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Innate immunity in chicken lines developed by EMBRAPA Suínos e Aves: antimicrobial activity of macrophages and serum

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ABSTRACT - The objective of this study was to compare the antimicrobial activity of macrophages and serum in laying hen (MM, CC, and CCc) and broiler chicken lineages (TT and LL). Macrophages were evaluated for phagocytic and antimicrobial activity. Microbicidal serum activity was evaluated by the resistance test for serum and the agar test. The results showed that phagocytic activity was higher in males of the MM strain, with 13% of macrophages presenting phagocytosis, while the other lineages studied, and even female MM, presented a rate of 6% of phagocytic cells. However, antimicrobial activity in macrophages from males of CCc lineage and females of TT lineage were higher, eliminating more than 30% of the *Salmonella enterica* inoculum, while in the other strains, the results were similar, with inoculum reduction below 30%. In the serum resistance assay, female laying lines presented higher antibacterial activity than female broiler lines. In the trials to evaluate the microbicide activity of the serum, females of both broiler and laying lineages presented higher performance when compared with males of the same lineage. Females of laying hen lines (MM and CC) present a greater antibacterium activity than males. These results can contribute to a better understanding of the immune response in broiler chicken and laying hen lineages, to aid development of lineages of birds more resistant to pathogens.

Keywords: complement system, fowls, genetic selection, natural immunity

1. Introduction

The poultry industry has been investing in genetic improvement for more than 50 years. This effort has resulted in significant advances in production, such as increase in live weight, broiler growth, and yield of *pectoralis minor* and *major* (Zuidhof et al., 2014). However, this genetic improvement has focused on production without evaluating resistance to diseases (Collins et al., 2014).

As infectious diseases are the major cause of mortality, the use of vaccines and antibiotics is necessary for disease prevention (Jacob et al., 2013; Blake and Tomley, 2014). However, the low efficiency associated with some vaccines, as well as concern regarding the use of antibiotics as prophylactic drugs, are important problems faced by the poultry industry (Ghunaim et al., 2014; Lhermie et al., 2017).

Breeding to produce animals more resistant to infection has been developing in recent years. However, the results achieved so far have been disappointing. Studies have shown that a lineage that is more resistant to a particular pathogen, in general, has low resistance to other pathogens or presents

lower productivity (Pavlidis et al., 2007). Considering that the specificity of the immune response is directly related to the development of the so-called adaptive immune response, these studies suggest that genetic improvement based on parameters related to adaptive immunity may not be compatible with animals with high productivity and resistance to infectious diseases (Swaggerty et al., 2019). On the other hand, studies show that innate immunity is a critical factor for disease resistance or susceptibility (Swaggerty et al., 2019). Innate immunity is the first line of defense of an animal, being activated without the need for prior exposure to the pathogen (Swaggerty et al., 2019). In addition, genetic selection programs that have resulted in increased productivity may be associated with an increase in innate immunity (Chema et al., 2003).

In Brazil, the Embrapa Swine and Poultry National Research Center has developed and maintained, under multitrait selection, several laying hen and broiler chicken lineages. Although these lineages have been evaluated regarding production traits (Figueiredo et al., 2012a,b), there are no studies on their immune system.

Thus, in the present study, we carried out a comparative analysis on the antimicrobial activity of macrophages and peripheral blood serum of laying hen (MM, CC, and CC control) and broiler chicken lineages (TT and LL control).

2. Material and Methods

2.1. Animals

In this study, 50 animals of 17 weeks of age, 25 males and 25 females, 10 of each [MM, CC, CC control (CCc), TT, and LL control (LLc)] were used. The Embrapa Swine and Poultry National Research Center (Concórdia, Santa Catarina, Brazil) produced the animals. The animals were kept in cages (two animals/cage) on a farm localized in Londrina, Paraná, Brasil (23°17'34" S, 51°10'24" W, elevation 550 m) with water and feed *ad libitum*. Briefly, MM is a Rhode Island Red semi-heavy, brown egg laying hen line; CC is a laying hen originated from the White Leghorn breed. Both lineages have been under selection for egg production traits since the mid-1980s. CCc is a control lineage with the same genetic basis as CC but has not been selected since 1989; TT is a paternal broiler line developed and maintained by Embrapa under multitrait selection since 1992; and LLc is a paternal broiler control lineage maintained with no selection since the beginning of the 1980s. Research on animals was conducted according to the institutional committee on animal use (protocol 62/12).

2.2. Bacterial strains

Bacterial strains of *Escherichia coli* K12 strain 711, *E. coli* K12 strain HB101, and *Salmonella enterica* serovar Enteritidis ATCC 13076 were used. The strains were stored at a temperature of −80 °C in Brian Heart Infusion broth (BHI - Difco®) containing 20% glycerol (Merck®) until use.

2.3. Obtaining phagocytes

Initially, the animals received an intra-abdominal injection of a sterile 3% Sephadex G-50 Fine solution in 0.9% NaCl. Two days later, each animal was weighed, and samples of phagocytes were obtained by washing the abdominal cavity. Blood samples were collected to obtain serum and kept at −20 °C until use. Abdominal lavage was obtained by inoculating 20 mL of sterile RPMI-1640 medium (RPMI) into the abdominal cavity of the animals and counted in a hemacytometer. The cells were diluted to 1×10^6 cells/mL and incubated on coverslips for 30 min at 37 °C to allow adherence of phagocytic cells to the glass surface.

2.4. Evaluation of phagocytic activity

Monolayers of phagocytes, obtained as previously described, were co-incubated with 1×10^7/mL of *S. enterica* Enteritidis ATCC 13076 for 30 min in 5% CO_2 at 37 °C. The coverslips were washed with RPMI

for elimination of non-internalized bacteria, fixed with pure methanol for 20 min at room temperature (RT), and stained with Giemsa (Laborclin) at RT. The slides were assembled and observed under an optical microscope at 1000X magnification. A total of 20 fields were counted and then the percentage of macrophages containing bacteria was calculated.

2.5. Antibacterial activity of macrophages

In 24-well sterile plates, a solution of RPMI containing 1.5×10^6 bacteria of the abdominal lavage/mL and 1.5×10^7 of *S. enterica* serovar Enteritidis ATCC 13076/mL was incubated at 37 °C, 5% CO_2, for 1 h. The cells were then lysed with sterile distilled water, and aliquots were seeded on Mac Conkey agar (Oxoid®). After 18 h at 37 °C, the number of colony-forming units (CFU) was determined. The data are expressed in % of the reduction in the number of CFU.

2.6. Serum antibacterial activity in agar

A suspension of 1.5×10^8 bacteria/mL (*E. coli* K12 strain 711 and *E. coli* K12 strain HB101, 0.5 on the McFarland scale) was seeded on plates with Muller-Hinton agar (MH-Difco®). Posteriorly, 10 µL of the serum from each individual were added, and plates were incubated at 37 °C for 18-24 h. The presence or absence of inhibition halos was observed and measured (diameter in mm). The results are expressed in number of animals whose serum resulted in the formation of a halo of bacterial growth inhibition.

2.7. Serum resistance

For the serum resistance assay, *E. coli* K-12 strain 711 was cultured in Luria-Bertani broth (Difco®) for 24 h at 37 °C. Subsequently, the bacterial culture was diluted at 1:100 and grown to the log phase (OD 0.5). Volumes of 87.5 µL of the bacterial suspension were incubated with 50 µL of poultry serum at 37 °C. Optical density (OD) was determined at 630 nm at 0, 30, 60, 90, 120, 150, and 180 min of incubation. The result was expressed as the ratio of OD obtained after 30 min and zero min of incubation.

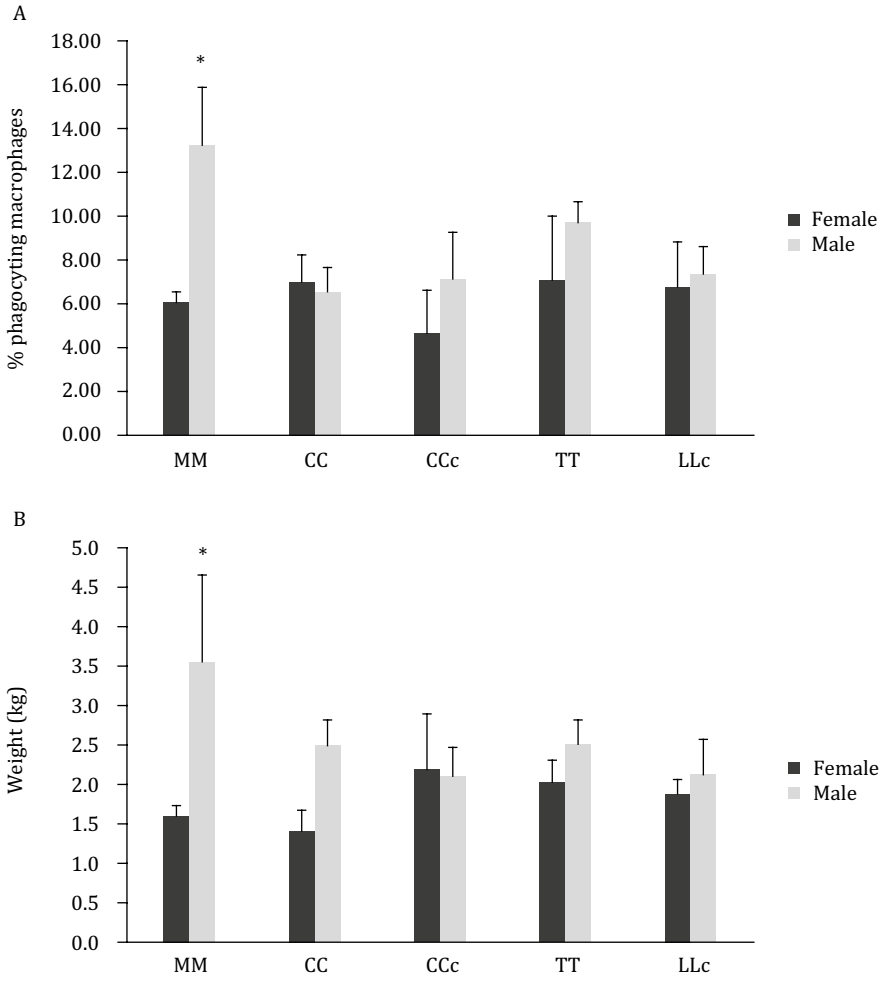
2.8. Statistical analysis

The Mann-Whitney test was used for comparisons between laying hens and broiler chickens, between TT and LLc lines, and between males and females of the same line. To compare the laying hen lines (MM, CC, and CCc), we used the Kruskal-Wallis test, followed by the Dunn test. Values of $P<0.05$ were considered significant. The software used for the analysis was GraphPad-Prism 5.0. For analysis of the antibacterial activity of the serum in agar, the logistic regression test was applied, for which the R program was used (R Core Team, 2013).

3. Results

3.1. Phagocytic and antibacterial activity of abdominal macrophages

Changes in reaction time and number of phagocytes can predispose a vertebrate host to diseases. The phagocytic activity assay aims to evaluate the capacity of macrophages of the studied lineages. The results showed that there were no significant differences in percentage of macrophages in the majority of the strains studied. Only males in the MM lineage presented a significantly higher number of phagocyting macrophages when compared with other strains, as well as in relation to the females of the same lineage (Figure 1A). This result is probably a consequence of the great weight that the animals reach (Figure 1B).



MM is a Rhode Island Red semi-heavy, brown egg laying hen line; CC is a laying hen originated from the White Leghorn breed. Both lineages have been under selection for egg production traits since the mid-1980s. CCc is a control lineage with the same genetic basis as CC but has not been selected since 1989; TT is a paternal broiler line developed and maintained by Embrapa under multitrait selection since 1992; and LLc is a paternal broiler control lineage maintained with no selection since the beginning of the 1980s.
Data are shown as mean ± standard deviation of the percentage of abdominal macrophages in phagocytosis and body weight in kg.
* P≤0.05.

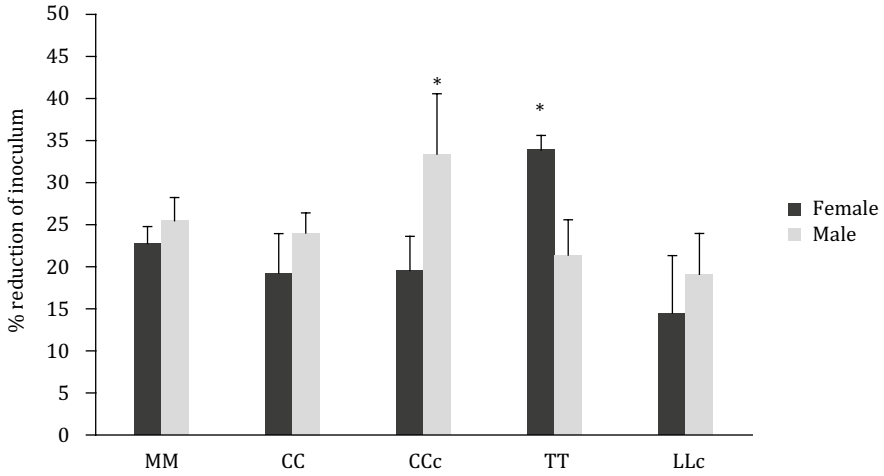
Figure 1 - Percentage of phagocyting macrophages (A) and weight of the studied strains (B).

Incubation of macrophages with bacteria from *Salmonella enterica* serovar Enteritidis ATCC 13076 was used to analyze the microbicidal action of abdominal macrophages. The results demonstrated a different profile from Figure 1A. The male CCc strain and female TT lineage eliminated more than 40% of the inoculum, while the other animals analyzed eliminated below 30% (Figure 2).

3.2. Antibacterial activity of serum in agar and serum resistance test

Analysis of the antibacterial activity of the serum resistance test showed that females of laying hen lineages demonstrated more intense antibacterial activity in the first 120 min of the test when compared with the broiler chicken lineages (Figure 3A). In serum, there were no significant differences when comparing males in different lines (Figure 3B). Furthermore, after 180 min of incubation, no significant differences were observed between the sera of any strains.

The evaluation assay of antimicrobial activity in agar demonstrated that the females of the laying hen lines (MM and CC) present a greater ability to inhibit bacterial growth when compared with males MM and CC. Males of all lineages demonstrated similar capacity when compared to each other (Table 1).



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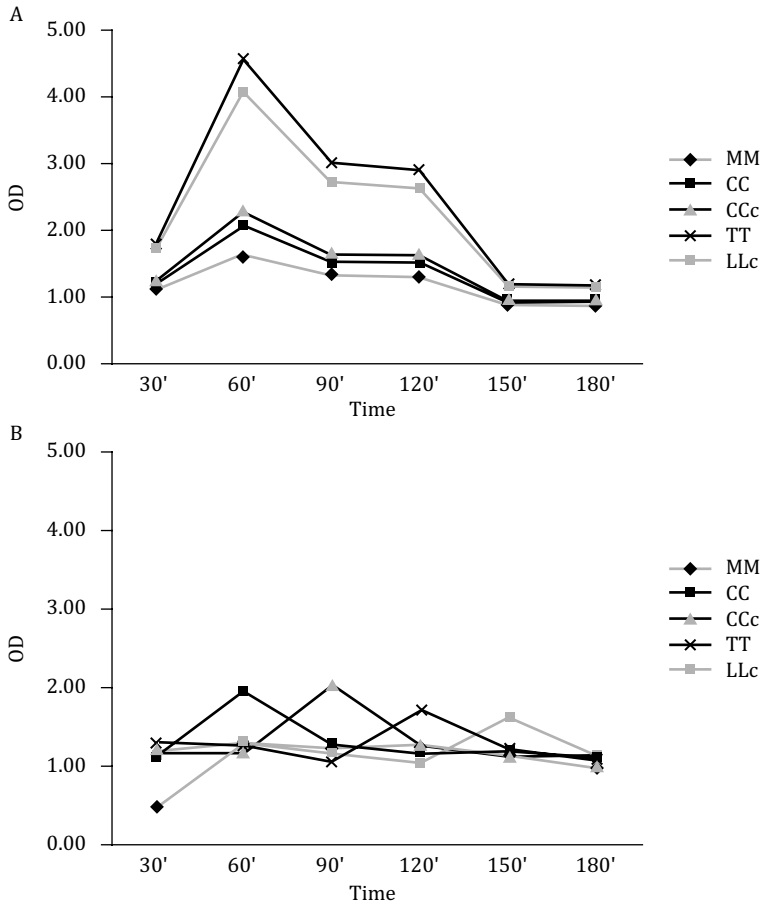
Data are shown as mean ± standard deviation of % reduction in the number of colony-forming units of *Salmonella enterica* Enteritidis ATCC 13076 post exposure to abdominal macrophages.
* P≤0.05

Figure 2 - Antibacterial activity of abdominal macrophages.



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Data are shown as mean of the ratio optical density (OD) at 30, 60, 90, 120, 150, or 180 min/OD initial.

Figure 3 - Serum resistance assay of females (A) and males (B).

Table 1 - Antibacterial activity of the serum in agar[1]

Line	Sex	n	*E. coli* K12 strain 711	*E. coli* K12 strain HB101
MM	Female	5	3	5
	Male	5	0	0
CC	Female	5	3	4
	Male	5	0	0
CCc	Female	5	1	2
	Male	5	1	1
TT	Female	5	0	0
	Male	5	0	0
LLc	Female	5	1	1
	Male	5	2	2

MM is a Rhode Island Red semi-heavy, brown egg laying hen line; CC is a laying hen originated from the White Leghorn breed. Both lineages have been under selection for egg production traits since the mid-1980s. CCc is a control lineage with the same genetic basis as CC but has not been selected since 1989; TT is a paternal broiler line developed and maintained by Embrapa under multitrait selection since 1992; and LLc is a paternal broiler control lineage maintained with no selection since the beginning of the 1980s.
n - number of animals per lineage.
[1] The results are expressed as the number of animals of the MM, CC, CCc, TT, and LLc lines, whose sera are capable of inhibiting bacterial growth of *E. coli* K12 711 and *E. coli* K12 HB101 strains.

4. Discussion

The early stage of infection is an important factor for disease resistance or susceptibility (Swaggerty et al., 2019), and the first line of defense against pathogens is innate immunity, which may limit or prevent the infectious process (Kaiser, 2010). Innate immunity depends on several factors, such as the antimicrobial activity of tissue macrophages and the complement system. Macrophages are innate immunity cells capable of recognizing, phagocytizing, and destroying microorganisms (Kaiser, 2010).

In this study, the number of abdominal macrophages did not differ significantly among lineages as described by Cheema et al. (2003). However, we observed that males of the MM lineage presented a significantly higher number of abdominal macrophages than females, probably due to large weight of these animals. As far as we know, there is no study on chickens showing a relationship between sex and number of macrophages.

The phagocytosis process does not always lead to the death of the pathogen. The ability of the macrophage to kill a microorganism is assessed by its microbicidal activity. Loyola et al. (2002) demonstrated that some strains of microorganisms might be more resistant to microbicide mechanisms of macrophages. The invading microorganisms are trapped in phagosomes, which fuse with lysosomes. However, there are certain avian bacteria, such as *Staphylococcus aureus* and *Pasteurella multocida*, that are resistant to phagocytosis by macrophages (Qureshi, 2003).

In this work, we compared the microbicidal activity of macrophages of lineages and showed a reduction below 30% in all lineages. However, males of the CCc lineage and females of the TT lineage have greater microbicidal capacity, eliminating more than 40% of the inoculum. Although there are studies comparing phagocytic capacity in different chicken lineages (Cheema et al., 2003; Guimarães et al., 2011), this is the first one that observed this difference between males and females of the same lineage.

The complement system is one of the main effector mechanisms of innate and humoral immunity. It is activated by the presence of the microorganism and results in the destruction of the pathogen through activation of the inflammatory response, by facilitating phagocytosis or by direct lysis (Bianchini et al., 2009; Shokal and Eleftherianos, 2017). Therefore, one of the main mechanisms of virulence of bacteria is the ability to resist the lytic action of the complement system (Barbieri et al., 2015). Serum resistance is related to virulence factors that lead to a failure in the action of the complement system, which may be the result of activation blockade or non-formation of the membrane attack complex (Binns et al., 1982). Bacteria isolated from chickens that developed sepsis generally have resistance to the complement

system, while bacteria isolated from healthy animals are sensitive (Barbieri et al., 2015). In the present study, the results demonstrate that the lines have difference in the action of the complement system (Baelmans et al., 2005).

In the serum resistance test, it was observed that the serum obtained from laying hens had a more efficient action when compared with the serum from broiler chickens after 30 min of incubation. This result was influenced by serum of animals of the MM line, which demonstrated faster complementary activity than the serum of animals from the other laying hen lines. Whether this faster activity is associated with variations in genes already related to complement system activity, such as IL-17A and MHC (Biscarini et al., 2010), is something to be investigated in future studies.

Regarding the antibacterial activity of serum, another influencing factor was the sex. Our results showed that, in the initial part of the experiment, males presented a larger action of the complement system than females of the same lines. On the other hand, in the final part of the experiment, females of the MM and CC laying hen lines presented higher antibacterial activity than males of the same lines. These results show that there are significant differences in complement system activity according to the sex of the animals. However, our results are different from previous studies that failed to find significant differences in complement system activity when the sex of the animal was considered (Stewart et al., 1985). This is probably due to the significant methodological differences between studies.

5. Conclusions

The results obtained in the present study suggest that the innate immune response in poultry may be influenced by genetic background. In particular, it is important to observe that the MM line presents higher number of macrophages than the other analyzed lines, but have not demonstrated the same ability to kill microorganisms. Furthermore, sex is a factor that impacts the complement system-mediated response. Regarding the importance of the complement system for immune response in poultry, the identification of genetic characteristics associated with differences in the complement system activity observed in this study may contribute to a better understanding of the innate immune response in poultry and to the development of new chicken lines resistant to pathogens.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: M.C. Ledur, W. Loyola and E.J. Venancio. Data curation: W. Loyola and E.J. Venancio. Formal analysis: M.C. Silva, G. Nakazato, W. Loyola and E.J. Venancio. Funding acquisition: M.C. Ledur and E.J. Venancio. Investigation: M.C. Silva, I. Conchon-Costa, G. Nakazato, J.W. Pinheiro, M.C. Ledur and E.J. Venancio. Methodology: M.C. Silva, I. Conchon-Costa, G. Nakazato, J.W. Pinheiro, W. Loyola and E.J. Venancio. Project administration: W. Loyola and E.J. Venancio. Resources: M.C. Ledur and E.J. Venancio. Supervision: W. Loyola and E.J. Venancio. Validation: M.C. Silva, I. Conchon-Costa, G. Nakazato and J.W. Pinheiro. Visualization: M.C. Silva. Writing-original draft: M.C. Silva, W. Loyola and E.J. Venancio. Writing-review & editing: M.C. Silva, M.C. Ledur, W. Loyola and E.J. Venancio.

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