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Analysis of Chicken Gut Microbiome Fed by *Phyllanthus urinaria* as Phytobiotic Using 16S rRNA Metagenome

Research Article

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ABSTRACT

Applying of antibiotic growth promoters (AGPs) in chicken has been forbidden due to leaving residues and resistance in people who consume them, especially in livestock products. The provision of phytobiotics as AGPs substitute for chickens increases their immunity and productivity. Phytobiotics may threaten pathogenic bacteria or promote colonization of beneficial bacteria for chickens. This study analyzed the effect of meniran leaves (*Phyllanthus urinaria*) as a phytobiotic and an alternative for AGPs on the abundance, diversity, and composition of the chicken gut microbial community. The microbial ecology of chicken gut used the molecular markers of the 16S rRNA amplicon sequencing V3-V4 region. Samples were 20 chickens maintained for 21 days and fed according to basal requirements that were divided into two treatments, namely 0% phytobiotic provision (T0) and 2% meniran leaves phytobiotic provision. The sequencing libraries were constructed by utilizing the Ion Plus Fragment Library Kit 48 rxns. The sequencing result was evaluated by performing single-ends reads quality control, operational taxonomic units (OTUs) clustering, species annotation, and diversity within groups (alpha diversity). The outcome data revealed that Firmicutes was the predominant phylum in both samples. Based on the class level, T0 was identified to have 100% *Bacteroidia*, while T1 was detected to have 78% *Bacteroidia* and 22% *Flavobacteriia*. Based on the order level, T0 was dominated by the *Negativicutes* and T1 was dominated by the *Selenomonadales*. Based on the genus level, T0 was dominated by the *Megamonas* and T1 was dominated by the *Lactobacillus*. The OTUs of T0 and T1 were 126 and 144. This study concludes that the *Phyllanthus urinaria* provision as a phytobiotic influences the diversity, relative abundance, and composition of the chicken gut microbiota.

KEY WORDS broiler, meniran leaf, metagenomic, microbiota.

INTRODUCTION

Antibiotic growth promoters (AGPs) are commonly used to enhance weight gain in poultry production. However, the increasing interest across AGP's impact on the development of antibiotic persistent in pathogens, including zoonotic bacteria in the chicken gut microbiota (Lekshmi *et al.* 2017). In several countries, using antibiotic as growth promoter is not permitted such as the European Union, which has banned the use of several antibiotics as growth promoters for poultry (Castanon, 2007). This issue has a major concern in China, which has banned antibiotics as animal feed since July, 2020 (Zhang *et al.* 2021). Indonesian government is also concerned with the following issue regarding the Classification of Veterinary Drugs based on the Permentan No. 14/2017, which bans AGPs for poultry production. AGPs are dangerous due to leaving residues and resistance in people who consume the poultry product,

which can obstruct the consumer's metabolism (Brower *et al.* 2017; Muaz *et al.* 2018).

A recommended method to overcome this obstacle is using the herbal plant as feed additives, known as phytobiotics. The application of phytobiotics can improve production, reproduction, and health (Ogbuewu *et al.* 2020).

Meniran (*Phyllanthus urinaria*) leaves are parts of the medical plants that contain various bioactive components, such as triterpenes, flavonoids, tannins, alcohols, phenolic acids, and lignans, i.e. filantin, hypofilantin, phenolic, and flavonoids (Nandhakumar and Indumathi, 2013). Meniran leaves can also supress the growth and development pathogenic bacteria, including gram-negative and gram-positive bacteria that infect the skin, urinary tract, and digestive tract, and can be found in soil, and water area (Eldeen *et al.* 2011). The use of meniran leaves in poultry can stimulate immunity by improving disease resistance and excellent performance (Hidanah *et al.* 2018; Astuti and Suripta, 2020). As an antimicrobial, the use of meniran leaves as a phytobiotic has formidable effect in the population and diversity of gastrointestinal microbiota, especially microbes, which benefits the host. Moreover, microbiota in gastrointestinal is mostly symbiotic with the host through various mechanisms. Microbiota assists the host to absorb nutrients, digest food, and perform a function in the immune system (Stanley *et al.* 2014). This interaction between the host cells and bacteria can emerge, known as "crosstalk" that strengthens their symbiotic relationship (Allaire *et al.* 2018).

Technological development at the molecular level is developing rapidly to easily explore the chicken gastrointestinal microbiota through sequencing analysis, which provides more prominent and more complete data without limitations for the uncultured microbes. The 16S rRNA gene sequence consists of nine hypervariable areas distributed with the conserved sections. Variations in the 16S rRNA gene can broadly characterize the various microbial populations and communities (Hess *et al.* 2011; Bhogoju *et al.* 2018). Studies of the chicken gut microbial community were conducted to identify the dynamics of chicken gut (Asrore *et al.* 2015), discover the gene pathway, and alter microbial diversity (Huang *et al.* 2018). Some management alterations by housing environment and protein source substitution can change the microbial population and diversity (Hubert *et al.* 2019). The dynamics of chicken gut microbiome are diverse along with the different age (Asrore *et al.* 2015). After the provision of anticoccidial and AGP, the chicken gut microbial modulation obtained different bacterial diversity and composition (Danzeisen *et al.* 2011). Therefore, this study aimed to analyze the population and abundance of chicken gastrointestinal microbiota fed by meniran leaves as an alternative for AGP using the 16S rRNA sequencing techniques to obtain the diversity, abundance, and composition of the microbial community, including a phylogenetic tree of microbiota in the chicken gut ecology.

MATERIALS AND METHODS

Sample collection

This study was carried out by maintaining the 20-day-old chickens of strain Cobb 500 obtained from the reputable farm. The chickens were inspected and ensured to be free from infections and disease symptoms. Before performing the experiment, the house, cage, feeder, and drinker were cleaned, disinfected, and fumigated. The chickens were maintained in an open-house. However, the temperature and humidity levels in the houses were controlled according to the chicken age and condition following the Standard Operating Procedure of Umiarti (2020). The chickens were maintained in two groups of treatments, namely T0, which contained 10 broilers fed without phytobiotics in the ration and T1, which contained 10 broilers fed with 2% meniran leaves phytobiotic in the ration. The ration fed to the chicken was produced according to NRC (1994). Each group was assigned in individual pen, which provided independent replication, contained feeder and drinker. The chickens were maintained until they reached 21 days age. At the end of the experimental period, all chickens have fasted for 8 hours, then randomly selected 3 chicks of each group and slaughtered.

The intestinal content from ileum, jejunum, duodenum and cloaca were collected and pooled for each group using 15 mL corning tube containing the DNA storage medium (Zymoresearch, USA). This fluidic samples were then used as microbial DNA extraction and amplicon sequencing analysis source.

Amplification and sequencing

The genomic DNA was isolated from 5 mL of the intestinal fluid samples using ZymoBIOMIC DNA Miniprep Kit (Zymoresearch, USA). The gene target amplification was performed using the 16S rRNA primer pair on the V3-V4 region containing forward and reverse sequence, namely (5 ′CCTAYGGGRBGCASCAG 3′) and (5′ GGACTACNNGGGTATCTAAT 3') (Youssef *et al.* 2009). The amplification was conducted using the PCR Biorad T100 by adopting the PCR technique of the New England Biolabs Phusion High Fidelity Master Mix. The amplification stage was started first denaturation at 95 ˚C for 30 seconds, followed by 25 cycles of denaturation at 95 ˚C for 10 seconds, amplification at 62 ˚C for 30 seconds, elongation at 72 ˚C for 30 seconds, and final elongation at 72 ˚C for 5 minutes. The PCR results were run in 1% agarose gel to sustain the data quality control's reliability.

The purification process was achieved using the Themofisher Ion Plus Fragment Library Kit 48 rxns, and the quantification was conducted using the Qubit™ 3 Fluorometer. Moreover, the purified samples were inserted toward 1.5 mL parafilm sealed tube, and the sequencing was completed with the IonS5TMXL (Thermofisher) using the NovogenAIT service, China.

Data analysis

The data sequencing results (raw reading) were cleaned up according to Bokulich *et al.* (2013) using the QIIME. The readings were then aligned and matched against NCBI database according to Caporaso *et al.* (2010). The Chimera detection and removal were performed according to Edgar *et al.* (2011). The OTUs clustering utilized the Uparse (Uparse v7.0.1001 accessed at http://drive5.com/uparse/). The effective reading was cut-off by 97% similarity of OTUs. The species annotation was completed using the Mothur SSUrRNA database and SILVA Database according to Edgar *et al.* (2011). The phylogenetic trees (kingdom, phylum, class, order, family, genus, species) were built with the MUSCLE software according to Edgar (2004). The group abundance was examined using the alpha diversity via the Chao1, ACE, and Shannon and Simpson estimators (http://scikit-bio.org/).

RESULTS AND DISCUSSION

The sequencing data were obtained by filtering the reading quality score, removing the non-biological contaminant sequences, and picking up the OTUs. The Chimera filter was used to obtain the adequate data. Clustering sequences defined as OTUs were analyzed according to the similarity level (Hugerth and Andersson, 2017). Simultaneously, during the OTUs building, all of information from each sample was obtained based on the effective and low frequency of reading data annotation. The species diversity investigation in each unit and all effective readings were stratified by 97% similarity of DNA sequences to OTU. The analytical dataset of T0 and T1 treatments were slightly different and are presented in Figure 1. The total average of Tags was 104615, the average of Taxon Tags was 83843, the average of Unique Tags was 20772, the average of OTUs was 135, and there were no unclassified data. Furthermore, each sample's difference and similarity were analyzed by building the Venn diagram, and the unique OTUs are displayed in Figure 2. The diagram displays the diverse microbiome, and the overlay area was a shared OTUs. The shared OTUs in both samples were 35. The OTUs annotation tree can be shown in Figure 3. This graph represents the taxonomic rank range in each sample. The diagram size indicates the relative abundance of species.

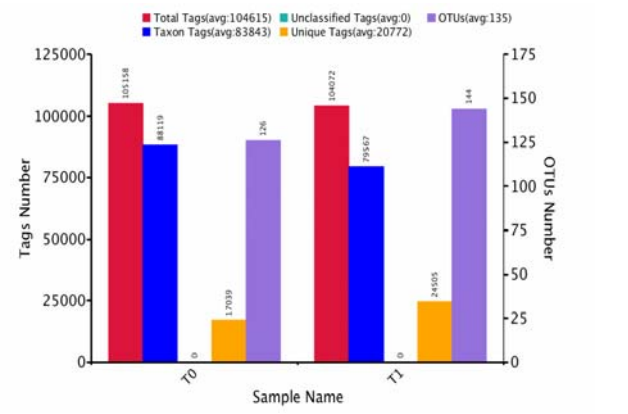

Figure 1 The data reads and OTUs number of samples
T0: chicken feed as basal diet (without *Phyllanthus urinaria*) and T1: chicken supplemented with 2% *Phyllanthus urinaria*

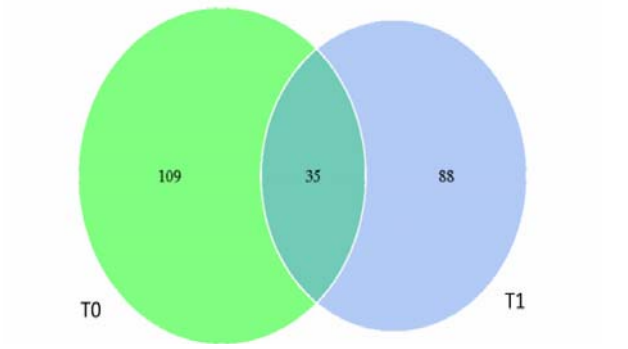

Figure 2 Venn diagram illustrating shared and unique OTUs
T0: chicken feed as basal diet and T1: chicken supplemented with 2% *Phyllanthus urinaria*

The T1 treatment had 3 phyla, while the T0 treatment had 4 phyla including *Tenericutes* with firmicutes as dominant phylum. Figures 4 and 5 display the taxonomic tree of top 10 genera in the T0 and T1. The alpha diversity analysis is extensively applied to evaluate microbial diversity within communities, including species diversity curves and species accumulation plots (Li *et al.* 2013). In general, 97% of the OTUs produced are considered homologous in species. The alpha diversity sample's complexity results can be seen in Table 1. The alpha diversity was applied for analyzing the complexity of species sample diversity through several indices, including the Shannon, Simpson indices Chao1 and abundance-based coverage estimator (ACE). Descriptively, the present investigation found that supplementation of meniran leave significantly modulates the microbial richness and evenness which is OTUs of T1 was lower than T0. The modulation of the gut microbiome could be through various mechanisms such as promotion and inhibition (direct effect) or colonization of new organisms (indirect effect). According to Zhang *et al.* (2021) herbal medicine can promote beneficial gut bacteria.

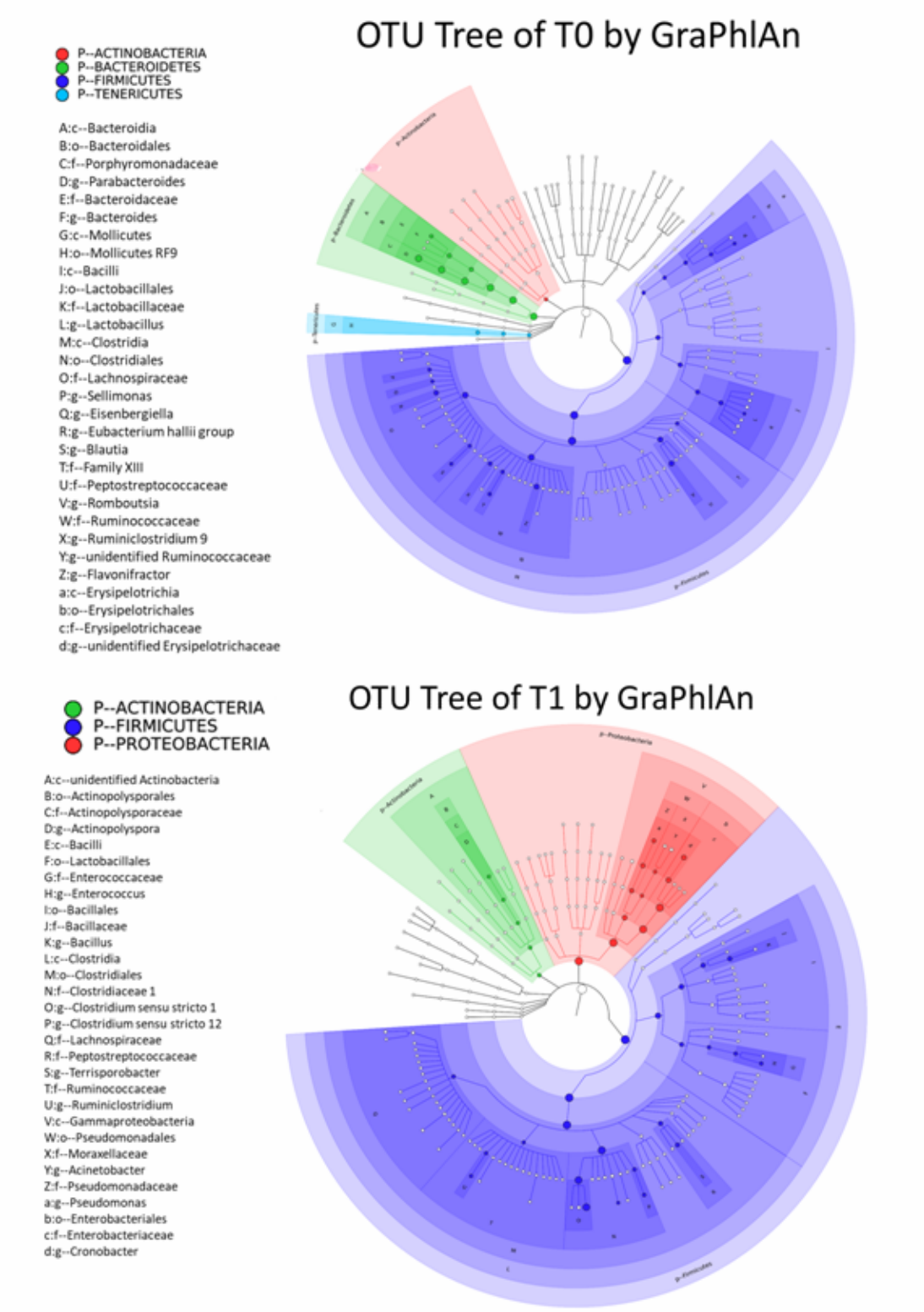

Figure 3 Tree diagram of OUT annotation
Different color shows different phylum and size of circle shows the species abundance
T0: chicken feed as basal diet and T1: chicken supplemented with 2% *Phyllanthus urinaria*

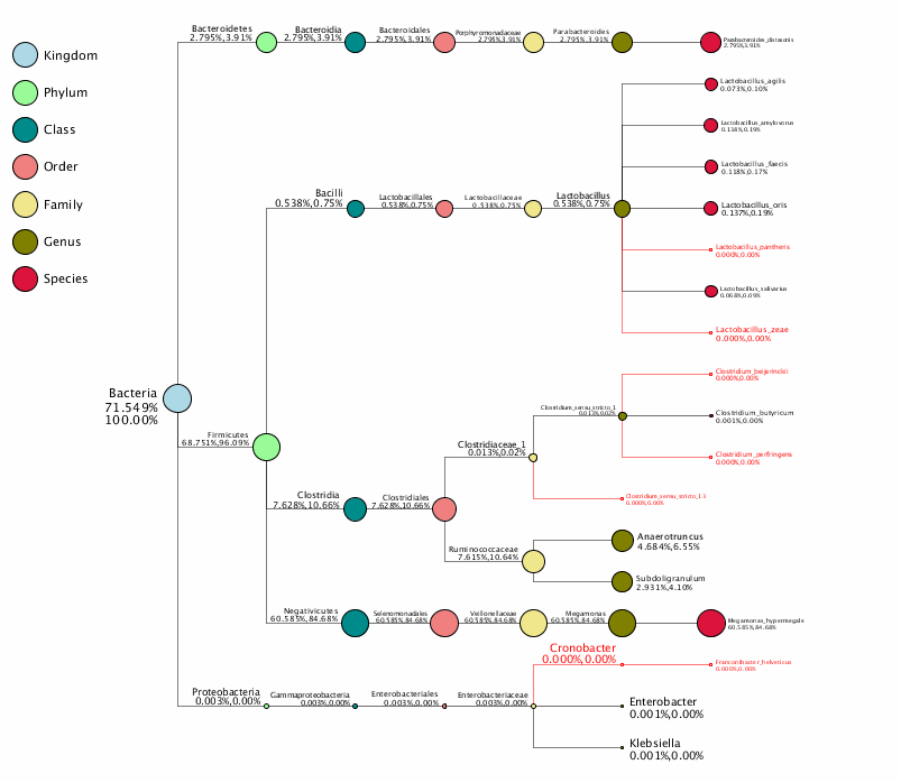

Figure 4 Species spesific of T0 (feed as basal diet) sample from the top 10 genus

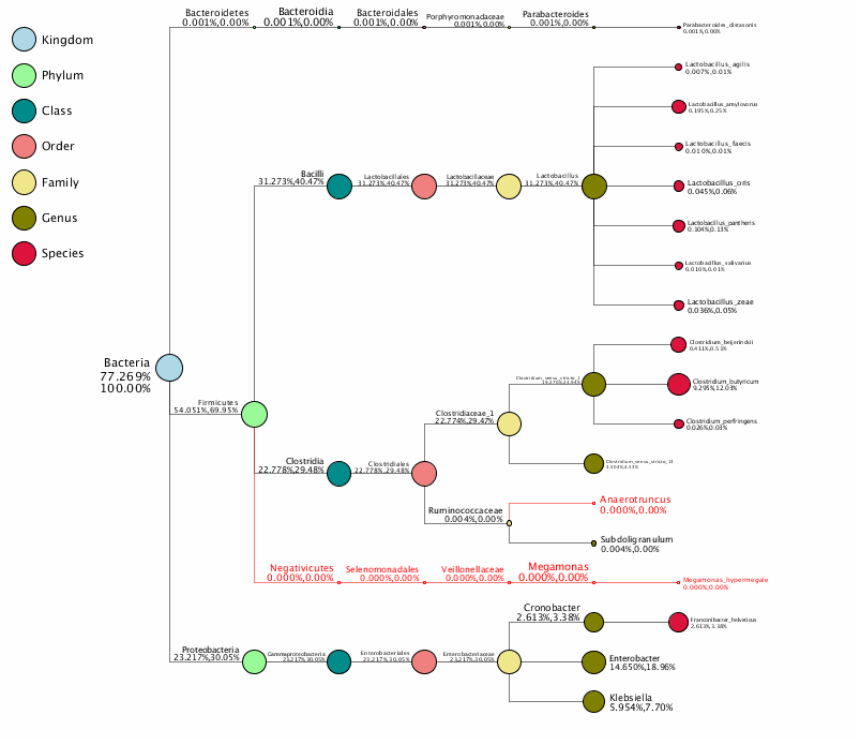

Figure 5 Species specific of T1 (supplemented with 2% *Phyllanthus urinaria*) from top 10 genus

Table 1 Alpha diversity indices

Sample name	Observed species	Shannon	Simpson	Chao1	ACE
T1	123	4.463	0.928	128.250	125.649
T0	144	3.837	0.734	149.250	149.699

T0: chicken feed as basal diet (without *Phyllanthus urinaria*); T1: chicken supplemented with 2% *Phyllanthus urinaria* and ACE: abundance-based coverage estimator.

The present finding may be dued to meniran leaves generally use as herbal medicine and it contain various compound including flavonoid, saponin, tannin and polysaccharide (Harvey *et al.* 2015; Huang *et al.* 2016; Zhang *et al.* 2017; Ali *et al.* 2018) could be an inhibitor of some bacteria including *Escherichia coli*, *Salmonella typhi*, *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* (Nguyen and Nguyen, 2013; Hidanah *et al.* 2018). The antimicrobial activity of meniran leaves is associated with phyltetralin, phyllanthin, routine, quercetin, methyl brevifolincarboxylate, and trimethyl-3,4-dehydrochebulate, which can bind to the protein in bacterial cell membranes to form a water-soluble protein complex, which causes bacterial death (Geethangili and Ding, 2018).

This study showed that, addition of *Phyllanthus urinaria* at the level of 2% could increase the evenness of the genus of *Lactobacilli*. This result may be due to the *P. urinaria* has been known as prebiotic that could promote beneficial microbe in chicken gut (Sinurat *et al.* 2018). Furthermore, the gut microbiome also have a key role on mediating medical compound biotransformation into coumpound that may have bioactivity such as flavonoid into baicalin, naringin, rutin; terpenoid into genisiposide, phorbol; alkaloid into aconitine and berberine; tannin into pedunculangin, procyanidin dimer (Feng *et al.* 2019). These compound also has bioactivity that could develop mutualism between the gut microbiome and the host. Previous studies about phytobiotic showed that the provision of *Macleaya cordata* extract affects the diversity of microbiota in the chicken foregut, enhancing the *Lactobacillus* population.

Using bioactive compounds from blueberry and blackberry phenolic extracts as AGPs substitute could increase the chicken body weight to 5.8%, which was higher than control treatment by showing a higher ratio of *Firmicutes* to *Bacteriodeites* than control treatment (Salaheen *et al.* 2017). In addition, Salaheen *et al.* (2017) also described that the provision of phenolic bioactive-based supplements could be used as enhancers in the regulation of livestock growth by supporting the formation of carbohydrate metabolism corresponding genes and energy production. From the of the alpha diversity, a rare faction curve was obtained (Figure 6).

The biodiversity was generally shown from the rare faction curves and relative abundance rate curves. The abundance curve rank was used to present relative species abundance and this condition could be promoted to formulate species abundance and uniformity. This method overwhelms the lack of a biodiversity index that does not serve a variable role (Lundberg *et al.* 2013).

This study showed relative abundance improvement at the level of genus, then declined at the species level (Figure 7). The distribution of the top 10 microbiota based on the phylum, class, order, family, and genus in this study was presented in Figure 8. *Firmicutes* were found to have the greatest relative abundance at the phylum level of phylum in the two treatments, namely 94% (T0) and 64% (T1), followed by the *Proteobacteria* at 3% (T0) and 35% (T1), *Bacteroidetes* at 3% (T0) and 0.05% (T1), *Actinobacteria* at 0.2% (T0) and 0.8% (T1), and *Tenericutes* at 0.3% (T0) and 0% (T1). Other phyla that were only found in small numbers on the T1 treatment were *Acidobacteria* (0.008%), *Cyanobacteria* (0.006%), and *Chloroflexi* (0.003%). T1 treatment showed more varied results than the T0 (Figure 8). In the *Firmicutes* phylum, the T0 showed more diverse classes, namely *Negativicutes* (65%), *Clostridia* (32%), and *Bacilli* (3%). In contrast, the T1 were only found *Bacilli* (55%) and *Clostridia* (45%). In the *Proteobacteria* phylum, *Grammaprobacteria* was found with the relative abundance percentage in the T0 and T1 samples of 100% and 99%, respectively. In the *Bacteroidetes* phylum, the class in the T0 was 100% *Bacteroidia*, while the T1 were found two classes, namely 78% *Bacteroidia* and 22% *Flavobacteria* 22%.

Our study determined that different order relative abundance among the T0 samples found *Negativicutes* with the class levels of *Selenomonadales* (65%), *Clostridiales* (32%), and *Erysipelotrichales* (0.8%). The Genus in the *Selemionadales* order was dominated by *Veillonellaceae* genus (65%) and *Megamonas*, as also the dominant class level at 65%.

The *Bacilli* class in the T0 and T1 was not significantly different at the order level, specifically *Lactobacilales* at 97% (T0) and 92% (T1). The *Bacilales* order was also found at 3% (T0) and 8% (T1).

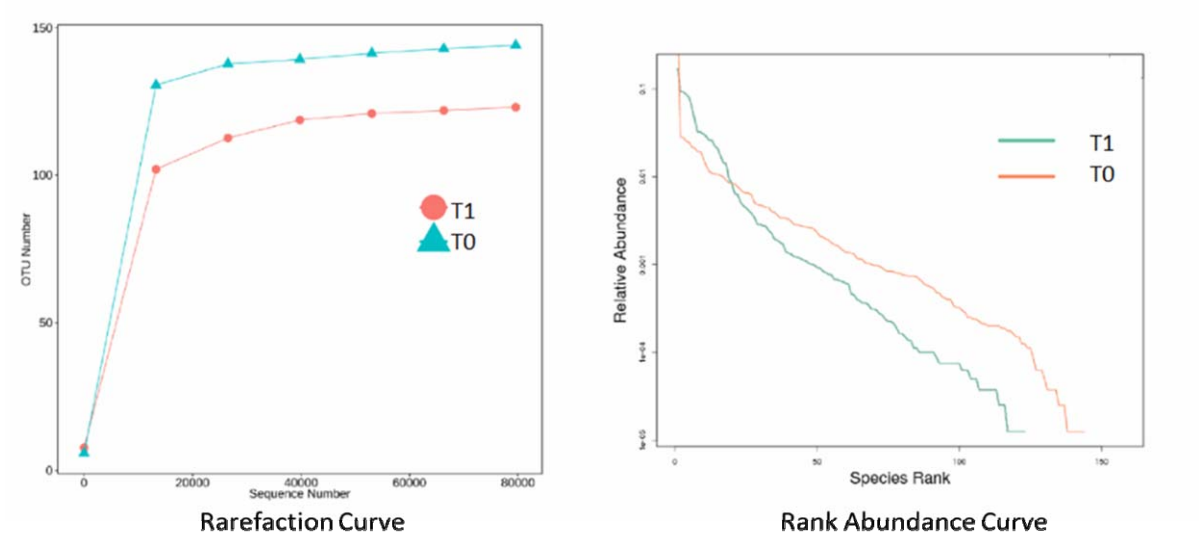

Figure 6 Rarefaction and rank abundance curve
T0: chicken feed as basal diet and T1: chicken supplemented with 2% *Phyllanthus urinaria*

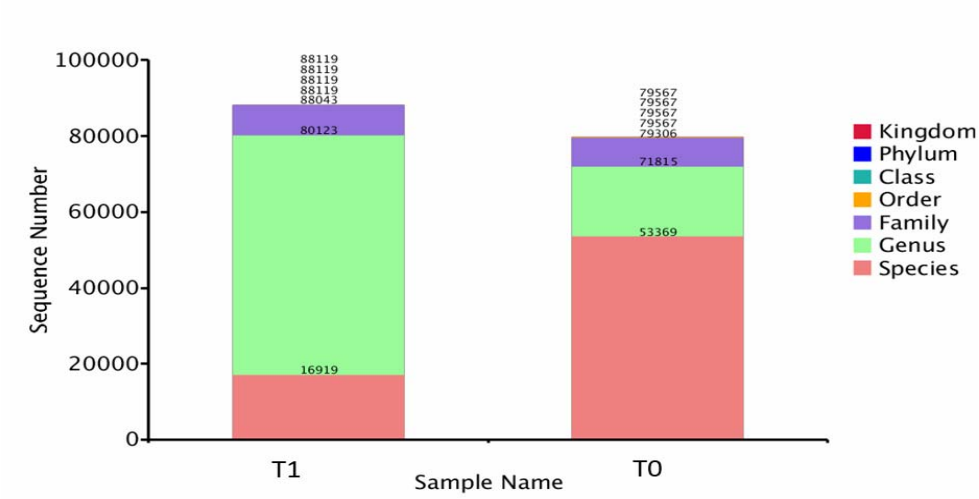

Figure 7 The taxonomic composition of microbiota abundance
T0: chicken feed as basal diet and T1: chicken supplemented with 2% *Phyllanthus urinaria*

In the *Lactobacillales* order, the T0 had 75% *Enterococcaceae* family, 23% *Lactobacillaceae* family, 0.2% *Leuconostocaceae* family, and 0.1% *Streptococcaceae* family. In the T1, the *Lactobacillaceae* family dominated the samples at 97%, followed by *Enterococcaceae* (3%), *Streptococcaceae* (0.4%), and *Leuconostocaceae* (0.02%). In the *Lactobacillaceae* family, the T0 were found the *Enterococcus* (76%), *Lactobacillus* (23%), *Weissella* (0.2%), and *Streptococcus* (0.1%). Meanwhile, in the T1, only 97% of the *Lactobacillus* genus were found, followed by 3% *Enterococcus*. The largest genus in the T1 was *Lactobacillus*, followed by the unclassified bacteria, *Clostridium sensu stricto* 1, *Enterobacter*, *Klebsiella*, *Clostridium sensu stricto* 13, and *Cronobacter*. Whereas in the T0, the highest species abundance was *Megamonas*, followed by unclassified bacteria, *Anaerotruncus*, *Subdigranulum*, *Parabacteroides*, and *Lactobacillus* in small numbers (Figure 8). The most dominant genus in the chicken gut was *Lactobacillus*, besides Clostri*diaceae*, *Enterococci*, *Streptococcus*, *Enterobacter*, and *Coliform* (Stanley *et al.* 2014).

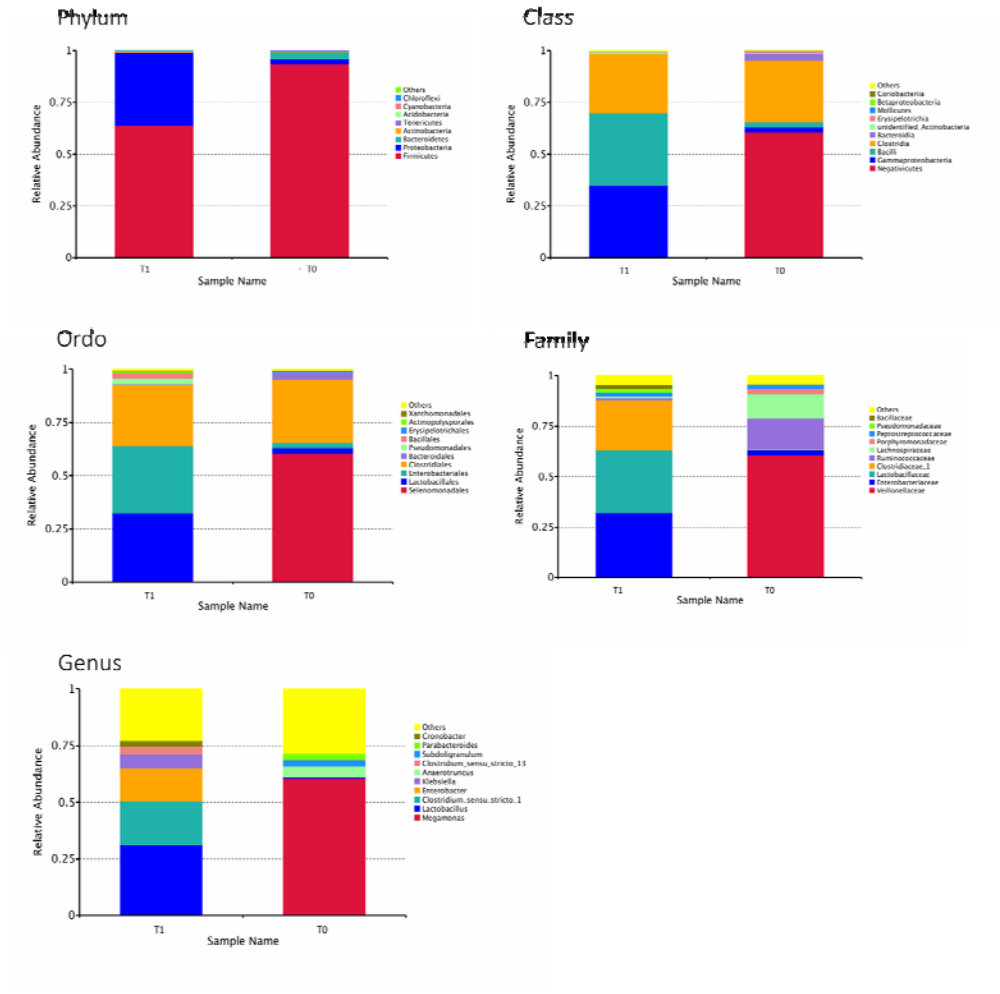

Figure 8 The Relative abundance of chicken gut microbiota at level of phylum, class, order, family and genus
T0: chicken feed as basal diet and T1: hicken supplemented with 2% *Phyllanthus urinaria*

These results were different from (Asrore *et al.* 2015), who explained that the unclassified bacteria were found to be the most abundant species at the genus level in chicken ileum and cecum. Moreover, at the genus level, the abundance of *Lactobacillus* was more abundantly found in the chickens fed with the T1 treatment, while the number of *Lactobacillus* was less found than the *Megamonas* in the T0 treatment. According to Rychlik (2020), the microbiota present in the small intestine including *Lactobacillus, Turicibacter*, *Enterococcus*, *Clostridium sensu stricto*, *Escherichia coli* and *Helicobacter* from phylum *Proteobacteria* and colonizing bacteria commonly from *Coriobacteriaceae* with genera *Olsenella* and *Collinsella* and family *Bifidobacteriaceae* with genus *Bifidobacterium*. In caecum, some appear bacteria in hens include *Fusobacteria*, *Synergistetes*, *Elusimicrobia*, *Spirochaetes* or *Verrucomicrobia*.

The population of T1 microbiota was dominated by the *Lactobacillus* genus, which is a beneficial microbe for the chicken as a probiotic and can produce bacteriocin which can attack the pathogenic bacteria, such as *S. aureus*, *E. coli*, *B. Subtillis*, *S. Typhi*, *B. megaterium*, *B. cereus*, *K. pneumoniae*, and *E. fecalis* (Chandra Das *et al.* 2020). In addition, (Chandra Das *et al.* 2020) also explained that the *Lactobacillus* genus was resistant to the Erythromycin and Ciprofloxacin antibiotics. This proves that the *Lactobacillus* is more resistant when being exposed to the meniran leaves phytobiotics than the other bacteria. Microbiota in the gastrointestinal has an essential role in the nutrient digestion process and affects the host performance. According to Stanley *et al.* (2014), chickens with low and high FCR are identified as having a different abundance of microbes in fecal and caeca regions.

CONCLUSION

This study concludes that the provision of meniran leaves as a phytobiotic affects the diversity, abundance, and composition of broiler chicken gut microbiome. The average of OTUs in the samples was 135, and the shared OTUs were 35. The richness level of T1 was lower than T0, and the

abundance level of T1 was higher than T0. In both samples, the dominant phylum found was *Firmicutes*. The T1 treatment sample consisted of large *Lactobacilli*, which could benefit the host. This study suggests performing a beta diversity analysis with the number of samples in more than three groups for further analysis.

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