification of V4 hypervariable region of the 16S rRNA (~300 bp) was performed by polymerase chain reaction (PCR) with Primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′)

and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Quality control was conducted using agarose quantification, and the PCR product was run on a 1.7% TAE agarose gel. Library preparation was performed using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina, and sequencing on an Illumina HiSeq platform was conducted by NovoGene (Singapore). DADA2 was used to correct sequencing errors, remove low-quality sequences, and address chimera errors. The resulting ASVs (Amplicon Sequence Variants) data were used for taxonomic classification against SILVA (silva_nr99_v138.1). Downstream analysis and visualizations were performed using packages in RStudio (R version 4.2.3) (https://www.R-project.org/) and Krona tools (https://github.com/marbl/Krona).

3. Results

3.1. Analysis of Microbiome Abundance of *L. borneensis*

Identification of 16S rRNA through NGS in the V4 region of three samples resulted in bacterial taxonomy, which was divided into 20 phyla, 38 classes, 80 orders, 155 families, and 225 genera. The most dominant phyla are Proteobacteria, Bacteroidota, and Firmicutes, as shown in Figure 1A. There were differences in the abundance of phyla among the three treatments: in the earthworm and fish treatments, Proteobacteria and Bacteroidota were very abundant, while the shrimp treatment was dominated by Proteobacteria and Firmicutes. The abundance of the Firmicutes phylum in earthworms and fish treatments is relatively low, as seen in the Figure 1A. In the earthworm treatment, Proteobacteria exhibited the highest dominance (Figure 1A).

Further differences can be observed at the class level. The microbiota dominating the earthworm and fish treatments are relatively similar, comprising mainly the Gammaproteobacteria and Bacteroides classes, while the shrimp treatment is dominated by Bacilli. However, the abundance of the Clostridia class was higher in the fish treatment compared to earthworms and shrimp samples (Figure 1B). At the family level, the highest percentages in the earthworm and fish treatments are predominantly represented by Alcaligenaceae and Enterobacteriaceae. The family heatmap (Figure 2A) shows lighter colors for the families Weeksellaceae, Vibrioceae, Pseudomonaceae, and Aeromonadaceae in the fish and shrimp treatments, whereas these bacteria are present in very low abundance in *L. borneensis* treated with earthworms.

In fish treatments, the most common bacterial genera were *Achromobacter, Citrobacter, Alcaligenes, Bacteroides, Acidaminobacter*, and *Pseudoalteromonas* (Figure 2B). The genera frequently found in *L. borneensis* treated with earthworms were *Alcaligenes, Achromobacter, Petrimonas, Salmonella,* and *Campylobacter*. The presence of *Proteocatella* and *Lactobacillus* bacteria was observed only in the shrimp feed treatment (Figure 2B).

The abundance of microbiota at the genus level varied in diversity and dominance across different samples. In the earthworm, the genus *Alcaligenes* was dominant

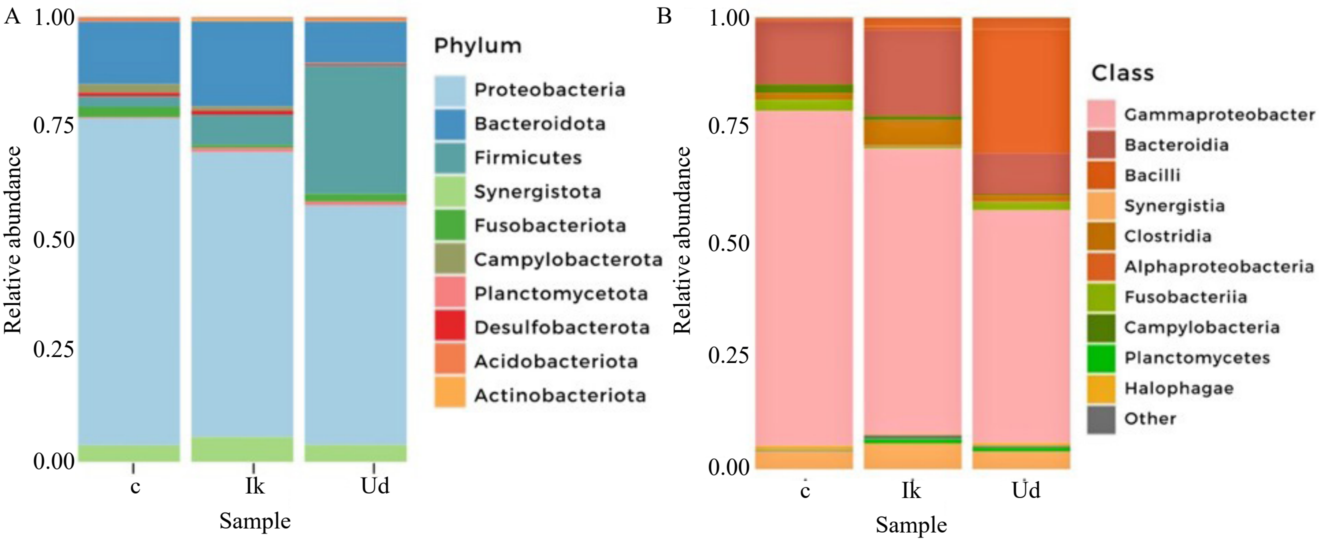

Figure 1. Distribution of bacterial abundance in the microbiome of *Lanthanotus borneensis* at the taxonomic level (A) phylum, (B) class

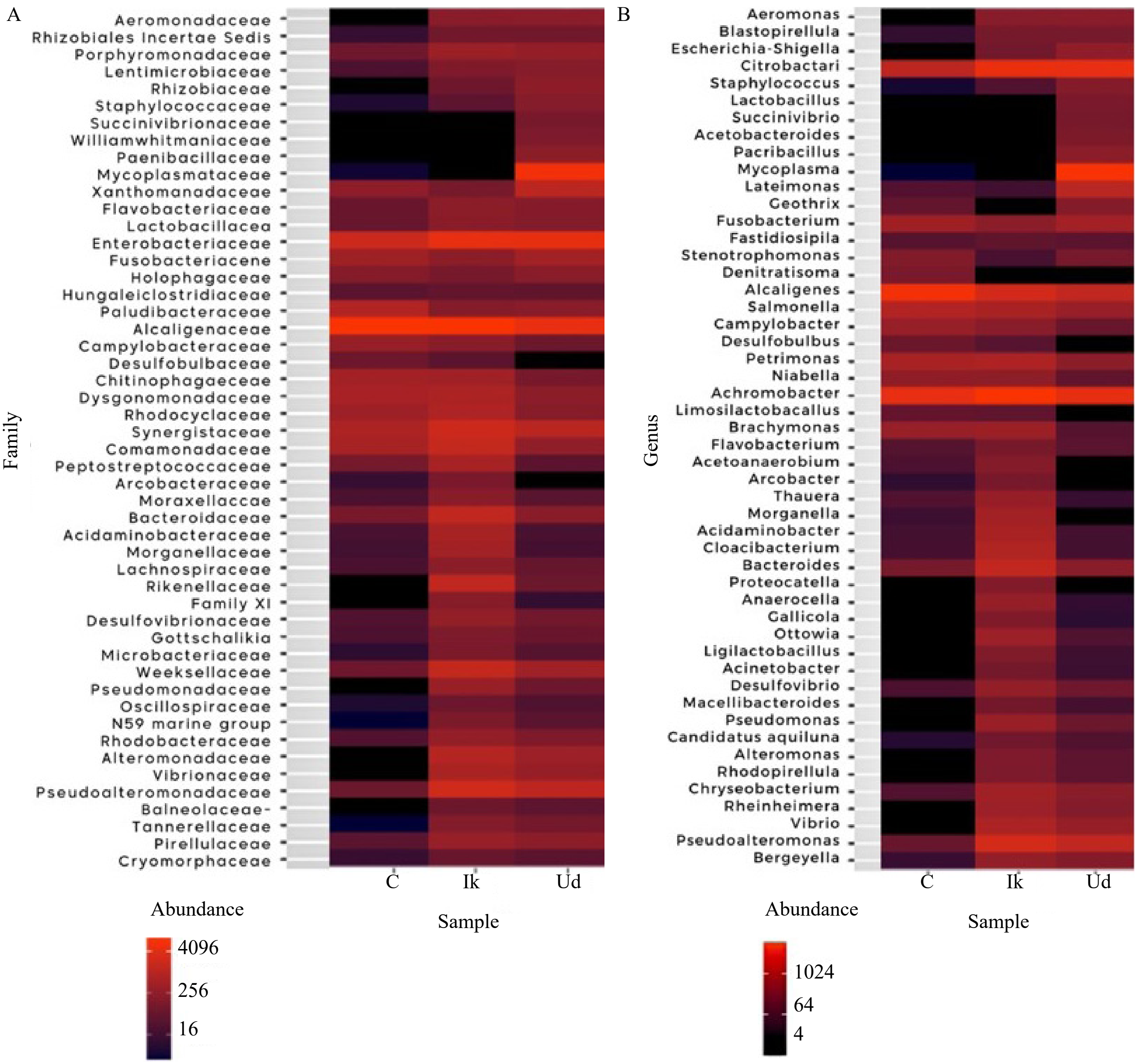

Figure 2. Heatmap showing the comparison of microbial abundance in each treatment group in taxon (A) family, (B) genus

(Figure 3A), where this genus was less numerous in the fish and shrimp treatments. Meanwhile, in the fish treatment, the genus *Achromobacter* was most prevalent, whereas in the shrimp treatment, the genus *Mycoplasma* was more dominant but very low numbers in the earthworms treatment and not found in the fish treatment (Figure 3A). *Salmonella*, *Campylobacter*, and *Fusobacterium* were most abundant in the earthworm treatment while *Desulfovibrio, Bacteroides, Bacillus* and *Lactobacillus* were more prevalent in the shrimp treatment (Figure 3B).

3.2. Diversity Among Group

The level of bacterial diversity in each sample was measured using several indices, namely observed results (Figure 4A), Shannon index (Figure 4B) and Simpson (Figure 4C). The analysis of diversity values based on these indices revealed different levels of diversity in each treatment. The observed values presented in Figure 4A show that the bacterial diversity in fish samples exceeds 240, which is significantly higher than the values for shrimp and earthworm treatments, which are below 200 (Figure 4A).

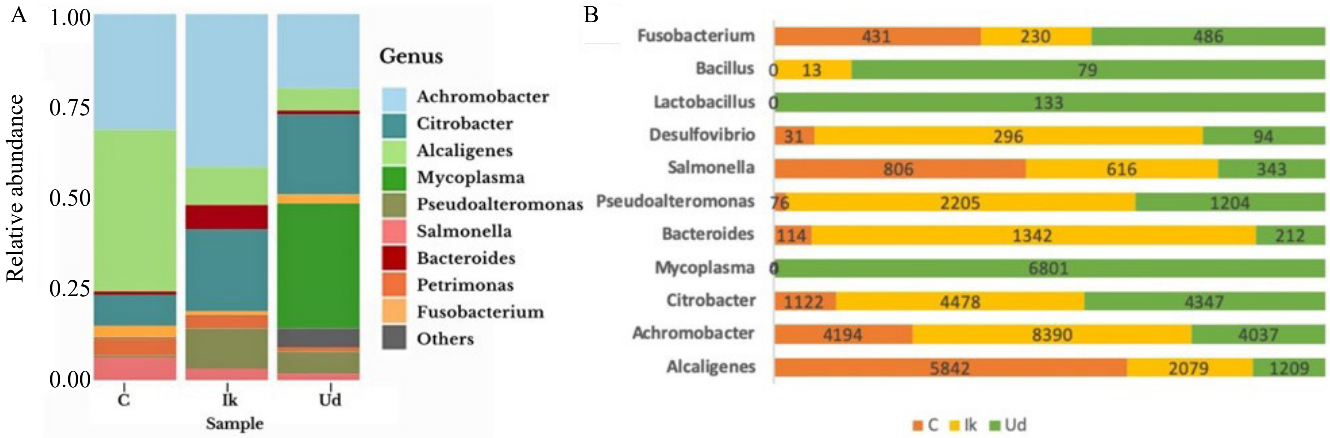

Figure 3. Abundance of bacteria at the genus level in each treatment, C: earthworms; Ik: fish; Ud: shrimp. (A) Top 10 taxa from 3 treatments, (B) Comparison of specific bacterial counts from three treatments

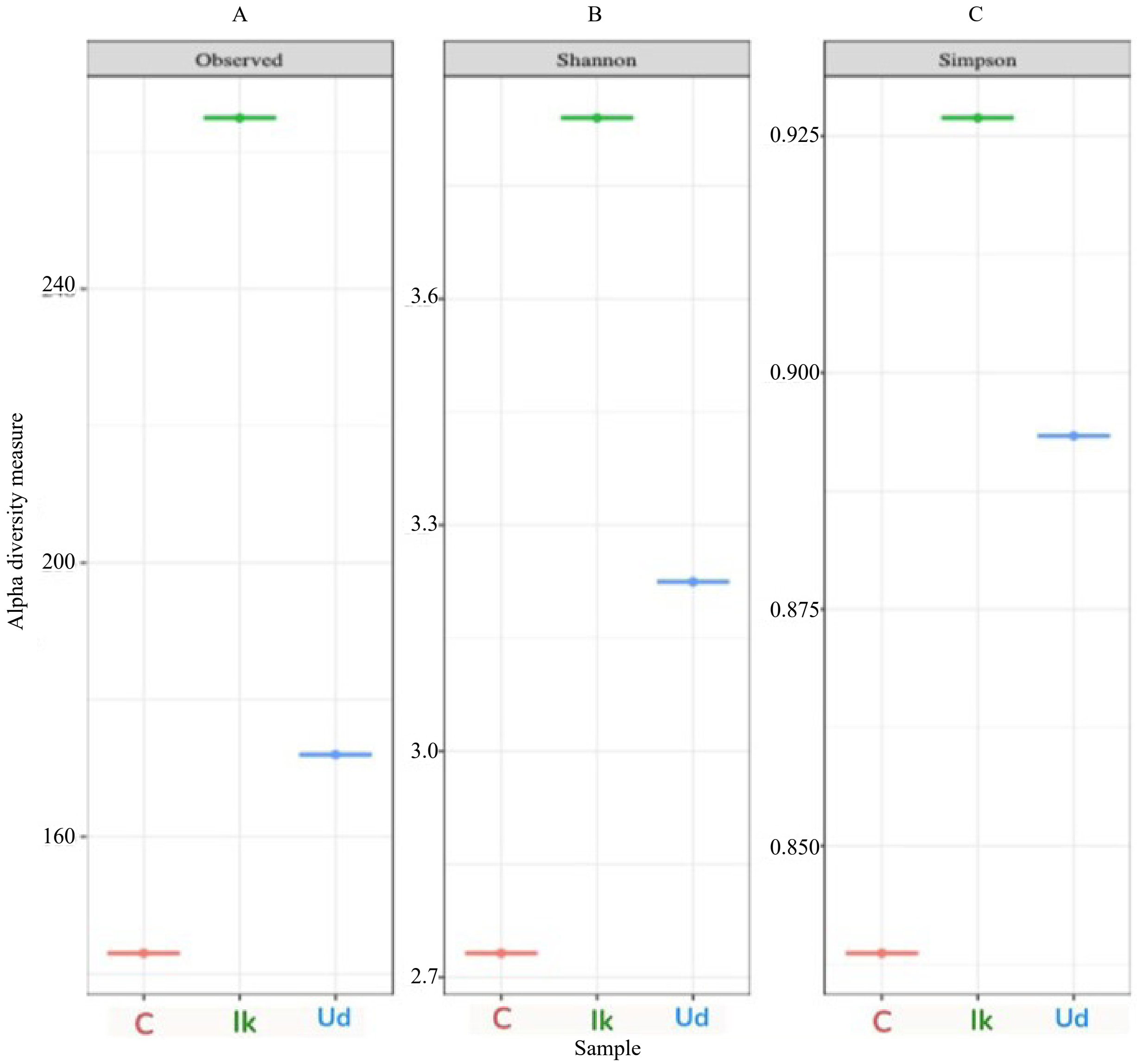

Figure 4. Alpha diversity index value. alpha diversity index (A) observed value, (B) index Shannon, (C) Simpson index in treatment (C) earthworms; (Ik) fish; (Ud) shrimp

Based on the calculation results, it shows that in general the Shannon-Winner diversity index of bacterial communities from the fish treatment samples was the highest, at 3.9, followed by the shrimp and earthworms (Figure 4B). The Simpson index value showed that the highest value is in the treatment with fish feed, around 0.925, followed by shrimp feed and earthworms feed. Apart from that, the alpha diversity value for each sample was also shown by the rarefaction value. Figure 5 showed that the highest species richness is in the fish sample with a value of 260 and the curve becomes a plateau at a sample size exceeding 30,000, on the other hand, earthworms have the lowest value around 142 in a sample size below 20,000.

4. Discussion

Previous studies on the microbiome in several reptiles, such as turtles, lizards, and crocodiles, reported that Bacteroidota, Proteobacteria, and Firmicutes are the dominant phyla that inhabit the gut (Colston 2017; McKnight *et al.* 2020). For example, in the gut microbiome of the reptile Chinese Crocodile Lizard (*Shinisaurus crocodilurus*), it was also reported that the dominant phyla were Proteobacteria, Bacteriodota, and Firmicutes (Jiang *et al.* 2017). Bacteria in this phylum have also been found to dominate the microbiome in the gut of humans, mammals, and birds (Bradley and Pollard 2017; Moon *et al.* 2018). The same thing was also found in *L. borneensis* the most dominant phyla were Proteobacteria, Bacteroidota, and Firmicutes.

Proteobacteria generally play a role in the catabolism and biosynthesis of vitamins in their hosts (Colston and Jackson 2016). The abundance of the Proteobacter phylum was highest in *L. borneensis* treated with fish feed, and this was also accompanied by relatively low diversity values when seen from the calculation results of the Shannon and Simpson index (Figure 4). However, the high presence of Proteobacteria in the gut is a symptom of an imbalance in the microbial community, metabolic disorders, and a high abundance of Proteobacteria which is not accompanied by the diversity of other microbiota in a species, indicating the presence of dysbiosis in the gut (Méndez-Salazar *et al.* 2018).

A high abundance of bacteria from the phyla Firmicutes and Bacteriodota (F:B) is closely related to body weight gain and fat synthesis (Dreyer and Liebl 2018). In this study, the observed differences in weight gain across the different diet treatments are significant. The results indicate that feeding with fish (16.8 g) and shrimp (21.4 g) produced better weight gain compared to feeding with worms (2.9 g). Firmicutes play an important role in fermentation in the gut by utilizing fiber as a substrate (Dalile *et al.* 2019). However, suppose fiber in the body is limited, in that case, these bacteria will utilize the amino acids available in the host body as a carbon source through the glycolysis pathway and produce products in the form of short-chain fatty acids or SCFA (short-chain fatty acid) such as acetate, iso-butyrate, iso-valerate, and propionate (Duncan *et al.* 2021). Bacteria that are known to be able to ferment with peptide and amino acid substrates are distributed in the Bacteroidota and Firmicutes phyla, such as Bacterioides, Prevotella, Clostridia (Duncan *et al.* 2021). SCFA, produced in abundance, will be absorbed into the host's tissues as a food reserve (Martin-Gallausiaux *et al.* 2021). The highest crude protein content is in shrimp; this protein can be a carbon source for the Firmicutes group to produce SCFA.

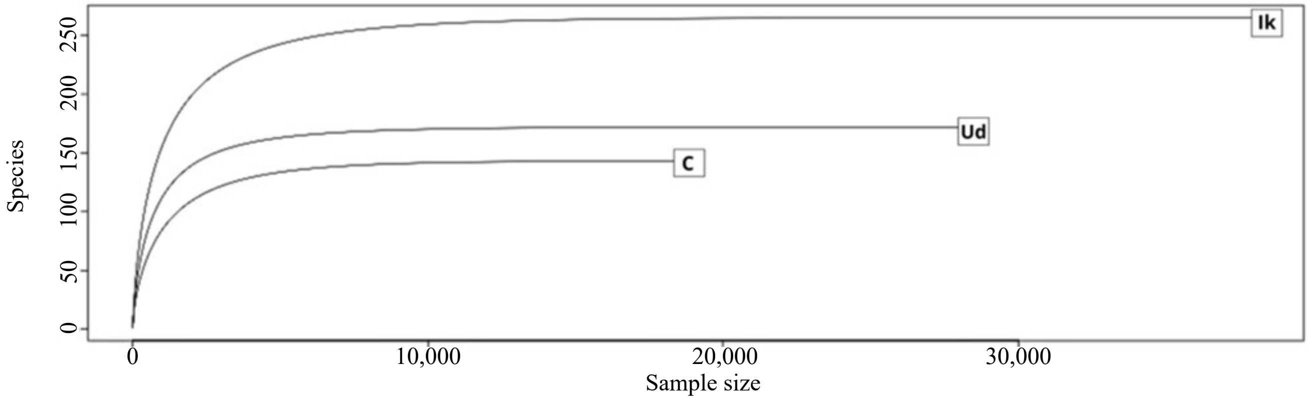

Figure 5. Rarefaction curve showing the difference in the size of the sample read and the numbercof species found in the cloaca samples of *L. borneesis* when treated with earthworms (C), shrimp (Ud) and fish (Ik)

Moreover, *Lactobacillus* and *Bacteroides* plays an anti-inflammatory role by producing interleukin 10 and transforming growth factor ß as well as promoting the differentiation and proliferation of Treg (regulatory T) cells (Hayashi *et al.* 2013). The presence of bacteria from the class Bacilli, such as *Bacillus* and *Lactobacillus* plays a role in inhibiting the colonization of pathogens in the gut (colonization resistance) by competing for the use of lactate as a carbon or nutrient source and also releasing antimicrobial compounds (Louis *et al.* 2022).

Weeksellaceae, Vibrioceae, Pseudomonaceae, and *Aeromonadaceae* bacteria are commonly found in the gut of marine animals such as white sea fish and turtles. *Vibrio* and *Pseudomonas* have high proteolytic and cellulolytic lipolytic abilities (Burtseva *et al.* 2021). The abundance of marine bacteria in the gut of *L. borneensis* is related to the transmission of bacteria from their food habitat, namely fish and shrimp from marine waters. Moreover, complex compounds such as sulfate from food and water consumed by the host will be utilized by fermentative bacteria such as sulfate-reducing bacteria (Duncan *et al.* 2021). These fermentative bacteria consist of *Desulfovibrio* sp., *Staphylococcus* sp., *Bacteriodes* sp., *Enterobacter* sp., *Clostridium* sp., (Kuskevych 2020). Bacterial members of the *Lachnospiraceae, Bacteroides*, and *Desulfovibrio* as sulfate-reducing bacteria significantly contribute to hydrogen removal where hydrogen removal allows a more complete oxidation of the organic substrate and, therefore, a higher energy yield than anaerobic fermentation (Lazarkevich *et al.* 2024).

At the genus level, the microbiome of *L. borneensis* has differences; in *L. borneensis* treated with earthworm food, it is dominated by the genus *Alcaligenes. Alcaligenes* are gram-negative bacteria and are commonly found in the intestines of various mammal species, such as monkeys, humans, and mice. Gram-negative bacteria, such as *Alcaligenes*, and bacteria from the bacterioles class, such as *Salmonella* and *Escherichia*, are classified as opportunistic bacteria, where their cell walls consist of Lipid A, which is endotoxin if the amount is too abundant (Kunisawa and Kiyono 2012).

The abundance of *Achromobacter* accumulated in *L. borneensis* with all types of treatment, but it was most abundant in the fish treatment. *Achoromobacter* is a Gram-negative bacterium belonging to the class Bacilli and is most commonly found in water and soil habitats (Abdouchakour *et al.* 2015). This bacterium is also found abundantly in Zebrafish (*Denio rerio*) and Blue Sea Snakes (*Hydrophis cyanocinctus*) (Zhong *et al.* 2022; Scott *et al.* 2023). The presence of *Achromobacter* in each treatment may be influenced by the association of microbiota from food and habitat, as the habitats of earthworms, fish, and shrimp are closely related to soil and water, which are primary habitats of *Achromobacter*. Gut microbiota is not only influenced by the nutritional and chemical composition of the host's feed but also the microbiota content in the food; thus, it is possible that transmission occurs between the microbiota of predators and the microbiota of their prey (Dion-Phénix *et al.* 2021).

The high abundance of *Mycoplasma* in *L. borneensis* with shrimp treatment was also found in crocodile monitor lizards (*Shinisaurus crocodilurus*), as reported by Jiang *et al.* (2017). The most dominant *Mycoplasma* in microbiome of *L. borneensis* fed shrimp is *Mycoplasma synoviae*. This bacteria is reported as a pathogenic bacteria that infects poultry and cattle farms, causing inflammation in the host (Yadav *et al.* 2022). Reptiles have innate and adaptive immune systems, consisting of non-specific leukocyte components and antimicrobial peptides, and in some cases, the immune response in reptiles is stronger than in mammals (Zimmerman 2020; Siddiqui *et al.* 2022). In addition, *Fusobacterium* was detected in each treatment, but it was most abundant in monitor lizards in the fish and earthworm treatments. The abundance of *Fusobacterium* was also found in the Beautiful Japalure lizard (*Diploderma splendidum*) (Tian *et al.* 2020). Although *Fusobacterium* is a normal microflora in the gut, there have been several reports linking it to gut infections that cause colon cancer and tumor inflammation (Cuellar-Gómez *et al.* 2022).

Shannon-Wienner diversity analysis is used to measure biodiversity by looking at the level of richness. In the context of species richness and diversity, both the observed results, the Shannon index and the Simpson index presented the same value, namely the highest species diversity in the fish treatment. At the same time, the lowest was the diversity value in the earthworm treatment. The diversity value through the Shannon index is measured in the range of 0.0 to 5.0. Usually, the resulting value is between 1.5-3.5 and rarely reaches a value of up to 4.5, a value above 3 indicates that the diversity in the habitat is very rich and stable and a value close to 1 indicates contamination and changes that make the environment unstable (Khatri *et al.* 2020). Apart from that, the Simpson index measures the level of biodiversity through the level of evenness of the number and types of species with each other or the balance of species proportions (relative abundance) (Jasirwan *et al.* 2021). The range of diversity for the Simpson index is 0–1, where 0 is categorized as no diversity and 1 as having high diversity (Chiffele *et al.* 2020).

Rarefaction is generally used to determine species richness during sampling by analyzing the uniqueness of the organisms found in a habitat and producing a curve. This curve will grow as common species are discovered and will eventually plateau when only rare species remain (Xia 2023). The condition of the curve becoming a plateau at a certain sample size represents the number of species detections in that treatment. As a result, the fish treatment contained the most varied species.

In summay, the gut microbiome of *L. borneensis* in earthworms and fish treatments was dominated by the phyla Proteobacteria and Bacteroidota, while in the shrimp feed treatment there was a high abundance of Firmicutes. Additionally, the earthworms treatment was predominantly inhabited by bacteria from the genus *Alcaligenes*, while the fish treatment had the highest proportion of *Achromobacter*, and the shrimp treatment was dominated by *Mycoplasma*. Fish and shrimp feed have the highest bacterial diversity compared to earthworms, indicating that fish and shrimp are more favorable for *L. borneensis*. Further research regarding mixed feed formulation, immune profiles, and comparison of the microbiome with wild *L. borneensis* also needs to be carried out.

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