Metagenome analysis of gut microbial in both the caged and non-caged ducks

R Susanti¹, A Yuniastuti¹, F Fibriana²

¹Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang. Gedung D6 Lantai 1, Kampus Sekaran, Gunungpati, Semarang 50229, Central Java, Indonesia.
²Department of Integrated Science, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang. Gedung D5 Lantai 1, Kampus Sekaran, Gunungpati, Semarang 50229, Central Java, Indonesia

Corresponding author: r.susanti@mail.unnes.ac.id

Abstract. The different duck husbandry techniques, the type of feeds, and environments are believed to have an impact on the diversity of duck's gut microbiome. This research has the objective to examine the diversity of gut microbiome of duck in different husbandry models, i.e., cage and non-cage. The research design was an exploratory study. Duck samples were taken purposively from both cage (CG) and non-cage (NCG) methods at local duck farms in Gunungpati, Semarang, Central Java, Indonesia. Five grams of duck intestinal samples were prepared for DNA isolation, 16S rRNA gene amplification in the V3-V4 region and were sequenced with Next Generation Sequencing (NGS) method: metagenomics. The 16S metagenomic analysis was performed using QIIME2 (Ver. 2019.4). The most dominant gut bacteria that found in both CG and NCG was Family Enterobacteriaceae, Phylum Proteobacteria and followed by Lactobacillaceae from Phylum Firmicutes. However, the bacterial diversity map in family level was not shown a significant trend between CG and NCG. There were 851 shared species belongs to NCG and CG; it means that more than 800 same bacteria species build up the gut ecosystem both in NCG and CG. It is likely because of similar feed and environmental condition from the two husbandries. The diversity of duck gut microbiome in Gunungpati may be closely related to feeding, rearing techniques, and environments. The number of bacteria species in the non-caged duck (NCG) was higher than CG or caged duck.

1. Introduction
Gut microbiota is bacterial communities live in the digestive tracts of all living things. The microbial composition has been proven to affect the evolution, nutrients absorption, physiology, and immunity regulation in living things, including duck [1]. A recent research in the gut microbiome of duck has shown that husbandry techniques (cage and no cage) and feed compound (organic or pellet) have the impact on the gut microbiome composition and also have the role as immune system which protects the host from parasites and pathogens infection [2]. On the other side, microbiome diversity affects metabolism and host viability, directly.

Indonesian local duck farm is divided into two techniques of husbandry, i.e., cage and non-cage. Usually, the non-caged duck (free forage-duck) has more diverse habitat conditions that likely provide them wide access to collect various foods that influence their gut microbiome [3], high meat quality and healthier than caged ducks [4]. Also, dysbiosis condition increases pathogen infections and decreases
bird resistance against metabolic disease, thus threatening food security for humans [1]. However, there is still no prove on the relationship of duck husbandry technique, environment, and feed in Indonesia with the microbiota composition in the gastrointestinal tract and also metabolic systems. Therefore, based on those cases, this research aimed to understand how the relationship of husbandry technique, feed, and environment contributes to the gut microbiome composition with the metabolisms. This research is an effort to find the right husbandry model for optimizing the composition of gut normal flora to support the function of the gut as well as duck growth and health.

2. Methods

The research samples of caged and non-caged ducks were obtained from local farmers in Gunungpati District, Semarang, Central Java, Indonesia. DNA isolation was performed using the QIAamp DNA Stool Mini Kit (Qiagen, San Diego, California, US). Gene library running kit was obtained from Nextera XT DNA Library Preparation Kit (Illumina, San Diego, California US).

2.1. Research design

This research was an exploratory observational study to overview and analyze composition and abundance of the gut microbiome in both caged and non-caged duck. This research evaluated the correlation among gut microbiomes with the feeds, habitats, and environmental conditions of ducks. Samples were obtained from local duck husbandry in Gunungpati District, Semarang City. The DNA isolation and gene purification were carried out at the Laboratory of Molecular Biology and Laboratory of Molecular Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang. Metagenomic analysis was carried out at Genetika Science, Jakarta. Samples were obtained purposively with the criteria for sampling (inclusion), 1) cage and non-caged ducks, 2) At least three months old-male (drake) or female (hen) ducks, 3) no antibiotics supplementation in animals within 2 weeks before the examination.

2.2. Feed, habitat and cultivation documentation

Sample information, such as sex and ages, husbandry methods, environmental conditions, and feeds, were recorded by observation and the farmer in Gunungpati District. The duck husbandries are usually in the area of the owner's house; the cage is located in the backyard of the house or just let them free or non-caged. Then, duck samples were sacrificed, and 1 g of craw content were collected for feed analysis. After that, intestine and feces were collected aseptically and were kept in sterile vial tubes containing later RNA (Life Technologies AM7021). Subsequently, the samples were stored at -20 °C before further analysis.

2.3. DNA isolation and Next Gene Sequencing (NGS)

Bacterial DNA was extracted from both caged and non-caged duck's intestine and feces using QIAamp DNA Stool Mini Kit (Qiagen, San Diego, California, US) following the manufacturer's protocol. Extracted DNA was stored in a -20 °C freezer before being used for further analysis.

The 16S rRNA gene markers were taken from the V3-V4 region [5,6] and were amplified by PCR (denaturation 94 °C for 3 min, continued by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and the final extension at 72 °C for 10 min. The primers used are 338F (5′-GGACTACHVGGGTWTCTAAT-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) [5]. The PCR reaction was carried out with a total volume of 20 μl containing a mixture of 0.5 μl containing of 5U/μl Easy Taq DNA polymerase, 2 μl of 10× Easy Taq buffer, 2 μl of 0.25 mmol/L dNTPs, 0.2 μmol/L of 16SrRNA primer, 10 μmol of DNA templates, and ddH2O to 20 μl.

The amplicon detection of PCR results was run in 2% of agarose (w/v) in 1× of Tris-Boric acid-EDTA (TBE) buffer. Amplicons were purified in equimolar and paired-end sequencing (2×300) on the MiSeq Illumina platform following the standard protocol. Gene library was run using Nextera XT DNA Library Preparation Kit (Illumina, San Diego, California US) using a sequence of eight special bases as
barcodes or adapters according to the manufacturer's instructions [7] and then was confirmed to the online database.

2.4. Bioinformatics analysis
The sequencing result was performed in fastqc format, and read based on the unique barcode, and primary sequences based on following criteria: (1) matching the correct amplicon code, (2) two nucleotide mismatches in primary matching, (3) read containing ambiguous characters deleted. The reading of the amplicon gene is run through Trimmomatic (version 0.33) [8] to eliminate the base pair of the amplicon from the low-quality PCR using these parameters. The trimmed reading was then combined again using the FLASH program (version 1.2.8) [9] with the parameters [-m 10 –x 0.2 –p 33 –r 300 –f 450 –s 150] [10].

Metagenomic Analysis was conducted using QIIME2 (Ver. 2019.4) [11]. The paired-end file was demultiplexed using Plugin Demux. Then, quality control was run using plugin Dada2 [12]. Six diversity index was collected such as Shannon [13], Simpson [14], Pielou evenness [15], Margalef [16], Chao1 [17], and observed OTUS [18]. Bacteria taxonomy was arranged based on the Greengenes 13_8 99% OTU database [19]. The phylogenetic tree from every sample constructed using MAFFT [20] and FastTree [21]. The presentation of heat-map graphic [12], was conducted using Plugin Heatmap and bar-plot taxa were visualized using Microsoft Excel 2010.

3. Result and discussion
Based on the field observation in Gunungpati District, the husbandry techniques, cage, and non-cage may contribute to foraging behavior and feed preference in duck. It promoted the differences in feed types, where non-caged ducks have more diverse feed composition compared to caged ducks. The diverse environment during foraging activity makes non-caged ducks obtain various types of nutrition from surroundings. However, husbandry of caged duck is classified to be an intensive care model; it allowed the duck to forage only in the cage area that provides food. Also, the majority of duck farmers both in a cage and non-cage technique in Gunungpati provide additional pellet mixed with bran, rice, and tofu dregs (Table 1). Various feed conditions gave effect to organic material that is digested by ducks. Even so, the caged ducks eat starch sources higher than the non-caged ducks.

### Table 1. Environment conditions and feed of ducks in different husbandry models

| Parameter              | Caged Ducks (CG)               | Non-caged Ducks (NCG)          |
|------------------------|--------------------------------|--------------------------------|
| **Environment**        |                                |                                |
| Mixed farm with other animals | No                            | No                            |
| Watery areas           | Muddy pond inside the cage     | Land, paddy field, and rivers  |
| Water pH               | 7.0                            | 7.0-7.1                        |
| Muddy soil             | Yes                            | Mostly                         |
| Vegetation             | No                             | Grassy                         |
| Light penetration      | Indirect                       | directly exposed               |
| **Feed**               |                                |                                |
| Feed type              | Bran and rice                  | Waste food, mostly rice        |

Ducks farmers are kept duck population in large quantities in more than 100 ducks, was practiced both in the cage and non-cage techniques. Based on climate conditions and habitat characteristics, the two types of ducks were not different because they were still in one area, both geographically and administratively. In intensive cage farming (CG), ducks were kept in a permanent cage with water pond in it. Whereas, in non-caged ducks (NCG), ducks were herded to the paddy fields or river banks. The grazing process allows ducks to consume various types of an organic compound from gravel to, as a source of fiber [22-24]. The craw contents were also revealed that the CG group contained plant roots and left more abundance than NCG.
The duck's claw contents were quite different in both duck, mostly, big gravel and organic material such as wood, roots, and leaves were commonly found in NCG, then smaller and less variation of food in CG. Food composition shows the diversity of material that was richer in the NCG group, because of the foraging area was wider. The composition of gravel and soil in NCG has a smooth surface that characterizing specific gravel from river or watery areas. Unlike in the NCG, the claw content in CG consisted of small-single sized gravel and fewer. The organic material was more refined and unvarying, which probably came from bran or additional feed. Also, high fiber intake is likely affecting duck productivity and metabolism compared to carbohydrates and lipids-riched-pellet. As a result, various kind of feed components possible affects gut microbiome diversity, growth, and quality of duck metabolism [25-26].

3.1. Gut microbiome composition

The metagenomic approach is the most appropriate technique to determine the gut microbiome composition of ducks. This technique can be used to understand various species of gut microbiome that help metabolic processes [27]. Based on the results of metagenomic analysis shown that gut microbiome differed between CG and NCG (Figure 1). Data analysis showed that the gut microbial of CG ducks at the phylum level was dominated by Proteobacteria (69.89%), Firmicutes (21.96%), Actinobacteria (4.02%), TM-7 (1.11%) and Planctomycetes (0.96%). While the NCG ducks, microbial gut ducks were dominated by Proteobacteria phyla (62.18%), Firmicutes (25.77%), Actinobacteria (8.49%), Planctomycetes (1.23%), and TM-7 (0.78%).

![Figure 1. The gut microbiome diversity (Phylum level) in ducks.](image)

At the family level, the gut of CG ducks were dominated by Enterobacteriaceae (72.5%), Lactobacillaceae (7.18%), Enterococccaceae (3.31%), Ruminococcaceae (3.21%) and Coriobacteriaceae (1.47%); whereas gut microbial NCG ducks were dominated by Enterobacteriaceae (65.65%), Coriobacteriaceae (6.17%), Lactobacillaceae (5.48%), Ruminococcaceae (4.37%), and Bacillaceae (3.54%). The most dominant gut bacteria that found in both CG and NCG was Family Enterobacteriaceae, Phylum Proteobacteria and followed by Lactobacillaceae from Phylum Firmicutes. However, the bacterial diversity map in family level was not shown a significant trend
between CG and NCG (Figure 2). It is likely because of similar feed and environmental condition from the two husbandries.

Mostly, the gut microbiome composition has the same bacteria composition, which was dominated by *Proteobacteria* and *Firmicutes*. However, in particular, some bacterial phyla showed specificities in NCG and do not appear in a large percentage of CG, vice versa. The interesting thing found was the small percentage of bacteria families of *Paenibacillaceae*, *Bacillaceae*, and *Gemmataceae* (*Firmicutes*) in CG, but abundant in NCG. Then, a high percentage of *Planctomycetaceae* and *Verrucomicrobiaceae* families in CG, however, not found in NCG (Figure 2). This picture was seen in the heat map diagram.
(Figure 3), where the absence of the bacterial family is performed by black color, and bright colors indicate the abundant bacteria.

Based on the family, *Enterobacteriaceae* was the most abundant bacteria in both NCG and CG, followed by *Lactobacillaceae* and *Coriobacteriaceae*. The three families compose three different types of phyla, respectively, Phylum *Proteobacteria*, *Firmicutes*, and *Actinobacteria*, where the abundant species of both ducks were *Enterococcus sp.*, *Lactobacillus sp.*, *L. pontis*, *Pediococcus sp.*, *Family Ruminococcaceae*, and several bacteria families, which has a fewer percentage. The bacteria found only in NCG were *Colinsella sp.*, *Family Coriobacteriaceae*, *Bacillus sp* and *Bacillus coagulans*, *Aneurinibacillus*, *P. acidilactis*, *Clostridium sp*, *Blautia sp*; whereas bacteria that have percentage less
than 0.05% (deficient) from total bacteria species of gut microbiome were Family *Lacnospiracae*, which only has eight species, and *Gemmata* from Family *Planctomycetaceae*, which has four species.

Duck farming from Gunungpati shows that gut microbiome was dominated by *Proteobacteria* both in NCG and CG. It was possible because of a similar feed intake. The dominant feed is pellets mixed with bran and corn, increasing glucose content. Also, duck farmers added some more composition such as leftover oily food and rice.

There were 851 shared species belongs to NCG and CG; it means that more than 800 same bacteria species build up the gut ecosystem both in NCG and CG. It is probably due to they live in similarity habitat conditions and types of feed, which resulted in the emergence of the same species. The number of bacteria species in the NCG was higher than CG. It indicates that may affect habitat, and foraging area contribute to duck’s gut microbiome (Figure 4).

**Figure 4.** Number of species of bacteria that intersect between different species of treatment patterns

Ducks are commonly kept in the community and the essential source of food production. Ducks have proportionally smaller intestines and shorter food transit times than mammals. It does not mean that water digestion is less efficient. The digestive tract of ducks is a community habitat for complex microbes. The gut microbiome also has a link between diet and health because they play a role in assimilating nutrients from food [28].

Environmental conditions and community strongly influence duck husbandry in Gunungpati. Most of the non-caged duck farmers build their farm near the community but practicing duck herding in the rice-field and rivers. Meanwhile, caged duck is a closed-intensive farm that restricts access out of the cage; it makes fewer divers feed profile. Plant roots and grass leaves were the dominant types of organic material found in NCG. The high level of organic compounds provides a high fiber intake than complex carbohydrates. Recent research reveals that fecal composition depicts host interaction with their environment and gut microbiome [29-31]. It correlates with this research finding that NCG ducks have more abundant gut microbiome than CG. It indicates that interaction with the environment is one of the determinants of the diversity of gut microbiome in ducks [32]. In the other side, feed interventions are also used by farmers to increase duck growth and reduce the risk of enteric pathogenic infections [31, 33].

3.2. *Fatty acid in duck's intestine*

Gut microbiome runs metabolic activities that may provide various beneficial metabolic compound tor duck metabolism. Identification of volatile short-chain fatty acids (SCFA) (Table 2) shows that each duck contains SCFA compounds with various compositions.
Table 2. Compound contents in intestinal samples identified using the GCMS method

| Compound                                      | Levels (%)  |
|-----------------------------------------------|-------------|
|                                               | CG          | NCG         |
| Hexadecanoic acid/ Palmitic acid              | 53.62       | 28.85       |
| 9,12-Octadecadienoic acid (Z,Z)/ Linoleic acid| 46.38       | -           |
| Oleic Acid                                    | -           | 71.15       |
| trans-13-Octadecenoic acid                    | -           | -           |
| 9-Octadecenoic acid (Z), methyl ester/ Methyl Oleate | -           | -           |

SCFA compounds that can be found both in NCG and CG were palmitic acid or hexadecanoic acid with an average composition of 53.62% at CG and 28.85% at NCG. Oleic acid compounds which were unsaturated fatty acids are found in NCG, while 9-Octadecenoic acid is found in CG.

The gut microbiome plays an important role in the production of essential vitamins, essential amino acid and short-chain fatty acids (SCFA), prevent infection and provides selective habitat for pathogenic bacteria and or antibiotic resistance viruses. The type and amount of SCFA produced by gut microbiome are determined by total consumed fiber and diversity of the intestinal microbiota. Diet intervention change the intestinal microbiota, and these changes can contribute to the host metabolic phenotype. Fiber fermentation is the main mechanism for producing important energy for some members of the gut microbiome; For example, high fructans consumption increases Bacteroidetes colonies. However, from metagenome analysis, Bacteroides composition was very small compared to other bacteria family. It indicates that fiber consumption in duck is lower than starch or other organic components [34-35].

Firmicutes diversity is associated with gut microbiome in adult birds [36], which actively expresses enzymes to produce SCFA from organic compound. From previous research, shown that variant species of genus Blautia tend to produce propionate-producing enzymes compared to butyrate and other SCFA compounds [37]. Firmicutes also express L-fucose isomerase, which composes mucins in the digestive tract [38] and acts as a gut microbiome adhesion receptor [39]. Thus, the high diversity of Firmicutes correlates with the composition and survival of other gut microorganisms. Phylum Firmicutes and Bacteroides (low amounts of both NCG and CG) have amylolytic and cellulase activity [40]. Therefore, low fiber feeding tends to reduce the composition of bacteria.

The maintenance pattern of duck farms in Gunungpati tends to be intended for fattening and eggs production. So, it may explain that the ducks eat more carbohydrate and lipids, such as rice and rice, leftover oily food. However, organic material consumed by loose livestock ducks is likely to be an additional supply of fiber. Fiber is a non-starch component of polysaccharide (NSP) as the main material that is digested or fermented by the gut microbiome. Breaking product fermentation, NSP produces short-chain volatile fatty acids (SCFA), which are absorbed by the mucosa and are catabolized. In some experimental animals, consumption of high-fiber foods showed a significant increase in body size [25].

In particular, butyrate has proven able to reduce Salmonella infection and decay of epithelial cells. Also, SCFA is an energy substrate for metabolism in the colon (butyrate) epithelial and peripheral tissue (acetate and propionate) [41], and acetate and propionate are metabolized by the liver and used as a substrate for lipogenesis and gluconeogenesis. The hydrogen molecule produced during the butyrate fermentation process is a strong free radical that able to destroy epithelial cell wall. In host intestine, the negative effect of H+ eradicates by free H+-fixing bacteria. Although the symbiotic relationship of bacteria is very profitable to prevent pathogenic infections and viruses, environment, husbandry technique, and feed can affect this ability [42].

In general, the four main bacterial phyla most often associated with animal intestinal systems are Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria [29]. However, samples obtained from farms in Gunungpati show that Bacteroidetes was a deficient proportion of gut microbiome in both CG and NCG. High gut microbiome communities Firmicutes and Proteobacteria play a role in increasing poultry weight caused by increased fat accumulation in adipose cells [43-44].
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