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**Research Article**

# Next-Generation Sequencing of the Microbial Community Profile In Free-Range Chicken (*Gallus gallus domesticus*) Cecum from East Nusa Tenggara Province

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**ABSTRACT**

Free-range chicken is livestock reared to support the humans' need for protein alongside its ritualistic use in traditional medicine to treat diseases. This study investigates the diversity of bacterial communities in the free-range chicken cecum reared in different East Nusa Tenggara Province localities comprising Sambi 1, Sambi 2 villages, Labuan Bajo, and Kupang City. The extracted chromosomal DNA was subjected to next-generation sequencing using the V3-V4 region primers. Results revealed that the Kupang chicken cecum had the highest total tags, while the Sambi 2 village recorded the lowest. Similarly, Sambi 2 chicken cecum exhibited the highest unique tags (6662) and OTUs number (1261), while the Kupang samples gave the lowest at 2550 and 745, respectively. The Shannon diversity index for bacterial diversity demonstrated that cecum samples from Labuan Bajo (5.679) were more diverse than Sambi 1 (5.378), Sambi 2 (5.653), and Kupang samples (3.77). The bacteria with the highest dominance index (0.935) was found in Sambi 2, while the lowest was observed in the Kupang samples (0.082). The three bacterial phyla showing the highest relative abundance were those from Sambi 1, Sambi 2, and Labuan Bajo cecum samples, comprising Firmicutes, Bacteroidota, and Actinobacteriota.Conversely, the Kupang samples showed an abundance of Firmicutes, Bacteroidota, and Campilobacterota, compared to the Lactobacillus-dominated Kupang, Sambi 1, and Sambi 2 chicken cecum samples. The highest relative abundance for Bifidobacterium occurred in Sambi 1 and Sambi 2 chicken cecum samples, the Kupang samples were Campylobacter dominated, and *Olsenella* was abundant in the Labuan Bajo samples. Intriguingly, the bacterial composition in the tested chicken cecum samples largely comprised beneficial bacteria such as the lactic acid bacteria group. This bacterial group can be further characterized for obtaining probiotic cultures that could improve the health of free-range chickens.

*Keywords: Cecum, Diversity, Firmicutes, Free-range chicken, Next-generation sequencing*

## Introduction

Free-range chicken (*Gallus gallus domesticus*) is a local poultry breed in Indonesia, locally referred to as '*ayam kampong*' and its meat is well known for its unique flavor. Free-range chicken is produced widely throughout the Indonesian provinces, such as East Nusa Tenggara, especially in East Manggarai Regency. In the West Manggarai regency and Kupang city, 130, 655 and 164,574 free-range chickens are produced, respectively, with the free-range variety topping broiler chicken in 2022 [1]. Free-range chicken production in these two places has shown a steady annual increase in response to higher community demand.

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Free-range chickens are class Aves endowed with unique morphological features, in which their feathers are a combination of white, black, orange, and brown, with the legs either grey or yellow. Their height from the back to the feet ranges from 20-24 cm, while their bodies from head to tail are between 23-30 cm. A 15-week-old free-range chicken normally weighs between 450-650 grams.

Free-range chicken uses are not limited to a source of protein but for medical purposes in traditional ritual ceremonies. These chickens are usually fed household food waste and other naturally derived food sources. While these free-living chickens get to perch on trees at night, this traditional way of rearing chickens makes them susceptible to infectious diseases caused by pathogenic microorganisms [2, 3], although treatable with antibiotics. The heavy dependence and continuous use of antibiotics on these chickens run the risk of pathogens developing resistance to antibiotics and upsetting the delicate balance of microbes in the intestines [4, 5].

Microbes that live in gastrointestinal tracts, such as the cecum of free-range chicken, are diverse, especially the lactic acid bacteria (LAB). LAB is chickens' most frequently applied probiotic microorganism to improve their health [6, 7, 8]. The literature has shown that the cecum of chickens contains an array of microbes, including *Bacteroides, Eubacteria, Lactobacilli, Bifidobacteria*, and *Clostridia* [9]. Another study reported a more diverse microbial community in chicken cecum samples and was dominated by anaerobes [10, 11, 12]. It was estimated that ~10% of indigenous bacteria in the chicken cecum were culturable, with the *Lactobacillus* rated among the most culturable cecal microbiota (24%) [9, 13].

In the present study, cecum samples from free-range chickens were subjected to next-generation sequencing (NGS) to uncover the bacterial communities that reside in them. NGS is a type of DNA sequencing technique that identifies sequences through parallel sequencing of multiple small fragments of DNA [14]. Consequently, this study aimed to identify the bacterial communities that exist in the cecum of free-range chicken reared from different localities throughout the East Nusa Tenggara Province localities comprising Sambi 1, Sambi 2 villages, Labuan Bajo, and Kupang city. Pertinently, this work would lead to a better understanding of the diverse bacterial communities in cecum samples of free-range chickens within the tested region.

## Material and Methods

### *Ethics statement*

This research was approved by Animal Care and Use Committee, Universitas Brawijaya (No: 017-KEP-UB-2022).

### *Sample collection*

Free-range chickens aged 15 weeks were collected from four localities of the East Nusa Tenggara Province: Kupang city, Labuan Bajo, Sambi 1 village, and Sambi village 2. Cecum samples of free-range chickens from Sambi 1 village were home-reared, while those from Sambi 2 were reared on agricultural land. Each cecum sample was labeled as Kupang (SK), Labuan Bajo (SLB), the cecum of Sambi 1 (SS1), and the cecum of Sambi 2 (SS2). The samples were transported to the Faculty of Mathematics and Natural Sciences Microbiology Laboratory, Catholic Widya Mandira Kupang University, for further analysis. The chickens were dissected under sterile conditions to obtain the cecum samples before storing them a DNA shield.

### *DNA Extraction*

DNA from free-range chicken cecum samples was isolated using CTAB/SDS method. Genomic DNA was purified, DNA concentration was quantified on a Nanophotometer, and the DNA was resuspended in a tris-EDTA buffer before storing at -20 °C until further analysis [15].

### *Analysis of chromosomal DNA by NGS method*

The 400-450bp size of the band was analyzed by Illumina (MiSeq) platform (paired-end reads). The primers used in this sequencing were the V3-V4 region 515F (5'CCTAYGGGRBGCASCAG 3') dan 806R (5'GGACTACNNGGG-TATCTAAT 3') [16]. The Illumina protocol was adopted for the V3–V4 (V34) region following their wide usage in gut microbiota studies [17, 18, 19, 20]. The V34 primer-pair combination amplifies artifacts and has a different composition than other regions, including the V12 [21]. In contrast, the V34 region is more suitable for gut microbiota analysis than V12 because of its higher potential to detect the order of Bifidobacteriales [22]. The optimal variable regions might vary according to the analysis target, primers specificity, GC

contents of the selected region, and the bacterial compositions of different samples [23].

In this study, the operational taxonomic units (OTUs) were clustered at a threshold level of 97% sequence identity. Only sequences with the highest frequency were selected as the representative sequences of OTUs and annotated using the Green Genes database [24]. The Shannon diversity and Simpson indices were measures used for alpha-diversity to indicate the evenness of community structure, richness, and the observed number of OTUs. Correlations between temperature, chicken age, tags, OTUs number, richness, and diversity of bacteria were measured by Pearson correlation coefficient. This study used a paired modification of the Wilkinson-Mann-Whitney non-parametric criterion to determine the significance of differences between the average value of Shannon, Simpson, Chao1, and ACE indices identified by the V3-V4 fragments. Sequencing data were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME v1.9.0). The paired-end reads were joined with a fast length adjustment of short reads (FLASH v1.2.11). Qualitative and quantitative comparisons of the community composition at different taxonomic organization levels (phylum, class, order, family) were made using the Nonmetric Multidimensional Scaling-NMDS with Bray-Curtis distance-, Gower distance-, and the Jaccard distance metric. Estimates of taxon representation in samples (number of reads per taxon) were visualized as heat maps. The rows and columns were clustered using the average method based on the calculated distance matrix [25].

## Results and Discussion

### *Sample characteristics and bacterial diversity*

The 15 weeks old free-range chickens, namely hens reared in the Sambi 1, Sambi 2, Labuan Bajo, and Kupang localities, were reared at different temperatures. Free-range chickens reared in the Kupang locality were subjected to the highest temperature (34.6°C), while the lowest was in the Sambi 2 village (31.8°C) (Table 1).

The study discovered that the highest total tags and taxon tags occurred in the cecum of Kupang chickens, corresponding to 125679 tags and 123121 tags. Conversely, cecum samples from Sambi 2 showed the lowest total tags (97991 tags) and taxon tags (91324 tags). The Unique tags that appear once and only exist in a single sample were identified in cecum samples of Kupang free-range chicken (2550 tags), while the highest was noted in the Sambi 2 samples (6662 tags). The OTUs number from Sambi 1, Sambi 2, Labuan Bajo, and Kupang chicken cecum were 1014, 1261, 945, and 745, respectively (Table 2).

The Shannon index representing microbial diversity, and the Simpson index for dominance in the four tested samples, were performed using the Chao1 and ACE indices. Sambi 2 chicken cecum samples exhibited the highest number of dominant species. Microbial diversity ranked from the highest to the lowest was observed for cecum samples obtained from free-range chickens reared in Labuan Bajo, Sambi 2, Sambi 1, and Kupang, respectively. Contrariwise, the highest to lowest microbial abundance was noted for the Sambi 2, Sambi

Table 1. Environmental temperature and sample characteristics

| Parameter | Sampling location | | | |
|---|---|---|---|---|
| | Sambi 1 | Sambi 2 | Labuan Bajo | Kupang |
| Temperature | 32.4°C | 31.8°C | 34°C | 34.6°C |
| Chicken age (week) | 15 | 15 | 15 | 15 |
| Type of chicken | Hen | Hen | Hen | Hen |

Table 2. Tags, OTUs number, bacterial diversity, and bacterial richness

| Parameter | Sampling location | | | |
|---|---|---|---|---|
| | Sambi 1 | Sambi 2 | Labuan Bajo | Kupang |
| Total tag | 107445 | 97991 | 109067 | 125679 |
| Taxon tag | 101422 | 91324 | 102845 | 123121 |
| Unique tag | 6018 | 6662 | 6219 | 2550 |
| OTUs number | 1014 | 1261 | 945 | 745 |
| Shannon diversity index | 5.378 | 5.653 | 5.679 | 3.77 |
| Simpson diversity index | 0.916 | 0.935 | 0.93 | 0.0822 |
| Chao1 richness index | 1016.522 | 1551.624 | 929.279 | 736.745 |
| ACE richness index | 1023.601 | 1403.289 | 943.987 | 773.008 |

Table 3. Pearson correlation coefficients among temperature, chickens age, tags, OTUs number, richness, and diversity of bacteria

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.000 | 0.999** | -0.951* | -0.968* | -0.915 | -0.934 | -0.891 | -0.915 | 0.881 |
| 2 | | 1.000 | -0.963* | -0.956* | -0.931 | -0.948 | -0.871 | -0.897 | 0.868 |
| 3 | | | 1.000 | 0.842 | 0.993** | 0.998** | 0.710 | 0.747 | -0.748 |
| 4 | | | | 1.000 | 0.785 | 0.814 | 0.975* | 0.986* | -0.924 |
| 5 | | | | | 1.000 | 0.999** | 0.645 | 0.682 | -0.666 |
| 6 | | | | | | 1.000 | 0.680 | 0.717 | -0.701 |
| 7 | | | | | | | 1.000 | 0.998** | -0.879 |
| 8 | | | | | | | | 1.000 | -0.900 |
| 9 | | | | | | | | | 1.000 |

Parameter code: 1 = Total tag, 1 = Taxon tag, 3 = Unique tag, 4 = OTUs number, 5 = Shannon diversity index, 6 = Simpson diversity index, 7 = Chao1 richness index, 8 = ACE richness index, 9 = temperature
**. Correlation is significant at the 0.01 level (2-tailed)
*. Correlation is significant at the 0.05 level (2-tailed)

1, Labuan Bajo, and Kupang chicken cecum samples (Table 2).

The study found that the number of OTUs was negatively correlated with the total tag and tag taxon because the OTUs number was grouped based on 97% sequence similarity to represent the genera or species number. The Shannon diversity index was significantly (p-value < 0.05) correlated with unique tags, OTUs number, Simpson diversity index, Chao1 richness index, and ACE richness index. In contrast, the temperature, total tags, and taxon tags were negatively correlated with the bacterial diversity index (Table 3). In general, the increase in species richness and evenness corresponded to an increase in diversity [26]. Meanwhile, Simpson's diversity index negatively correlated with tag total, taxon, and temperature. It is pertinent to indicate here that the Shannon index prioritizes species richness, while the Simpson index considers species evenness rather than species richness in its measurement. This is because Simpson's diversity index also shows the dominance of certain species in a community [27, 26]. This study also observed that the rearing temperature positively correlated with the tag total and tag taxon, as temperature affects the number of species. The living microbes in chicken cecum are those that survive at the host temperature (Table 3).

Interestingly, the Labuan Bajo and Sambi 2 chicken cecum exhibited a higher bacterial diversity, probably because the free-range chickens were reared on agricultural land. Their diet expectedly comprised natural foods: soil, fresh grass, insects, wild seeds, fruit, berries, worms, etc. On the other hand, age is another key factor in the difference in bacterial diversity. Chickens between 15-20 weeks old have a more diverse bacterial community than those less than 15 weeks. Other factors influencing the differences seen here are behavior patterns, overall health, species, and rearing environment [28, 29].

The decreased bacterial diversity could be due to the overabundance of a few dominant bacterial genera in the chicken cecum. Also, the prevalence of competitive bacterial communities has been shown to affect the richness and abundance of a bacterial community [30, 29]. Another possible reason is that the free-range chickens have adapted digestion of food sources from the surrounding environment, bringing about a more diverse bacterial community in the cecum [9].

### *Bacterial community profile*

Figure 1 illustrates the proportion of 10 phyla with higher relative abundance in each sample. As can be seen, Firmicutes were the dominant phyla in Sambi 1 (SS1) and Kupang (SK), corresponding to 37.8% and 57.6%, respectively. In contrast, Bacteroidota was the dominant phylum in Sambi 2 (SS2) (30.2%) and Labuan Bajo (SLB) (35.1%) cecum samples. The Campilobacterota phylum was the highest relative abundance in Kupang chicken cecum samples (30%). Contrariwise, seven phyla showed relatively low abundances (total OTUs < 1%) in the SS1, SS2, and SLB chicken cecum samples for Actinobacteria, Campilobacterota, Patescibacteria, Proteobacte-

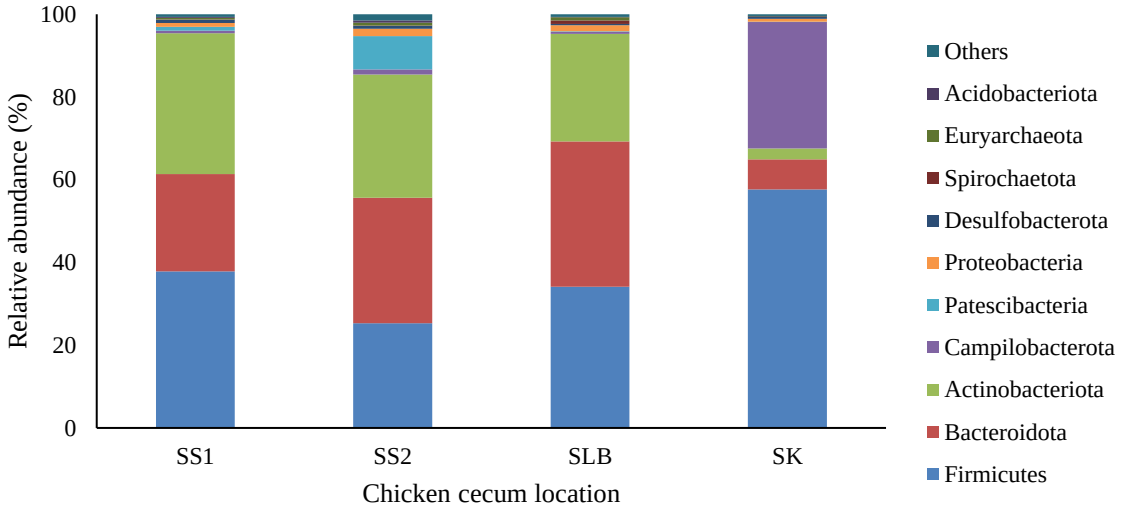


Figure 1. Relative abundance of bacteria sequence read at the phylum level

ria, Desulfobacterota, Spirochaetota, Euryarchaeota, and Acidobacteriota. SK chicken cecum samples exhibited relatively low abundances for eight phyla comprising Bacteroidota, Actinobacteriota, Patescibcteria, Proteobacteria, Desulfobacterota, Spirochaetota, Euryarchaeota, and Acidobacteriota (Figure 1).

The findings seen here corroborated earlier observations by similar studies, which showed Firmicutes and Bacteroidetes as the top two most abundant bacteria at the phylum level [31, 32, 33, 29]. Moreover, Firmicutes and Bacteroidetes comprised over 25% of all microbiota in the tested chicken cecum samples. [34, 35]. Literature has shown that initially, after hatching, the gastrointestinal of free-range chickens is dominated by Enterobacteriaceae. Soon, the Firmicutes dominate the gastrointestinal of chickens by the seventh day after hatching [36]. After that, the gastrointestinal tract is colonized by other bacterial species, which originate from the surrounding environment and other bacteria in food and water [37, 38]. It is germane to indicate here that the phylum Firmicutes has an anti-inflammatory role in the gastrointestinal of chickens, namely involved in immune homeostasis. Consequently, microbial imbalances in the gastrointestinal of free-range chickens can destabilize the immune system and increase the chickens' susceptibility to diseases [38].

In the case of Bacteroidetes, these are Gram-negative bacteria that aid in starch and fiber digestion [39]. This phylum is typically dominant in the chicken cecum samples because of its fiber-degrading ability. Moreover, the cecum's role is to digest feeds containing fiber with the help of microbes [40]. It was described that chickens fed a high-fat diet increase the abundance of Firmicutes, while a high-fiber feed favours Bacteroidetes growth [34]. Bacteroidetes are common butyric acid-producing bacteria closely linked to the predicted polysaccharide biosynthesis and metabolic functions in the Kyoto Encyclopedia of Genes and Genomes (KEGG) [41].

As can be seen, the relative abundance of Firmicutes increased with the age of the chicks, consistent with the report showing Firmicutes being the largest microbiome component in the chicken cecum. In general, these microbes digest starch and carry out fermentation related to metabolic processes for energy production. In contrast, the relative abundance of Bacteroidetes decreased with the age of chickens [42, 43, 44].

Results revealed that the Actinobacteriota phylum was abundant in Sambi 1, Sambi 2, and Labuan Bajo cecum samples, thus corroborating its high productivity in such a sample [34, 38]. These cellulose-degrading microbes produce specialized hydrolytic enzymes that degrade lignocellulose [45]. This explains their low energy consumption following their ability to utilize sugars from the cellulose and hemicellulose degradation in the gut [46, 47].

This study found that at the genus level, there are a total of 8811 genera in the Sambi 1, 14774 genera in the Sambi 2, 10850 genera in Labuan Bajo, and 4518 genera in the Kupang chicken cecum samples. *Lactobacillus* was the dominant bacteria in the Kupang samples (46%), while

Sambi 1 samples were dominated by *Bifidobacterium* (29%). *Olsenella* was dominant in Sambi 2 (16%) and Labuan Bajo (23%) cecum samples. *Lactobacillus*, *Olsenella*, *Bacteroides*, *Rikenellaceae* RC9 gut group, and *Streptococcus* exhibited relative abundances above 4% in Sambi 1 samples. Meanwhile, the *Lactobacillus* A genus gave above 1.3% relative abundance in Sambi 2 samples, compared to the *Bifidobacterium*, *Bacteroides*, *Rikenellaceae* RC9 gut group, and *Saccharimonadales*. In contrast, cecum samples from Labuan Bajo showed relative abundances above 7% for *Lactobacillus*, *Bacteroides*, *Rikenellaceae* RC9 gut group, *Ruminococcus* torques group, and *Enterococcus*. In Kupang cecum samples, relative abundances exceeding 1% were noted for *Bifidobacterium*, *Campylobacter*, *Olsenella*, and *Bacteroides* (Figure 2).

The literature has shown that indigenous bacteria in chicken cecum compose bacteria suited to survive in such an environment [48]. When the intestinal microbiota matures, the dominant microorganisms in the duodenal intestine are *Lactobacillus*. In contrast, chickens' dominant microorganisms in the cecum and colorectum are more complex, mainly comprising the Bacteroides, Odoribacter, and Clostridiales vadin BB60 group [11, 12]. On the other hand, chicken cecum is also dominated by anaerobic bacteria. Anaerobic bacteria are more commonplace in the cecum than the *Lactobacillus*, which the latter is more abundant in the ileum [13, 9].

The literature has shown that the genus *Lactobacillus* is bacteria indigenous to the chicken gut and particularly abundant in the intestines. This genus plays a role in the digestive process and passes to the chicken cecum, which explains its abundance [48]. Compared to pathogenic bacteria, *Lactobacillus* can strongly adhere to the intestinal wall of animals and competitively inhibits the adhesion of pathogenic bacteria. Also, this capability effectively inhibits pathogenic bacteria's reproduction, maintaining intestinal flora's balance [49, 50]. The *Lactobacillus* also produces lactic acid and short-chain fatty acid in chicken intestines, reducing the intestine's pH value [51], thus inhibiting pathogenic bacteria growth but supporting the growth of indigenous *Lactobacillus* and other diverse bacteria [52, 48].

The study also noted that the *Bifidobacterium* was also dominant in Sambi 1 and Sambi 2 cecum samples. These Gram-positive, catalase-negative bacteria cannot grow under aerobic conditions and showed fructose6-phosphate phosphoketolase activity [53]. *Bifidobacterium* typically utilizes carbohydrates as a substrate for its growth. When the un-degradable oligosaccharides in the chicken feed reach the large intestine and cecum, the *Bifidobacterium* and lactic acid bacteria catabolize them for growth. These conditions are also unfavorable for pathogenic bacteria such as *Salmonella* to grow [53, 54]. Previous studies reported that *Bifidobacterium* is abundant in chicken cecum [53, 24, 55, 56]. Conversely, the high population of *Bifidobacterium* also inhibits the growth of pathogenic bacteria such as *Salmonella* typhi N15 and *Escherichia coli*-EHEC (enterohaemoragic *Escherichia coli*) by producing antimicrobial

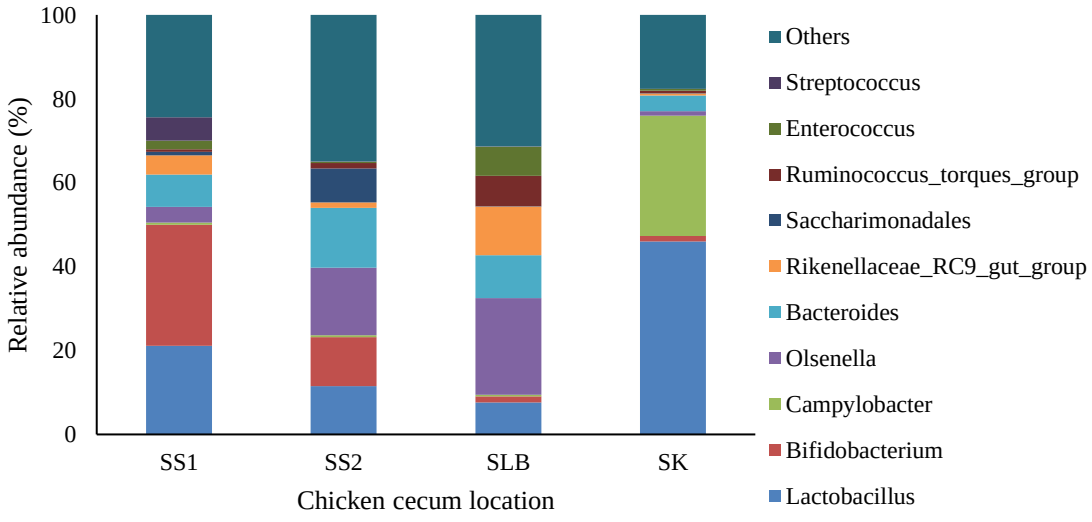


Figure 2. Relative abundance of bacteria sequence read at the genus level

compounds such as lactic and acetic acids. These compounds reduce intestinal pH, increase fermentation and boost the host immunity [55, 56, 57, 58].

A noteworthy outcome seen in this study was the abundance of *Campylobacter* in Kupang chicken cecum. This genus was described in several previous studies that sampled chicken cecum [59]. This zoonotic pathogen is the causal agent of gastroenteritis [60, 61] by colonizing the colon and cecum of chickens [62, 63]. The bacteria are abundant in the cecum as they spread through contaminated feed and water consumed by chickens. In this study, the prevalence of *Campylobacter*ia in the Kupang cecum samples has to do with the source of chicken feed from household waste that might have been contaminated with Campylobacteria.

Our observation of the abundance of *Olsenella* in the Sambi 1 and Labuan Bajo cecum samples reveals that this phylum Actinobacteriota group is strictly anaerobic, Gram-positive, and non-motile. This group of bacteria is catalase-negative and non-spore-forming, rod-shaped bacteria with a DNA G+C content of 62–64% [64]. This genus typically inhabits the oral cavity, gastrointestinal of humans and animals and favors the anaerobic environment [65, 66]. The genus *Olsenella* was previously documented to be abundant in the chicken cecum by metagenomic methods [67, 68, 69, 70, 71]. This genus utilizes arbutin, cellobiose, dextrin, D-fructose, L-fucose, D-galactose, α-D-glucose, maltose, D-mannose, D-melibiose, D-raffinose, salicin, sucrose, and turanose as carbon sources. They also produce a volatile fatty acid, namely acetic acid, which has antimicrobial properties [68].

## Conclusion

Our findings showed that the number of OTUs in the Sambi 1, Sambi 2, and Kupang cecum samples was proportional to the increased diversity. Higher OTUs led to higher microbial diversity. Interestingly, this was different for the Labuan Bajo samples, which showed an inverse correlation to the number of OTUs. The study also found that Firmicutes, Bacteroidota, and Actinobacteriota were the highest phyla number in Sambi 1, Sambi 2, and Labuan Bajo samples. At the same time, Firmicutes and Campilobacterota were the highest phyla number in Kupang samples. *Lactobacillus*, *Bifidobacterium*, and *Olsenella* exhibited high relative abundances in Sambi 1, Sambi 2, and Labuan Bajo, while *Lactobacillus* and *Campylobacter* yielded high relative abundances in Kupang cecum samples. If further investigated, the highest bacterial composition, which in this study, turns out to be group of beneficial bacteria, could aid in identifying bacteria with probiotics potential. Hence, it can be construed that the diversity of stable gut flora depends on dietary composition or treatments, breed, environmental factors, sequencing approach, primers, and geographical distribution of the free-range chickens. Therefore, further research will focus on the specific factors that influence microbial composition and determine their interactions in altering the host's phenotypic nature or physiological status.

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