Immunohistochemical distribution of Immunoglobulin-A in relation to the intestinal microbiota of *Cairina moschata* (Muscovy) duck

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Abstract. The intestinal mucosal was a physical barrier of host defense against foreign pathogens. This research was conducted to elaborate the distribution of IgA and its relation to the abundance of muscovy duck intestinal microbes. The muscovy duck samples were obtained from local community farms in Gunungpati Subdistrict, Semarang City, which were maintained in the barn (non-cage). Each muscovy duck sample was slaughtered and dissected the chest cavity to the abdomen and then taken the intestinal organs. A total of 5g of intestinal contents was taken aseptically and used for NGS analysis. Furthermore, intestinal tissue was made into histology slides for immunohistochemical IgA analysis. The results of the immunohistochemical analysis showed that the IRS score of the small and large intestine were 4 (moderate). Muscovy duck in this study was healthy or normal, so the IRS score was in position 4 (moderate). Intestinal bacteria were dominated by Firmicutes phyla (48.71%), followed by Proteobacteria (32.87%) and Actinobacteria (8.32%). At the ordo level, bacterial composition was dominated by the ordo Enterobacteriales (32.08%), Clostridiales (21.04%), Bacillales (14.84%) and Lactobacillales (13.41%). In this intestinal muscovy duck, there was an equilibrium of microbiota components and there was no exogenous microorganisms that stimulate the overexpression of IgA production.

1. Introduction
The intestinal mucosal surface was a physical barrier of host defense against foreign pathogens [1]. The intestinal mucosal was also involved in the mechanism of tolerance to commensal microbes or harmless food antigens. The intestinal mucosal structure was very thin and permeable. It’s related to the physiological function of the intestine in the food absorption process. Although the intestinal mucosa has a thin and permeable structure, it has resistance to infection by pathogens. In fact, most pathogenic agents enter the host body through the intestinal tract. The ability of the intestinal mucosa to maintain a balance of immunity between commensal microorganisms and defense against pathogens, due to the role of sIgA [2]. The results of the study of Pabst et al. [3] showed that commensal microorganisms were able to induce sIgA secretion. The sIgA has a dual function to maintaining homeostasis in the mucosal site, i.e. immune response to pathogens and induction of tolerance to harmless food antigens or commensal bacteria.

Immunoglobulin A (IgA) was the most commonly found in the mucosa of the digestive tract, respiratory tract, vaginal tract, tears, saliva, and colostrum. Mucosal IgA was a secretory immunoglobulin. It’s structure was arranged of two monomeric IgA, J-chain (as IgA secretory) and...
secretory component (SC) [4]. In the upper respiratory tract, there are also trimeric slgA, tetrameric slgA, and greater IgA polymer. As a class of primary antibodies in several extrinsic secretions, slgA has a distinctive structure and function. The ability of slgA to eliminate pathogens is not only through non-specific immunity [2]. IgA also plays a significant part in the adaptive immunity caused by pathogens [5,6]. One characteristic of the mucosal immune system was microbial colonization [7]. The role of IgA in microbial colonization in the intestinal mucosa was very unique. IgA acts to bind and 'coat' commensal bacteria in the intestine, but but at the same time, the IgA layer forms the composition of the intestinal microbiota [8]. Although T-independent and T-dependent immune reaction are also embroiled in the lining of commensal bacteria, the mechanism is different from slgA [9]. The formation of intestinal microbiota by IgA was done by limiting the growth of pathogenic organisms, as well as promoting colonization or expansion of certain species [8,10].

Muscovy duck was a waterfowl that acts as a reservoir for the avian influenza virus. The role of this muscovy duck was played by the immunity of the intestinal mucosa. This research was conducted to elaborate the distribution of IgA and its relation to the abundance and types of *Cairina moschata* (muscovy) duck intestinal microbes.

2. Methods

This research was an observational exploratory study to analyze the distribution of IgA and its relation to the abundance and composition of healthy muscovy duck intestinal microbiota. Muscovy duck samples were acquired from local community farms in Gunungpati Subdistrict, Semarang City which were maintained in the barn (non-cage). Muscovy duck samples were acquired purposively with inclusion criteria, i.e. 1) healthy muscovy duck, 2) males or females aged at least three months, 3) did not receive feed or drugs containing antibiotics within 2 weeks. Samples were excluded from the study if known to be laying eggs.

Each muscovy duck sample was slaughtered and dissected the chest cavity to the abdomen and then taken the intestinal organs. A total of 5g of intestinal contents was taken aseptically, collected in a microtube, and stored frozen at -20°C until testing for NGS analysis. The intestinal organs are cleaned with sterile aquades and put in 10% formalin (in PBS). Furthermore, intestinal tissue was made into histology slides for immunohistochemical IgA analysis.

2.1. Metagenomic analysis

Microbial DNA was isolated from the intestinal contents of each muscovy duck, followed by amplification of the 16S rRNA gene in the V3-V4 region. Furthermore, the amplicon was sequenced by the Next-Generation Sequencing (NGS) method: metagenomic. Microbial DNA was interested from intestinal contents samples using the QIAamp DNA Stool Mini Kit (Qiagen, San Diego, California, US) corresponding to the producer's procedure. The isolated DNA was stored in the -20°C freezer before being used for more distant analysis. DNA samples were amplified in the 16S rRNA region V3-V4 gene [11,12]. PCR amplification consisted of an initial denaturation for 3 min at 94°C, followed by 27 cycles of 30 sec denaturation at 95°C, annealing for 30 sec at 55°C and extension for 45 sec at 72°C. The final extension step was carried out at 72°C for 10 min. The primers used were 338F (5′-GGACTACHVGGGTWTCTAAT-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) [11]. Reaction of PCR was undertaken with a overall volume of 20 mL containing 0.5 μL (5 U / μL) Easy Taq DNA polymerase, 2 μL 10 × Easy Taq buffer, 2 μL 0.25 mmol/L dNTPs, 0.2 μmol/L primer, 10 ng DNA template, and ddH2O to 20 μL.

The 16S metagenomic exploration was performed using QIIME2 (Ver. 2019.4) [13]. Paired-end files were demultiplexed using the demux plugin. Quality control in each sample was analyzed using the Dada2 plugin [14], and the diversity index value was generated using 6 diversity indices namely Shannon [15], Simpson [16], Pielou evenness [17], Margalef [18], Chao1 [19], and Observed OTUS [20]. Barplot taxa preparation was done using Microsoft Excel 2010.

2.2. Immunohistochemical staining of IgA in the intestine
Histological samples were obtained from the small and large intestine of muscovy duck. Before the intestinal organ sample was sectioned, it was fixed in a 4% neutral paraformaldehyde solution for more than 15 days. Paraffin-embedded parts were made using standard methods and were immunohistochemically examined. Paraffin-embedded intestinal sections of muscovy duck were deparaffinized, blocking endogenous enzyme, antigen retrieval (heat-induced epitopes retrieval), blocking background staining, and stained with goat anti-chicken IgA antibody HRP conjugated (A.30-103P; Bethyl laboratories Inc) by sequential incubation. The working concentration optimal of these antibodies was 1:500. The slides were rinsed three times with PBS for 5 min. The slides were added DAB (diaminobenzidine) as a chromogen substrate, and incubated for 3-10 min. The slides were rinsed with aquades, and each section was counterstained with hematoxylin. After mounting, observations were made for the presence of brown stain antigen (IgA).

Each intestinal tissue was observed 3 times in a different field of view. The observations were calculated as a percentage of positive cells and intensity of staining. The immunoreactive score (IRS) provides a range of 0-12, as a result of multiplication between the positive cells score (0-4) and staining intensity score (0-3). The IRS and its category were determined according to Fedchenco & Reifenrath [21] as in Table 1.

Table 1. The percentage of positive cell, intensity of staining and immunoreactive score

| A (percentage of positive cells) | B (intensity of staining) | IRS score (multiplication of A and B) |
|---------------------------------|--------------------------|-------------------------------------|
| 0 = no positive cells           | 0 = no color reaction    | 0-1 = negative                      |
| 1 = <10% of positive cells      | 1 = mild reaction        | 2-3 = mild                          |
| 2 = 10-50% positive cells       | 2 = moderate reaction    | 4-8 = moderate                      |
| 3 = 51-80% positive cells       | 3 = intense reaction     | 9-12 = strongly positive            |
| 4 = >80% positive cells         |                          |                                     |

3. Results and Discussion

3.1. Immunohistochemical staining of IgA in the small intestine

Immunohistochemistry (IHC) is a routine method for detecting the presence and location of proteins and other antigens in tissue sections, using antibodies. Antigen-antibody interactions were visualized by the chromogenic method or fluorescent method. The concept of the immunohistochemical method used in this study was direct immunohistochemistry, using a primary antibody (goat anti-chicken IgA antibody) labeled with an enzyme reporter (HRP). This primary antibody will bind directly to the target antigen. In this study, the IgA as a target antigen. The IgA which binds to the goat anti-chicken IgA antibody, visualized with chromogen 3,3’-diaminobenzidine (DAB). This chromogen was a substrate of horseradish peroxidase (HRP) which was used for detection of HRP conjugated primary antibodies. DAB was the most sensitive chromogen that produces dark brown color.

All intestinal organs in this study described histologically normal conditions. The immune- labelled IgA revealed a brown cytoplasmic staining. This result showed that the IgA was detected in small intestine and large intestine of muscovy duck, as illustrated in Figure 1. The distributions of IgA in small intestine were similar in the large intestine. IgA were dispersed in the lamina propria, and most of the cells concentrated around the intestinal crypts (Figure 1).

The results of the immunohistochemical analysis showed that the average proportion of positive cells was 36.6% (score = 2) and 14% (score = 2) in the small intestine and large intestine, respectively. In the small intestine and large intestine, both of them showed the intensity of staining was moderate (score = 2). IRS score (multiplication between the percentage of positive cells and intensity of staining) of the small and large intestine were 4 (moderate). IgA production increases if there is an infection of a pathogen in the intestinal tract. *Salmonella enteritidis* infection in broiler chickens can lead to increased of IgA production. [22]. Deficiency of methionine also significantly reduces the amount of IgA duodenal
of broiler chickens [23]. Muscovy duck in this study was healthy or normal, so the IRS score was in position 4 (moderate). In this intestinal muscovy duck, there was an equilibrium of microbiota components and there was no exogenous microorganisms that stimulate the overexpression of IgA production. The IgA response is able to stabilize the colonization of symbiotic microorganisms and provide resistance to invasion of exogenous microorganisms [24]. IgA also plays an important role in the selection and stabilization of specific symbionts in hosts [25]. The involvement of IgA in the microbiota composition was very significant. This was indicated by the appearance of dysbiosis in individuals with IgA deficiency [26].

![Figure 1](image1.jpg)

**Figure 1.** Immunohistochemical staining of IgA in the small intestine (A) and large intestine (B) of muscovy duck. The IgA were dispersed in the small and large intestinal lamina propria, and some cells concentrated around the crypts (magnification: 400×).

### 3.2. Metagenomic analysis of intestinal microbiota

The results of metagenomic analysis identified 8 bacterial phyla from the contents of the muscovy duck intestine (Figure 3). Intestinal bacteria were dominated by Firmicutes phyla (48.71%), followed by Proteobacteria (32.87%) and Actinobacteria (8.32%). Four other phyla (Planctomycetes, Bacteroidetes, TM7 and Verrucomicrobia) were in the range of 0.32-7.93. While one other phylum has not been identified. At the ordo level, there were 10 ordos of bacteria in the muscovy duck intestine. Bacterial composition was dominated by the ordo Enterobacteriales (32.08%), Clostridiales (21.04%), Bacillales (14.84%), Lactobacillales (13.41%), Gemmatales (8.08), and Coriobacteriales (6.28%) (Figure 3). Four ordo (Actinomycetales, Desulfovibrionales, CW040, and Bacteriodales) were in the range of 0.33-2.05%. The diversity index of muscovy duck intestinal bacteria were 4.82 (Shannon), 0.89 (Simpson), 0.71 (Pielou Evenness), 11.46 (Margalef) and 109 (Chao1).

IgA is involved in the selection of certain species (even strain) as a symbiont and has implications for the co-evolution of host-microbiota [25]. Each animal has the characteristics of the intestinal microbiota, depending on the environment, feed and maintenance patterns. Domestic duck (*Anas platyrhynchos*) microbiota which were maintained in barn (non-cage), were dominated by *Proteobacteria* phyla (62.18%), *Firmicutes* (25.77%), and *Actinobacteria* (8.49%) [27]. Meanwhile, for domestic geese (*Anser cygnoides*) that were maintained in barn, the intestinal microbiota composition dominated by phylum TM7 (53.18%), *Firmicutes* (32.51%) and *Bacteroidetes* (5.42%) [28]. *Firmicutes* is one of the dominant bacterial phyla in domestic of waterfowl (muscovy duck, duck and geese) [27,28], also in chicken [29]. In humans and animals, the ratio of *Firmicutes* abundance to *Bacteroidetes* abundance in the intestinal microbiota is associated with energy harvesting efficiency [29]. *Firmicutes* bacteria are linked with the decipherment of polysaccharides and the generation of butyrate [30].
role of Proteobacteria in the digestive tract of poultry, until now remain undetermined. Actinobacteria was the third most plentiful phylum of microbes in the muscovy duck intestinal, but no studies have examined the benefit of Actinobacteria in avian. Bacteroidetes bacteria perform a mechanism in the decomposition of complex carbohydrates and the production of propionate via the succinate pathway [30].

Figure 2. Composition of muscovy duck intestinal microbiome (Phylum taxa and Ordo taxa)

4. Conclusion
In healthy muscovy ducks maintain in granary, Ig A was detected positively in the small and large intestines with an IRS score = 4 (moderate). IgA was involved in the selection of certain species (even strain) as a symbiont and has implications for the co-evolution of host-microbiota. Intestinal bacteria were dominated by Firmicutes phyla (48.71%) followed by Proteobacteria (32.87%) and Actinobacteria (8.32%). At the family level, there were 24 families of bacteria in the muscovy duck intestine. Bacterial composition were dominated by the ordo Enterobacteriales (32.08%), Clostridiales (21.04%), Bacillales (14.84%), Lactobacillales (13.41%), Gemmatales (8.08), and Coriobacteriales (6.28%). In this intestinal muscovy duck, there was an equilibrium of microbiota components and there was no exogenous microorganisms that stimulate the overexpression of IgA production.

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