

Effects of production system on the gut microbiota diversity and IgA distribution of Kampung chickens, Indonesia

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Abstract. *Susanti R, Christijanti W. 2022. Effects of production system on the gut microbiota diversity and IgA distribution of Kampung chickens. Biodiversitas 23: 1082-1090.* Native chickens (Kampung chickens) are poultry in Indonesia that is raised using a traditional production system. This study aimed to analyze the differences of the Kampung chicken production system (extensive/E and semi-intensive/SI) on the diversity and abundance of gut microbiota and the distribution of IgA. Each sample of Kampung chickens was slaughtered, then their thoracic and abdominal cavities were dissected, and their intestines were collected. Furthermore, the intestinal tissue was processed to make histological preparations for immunohistochemical analysis of IgA. Gut contents of 25 g were taken aseptically and used for next-generation sequencing (NGS) and GC-MS analysis. The results showed 12 bacterial phyla in the intestines of SI and E chicken. E chickens had a higher abundance and diversity of microbiota than the SI chickens. The phylum Firmicutes dominated the E and SI chickens' gut microbiota (>50%). SI chickens have a higher Firmicutes/Bacteroidetes ratio than the E chickens. The SIgA distribution showed an IRS score of 4 (moderate), both in E and SI chickens. It was concluded that the production system affects the intestinal microbiota's abundance and diversity but not on the intestine IgA of Kampung Chickens. This study highlights that based on the Firmicutes/Bacteroidetes ratio, the semi-intensive system is more suitable for Kampung chicken meat and eggs than the extensive system.

Keywords: Gut microbiota, IgA, kampung chickens, production system

INTRODUCTION

The domestic chicken, *Gallus gallus domesticus* is a model organism which commonly used to study human biology (Oakley et al. 2014). Kampung chickens are domestic chicken raised using the traditional production system by Indonesians. Commonly, there are three types of native chicken rearing systems in Indonesia: the traditional extensive system, semi-intensive system, and intensive system. In the traditional extensive system, all chicken activities are carried out naturally. This traditional system is the most popular in Indonesia because most farmers do not have capital or access to financial institutions, so they cannot afford to buy feed, supplements, or medicines. This system is considered unsuitable for elevating the productivity of native chickens because it is difficult to manage their feed consumption (FAO 2008). And domestic chickens that are released to find their own food will be vulnerable to ectoparasite attacks (Riwidharso et al. 2020).

Semi-intensive system is considered more effective than extensive system. In this system, Kampung chickens are placed in an open area bordered by a fence, which is mostly developed in the courtyard or backyard of the farmer's house. The farmer routinely provides feed and water for drinking but does not carry out daily medical care, and no technology is applied. Kampung chickens are usually raised for non-commercial purposes, only for sale when there is an urgent need. In the intensive system, chickens are managed more professionally. Chickens are

kept in cages and provided with feed, water, supplements, and regular medical applications. The chicken population is also separated by age. Chicken production is generally for profit-oriented objectives which is a completely commercial purpose.

Public concern about the negative impact of poultry meat and egg products is very high, especially on food safety. Animal health is a prerequisite for high productivity and to produce a product that is safe for human consumption. Poultry health is determined by a fully functional immune system. Various stressors can have a negative influence on the immune system. Environmental factors such as level of biosecurity, type of cage, access to feed and climate also influence the conformation of the intestinal microbiota. The intestinal microbiota is a significant and complex ecosystem for the chicken host. The abundance and types of gut microbiota can increase the productivity of chickens (Xu et al. 2016). The variety and abundance of intestinal microorganisms provide important information regarding metabolism, nutrient absorption (Khosravi and Mazmanian 2013), immune system (Thaiss et al. 2016a), and the body weight (Thaiss et al. 2016b). The interaction between the components of the microbiota causes bacteria to generate bacteriostatic or bactericidal substances for competition between bacteria. The interactivity of the microbiota and the host's innate immune system will trigger an adaptive immune response. The immunoglobulin (Ig)A response directly determines the composition of commensal bacteria that colonize the

intestine, but the production of IgA requires energy from metabolic resources (Penny et al. 2021). Therefore, there is an interplay between the composition of the microbiota, immunoglobulin A, and diet.

The production system greatly affects the conformation and diversity of the gut microbiota, especially in coprophagic animals such as mice (McCafferty et al. 2013; Laukens et al. 2016) and chickens. Particles around chickens and feathers that are contaminated with intestinal microbiota (in feces), if eaten by chickens, will influence the composition of their gut microbiota (Meyer et al. 2012; Zhao et al. 2013; Org et al. 2015). Dagu Chickens reared in cages indicated a decreased portion of Bacteroidetes in the caecum, and a bigger ratio of Firmicutes/Bacteroidetes than those reared cage-free. The microbiota in the caecum of cage-free Dagu chickens has a bigger abundance of organisms embroiled in the metabolism of amino acids and glycans (Xu et al. 2016). The gut bacterial community in cage-free geese is dominated by Phylum TM7 (*Saccharibacteria* candidate) (>50%), followed by Firmicutes and Bacteroidetes. Meanwhile, geese kept in cages are dominated by Firmicutes (close to 50%), followed by TM7 and Actinobacteria (Susanti et al. 2020).

Kampong chickens have less meat and are redder in color than the broilers. Kampong chicken contains more protein and has a better water holding capacity (Mikulski et al. 2011; Chen et al. 2013). The price of Kampong chicken meat is also more expensive than broiler chickens. Moreover, people choose the Kampong chicken meat and eggs because they are believed to have high nutritional contents and many essential amino acids and have a chewy texture and not fatty. This is one of the causes of the high demand for native chickens. Based on research by Fanatico et al. (2007), outdoor-reared chicken meat has more protein than indoor-reared chicken.

The health of Kampong chickens is one of the prerequisites for the safety of meat and egg products for consumption. The health of chickens is determined by the production system. Kampong chicken production system that many people use are the extensive and the semi-intensive system. This research aimed to analyze the distinction in the diversity and abundance of the gut microbiota as well as the distribution of intestinal IgA of Kampong chickens reared in the extensive and semi-intensive systems.

MATERIALS AND METHODS

Study design and sample

This research is an exploratory observational study to investigate the abundance and variety of gut microbiota and the distribution of intestinal IgA of Kampong chickens reared with extensive (E) and semi-intensive (SI) production systems. Kampong chicken samples were obtained from local community farms in Gunungpati District, Semarang City, Indonesia with extensive and semi-intensive production systems. E Kampong chickens' sample in this research are live freely around the farmer house to find food, carry out reproduction, play with other

birds, take care of their young, and do other activities. Kampong chickens grow and develop without farmer intervention, no special feed is provided, no cages are built, no health management is implemented, and no technology is applied. In this research, SI Kampong chickens' sample is placed in an open area bordered by a fence, which is mostly developed in the courtyard or backyard of the farmer's house. The farmer routinely provides feed and water for drinking but does not carry out daily medical care, and no technology is applied.

Samples were obtained purposively with inclusion sampling criteria as follows: (i) Kampong chickens that were raised extensively and semi-intensively, (ii) males or females chicken aged at least three months, (iii) chicken did not receive any feed or drugs containing antibiotics within 2 weeks. Samples were excluded from the research sample if it was found that the chickens were laying eggs.

Sample preparation

Each sample of Kampong chicken was slaughtered following the animal welfare procedures. The thoracic and abdominal cavities were dissected, and the intestinal organs were removed. Twenty-five grams of gut contents were taken aseptically, collected in a microtube, and stored at -20°C until NGS (Next-Generation Sequencing) and GC-MS examination was carried out. The intestinal organs (small and large intestine) were cleaned with sterile distilled water, and put in 10% formalin (in PBS), then histological preparations were made to identify IgA by immunohistochemical staining.

Next-Generation Sequencing (NGS) analysis

The intestinal microbial genome was extracted from samples of intestinal contents using the QIAamp DNA Stool Mini Kit (Qiagen, San Diego, California, US) following the manufacturer's instructions. The diversity and abundance of gut microbiota were analyzed was carried out based on the 16S rRNA gene marker region V3-V4 (Yarza et al. 2014). The DNA amplification operation used the Illumina HiSeq 2500 platform for 20 cycles according to a study by Holm et al. (2019). The primers used were 338F-forward primer (5'-GGACTACHVGGGTWTCTAAT-3') and 806R-reverse primer (5'-GGACTACHVGGGTWTCTAAT-3') (Holm et al. 2019), which bind to the barcodes, i.e., a sequence of eight specific bases in each sample.

Metagenomic analysis

Metagenomic analysis of 16S intestinal microbiota was performed using QIIME2 (Ver. 2019.4) (Caporaso et al. 2010). The paired end files were demultiplexed using the demux plugin. Quality control in each sample was conducted using the DADA2 plugin (Callahan et al. 2016). Furthermore, the diversity index value was obtained by using six diversity indexes, i.e., Shannon (Shannon and Weaver 1949), Simpson (Simpson 1949), Pielou's evenness (Pielou 1966), Margalef (Magurran 2004), Chao1 (Chao 1984) and observed OUT's (DeSantis et al. 2006). The taxonomy was compiled based on the Greengenes-13_8 99% OTU database (McDonald et al. 2012), heatmap

compilation (Hunter 2007) using the heatmap plugin, and taxa barplot preparation using Microsoft Excel 2010.

GC-MS analysis

GC-MS analysis was performed to predict the short chain fatty acids (SCFA) produced by bacteria. GC-MS analysis was carried out according to methods describe earlier by Susanti et al. (2020).

Detection and staining of SIgA by immunohistochemistry

The histological preparations of the small intestine and colon of native chickens in this study were carried out according to the procedure by Susanti et al. (2021). Each intestinal tissue was observed three times in a different field of view. The observed IgA was then quantified or scored based on the percentage of positive cells and the intensity of the staining, as shown in Table 1. The immunoreactive score (IRS) was counted out as the result of the multiplication of the positive cell proportion score (0-4) and the staining intensity score (0-4). IRS scores ranged from 0-12 with categories as used in the study by Fedchenko and Reifenrath (2014) and Susanti et al. (2021).

RESULTS AND DISCUSSION

Research on chicken intestinal microbiota can inform metabolic conditions, nutrient absorption, and immune function of the host. Compilation and comparison of microbiota metagenomics, metabolomics, and immunity is an effort to find formulas and markers of intestinal microbiota stability. This study focus was to investigate the microbiota metagenomics, metabolomics, and intestinal IgA of Kampong chickens reared in extensive and semi-intensive systems. This study results can be used as a basis for determining the production system, engineering the diet, and revealing potential antibacterial and antiviral compounds produced by each microorganism to improve the growth, productivity, and immunity of Kampong chickens.

The results of metagenomic analysis showed differences in the variety and abundance of intestinal bacteria in Kampong chickens reared with semi-intensive (SI) and extensive (E) systems. The diversity index value showed that the E chickens have a higher diversity than SI

chickens. All parameters of the diversity index stated that the E chickens have a higher diversity of intestinal bacteria than SI chickens (Table 1). Chickens in cages may experience stress due to limited access to space and feed, thus decreasing the variety of the gut microbiota. The diversity and abundance of gut microbiota is influenced by host and ecological constituents. Environmental factors as well as the level of biosafety, cages, litter, access to feed and climate also influence the conformation of the intestinal microbiota (Kers et al. 2018). Based on Bailey et al. (2010) research, mice that experience stress in the long term show a decrease in gut microbiota diversity.

Chicken intestines have a smaller size and shorter transit times than mammals (Stanley et al. 2015). The digestive system of chickens is adapted to form energy from food sources that are difficult to digest. This occurs because the digestive tract contains microbiota that supports the relationship between diet and health (Sergeant et al. 2014). Without microbial fermentation, chickens cannot digest and utilize complex polysaccharide substances in feed ingredients (Vrieze al. 2010). The gut microbiota of poultry is one of the ecosystems with the highest cell density, which is 10^7 to 10^{11} bacteria per gram of intestinal contents (Apajalahti 2004).

Bacteria in the intestines of SI and E chickens each consisting of 12 phyla. The abundance of the intestinal microbiota of the E chickens reached 24,220 Operational Taxonomic Unites (OTU), while the SI chickens reached 16,141 OTU. Intestinal bacterial density showed the presence of several OTUs in E chicken which had a darker color than SI chicken (Figure 1). This indicated that some bacteria were abundant in E chicken but lower in SI chicken. The relative composition of each bacterial phylum is shown in Figure 2. In the E Kampong chickens, it was dominated by phylum Firmicutes (55.48%), Actinobacteria (15.24%), and Bacteroidetes (14.18%). The other six phyla (Proteobacteria, Planctomycetes, Verrucomicrobia, Chloroflexi, k-Bacteria [k: kingdom] and Cyanobacteria) were in the range of 1.05-4.17%, while the other three phyla (Euryarchaeota, Synergistetes, TM7/Saccharibacteria) were only <1%. In the SI Kampong chickens, it was dominated by phylum Firmicutes (56.12%), Verrucomicrobia (12.29%), Actinobacteria (12.22%), and Bacteroidetes (11.73%). The other eight phyla (Cyanobacteria, Proteobacteria, k-Bacteria, Planctomycetes, Synergistetes, TM7, Euryarchaeota, and Chloroflexi) were in the range of 2.88-0.09%.

Table 1. Index diversity of gut microbiota of Kampong chickens with extensive (E) and semi-intensive (SI) production systems

Kampong chickens	OTU	Index diversity				
		Shannon	Simpson	Margalef	Pielou's evenness	Chao1
Extensive (E)	203	6.19	0.97	19.90	0.81	203.3
Semi-intensive (SI)	120	5.29	0.95	12.27	0.77	120.0



Figure 1. Heatmap diagram showing the abundance of intestinal bacteria in E and SI chickens

The results of this research indicated that the conformation of Bacteroidetes in both E and SI chickens was small (<20%). The ratio of Firmicutes/Bacteroidetes in E chickens was 3.91 and in SI chickens was 4.78. Previous studies indicated that adding a lot of fiber to the diet has the potential to elevate the amount of Bacteroidetes and reduce the Firmicutes/Bacteroidetes ratio (Parnell 2012; Trompette et al. 2014). The ratio of Firmicutes/Bacteroidetes in E chickens is smaller than SI chickens, this is probably due to the differences in the access to an environment that provides abundant food sources so that the opportunities to consume fiber-containing feed ingredients are also different.

A high Firmicutes/Bacteroidetes ratio increases chicken body weight, as more energy is absorbed (Turnbaugh et al. 2006). In chicken production, the bacteria associated with productivity are from phylum Firmicutes, along with Bacteroidetes and Proteobacteria (Torok et al. 2011). In adult chickens which are in the period of egg production, the microbiota in the caecum is dominated by the phylum Bacteroidetes and Firmicutes with a constant ratio (Videnska et al. 2014). An elevation in the number of Firmicutes in the intestinal microbiota is correlated with an increase in nutrient absorption, whereas an elevation in Bacteroidetes is correlated with a decrease in nutrient absorption (Jumpertz et al. 2011). SI chickens have a higher Firmicutes/Bacteroidetes ratio than E chickens, so that the semi-intensive (SI) method is more suitable for chicken meat and egg production purposes, compared to the extensive method. The conformation and abundance of the intestinal microbiota determine the metabolic function of the host. According to the study of Xu et al. (2016), the abundance of cecal microbiota of freely reared chickens is largely involved in the function of the amino acids and glycan metabolic pathway.

At the family level, the intestinal microbiota of SI and E chickens were dominated by Ruminococcaceae, o-Clostridiales, and Lachnospiraceae families, although with different proportions. The intestinal bacteria of E chickens were dominated by the Ruminococcaceae family (27.87%), followed by Coriobacteriaceae (13.38%), Clostridiales (13.21%), and Lachnospiraceae (11.09%) (Figure 3). The other twelve families were in the range of 1.15-5.85%. Intestinal microbiota in SI chickens was dominated by Ruminococcaceae (16.93%), Clostridiales (16.19%), Coriobacteriaceae (13.69%), Verrucomicrobiaceae (12.95%), and Lachnospiraceae (12.21%), while the other ten families were in the range of 1-4.5%, and only 1 family (Gemmataceae) is in the range <1.00. Overall, the amount of microbiota at the family level was higher in E chickens (18,001 OTU) compared to SI chickens (14,219 OTU).

Ruminococcaceae is the main butyrate producer. Butyrate is produced by Ruminococcaceae through the conversion of two molecules of acetyl-CoA to crotonyl-CoA (Eeckhau et al. 2016; Esquivel-Elizondo et al. 2017; Vital et al. 2017; Medvecky et al. 2018). Butyrate can also be produced by Genus *Flavonifractor* and *Pseudoflavonifractor* through succinate reduction or lysine fermentation. The genus *Pseudoflavonifractor* and *Anaerotruncus* are part of the motile gut colony. The

Lachnospiraceae and Ruminococcaceae families are very susceptible to oxygen, so that these two families cannot be observed from the gut microbiota during inflammatory disease in consequence of the formation of reactive oxygen species by macrophages and granulocytes (Thiennimitr et al. 2011). The dominance of Ruminococcaceae in chicken gut microbiota in this study, both in E and SI chickens showed that the intestinal chicken was normal, there was no inflammatory disease.

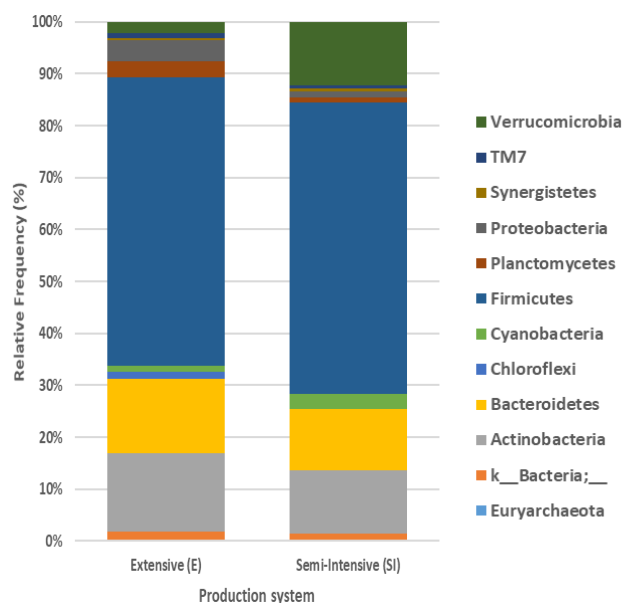


Figure 2. The gut microbiota diversity (Phylum level) in Kampong chickens with extensive (E) and semi-intensive (SI) production systems

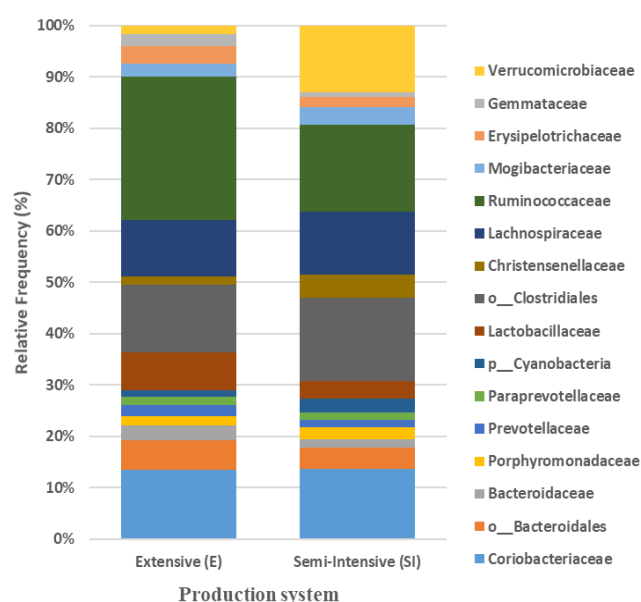


Figure 3. The gut microbiota diversity (Family level) in Kampong chickens with extensive (E) and semi-intensive (SI) production systems

Immunohistochemical staining of IgA in the intestine

The intestinal microbiota contributes to modulate various host physiological activities, including immunity (Levy et al. 2017). Intestinal bacterial species and their composition are controlled by IgA. Changes in the IgA reaction induce a decrease in the whole bacterial diversity. Secretory IgA (SIgA) in the intestine increases bacterial displacement to lymphoid tissue to promote antigen presentation (Palm et al. 2014; Fransen et al. 2015; Kubinak and Round 2016). SIgA performs a significant role in immune regulation and in defense system against microorganisms in the gut. The SIgA population is the largest immunoglobulin class in the intestinal mucosa.

All the intestinal tissue of Kampong chickens in this study showed normal tissue conditions. IgA is indicated by a brown color in the cytoplasm of the cell. It was observed that IgA was detected in the large and small intestine of chickens, as illustrated in Figure 3. The distribution and

amount of IgA in the large intestine were almost similar to those in the small intestine. IgA dispersed in the lamina propria, and most of the cells are concentrated around the intestinal crypts (Figure 3). SIgA is produced by plasma cells in the lamina propria section of the intestine, then this SIgA passes through the epithelium to the intestinal lumen.

Immunohistochemical analysis of the intestinal IgA of the E chickens showed an average proportion of positive cells by 19.67% (score = 2) and 14% (score = 2) in the large and small intestines, respectively. Meanwhile, the SI chicken showed an average proportion of positive cells by 29% (score = 2) and 20% (score = 2) in the large and small intestines, respectively. Both of the small and large intestines of the E and SI chickens showed moderate staining intensity (score = 2). The IRS score (multiplication of the percentage of positive cells and intensity of staining) of the small and large intestine was 4 (moderate) (Table 2).

Table 2. Immunoreactive score (IRS) of SIgA in intestinal chickens with immunohistochemical staining

Production system of Kampong chickens	Intestine	Intensity of staining	Average proportion of positive cells (%)	IRS
Extensive	Small intestine	2 (moderate)	14.00 (Score: 2)	4 (moderate)
	Large intestine	2 (moderate)	19.67 (Score: 2)	4 (moderate)
Semi-intensive	Small intestine	2 (moderate)	20.00 (Score: 2)	4 (moderate)
	Large intestine	2 (moderate)	29.00 (Score: 2)	4 (moderate)

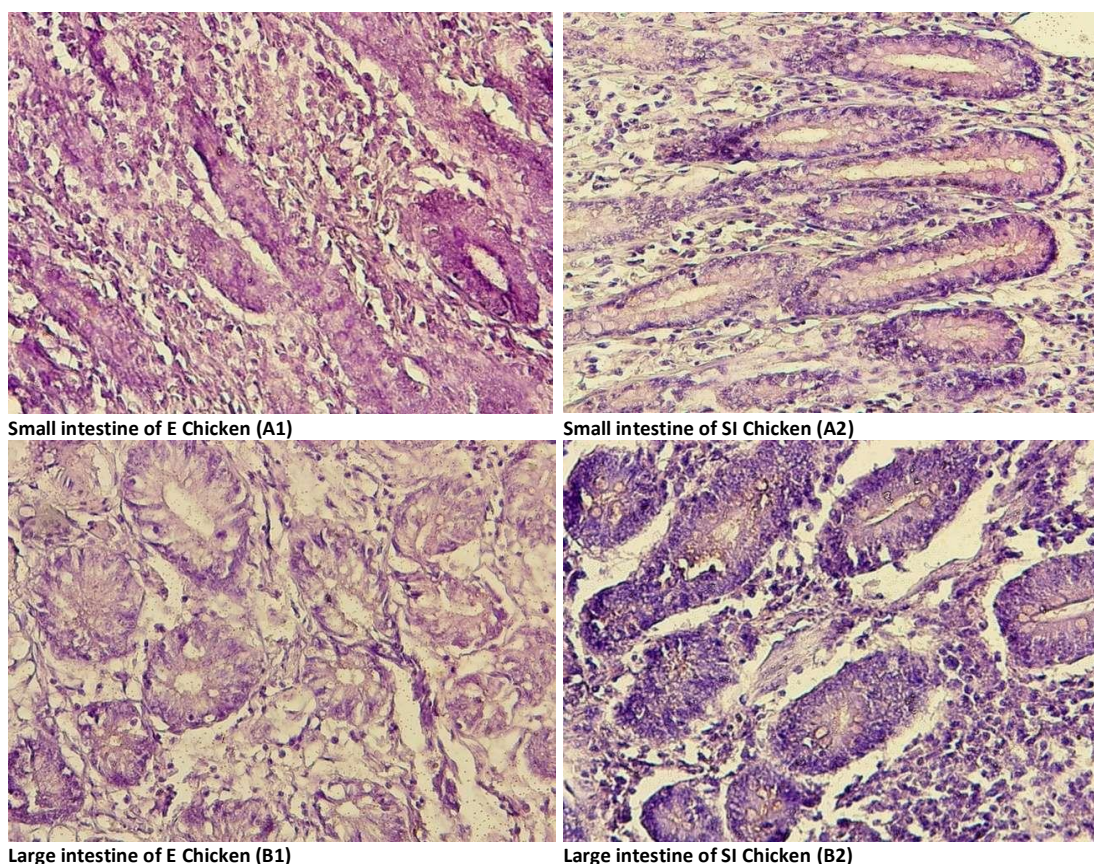


Figure 3. Immunohistochemical staining of SIgA in the small intestine (A) and large intestine (B) of Kampong chickens reared using an extensive (E) system (A1 and B1) and semi-intensive (SI) system (A2 and B2). The IgA dispersed in the large and small intestinal lamina propria, and some cells concentrated around the crypts (magnification: 400×)

Table 3. Compound contents in intestinal samples identified using the GCMS method

Sample	RT	Height	Area	% Area	Components
Extensive chickens					
E1	8.281	2.32E+08	15620809	26.42%	n-Hexadecanoic acid (Palmitic acid)
E2	9.091	3.81E+08	43502852	73.58%	9,12 Octadecadienoic acid (Z, Z)- (Linoleic acid)
Semi-intensive chickens					
SI1	8.346	1.3E+08	34427508	61.35%	n-Hexadecanoic acid (Palmitic acid)
SI2	9.136	1.5E+08	21687776	38.65%	9,12 Octadecadienoic acid (Z, Z)- (Linoleic acid)

The intestinal microbiota of Kampong chickens in this study was dominated by the phylum Firmicutes (55.48% in the E group and 56.12% in the SI group). Phylum Bacteroidetes in E and SI chickens were 14.18% and 11.73%, respectively (Figure 1). Previous research revealed that members of the phylum Firmicutes were the main producers of butyrate, while the phylum Bacteroidetes produced mostly acetate and propionate (Flint et al. 2015; Levy et al. 2016). Butyrate, propionate, and acetate are members of the SCFA or short-chain fatty acids groups (fatty acids with 1 to 6 carbon), which are volatile, and are resulted by bacteria in the large intestine through the fermentation process of undigested polysaccharides. The results of GCMS analysis showed that the intestines of Kampong E and SI chickens contained n-Hexadecanoic acid and 9,12 Octadecadienoic acid (Z, Z) - but with different proportions (Table 3). In E Kampong chickens, the proportion of 9,12 Octadecadienoic acid (Z, Z) - (73.58%) was more dominant than n-Hexadecanoic acid (26.42%). In contrast to SI Kampong chickens, the proportion of n-Hexadecanoic acid (61.35%) was more dominant than 9,12 Octadecadienoic acid (Z, Z) - (38.65%). The SCFAs produced by the chicken intestinal microbiota in this study, in particular by the phylum Firmicutes and Bacteroidetes, may be involved in the stimulation of SIgA production in the intestines. This is based on the results of research by Kim et al. (2016) which revealed that SCFA can modulate B cell gene expression through the prevention of histone deacetylase to increase antibody (SIgA) release. SCFA is capable of modulating metabolic sensors to increase mitochondrial energy production and increase B cell activation and differentiation and antibody production (Caromaldonado et al. 2014).

In conclusion, Kampong chickens reared using an extensive system had a higher abundance and diversity of microbiota than the semi-intensive system. The phylum Firmicutes dominated both extensive and semi-intensive chickens' gut microbiota (>50%). Semi-intensively reared chickens have a higher Firmicutes/Bacteroidetes ratio than extensively reared chickens, so that the semi-intensive system is more suitable for the purposes of chicken meat and egg production, compared to the extensive system. The SIgA distribution showed an IRS score of 4 (moderate), both in extensive and semi-intensive chickens. The SCFAs produced by the chicken intestinal microbiota in this study, particularly by the phylum Firmicutes and Bacteroidetes, may be involved in stimulating SIgA production in the intestine.

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