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Association between *in vitro* heterophil function and the feathering gene in commercial broiler chickens

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We recently showed that *in vitro* heterophil functional efficiency in commercial broiler chickens is genetically controlled and may be a sex-associated trait. To further characterize the genetic mechanism(s) of heterophil functional efficiency, we wanted to determine whether the feathering gene, present on the Z sex chromosome, contributes to heterophil functional efficiency. Heterophils from two pairs of broiler lines were evaluated; each pair contained a fast feather (FF) (lines A and X) and a slow feather (SF) line (lines B and Y). On days 1 and 4 post-hatch, heterophils isolated from two sets of pure line broilers (A and B, and X and Y) were evaluated for their ability to (1) phagocytize *Salmonella enteritidis*, and (2) exhibit bactericidal activity against *S. enteritidis*. On days 1 and 4 post-hatch, heterophils isolated from the FF lines were statistically ($P \leq 0.02$) more proficient at phagocytizing *S. enteritidis* than heterophils from SF lines. Bactericidal activity was also statistically ($p \leq 0.02$) greater on day 1 post-hatch in the heterophils isolated from FF lines compared to heterophils isolated from SF lines. These data indicate that the presence of the FF gene locus on the Z sex chromosome contributes to heterophil function and may contribute to the early innate immune competence of a flock.

Introduction

Heterophils, the primary polymorphonuclear leukocyte in chickens, are the avian counterpart to mammalian neutrophils, and function by ingesting and killing a variety of microbial pathogens within 30 min (Burton & Harrison, 1969; Maxwell & Robertson, 1998). These professional phagocytic cells are granular in appearance and exhibit an assortment of cytoskeletal and biochemical activities that can be easily assayed *in vitro* to evaluate functional efficiency. Functionally, heterophils rapidly kill bacterial pathogens by phagocytosing, degranulating and generating an oxidative burst (Desmidt *et al.*, 1996; Genovese *et al.*, 2000; Kogut *et al.*, 2001; Swaggerty *et al.*, 2003). Because of their early response and their ability to kill pathogens, heterophils are likely to be a useful tool for evaluating the innate immune response in lines of chickens.

Selection of birds based on rapid growth characteristics can adversely affect disease resistance and/or the immune response (Han & Smyth, 1972; Bayyari *et al.*, 1997). Instead of identifying birds resistant to a single pathogen, our laboratory is interested in identifying markers that will indicate which lines have the potential to mount the most effective immune response against multiple microorganisms. We recently showed that there are differences in *in vitro* heterophil functional efficiency between two distinct pure parental lines of

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broilers and between the reciprocal F1 crosses (Swaggerty *et al.*, 2003).

The feathering gene locus (*K*) is located on the Z sex chromosome and is a dominant sex-linked allele responsible for the slow feather growth in chickens, while the fast feather (FF), k^+, phenotype is the wild-type recessive trait (Bitgood, 1993; Chambers *et al.*, 1994). There is evidence to indicate slow feathering (SF) is a result of the incorporation of an endogenous retrovirus (EV) into the genome and that the feathering and *ev*21 genes are tightly linked (Bacon *et al.*, 1988; Levin & Smith, 1990). Genes that affect the feathering rate have been identified in Leghorns (Levin & Smith, 1990), broilers (Sabour *et al.*, 1992), turkeys (Asmundson & Abbott, 1961), and guinea fowl (Pal & Singh, 1997). There is evidence to suggest EV incorporations can adversely affect the immune competence of chickens. Furthermore, chicks hatched from EV-infected dams had a compromised immune response against avian leukosis virus (Smith & Crittenden, 1998).

The objective of this project was to compare *in vitro* heterophil functional activity between two pairs of broilers from different commercial providers within the first 4 days post-hatch to determine whether the feathering gene contributes to heterophil functional efficiency. Each pair of lines consisted of a FF and a SF line. Heterophils isolated on days 1 and 4 post-hatch were assayed for the ability to (1) phagocytize *Salmonella enteritidis*, and (2) exhibit bactericidal activity against *S. enteritidis*.

Materials and Methods

Experimental birds

Pedigree broiler chicks used in this study were obtained from two commercial breeders and were arbitrarily coded to maintain confidentiality. The first pair of broiler lines are designated A and B, and are FF and SF, respectively. The second pair of broiler lines are designated X and Y, and are FF and SF, respectively. Fertilized eggs were set in incubators (G.Q.F. Manufacturing Company, Savannah, GA, USA or Jamesway Incubator Company, Inc., Ontario, Canada) and maintained at wet and dry bulb temperatures of 32.2 and 37.8°C, respectively. After 10 days of incubation, the eggs were candled; non-fertile and non-viable eggs were discarded. The viable eggs were returned to the incubator until day 18, when they were transferred to hatchers (Humidaire Incubator Company, New Madison, OH, USA) and maintained under the same temperature and humidity conditions until day of hatch. Upon hatching, 200 birds per line were placed in their respective floor pens (10 feet × 10 feet) containing wood shavings, provided supplemental heat, water, and a balanced unmedicated corn and soybean meal based diet *ad libitum*. The feed was calculated to contain 23% protein and 3200 kcal metabolizable energy/kg diet, and all other nutrient rations met or exceeded the standards established by the National Research Council (1994).

Experimental design

On days 1 and 4 post-hatch, 90 and 45 birds, respectively, per line were used as blood donors. The blood from each day was pooled for the line to provide sufficient numbers of heterophils to run both assays on a given day to reduce variability. Each assay was conducted three separate times in replicates of four to eight, with pooled heterophils from the different lines of birds. The data from the repeated experiments were pooled for presentation and statistical analyses.

Isolation of peripheral blood heterophils

Heterophils were isolated from the peripheral blood of chicks on days 1 and 4 post-hatch as previously described (Kogut *et al.*, 2001). Briefly, blood from chicks was collected in tubes containing disodium ethylenediamine tetraacetic acid (EDTA) and mixed thoroughly. The blood and EDTA was pooled and diluted 1:1 with RPMI 1640 media containing 1% methylcellulose, and was centrifuged at 25 *g* for 15 min. The supernatant was transferred to a new conical tube and diluted with Ca^{2+}-free and Mg^{2+}-free Hanks balanced salt solution (1:1) and layered onto discontinuous Histopaque® gradients (specific gravity 1.077 over 1.119). The gradients were centrifuged at 250 *g* for 1 h. The histopaque layers were collected, and washed with RPMI 1640 (1:1), and the cells resuspended in fresh RPMI 1640, counted on a haemocytometer, and diluted to the desired concentration in RPMI. All tissue culture reagents and chemicals, including RPMI 1640, Hanks balanced salt solution, methylcellulose, Histopaque® 1119, and Histopaque® 1077, were obtained from Sigma Chemical Company (St Louis, MO, USA).

Bacteria preparation

A poultry isolate of *S. enteritidis* (97-11771) was obtained from the National Veterinary Services Laboratory (Ames, IA, USA). *S. enteritidis* was cultured in tryptic soy broth (TSB; Difco Laboratories, Becton Dickinson Co., Sparks, MD, USA) overnight at 41°C. The bacteria were pelleted (7700 *g*, 10 min), washed with cold phosphate-buffered saline (PBS) and centrifuged again (7700 *g*, 10 min). The supernatant was discarded and the pellet was resuspended in 1 ml cold PBS. A stock solution of *S. enteritidis* (1×10^9 colony forming units (CFU)/ml) was prepared in PBS. The bacterial concentration was determined spectrophotometrically (Spectronic 20D spectrophotometer; Milton Roy Co., Golden, CO, USA) using a standard curve with a reference wavelength of 625 nm. *S. enteritidis* were prepared fresh for each experiment and kept on ice until used.

Phagocytosis of S. enteritidis

Heterophils were assayed for the ability to phagocytize live *S. enteritidis* as previously described with the following modifications (Wigley *et al.*, 1999). Heterophils (2 ml) were diluted to 5×10^6 cells/ml in RPMI. *S. enteritidis* (2 ml of 5×10^7 CFU/ml) were added to the diluted heterophils (10 *S. enteritidis*:1 heterophil) and centrifuged at 2000 *g* for 15 min to promote contact between the heterophils and the *S. enteritidis*. The pelleted heterophils and *S. enteritidis* were then incubated for 1 h at 39°C to allow for phagocytosis, and then submerged in ice for 15 min to stop the reaction. The samples were washed with an equal volume of ice-cold RPMI and centrifuged at 2000 *g* for 15 min. The pellets were resuspended in ice-cold gentamicin (100 μg/ml prepared in RPMI; Sigma) and incubated for 1 h at 37°C on a rotary shaker. Heterophils were washed three times with ice-cold RPMI (3–5 ml per wash and centrifuged at 2000 *g* for 15 min), and the pellet was re-suspended in the original volume of media. Cytospin smears were prepared from each sample in a Shandon cytospin3 (Shandon Inc., Pittsburgh, PA, USA), stained with Hematology 3-step stain (Biochemical Sciences, Inc., Swedesboro, NJ, USA), and examined by light microscopy with an oil immersion objective (100 ×). Results were recorded as the phagocytic index: PI = percent heterophils containing *S. enteritidis* × the average number of *S. enteritidis* per heterophil × 100.

Bactericidal assay

The bactericidal activity of heterophils isolated from the different lines was evaluated as previously described (Stevens & Olsen, 1993) with the following modifications. Briefly, heterophils were diluted to 2×10^6 cells/ml in RPMI without phenol red. Heterophils, *S. enteritidis*, and heterophils plus *S. enteritidis* were plated onto a flat-bottomed 96-well plate and centrifuged at 400 *g* for 5 min at 4°C. The plate was incubated at 41°C for 30 min. Heterophils were lysed by incubation with 50 μl of 0.1% Triton X-100 (Sigma) for an additional 45 min at room

temperature on a shaker. Upon completion of the lysis, 50 µl co-enzyme Q (2.3-dimethoxy-5-methyl-1,4-benzoquinone)-XTT (2,3-bis [2-methooxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt; Sigma) was added to the plate and incubated for 30 min at 41°C. The OD (optical density) of each well in the plate was read at 450 nm on a Spectra MAX 340PC plate reader (Molecular Devices Corp., Sunnyvale, CA, USA). Bactericidal activity of heterophils isolated from each line of broilers was determined by comparison with a standard curve generated by four known concentrations of *S. enteritidis* (5×10^5 to 5×10^6 CFU). Results are expressed as the percent bactericidal activity.

Statistical analyses

Statistical analyses were performed using Microsoft® Excel 2000 version (Microsoft Corporation, 2000). Pairs of FF and SF lines were compared using Student's *t* test. All statistical analyses are based on comparisons between each pair (A and B or X and Y). No analyses were performed between the two pairs from different breeding companies.

Results

Phagocytosis of S. enteritidis

Heterophils isolated from lines A and B or lines X and Y were evaluated for their ability to phagocytize *S. enteritidis* on days 1 and 4 post-hatch (Table 1). At day 1 post-hatch, the PI of heterophils isolated from FF line A was significantly ($P \leq 0.02$) higher than the PI for heterophils isolated from the SF line B (601.0 and 410.7, respectively). Also on day 1 post-hatch, the heterophils isolated from the FF line X had a significantly ($P \leq 0.02$) higher PI when compared with heterophils isolated from the SF line Y (747.2 and 454.7, respectively). By 4 days post-hatch the heterophils isolated from the FF lines in each pair were still significantly ($P \leq 0.02$) higher than the PI for the heterophils isolated from the corresponding SF pairs. On day 4 post-hatch, the PI for lines A and B were 1094.7 and 737.5, and were 1081.1 and 733.7 for lines X and Y, respectively.

Table 1. *Phagocytosis of* S. enteritidis *by heterophils isolated from two pairs of FF and SF lines of broilers*

Line	Feather type	% heterophils with *S. enteritidis*	Average number of *S. enteritidis* per heterophil	Phagocytic index
Day 1 post hatch				
A	FF	72.9 ± 1.5	8.3 ± 0.3^{A}	601.0 ± 24.0^{A}
B	SF	70.8 ± 1.3	5.9 ± 0.5	410.7 ± 30.8
X	FF	76.9 ± 2.1^{A}	9.7 ± 0.4^{A}	747.2 ± 37.0^{A}
Y	SF	63.4 ± 2.1	7.3 ± 0.5	454.7 ± 42.7
Day 4 post hatch				
A	FF	84.5 ± 1.1^{A}	13.0 ± 0.2^{A}	1094.7 ± 24.6^{A}
B	SF	78.9 ± 1.4	9.4 ± 0.2	737.5 ± 14.3
X	FF	82.1 ± 1.1	13.2 ± 0.8^{A}	1081.1 ± 63.3^{A}
Y	SF	80.0 ± 2.5	9.2 ± 0.2	733.7 ± 17.7

Data expressed as mean ± standard error of the mean. Significant differences between the FF and SF lines for each pair (lines A and B or lines X and Y) are indicated by a superscript letter. No comparisons were made between the two FF lines nor between the two SF lines.

In addition to the calculated PI on days 1 and 4 post-hatch, heterophils from both FF lines had phagocytized significantly ($P \leq 0.02$) more *S. enteritidis* per cell when compared with the heterophils isolated from the SF lines (Table 1). On day 1 post-hatch there were no significant differences between the percentage of heterophils from lines A and B that phagocytosed *S. enteritidis*. However, there was a significant ($P \leq 0.02$) difference observed on day 4 post-hatch between heterophils isolated from lines A and B (84.5 and 78.9%, respectively). There was also a significant ($P \leq 0.02$) difference in the percentage of heterophils isolated from line X (76.9%) and from line Y (63.4%) that phagocytized *S. enteritidis* on day 1 post-hatch (Table 1). There were no significant differences observed on day 4 post-hatch in the percentage of heterophils from line X and line Y that phagocytized *S. enteritidis*.

Bactericidal activity

The bactericidal activity of the isolated heterophils was examined and the data are presented as percent bactericidal activity in Table 2. On days 1 and 4 post-hatch, heterophils isolated from the FF line A killed significantly ($P \leq 0.02$) more *S. enteritidis* than heterophils from the SF line B (day 1 = 68.7% and 46.7%, respectively; day 4 = 59.2% and 41.6%, respectively). A similar trend was observed on day 1 post-hatch between the heterophils isolated from the other FF and SF lines, X and Y. On day 1 post-hatch, heterophils from line X killed 71.5% of the *S. enteritidis* while heterophils isolated from line Y killed 56.7% ($P \leq 0.02$). However, by day 4 post-hatch, there were no statistical differences in bactericidal activity between heterophils isolated from lines X and Y (Table 2).

Table 2. *Bactericidal activity of heterophils isolated from two pairs of FF and SF lines of broilers*

Line	Feather type	% Bactericidal activity
Day 1 post hatch		
A	FF	68.7 ± 2.6^{A}
B	SF	46.7 ± 2.4
X	FF	71.5 ± 3.3^{A}
Y	SF	56.7 ± 3.1
Day 4 post hatch		
A	FF	59.2 ± 2.8^{A}
B	SF	41.6 ± 2.4
X	FF	56.7 ± 3.1
Y	SF	55.3 ± 4.2

Data expressed as mean ± standard error of the mean. Significant differences between the FF and SF lines for each pair (lines A and B or lines X and Y) are indicated by a superscript. No comparisons were made between the two FF lines or between the two SF lines.

Discussion

We recently showed that *in vitro* heterophil functional efficiency in commercial broiler chickens is genetically controlled and may be a sex-associated trait (Swaggerty *et al.*, 2003). To localize which region of the sex chromosome is involved in heterophil functional efficiency, we evaluated the feathering gene, present on the Z sex chromosome, and its effects on heterophil functional efficiency. The present study confirms *in vitro* heterophil functional efficiency can be attributed, in part, to the feathering gene located on chromosome 5. There is evidence to suggest that chromosome 5 contributes to the immune competence of chickens (Mariani *et al.*, 2001; Wigley *et al.*, 2002). A recent study identified the *SAL1* gene on chromosome 5 and showed that it was involved in conferring resistance to *Salmonella* infections in chickens (Mariani *et al.*, 2001). Furthermore, the presence of the *SAL1* locus accompanied by clearance of bacteria by macrophages correlates with resistance to *Salmonella* infections in chickens (Wigley *et al.*, 2002). Earlier work by our laboratory has shown the importance of the avian heterophil in responses to *Salmonella* infections in young chickens and turkeys (Kogut *et al.*, 1994a,b; Genovese *et al.*, 1998).

In the present studies, we obtained two different pairs of fast feathering and slow feathering lines to ensure the results were representative of FF or SF lines in general and were not an uncharacteristic finding observed in the initial lines obtained from the commercial provider. Based on the increased heterophil functional efficiency observed *in vitro*, the FF lines are more likely to control *Salmonella* infections at hatch. However, to ascertain the practical relevance of our findings to the poultry industry, *in vivo* challenge studies with *Salmonella* or other pathogenic microorganisms will need to be conducted in distinct FF and SF lines to determine whether the feathering gene contributes to the resistance and/or susceptibility in these two types of lines.

In the present study, the phagocytosis results provide the most convincing data to suggest that FF lines have a more efficient innate immune response compared with the SF lines. Heterophils from the FF lines consistently phagocytosed more *S. enteritidis* than heterophils from the SF lines. The phagocytosis data combined with the increased bactericidal activity of the heterophils from the FF lines strongly indicates a much more efficient innate immune response immediately post-hatch when compared with heterophils from birds of the SF lines. This is significant in that the innate immune response is critical in establishing the overall immune competence of the bird. The measurable differences in the ability of heterophils from FF and SF lines to phagocytize bacteria lends itself as a functional marker that can be used to evaluate commercial lines and screen for an increased innate immune response. Currently, the mechanism(s) involved in the difference in phagocytosis has not been fully explained in these lines of birds. Therefore, additional studies to dissect the mechanism(s) of phagocytosis should be considered.

The information gained from this study implies a role for using immunologically competent birds whose heterophils have an increased ability to phagocytize and kill bacteria. Selection for lines that have efficient heterophil function (or genetic marker(s) of said function) may, in turn, select for birds with a strong innate immune response. An effective heterophil and/or innate immune response will probably allow birds to eliminate or limit an infection within the first week post hatch and to direct the acquired immune response to elicit the appropriate secondary immune response. Evaluation of heterophils isolated from commercial FF lines for an innate immune response marker such as *in vitro* phagocytosis or bactericidal activity may be advantageous compared with further genetic selection for resistance against a single pathogen. Broilers with a reduced mortality rate to *Salmonella* infections shed bacteria for longer periods of time (Janss & Bolder, 2000), thereby increasing the probability of transmission to the entire flock. Selecting birds that are resistant to a single pathogen has proven to be complicated and ineffective, so it may prove beneficial for poultry breeders to select birds that have a generally efficient immune response.

The data presented herein indicate the presence of the feathering gene influences *in vitro* heterophil functional efficacy. Specifically, the data suggests the presence of the FF gene may enhance heterophil functional efficiency while the SF gene reduces the functional efficiency of isolated heterophils. Taken together these data suggest the feathering gene contributes, in part, to heterophil functional efficiency and may influence the early innate immune competence of a flock. By selecting birds with an efficient innate immune response, the primary breeder would probably select birds that are more equipped to mount an effective acquired immune response and to better control pathogens (Fearon & Locksley, 1996; Medzhitov & Janeway, 1997).

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RÉSUMÉ

Association entre la fonction hétérophile *in vitro* et le gène de l'emplumement chez les poulets de chair commerciaux

Récemment, nous avons montré que chez les poulets de chair commerciaux l'efficacité de la fonction hétérophile *in vitro* était contrôlée génétiquement et pouvait être associée au sexe. Pour caractériser davantage le mécanisme génétique de l'efficacité de la fonction hétérophile, nous avons voulu savoir si le gène de l'emplumement présent sur le chromosome sexuel Z contribue à l'efficacité de la fonction hétérophile. Les hétérophiles appartenant à deux paires de lignées de poulet ont été évalués, chaque paire contenait les lignées A et X à emplumement rapide (FF) et les lignées B et Y à emplumement lent (SF). Aux âges d'un et 4 jours après l'éclosion, les hétérophiles isolés des deux couples de lignées pures de poulet (A et B, X et Y) ont été évalués pour leur aptitude à: (1) la phagocytose de *Salmonella* enteritidis (SE) et (2) l'activité bactéricide vis-à-vis de SE. Aux âges d'un et 4 jours après l'éclosion, les hétérophiles isolés des lignées FF se sont avérés statistiquement plus compétents pour la phagocytose de SE que les hétérophiles des lignées SF ($P \leq 0.02$). L'activité bactéricide a également été statistiquement supérieure ($P \leq 0.02$) à l'âge d'un jour pour les hétérophiles des lignées FF comparés à ceux des lignées SF. Ces données montrent que la présence du locus du gène FF sur le chromosome sexuel Z contribue à la fonction hétérophile et peut contribuer à la compétence immune innée et précoce d'un troupeau.

ZUSAMMENFASSUNG

Verbindung zwischen der *in vitro*-Funktion von Heterophilen und dem Befiederungsgen in kommerziellen Broilern

Vor kurzem konnten wir aufzeigen, dass die funktionelle *in vitro*-Fähigkeit von Heterophilen in kommerziellen Broilern genetisch kontrolliert wird und dass sie vermutlich eine geschlecchtsgebundene Eigenschaft ist. Zur weiteren Charakterisierung der genetischen Mechanismen der Funktion von Heterophilen wollten wir feststellen, ob das Befiederungsgen, das sich auf dem Z-Geschlechtschromosom befindet, an der funktionellen Fähigkeit der Heterophilen beteiligt ist. Heterophile von zwei Paaren aus verschiedenen Broiler-Zuchtlinien wurden untersucht; jedes Paar bestand aus einer schnell befiedernden (FF) (Linien A und X) und einer langsam befiedernden (SF) (Linien B und Y) Zuchtlinie. Am 1. und 4. Tag nach dem Schlupf wurden Heterophile, die aus zwei Paaren reinliniger Broiler (A und B; X und Y) isoliert wurden, auf ihre Fähigkeit untersucht: (1) *Salmonella enteritidis* (SE) zu phagozytieren und (2) bakterizide Aktivität gegen SE zu zeigen. Am 1. und 4. Tag nach dem Schlupf wiesen die aus den FF-Linien isolierten Heterophilen eine signifikant ($p \leq 0.02$) höhere Phagozytose-

rate auf als die Heterophilen aus den SF-Linien. Ebenso war die bakterizide Aktivität der aus den FF-Linien isolierten Heterophilen verglichen mit den Heterophilen aus den SF-Linien am 1. Tag nach dem Schlupf signikant ($p \leq 0.02$) stärker. Diese Ergebnisse weisen daraufhin, dass die Präsenz des FF-Genlokus auf dem Z-Geschlechtschromosom die Funktion der Heterophilen mitbeeinflusst und dass sie zur frühen angeborenen Immunkompetenz einer Herde beiträgt.

RESUMEN

Asociación entre la funcionalidad de los heterófilos *in vitro* y el gen de plumaje en pollos de engorde comerciales

Recientemente demostramos que la eficencia funcional de los heterófilos *in vitro* en pollos de engorde comerciales estaba controlada genéticamente y que podía ser un rasgo asociado al sexo. Para caracterizar mejor el mecanismo genético de la eficacia funcional de los heterófilos se quiso determinar si el gen de plumaje, presente en el cromosoma Z del sexo, contribuye a la eficiencia funcional de los heterófilos. Se evaluaron los heterófilos de dos líneas de pollos de engorde, cada par contenía una línea de plumaje rápido (FF) (líneas A y X) y una línea de plumaje lento (SF) (líneas B e Y). A los días 1 y 4 tras el nacimiento, los heterófilos aislados de los dos lotes de líneas puras de pollos de engorde (A y B; X e Y) fueron evaluados para determinar su capacidad para: (1) fagocitar *Salmonella enteritidis* (SE) y (2) exhibir actividad bactericidad frente a SE. A los días 1 y 4 tras el nacimiento, los heterófilos aislados de las líneas FF fueron estadísticamente ($p \leq 0.02$) más eficientes en la fagocitosis de SE que los heterófilos de las líneas SF. La actividad bactericida fue también estadísticamente ($p \leq 0.02$) mayor el día 1 tras el nacimiento en los heterófilos aislados de las líneas FF en comparación con los heterófilos aislados de las líneas SF. Estos datos indican que la presencia del locus del gen FF en el cromosoma Z del sexo contribuye a la funcionalidad de los heterófilos y puede contribuir a la inmunocompetencia temprana en un lote.