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Original Article

Comparison of Characteristics and Differences in Early Immune Organ Development in Different Strains of Tianfu Broiler

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ABSTRACT

To effectively develop and utilize high-quality Tianfu broilers, this study evaluated the morphological and structural characteristics of the immune organs of such broilers with different strains (HS1 and HS2) at different developmental stages and analyzed the distribution of mast cells by toluidine blue staining. Moreover, the localization and expression of immunoglobulin, complement C3, C4 and CD3 in immune organs were also detected. The results showed that although there was no significant difference in the development of immune organs in the HS1 and HS2, the number of lymphatic follicles and capsule thickness in the spleen and bursa of Fabricius in HS1 were greater than those in HS2. Additionally, the number of mast cells in the spleen of HS1 was greater at Day 1 and Day 21 and was significantly higher than that of HS2 ($p<0.05$); the number of mast cells in the bursa of Fabricius reached 9.17 on Day 7, which was significantly higher than that of HS2 ($p<0.05$). Moreover, the serum IgA and IgM levels in HS1 were higher than those in HS2 on Day 14 and 21 ($p<0.05$). In addition, the complement C3 content in HS1 was significantly or extremely significantly higher than that in HS2 on Days 1, 14 and 21 ($p<0.01$, $p<0.05$), respectively, but significantly lower than in HS2 on Day 7 ($p<0.05$). These results indicated that the disease resistance of the HS1 line was stronger than that of the HS2 line, which lays a foundation for future disease- resistance breeding of Tianfu broilers.

INTRODUCTION

With the rapid development of the modern poultry industry, the impact of various diseases on poultry is becoming increasingly serious. To obtain poultry in a healthy growth state, people often use vaccination by injection to enhance the immune function and disease resistance of these animals (Ruan *et al.,* 2014; Peebles, 2018). However, with the proposed prohibition of antibiotics, methods of improving the natural resistance of poultry through disease-resistance genetic breeding has attracted extensive attention (Guo *et al.,* 2020), and the study of immunity and disease resistance has become a hot issue in modern poultry breeding research. Although in actual production the species of poultry, the breeding environment, and nutrition affect the disease resistance of poultry, the inherent immunity of poultry plays a critical role in disease resistance.

It is well known that the thymus, spleen and bursa of Fabricius are important poultry immune organs (Szenberg *et al.,* 1962; Ribatti *et al.,* 2019). The development and functional status of these organs directly determine the immune levels of birds, thus affecting the immune response of the poultry body to viruses, bacteria and parasites. As early as 1936, Selye pointed out that when animals are subjected to pressure,

immune organs and tissues such as the spleen, thymus and lymph nodes atrophy significantly (Selye, 1936). The spleen in particular is the center of cellular and humoral immunity because it is the largest peripheral lymphoid organ and the reservoir region of T and B lymphocytes in chickens; consequently, in recent years, an increasing number of studies have explored the immunological response of the chicken spleen (Kita, 2014). For example, Zhang *et al.* (2019) reported that with the development of spleen morphology and structure, the resistance of exogenous carbon particles was enhanced. At the same time, the number of T cells, B cells, and antigen-presenting cells in the spleen increases between hatching and adulthood. Moreover, previous studies have found that the thymus plays an important role in inducing T lymphocyte proliferation, differentiation, and selection, being a critical mediator of the innate immune response (Murray *et al.,* 2003; Thapa & Farber 2019). The bursa of Fabricius is a unique central immune organ of birds that plays an essential role in B lymphocyte development. To acquire a deeper perception of the molecular mechanism of the spleen and bursa of Fabricius development in poultry, an increasing number of researchers have screened and identified differentially expressed mRNAs, lncRNAs and circRNAs in poultry immune organs at different developmental stages through high-throughput sequencing in recent years. For example, Liu *et al.* (2020) identified 13689 circRNAs in the bursa of Fabricius on the 2nd day of incubation and Day 20 after hatching, indicating that circRNA is abundant and important in the development of the bursa of Fabricius. Furthermore, Zhang *et al.* (2018) revealed the differential regulation of the response of the two chicken strains to Newcastle disease virus by spleen transcriptome analysis. However, there are few studies on the development and changes in the tissue structure of immune organs in chickens of different breeds (strains).

The Tianfu broiler is a high-quality local chicken breed independently bred by the Poultry Research Breeding Group of Sichuan Agricultural University and Sichuan Banghe Agricultural Science and Technology Co., Ltd., of China, with good reproductive performance, fast growth rate, high feed conversion rate and survival rate (Tian *et al.,* 2021). In recent years, most studies have focused on the carcass traits and reproductive performance of Tianfu broilers, but there are relatively few studies on the morphology and histology of immune organs of Tianfu broilers. For example, Amevor *et al.* (2021) reported that dietary quercetin and vitamin E can increase hormone and receptor secretion through the liver-blood-ovarian signaling axis, and promote the development of reproductive organs, thus improving the reproductive performance of Tianfu broilers. Therefore, maximizing the immune function of Tianfu broilers in the process of growth and development, reducing the occurrence of disease, and improving their economic benefits are particularly important.

In view of the above, the aim of this study was to compare the morphological structure, the mast cell distribution and the localization and expression differences of immunoglobulin of two strains of chicken immune organs in different developmental periods. The results of this study provide basic data for the further development and utilization of the HS1 and HS2 strains of Tianfu broilers and supply a theoretical basis for improving the immune capacity of the two strains.

MATERIALS AND METHODS

Animals and management

The two strains of Tianfu broiler, namely HS1 and HS2, are used as research objects. HS1 has green feet, while HS2 has yellow feet. There is little difference between HS1 and HS2 in regards to body shape and appearance, but HS2 is more prone to disease than HS1. One hundred HS1 and HS2 eggs of similar sizes were selected and purchased from Sichuan Banghe Agricultural Science and Technology Co. Ltd. (Deyang, Sichuan, China). All birds were raised at the poultry breeding farm of Sichuan Agricultural University (Ya'an, China). The one-day-old HS1 (n=48) and HS2 (n=48) chicks were randomly allocated into 4 mixed-sex pens with a density of 4 chicks/m^2. Each pen was provided with pan feeders to ensure at least 2 cm/chick of front space and a line of automatic nipple drinkers. Artificial lighting for all chicks was provided at 20 lux with a duration of 23 h light for the first 3 d, which was then reduced to 16 h from Day 4 to Day 8, and to 11 h from Day 9 until Day 21. Meanwhile, all experimental chicks received routine vaccinations according to the requirements of the poultry breeding farm. All experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (license no. 2018-14).

Experimental design

At 1, 7, 14 and 21 days of age, 6 chickens from each strain (n=48) were randomly selected for weight

measurement and blood sample collection. The blood samples were stored in a -20 ºC refrigerator for future testing. Subsequently, each chicken was bled to death from the carotid artery, and immune tissues such as the thymus, spleen, and bursa of Fabricius were collected immediately for subsequent immune organ index measurement and HE staining observation.

Immune organ index

On Day 1, 7, 14 and 21, the weights of immune organs, including the thymus, spleen and bursa of Fabricius (with the attached tissue removed), of each chick were measured, and then the immune organ index was calculated. The formula is as follows:

$$\text{Immune organ index} = \frac{\text{Immune organ weight (mg)}}{\text{body weight (g) x 100\%}} \text{x100\%}$$

Histological observation

After the immune organs were weighed, tissue blocks of a certain size were selected and fixed in 10% buffered formalin for 24 h, followed by conventional histological procedures such as dehydration, transparency, waxing, embedding and fixation in paraffin (Histosec, Merck). Once the blocks were made, they were sliced into 4 μm thick slices using a Periodic Acid-Schiff (PAS) microtome, roasted, stained with hematoxylin and eosin (HE), sealed, and finally observed under a light microscope (Nikon, Japan).

Mast cell observation

The immune tissue sections were dewaxed and placed into water, toluidine blue solution was added to react with the sections for 20-30 min, and the sections were washed with distilled water for 30 s. Then the sections were placed in 0.5% glacial acetic acid for 30 s, and immediately removed and washed with distilled water. After the water was dried, the sections were dehydrated with anhydrous ethanol at different concentrations; that is, 95% anhydrous ethanol was applied for 3-5 s, 100% anhydrous ethanol was applied twice for 3 min each time, and finally, xylene was applied as the clearant twice, for 3 min each time. The sections were removed from the xylene solution, and the neutral resin was absorbed with a pipette gun and dripped onto the sections to cover the tissues and avoid bubbles. Mast cells were observed and counted under a 400× lens after the stained and sealed slides were fully dried. During counting, the number of mast cells in each filed was counted under the "zigzag" direction mirror, 3 sections were counted for each tissue, 5 fields were counted for each section, and the average value was calculated.

Determination of serum immunoglobulin and complement C3 and C4

The blood samples were centrifuged at 4 ºC and 3000 × g for 20 min to obtain the serum, which was stored at -20 ºC for subsequent immunoglobulin content and complement C3 and C4 detection. The contents of immunoglobulin A (IgA), immunoglobulin M (IgM) and immunoglobulin G (IgG) in serum were determined using an ELISA kit (Ruipan Biotechnology Co., Ltd, Shanghai, China), while complement C3 and C4 were detected by complement detection kits (Beijing Huayechang Biotechnology Co., Ltd, Beijing, China). All operations were carried out according to the kit instructions. At the same time, each indicator was measured three times.

Immunohistochemistry

The spleen and bursa of Fabricius samples were fixed with 4% formalin, embedded in paraffin, and sectioned into 5-μm-thick sections. Subsequently, after dewaxing and rinsing, the slides were immersed in PBS buffer for 5 min and then boiled in a microwave oven for 10 min. Then, the slides were incubated with PBS containing 5% hydrogen peroxide at room temperature for 15 min and washed with distilled water twice for 5 min each time. Next, 100 μL of blocking solution (normal goat serum) was added and incubated at room temperature for 15 min, and the appropriate amount of primary antibody (rabbit anti-CD3, 1:100) was subsequently added at 4 ºC overnight. After washing with PBS buffer 3 times, the cells were incubated with secondary antibody (sheep anti-rabbit IgG) and incubated with horseradish enzyme-labeled streptomycin, followed by counterstaining with Mayer's hematoxylin. To determine the expression level of CD3, an Olympus microscope was used to photograph the prepared slides, and then the Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to measure the integrated optical density (IOD) value of the immunohistochemistry section and the areas and density of the dyed region. Subsequently, the IOD/area formula was used to calculate the mean optical density, which reflected the concentration per unit area of CD3 protein. Five regions were randomly selected from each tissue for statistical analysis.

Statistical Analysis

The complete random design was carried out with the pen as the experimental unit. Data analysis was performed by one-way ANOVA using the general lines model (GLM) procedure of SPSS statistical software

(version 24.0, SPSS Inc., Chicago, IL, USA). Significant differences were determined by Duncan's multiple range tests. Pearson's correlation analysis test (SPSS, version 24.0) was used to analyze the relationship between body weight and immune organ indices. Differences were considered significant when $p<0.05$.

RESULTS

Comparison of weight between HS1 and HS2

As shown in Table 1, we found that the weight of the HS1 strain was higher than that of the HS2 strain on Day 1, 14 and 21 after hatching, but the difference was not significant ($p>0.05$). However, the weight of HS1 on Day 7 after hatching was significantly higher than that of HS2 ($p<0.05$). In addition, we also found that the weight difference between HS1 and HS2 reached the highest value at 21 days (20.22 g), while the weight difference reached the lowest value at 3.72 g on the first day of emergence. These results indicated that there is little difference between HS1 and HS2 at birth, but as time passes, the difference between them increases. In particular, the weight of HS1 increases, which is consistent with the large size of HS1 and the small size of HS2.

Table 1 – The mean weight of HS1 and HS2 chickens (g).

Age (day)	HS1	HS2	Difference (HS1-HS2)
1	38.62±3.35[a]	34.90±3.02[a]	3.72
7	85.30±5.50[b]	73.30±4.13[c]	12.00
14	176.10±13.04[d]	162.15±23.88[d]	13.95
21	312.42±31.96[e]	292.20±12.10[e]	20.22

Note: The same letter on the shoulder of the same line indicated no significant difference ($p>0.05$), and different indicated significant difference ($p<0.05$). Data were expressed as "mean ± standard deviation".

Comparison of immune organ development between HS1 and HS2

To understand the developmental differences in immune organs between HS1 and HS2 strains of Tianfu broilers, we first compared the weight of immune organs in the two strains. From Table 2, we can see that the weight of the immune organs of HS1 and HS2 increased significantly with age ($p<0.05$), and the weight of all three immune organs reached the maximum value on Day 21. The thymus weight of HS1 was significantly higher than that of HS2 at Days 1, 7 and 21 ($p<0.05$), and the spleen and bursa of Fabricius weights of HS1 were higher than those of HS2, but the difference was not significant ($p<0.05$). On Day 14, the thymus weight of HS1 was higher than that of HS2, while the spleen and bursa of Fabricius were slightly lower than those of HS2, and the difference was not significant ($p>0.05$). Therefore, HS1 has a certain advantage over HS2 in the weight of immune organs.

Table 2 – The weight comparison of immune organs between HS1 and HS2 (mg).

Age (day)	Thymus		Spleen		Bursa of Fabricius	
	HS1	HS2	HS1	HS2	HS1	HS2
1	58.78±8.45[a]	48.23±5.65[b]	13.70±1.30[a]	13.32±1.89[a]	45.52±5.83[a]	42.17±5.88[a]
7	255.48±26.61[c]	180.50±21.60[d]	147.68±27.01[b]	139.62±24.55[b]	167.85±17.05[b]	188.10±16.94[b]
14	551.65±71.69[e]	501.93±74.03[e]	331.47±38.87[c]	357.75±42.88[c]	440.55±73.73[c]	444.07±33.52[c]
21	1691.22±120.23[f]	1530.17±105.27[g]	604.90±78.53[d]	568.53±69.27[d]	1014.47±92.59[d]	991.65±97.43[d]

Note: The same letter on the shoulder of the same line indicated no significant difference ($p>0.05$), and different indicated significant difference ($p<0.05$). Data were expressed as "mean ± standard deviation".

Subsequently, we obtained the immune organ indices of HS1 and HS2 chickens at different ages. As shown in Figure 1A, the thymus index of HS1 was higher than that of HS2, but the difference was not significant ($p>0.05$). Meanwhile, the thymus index of HS1 increased significantly from Day 1 to 7 and from Day 14 to Day 21 ($p<0.05$), while it increased slightly from Day 7d to Day 14 without a significant difference ($p>0.05$). In contrast to that of HS1, the thymus index of HS2 increased with age, and reached its maximum value at Day 21. As shown in Figure 1B and Figure 1C, both the spleen index and bursa of Fabricius index of HS2 were higher than those of HS1, especially at Day 7 and Day 14, with significant differences between them ($p<0.05$). Curiously, compared with that at Day 14, the spleen index of HS1 decreased significantly at Day 21 ($p<0.05$), but the spleen index of HS2 increased slowly, and no significant difference was found ($p>0.05$).

Correlation analysis between body weight and organ index

To explore the potential relationship between immune organ development and body weight in HS1 and HS2 during the whole growth and development process, we conducted a correlation analysis of the body weight and organ index. The results indicated that the body weight of HS1 and HS2 was positively correlated with the indices of the immune organs, and

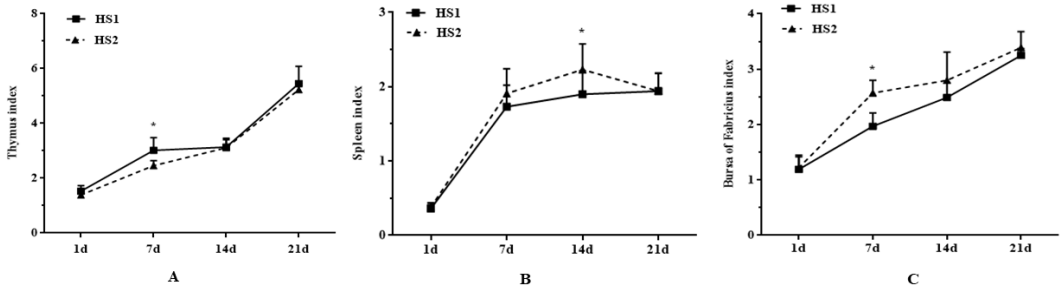

Figure 1 – Comparison of immune organ indexes between HS1 and HS2 in different periods. (A) Thymus; (B) Spleen; (C) Bursa of Fabricius.

this difference was extremely significant ($p<0.01$) (Table 3). Moreover, the correlation coefficients between the body weights of HS1 and HS2 and the thymus index, as well as the body weight of HS1 and the bursa of Fabricius index were both greater than 0.8, indicating a high correlation, while the correlation coefficients between the body weight of HS1 and the spleen index, as well as the body weight of HS2 and the bursa of Fabricius index, were both greater than 0.5, indicating a moderate correlation. In addition, we also found that the correlation coefficients between the body weight of HS1 and the indices of the three immune organs were all higher than those of HS2 (Table 3).

Table 3 – The correlation between body weight of HS1 and HS2 and organ index.

Index	Strains	Immune organ index		
		Thymus	Spleen	Bursa of Fabricius
Body weight	HS1	0.887**	0.650**	0.926**
	HS2	0.965**	0.555**	0.772**

Note: The number shoulder "**" in the table indicates a very significant difference of $p<0.01$.A correlation value of R>0.5 represents a moderate correlation, and a value of R>0.8 represents a high correlation.

Histological structure and developmental changes of the spleen

The morphological characteristics of immune system organs in HS1 and HS2 are shown in Figure 2. After HE staining, the marginal area and red pulp area of the spleen of HS1 and HS2 chickens were light pink, while the white pulp area was blue purple. On Day 1, the lymphocyte follicles of HS1 and HS2 were almost invisible, but as time went on, the lymphocyte follicles became increasingly obvious, from a small round dot on Day 7 to a large round or oval shape surrounded by several layers of reticular cells. The superficial layer was dense and mostly comprised B lymphocytes. Moreover, the area of the periarterial lymphatic sheath (PALS) increased with age. The nucleus of the splenic sinus was a purple spindle that protruded slightly into the sinus cavity, while the cytoplasm appeared as a red-stained linear connection. At Day 21, the volume of lymphatic follicles in HS1 was smaller than that in HS2, but there was little difference in the overall structure between HS1 and HS2.

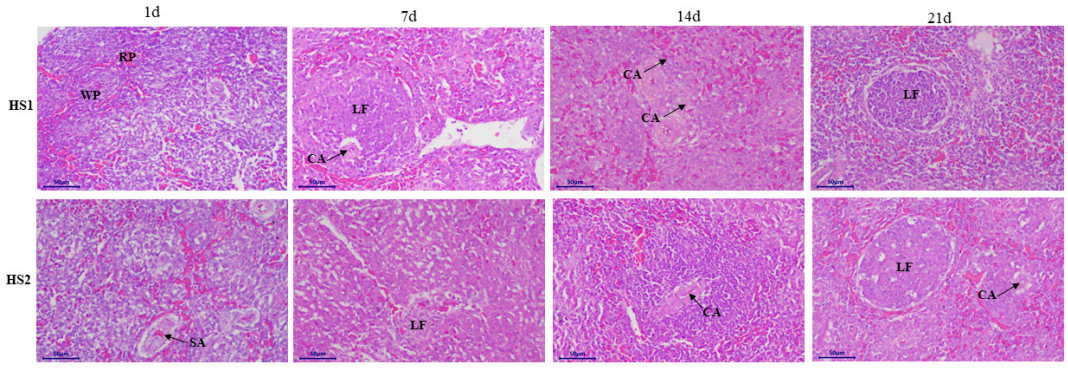

Figure 2 – Histological changes of spleen at different stages of HS1 and HS2.
Note: WP: White Pulp; RP: Red Pulp; CA: Central Artery; LF: Lymphatic Follicle; SA: Sheath Artery.

However, there were some differences in the number of lymphatic follicles per unit area of spleen and membrane thickness between HS1 and HS2 at different days of age. As shown in Table 4, we found that on Day 21, the number of lymphatic follicles in the HS1 and HS2 strains was significantly higher than on Days 1, 7 and 14 ($p<0.05$); however, the number of lymphatic follicles in HS2 first decreased and then increased on Days 1, 7 and 14, and there were no significant differences among the three points in time ($p>0.05$). Regarding membrane thickness, HS1 strains reached the maximum value of 27.17 μm on Day 21,

which was significantly higher than on Days 1, 7 and 14 (p<0.05). However, there was no significant difference in HS1 membrane thickness between Day 7 and Day 14 (p>0.05). In contrast to HS1, the membrane thickness of HS2 reached a maximum value of 20.96 μm at Day 7, which was significantly higher than that of the other three points in time (p<0.05). In addition, on Day 14, the membrane thickness of HS2 was significantly lower than that on Days 1, 7 and 21 (p<0.05).

Table 4 – Comparison of the number of lymph follicles per unit area and capsule thickness in spleen of HS1 and HS2 chicken at different periods.

Age (day)	Number of follicles per unit area (n/mm²)		Thickness of capsule (μm)	
	HS1	HS2	HS1	HS2
1	0.50±2.29^{c}	0.91±3.03^{b}	7.96±1.97^{c}	11.05±2.17^{c}
7	1.49±0.80^{ab}	0.88±0.58^{b}	18.35±3.98^{b}	20.96±3.87^{a}
14	1.76±0.74^{a}	1.03±0.29^{b}	18.36±4.08^{b}	14.71±5.75^{b}
21	1.31±0.48^{b}	2.38±0.86^{a}	27.17±2.67^{a}	18.66±5.99^{ab}

Note: The same letter on the shoulder of the same line indicated no significant difference (p>0.05), and different indicated significant difference (p<0.05).

Histological observation and developmental changes of the bursa of Fabricius

The results of the histological analysis of the bursa of Fabricius are presented in Figure 3 and summarized in Table 5. On Day 1, the bursa of Fabricius of both HS1 and HS2 had obvious wrinkling, and the mucosal epithelium was well developed. Meanwhile, there were scattered lymphocytic cells with indistinct differentiation characteristics in reticular scaffolds composed of many cells in the bursa of Fabricius, and the boundary between the cortex and medulla was not obvious (Figure 3A, A'). On Day 7, an obvious four-layer structure could be clearly seen on the HS1 bursa of Fabricius; the volume of lymphatic follicles was significantly larger than that on Day 1, the cells were closely arranged, the proportion of the cortex was very small, and each nodule was almost occupied by the medulla, while the four-layer structure of the HS2 bursa of Fabricius was not as clear as that of HS1 (Figure 3B, B'). On Day 14, the arrangement of follicles was relatively close, and the distribution of some loose connective tissue in the early stage was slightly reduced, but an obvious trabecular structure could be seen, and the cortex and medulla began to exhibit distinct boundaries (Figure 3C, C'). On Day 21, the size of follicles in the bursa of Fabricius was different between HS1 and HS2, both of which were composed of an obvious dark cortex and light medulla (Figure 3D, D'). Based on these experimental results, we identified that there was no significant difference in the structure of the bursa of Fabricius between HS1 and HS2.

However, the number of lymphatic follicles per unit area and capsule thickness of the bursa of Fabricius of HS1 and HS2 were different at different days of age. In contrast to the spleen, the number of lymphatic follicles in the HS1 and HS2 strains decreased with time. On Day 21, the number of lymphatic follicles in both strains reached the lowest value, 9.07/mm² and 9.37/mm², respectively, which was significantly lower than that on Days 1, 7 and 14 (p<0.05). Furthermore, the number of lymphatic follicles per unit area of bursa of Fabricius in HS1 was slightly higher than that in HS2 on Days 1, 7 and 14, but slightly lower than that on Day 21; however, the difference was not significant (p>0.05) (Table 5). Similar to the development and change in spleen capsule thickness, the thickness of the bursa of Fabricius increased with time in both HS1 and HS2. On Day 21, the thickness of the bursa of Fabricius of the two strains reached maximum values of 42.94 μm and 47.23 μm, respectively, which were significantly higher than those on Days 1, 7 and 14 (p<0.05). In addition, the thickness of the bursa of Fabricius was higher in HS2 at different ages than in HS1, but there was no significant difference between them (p>0.05) (Table 5).

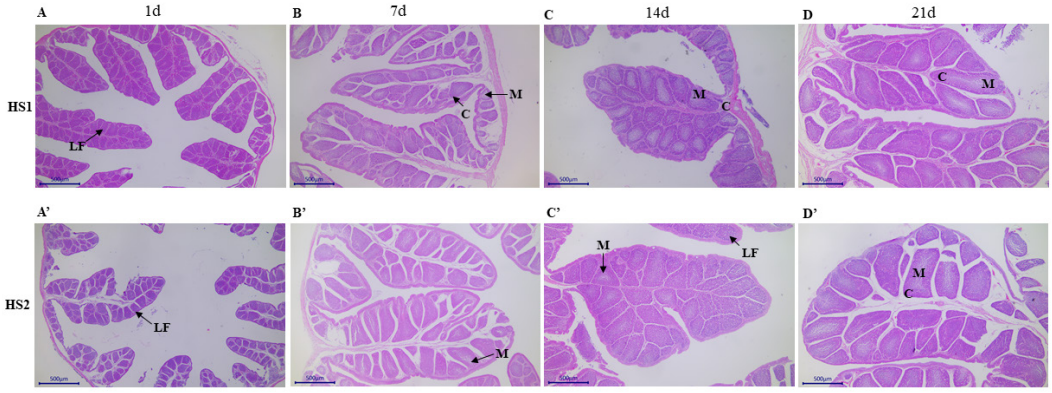

Figure 3 – Histological changes of bursa of Fabricius at different stages of HS1 and HS2. (A, A') 1d; (B, B') 7d; (C, C') 14d; (D, D') 21d.
Note: M: Medulla; C: Cortex; LF: Lymphatic Follicles.

Table 5 – Comparison of the number of lymph follicles per unit area and capsule thickness in bursa of Fabricius of HS1 and HS2 chicken at different periods.

Age (day)	Number of follicles per unit area (n/mm²)		Thickness of capsule (μm)	
	HS1	HS2	HS1	HS2
1	90.35±9.52^{a}	85.22±12.17^{a}	3.56±0.83^{d}	4.40±0.43^{d}
7	25.77±2.91^{b}	22.62±1.45^{b}	16.89±4.74^{c}	27.34±2.97^{c}
14	16.09±3.04^{c}	15.37±3.41^{c}	28.69±5.96^{b}	31.49±3.35^{b}
21	9.07±1.70^{d}	9.37±1.62^{d}	42.94±7.65^{a}	47.23±3.48^{a}

Note: The same letter on the shoulder of the same line indicated no significant difference (p>0.05), and different indicated significant difference (p<0.05).

Distribution of mast cells in immune organs

As shown in Figure 4A, the spleen mast cells of the HS1 and HS2 strains were mainly purplish red, with a relatively uniform size and round or oval shape, and were mainly distributed at the junction of red pulp and white pulp, as well as around the blood vessels and trabeculae of the red pulp and splenic nodules. Meanwhile, the number of mast cells in the spleen of HS1 was greater than that of HS2 at different developmental stages. However, the mast cells in the bursa of Fabricius were larger and mainly distributed in the mucous epithelium and epithelial plexus (Figure 4B). From Figure 4, it can be seen that mast cells were rare in the follicular cortex and medulla, with one or two cells occasionally appearing. In addition, mast cells were distributed sporadically in connective tissues, and they were of different sizes and shapes, such as round, oval, or spindle-like. Thus, the distribution and morphological structure of mast cells in the spleen and bursa of Fabricius of HS1 and HS2 were similar, without any difference.

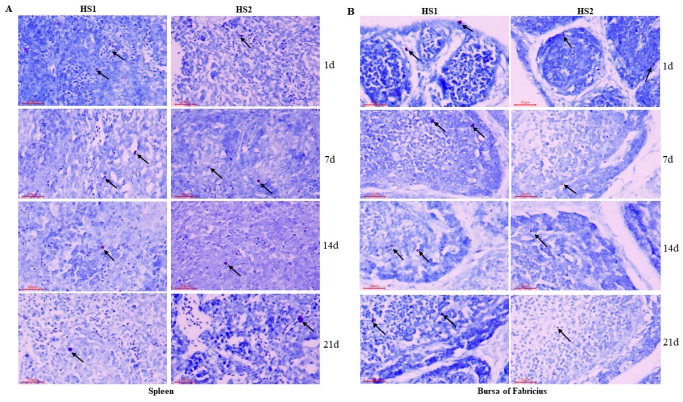

Figure 4 – Spleen and bursa of Fabricius mast cell distribution at different stages for HS1 and HS2. (A) Spleen; (B) Bursa of Fabricius. Arrows indicate mast cells.

However, the number of mast cells in the spleen or bursa of Fabricius in the two strains was different at different ages. As shown in Table 6, the number of mast cells in the spleen of HS1 was significantly higher than that of HS2 on Days 1 and 21 (p<0.05); on the contrary, the number of mast cells was significantly lower in HS1 than in HS2 on Day 7 (p<0.05). On Day 14, although the number of mast cells in the spleen of HS1 was higher than that of HS2, the difference was not significant (p>0.05). In addition, we also found that mast cells in the spleen of HS1 were most distributed on Day 1, while those of HS2 were most distributed on Day 7. Furthermore, on Day 7, the number of mast cells in the bursa of Fabricius of HS1 was significantly higher than that of HS2 (p<0.05); in contrast, on Days 1 and 21, the number of mast cells was significantly lower in HS1 than in HS2 (p<0.05).

Table 6 – Number of mast cells in HS1 and HS2 spleen and bursa of Fabricius.

Age (day)	Number of mast cells in spleen (n)		Number of mast cells in bursa of Fabricius (n)	
	HS1	HS2	HS1	HS2
1	12.83±5.64^{a}	6.83±0.90^{c}	7.00±1.15^{b}	8.83±0.90^{a}
7	6.00±0.58^{d}	7.33±1.11^{c}	9.17±1.77^{a}	6.50±1.26^{b}
14	7.00±1.41^{c}	6.67±1.25^{c}	8.83±4.78^{a}	8.33±3.25^{a}
21	8.50±2.36^{b}	6.33±1.11^{cd}	7.33±1.97^{b}	8.17±1.46^{a}

Note: The same letter on the shoulder of the same line indicated no significant difference (p>0.05), and different indicated significant difference (p<0.05).

Distribution and changes of CD3 in the development of immune organs

The CD3-positive signal of the spleen in the HS1 and HS2 strains was mainly distributed around the periarterial lymphatic sheath in the white medulla, while in the red pulp, the CD3-positive signal was less distributed, and the positive signal cells were yellow and round or nearly round (Figure 5A). Moreover, CD3-positive cells in the bursa of Fabricius were mainly distributed in the mucosal epithelium and epithelial plexus, less in the cortex, more in the medulla, and more in the lamina propria. The positive cells were tan, with a larger diameter, and the nuclei were not obvious (Figure 5B).

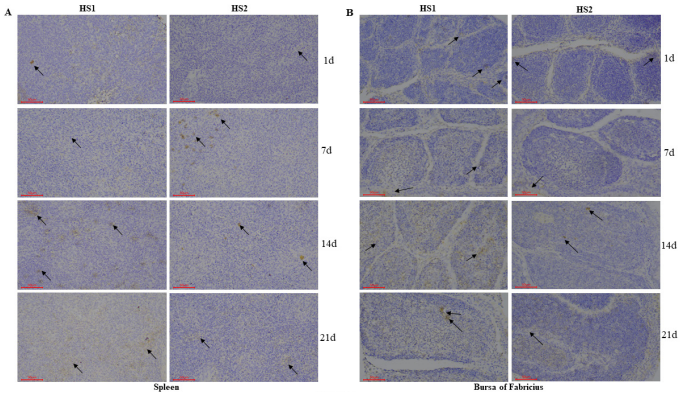

Figure 5 – CD3 expression in spleen and bursa of Fabricius of HS1 and HS2 at different stages. (A) Spleen; (B) Bursa of Fabricius. Arrows indicate CD3 positive cells.

The integrated optical density (IOD) and mean optical density (MOD) were different between different varieties at different ages. The MOD of CD3 in HS1 and HS2 spleens increased with time on Days 1, 7 and 14, but decreased significantly on Day 21 (p<0.05) (Table 7). Interestingly, the MOD of CD3 in HS1 and HS2 spleens was equal on Days 7, 14 and 21. However, on Day 14, the IOD of HS1 was significantly higher than that of HS2, while on Days 7 and 21, it was significantly lower than that of HS2 (p<0.05). Moreover, the MOD of CD3 in the bursa of Fabricius increased with time, and the MOD of HS1 and HS2 reached the maximum value on Day 21. At the same time, on Days 1 and 7, the MOD and IOD of HS1 were both higher than those of HS2 (p<0.05). In contrast, on Days 14 and 21, the MOD and IOD of HS1 were lower than those of HS2, but the difference between them was not significant (p>0.05) (Table 7).

Table 7 – Changes of CD3 in spleen and bursa of Fabricius of HS1 and HS2.

Tissue	Age (Day)	HS1		HS2	
		IOD	MOD	IOD	MOD
Spleen	1	28206.66±2841.35^{d}	4436.28±469.10^{d}	21542.32±2344.21^{d}	4964.56±541.26^{d}
	7	40128.96±5062.84^{c}	6311.29±796.20^{c}	50177.65±4970.36^{c}	6311.29±796.20^{c}
	14	102470.80±11702.00^{a}	16116.36±1840.46^{a}	88230.07±10823.06^{b}	16116.36±1840.46^{a}
	21	94577.50±9328.02^{b}	14874.92±1467.20^{b}	142570.67±14710.23^{a}	14874.92±1467.20^{b}
Bursa of Fabricius	1	8715.69±753.31^{bc}	1021.31±325.24^{c}	5338.16±1012.64^{d}	862.17±287.35^{c}
	7	9561.32±635.48^{b}	1324.35±405.71^{b}	6312.62±806.34^{c}	924.35±547.66^{c}
	14	8016.14±1024.35^{c}	1234.17±321.49^{b}	11680.66±2168.17^{b}	1534.17±664.94^{b}
	21	12164.21±2301.44^{a}	2016.36±534.18^{a}	19958.31±2234.82^{a}	3177.36±931.62^{a}

Note: The same letter on the shoulder of the same line indicated no significant difference (p>0.05), and different indicated significant difference (p<0.05). IOD represents the integrated optical density and MOD represents the mean optical density.

Comparative analysis of serum biochemical indices

To explore the difference in antiviral ability between the HS1 and HS2 strains, we detected the levels of IgA, IgM and IgG in their serum. The results showed that on Day 7, the IgA content of HS1 was significantly higher than that of HS2 (p<0.05), while on Day 1, it was lower than that of HS2, and the difference was not significant (p>0.05). Although the IgA content of HS1 was higher than that of HS2 on Days 14 and 21, the difference was not significant (p>0.05) (Figure 6A). Furthermore, the IgM content of HS1 was higher than that of HS2 on Days 1 and 7, but lower than that of HS2 on Days 14 and 21; meanwhile, the difference between the two strains was significant only on Days 7 and 21 (p<0.05) (Figure 6B). In addition, we found that the content of IgG in HS1 was higher than that in HS2 except on Day 7, and especially on Day 14 and 21, with a significant difference between them (p<0.05) (Figure 6C). Altogether, these results indicated that the contents of IgA, IgM and IgG in the serum of HS1 were higher than those of HS2.

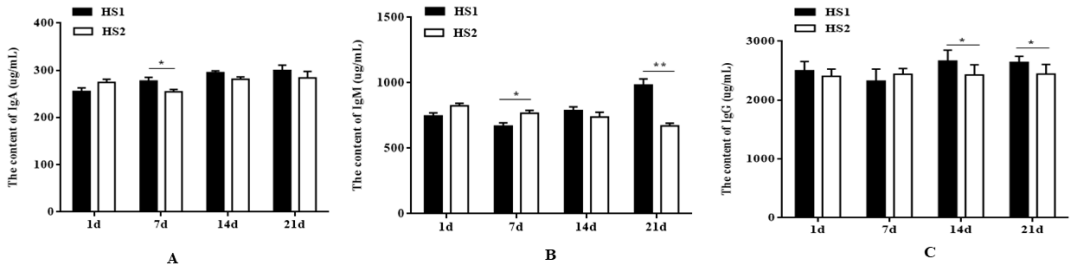

Figure 6 – Comparison of IgA, IgM and IgG contents in HS1 and HS2 serum. (A) IgA; (B) IgM; (C) IgG. Data are expressed as mean±SEM of three independent experiments. *p<0.05, **p<0.01.

Complement, as a defense factor in the body, participates in coordinating the stability of the internal environment and is an important auxiliary factor in the immune function of animals. Therefore, we further detected the levels of complement C3 and C4 in the serum of HS1 and HS2. As shown in Figure 7A, except on Day 7, the content of C3 in HS1 was significantly higher than that in HS2 (p<0.01 or p<0.05). Moreover, on Days 1 and 14, the content of C4 in the serum of HS1 was significantly lower than that in HS2 (p<0.05), but higher than that in HS2 on Days 7 and 21, especially on Day 21, and the difference reached a significant level (p<0.05) (Figure 7B).

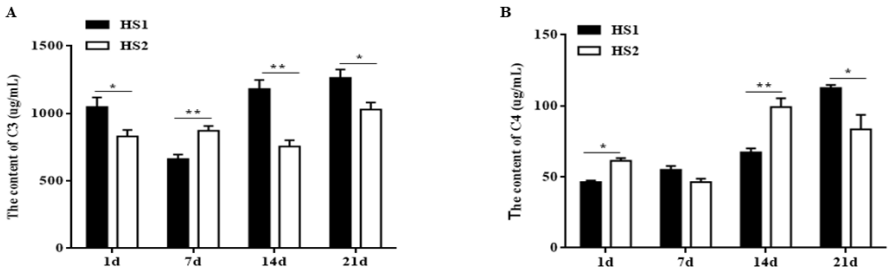

Figure 7 – Comparison of C3 and C4 content in HS1 and HS2 serum. (A) C3; (B) C4. Data are expressed as mean±SEM of three independent experiments. *p<0.05, **p<0.01.

DISCUSSION

Changes in immune organ development

The immune organs of poultry include the thymus, bursa of Fabricius and spleen. The thymus, whether in mammals or in poultry, is a vital immune organ. Not only can it play a role in the supervision and protection of the body, but it also prevents the body from external pathogens, tumors and antigens (Thapa & Farber 2019). Furthermore, the thymus is also the first developed lymphoid organ in chicken embryos and plays a critical role in T-cell development, in maturation and differentiation, and in humoral and cellular immunity (Peterson *et al.,* 1965; Song *et al.,* 2012). The bursa of Fabricius is a unique central immune organ of poultry and an important site for the early development of B lymphocytes. Because it contains a variety of cells such as lymphocytes, plasma cells and macrolymphocytes, it can produce corresponding immune responses to exogenous antibodies (Click *et al.,* 1956; Cooper *et al.,* 1966; Lydyard *et al.,* 1976). The spleen is the largest peripheral immune organ in poultry because, similarly to the bursa of Fabricius, it contains a variety of immune cells and factors; when the body is attacked by bacteria, this will also generate a corresponding resistance (Jeurissen 1991; Zhang *et al.,* 2019)

In recent years, some studies have shown that the development of immune organs is one of the key factors determining the immune level of poultry, and changes in their weight and organ index can reflect the maturity of the poultry immune system (Wang *et al.,* 2016). For example, Wang *et al.* found that the ratio of thymus and bursa of Fabricius to body weight of both Hyland brown laying hens and AA broilers reached its maximum at 18 days of age. However, for AA broilers, the ratio of thymus or bursa of Fabricius to body weight was lower than that of Hyland brown laying hens after 11 days of age, which was correlated with the rate of weight gain of laying hens and broilers (Wang *et al.,* 2004). Meanwhile, other researchers found that the weight of the immune organs of Shaoxing ducks and Jinyun ducks increased continuously with the increase in age. In addition, the immune organ weight, and the level of interleukin 2 (IL-2) of Shaoxing ducks at 70 days of age were higher than those of Jinyun ducks, while the immune organ weight, index and IFN-γ level of Jinyun ducks at 10 days of age were higher than those of Shaoxing ducks, which indicated that there were differences in immune system development and function among different ducks (Li *et al.,* 2008). Moreover, Xu *et al.* (2020) found that the spleen index of ducks continuously increased between 1 and 14 days of age, but after 14 days, the body weight gain rate of the ducks was higher than the organ weight gain rate of the spleen, and the spleen index decreased.

Similar to the results of Li *et al.* (2008), our results showed that the weight of immune organs in HS1 and HS2 strains of Tianfu broilers increased with age, and the weight of immune organs of HS1 strains was larger than that of HS2 strains, indicating that the development rate of immune organs of HS1 strains was faster than that of HS2 strains. Moreover, we also found that the organ indices of the thymus and bursa of Fabricius of the HS1 and HS2 strains reached maximum values at 21 days of age, and the organ indices of the spleen and bursa of Fabricius in the HS1 strain were greater than those in the HS2 strain. Interestingly, correlation analysis showed that the body weight of HS1 and HS2 strains was positively correlated with each immune organ index, and the difference was extremely significant (p<0.01). Most notably, the correlation coefficients between the body weight of HS1 and HS2 and the thymus index, as well as the body weight of HS1 and the bursa of Fabricius index of Tianfu broilers, were both higher than 0.8, indicating that body weight can indirectly reflect the development of immune organs.

In addition, differently from the spleen of ducks and geese, the spleen and bursa of Fabricius of Tianfu broilers were spherical and oval, respectively. Meanwhile, from the H&E results, we found that there

was no significant difference in the spleen between HS1 and HS2 strains of Tianfu chicken.

Difference in mast cells

Previous studies have shown that mast cells are unique secretory cells that can be found in almost all organs and tissues of the body, especially in blood vessels, lymphatic vessels, nerves, the gastrointestinal system, and the skin surface (Galli, 1990). Therefore, mast cells play an important role in host defense against pathogens such as bacteria, parasites and viruses (Crivellato *et al.,* 2005; Shelburne & Abraham 2011; Abraham & St John 2010).

In recent years, researchers have found mast cells in all vertebrates, including amphibians, reptiles, birds, fish and mammals (Baccari *et al.,* 2011). For example, in fish, Da'as *et al.* proved that zebrafish mast cells have the same functions as those of mammals in terms of both innate and adaptive immune responses, which laid a foundation for using zebrafish as an in vivo screening tool to screen new mast cell modulators (Da'as *et al.,* 2011). Interestingly, the density of mast cells resident in some organs will increase under pathological conditions (Shi *et al.,* 2015; Martino *et al.,* 2015), so this feature of mast cells has become a special concern, especially in recent years, and an increasing number of studies have shown that mast cells are closely related to the development of tumors (Dyduch *et al.,* 2012; Mao *et al.,* 2018). At present, the distribution and characteristics of mast cells in different parts of poultry have also attracted increasing attention. Ertugrul *et al.* (2018) reported the morphology, distribution, and heterogeneity of mast cells in the tongues of five different avian species, among which the number of mast cells was the highest in the tongue of the Gerze rooster. Meanwhile, Tachibana *et al.* (2019) found that mast cell degranulation is associated with nonspecific symptoms in chickens, although the mechanism is different between peripheral and central tissues.

Moreover, the results obtained in the present study showed that the splenic mast cells of the HS1 and HS2 strains of Tianfu chicken were purplish red (with a large number of small individuals), round or oval in shape, and mainly distributed at the junction of the red pulp and white pulp. In addition, the number of mast cells in the spleen of the HS1 strain was greater than that of the HS2 strain at different developmental stages, suggesting that the spleen of the HS1 strain of Tianfu broilers may release active substances through a large number of mast cells to resist the invasion of pathogenic microorganisms. Furthermore, we also found that the mast cells in the bursa of Fabricius were larger and mainly distributed in the mucous epithelium and epithelial plexus, and the cells were of different sizes and shapes, such as round, oval or spindle shapes. A similar result was reported by Guo *et al.* (2014), who reported that the size of mast cells in the spleen of Ningdu Yellow chickens is small, while the mast cells in the bursa of Fabricius are mainly distributed in the reticular tissue of the submucosa with uneven size and diverse volume. Therefore, mast cells show heterogeneity and may play an important role in the disease resistance of Ningdu Yellow chickens. In this study, the number of mast cells per bursa area of the HS1 and HS2 strains were different at different days of age. With increasing time, the number of mast cells generally showed a decreasing trend. The reason may be that in the later stage, the bursa of Fabricius increased, and with it the connective tissue, resulting in the apoptosis of lymphocytes; thus, the number of mast cells decreased, which was basically consistent with the previously observed change in mast cells in the thymus of Guangxi Yellow chickens (Wu *et al.,* 2016).

Comparative analysis of blood immune indices

Immunoglobulin is a kind of protein with antibody activity that exists in the serum and body fluids of the humans and animals. It has antibacterial and antiviral effects and can kill or dissolve pathogenic microorganisms with the coordination of complement, so it is an important component of the body to fight diseases. Previous studies reported that the main immunoglobulins in poultry include IgA, IgM and IgG (Mockett 1986; Choi *et al.,* 2019). Usually, IgM is a high molecular weight pentamer with a unit of μ2L2 in serum, and is generally produced in greater abundance than IgG. IgM is also the earliest antibody produced in the initial immune response, so it is of great significance in early diagnosis. Meanwhile, in most cases, IgA is the third type of immunoglobulin found in chicken serum and secretions, and is key to protecting mucosal surfaces from viruses, bacteria and toxins (Wieland *et al.,* 2004; Schroeder & Cavacini 2010; Liu *et al.,* 2013; Work *et al.,* 2015).

In recent years, the comparison of immunoglobulin content in the serum of poultry has mainly focused on the effect of nutrition on such content (Zhu *et al.,* 2019; Amevor *et al.,* 2021), but there are few analyses on the difference in immunoglobulin content among different breeds (strains) from the perspective of genetics. Our

present study indicates that the serum immunoglobulin IgA, IgM and IgG concentrations in HS1 and HS2 strains of Tianfu broilers at Days 14 and 21 were slightly higher than those at Days 1 and 7. At the same time, the immune indices in HS1 strains were higher than those in HS2. Similar findings reported by Zhao *et al.* (2020) showed that the concentrations of IgA, IgM and IgG in the blood of Cobb broilers were higher than those of Daweishan Mini broilers. Therefore, we speculated that the content of immunoglobulin in HS1 serum improves the humoral immunity level of HS1, enhances its resistance to disease, and makes it less susceptible to attack by external pathogens.

In addition to immunoglobulin, the complement system is widely involved in the antimicrobial defense response and immune regulation and is closely related to immune function, thus playing an important biological role in the body. Complement C3, the complement component with the highest serum content, plays a core role in the complement activation pathway and is a key component linking nonspecific and specific immunity, so it is often used as a basis to measure the level of humoral immunity (Chen *et al.,* 2003; Hangalapura *et al.,* 2004). As a chain in the complement system, C4 complement plays an important role in complement activation, pathogen clearance, virus neutralization and other aspects, participating in the immune defense response of the body together with C3 complement (Fearon & Carroll 2000; Mollnes *et al.,* 2002; Barrington *et al.,* 2002). Zhang *et al.* (2017) found that adding compound essential oils to the diet can increase the content of complement C3 and C4 in broiler serum, thus enhancing the immune regulation function of the body, and improving the ability to resist pathogen infection. In this study, overall, the levels of C3 and C4 in blood of HS1 strains were higher than those of HS2, which further verified the results of serum immunoglobulin, indicating that HS1 has stronger resistance to pathogen infection than HS2.

CONCLUSIONS

In conclusion, there were significant differences in the number of lymphatic follicles, the capsule thickness, and the number of mast cells in immune organs between HS1 and HS2 strains of Tianfu broilers. Meanwhile, the levels of immunoglobulin IgA, IgM, IgG, C3 and C4 in the serum of HS1 strains were all higher than those of HS2. Moreover, the number of mast cells in the spleen of the HS1 strain was greater than that of the HS2 strain at different developmental stages, indicating that HS1 strains had a stronger immune-regulatory ability than HS2 strains, thus resisting infection from exogenous bacteria. Our findings challenge us to further develop and utilize different strains of HS1 and HS2 of Tianfu broilers and lay a foundation for the breeding of disease-resistant Tianfu broilers in the future.

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