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Effects of a low crude protein diet with and without *Spirulina platensis* inclusion on the concentrations and proportions of circulating immune cells in broilers

Honors Thesis

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Table of Contents

Abstract:……………………………………………………………………………………4

Introduction:………………………………………………………………………………..5

Literature Review:…………………………………………………………………………7

Blood Leukocytes:……………………………………………………………………..7

Blood Lymphocytes:……………………………………………………………………..9

Materials and Methods:…………………………………………………………………..11

Animals and Treatment:…………………………………………………..…...11

Blood Sampling and Analysis:…………………………………………………………12

Lymphocyte Subpopulation Analysis by Immunofluorescent Staining

and Flow Cytometry:……………………………………………………………...13

Statistical Analysis:……………………………………………………………………..16

Results:………………………………………………………………………………………..16

Cell-Dyn Automated Hematology:……………………………………………...…..16

Concentration and Proportions among WBC Populations:…………………………17

Flow Cytometry:…………………………………………………….………………17

Discussion:……………………………………………………………………………..…..17

References:………………………………………………………………………..……….21

Tables and Figures:………………………………………………………………………....27

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Abstract

Immune system health is an important aspect of broiler production, especially with the reduction of the use of antibiotics. Nutritional aspects, like crude protein percentage, and natural alternatives to antibiotics are being investigated to see the effect they have on immunity. *Spirulina* is an alternative proteinaceous ingredient that producers are looking into, not only due to its high nutritional value, but also its immune boosting properties. The purpose of this study was to determine the effects of a low crude protein diet (LCP) and a *Spirulina*-supplemented low crude protein (LCP-SP) diet on broiler blood cell measurements, including, concentrations and proportions of individual white blood cell populations and various lymphocyte subsets. Ross 708 1-day old male broilers were assigned to one of three diet treatments: a standard crude protein diet (SCP), the LCP diet, and the LCP-SP diet, with 5 pens per dietary treatment. When the chickens were 37 days of age, heparinized blood samples were used to determine the concentrations of white bloods cells (WBC), thrombocytes, red blood cells (RBC), hemoglobin, and hematocrit by Cell-Dyn automated hematology analysis, prepare and Wright-stain blood smears for leukocyte counting by microscopy, and conduct immunofluorescent staining of peripheral blood mononuclear cells for lymphocytes population analysis by flow cytometry. Except for monocytes, none of the blood cell measurements conducted were affected ($P > 0.05$) by diet. The LCP diet resulted in increased ($P \leq 0.05$) monocyte concentration and proportion (% of WBC) compared to the standard CP diet, indicating heightened inflammatory activity with lower dietary protein content. The LCP-SP reversed the effect of the LCP diet, resulting in monocyte concentrations and proportions not different ($P > 0.05$) from those of the SCP diet. Natural feed additives can reverse the effects that low crude protein has on immunity in broilers, which is a promising sign for lowering feed costs without impacting the health of the bird.

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Introduction

Immune system health in broiler production is becoming increasingly important as producers move away from the use of antibiotics. In January of 2017, the United States Food and Drug Administration (FDA) removed all clearances for the use of medically important antibiotics for growth promotion [Smith, 2019]. This was in response to a growing emergence and spread in antimicrobial resistance due to the use of antimicrobials in broiler production [Roth et al., 2019]. In addition, there is a growing popularity in the marketing strategy that severely limits or entirely prohibits the use of antibiotics [Smith, 2019]. In 2014, sales of antibiotic-free chicken rose by 34% in the United States [Mainstream, 2015]. In April 2015, Tyson Foods announced it would no longer use human antibiotics to raise chickens by September 2017 [Mainstream, 2015]. Pilgrim's Pride also announced it would raise 25% of its birds without routine use of antibiotics by the end of 2018 [Mainstream, 2015]. NRDC estimates that more than one-third of the United States chicken industry has eliminated or pledged to eliminate routine use of antibiotics [Mainstream, 2015]. In the article, Nearly 60% of US broilers now raised without antibiotics, but that number may have peaked, in 2019 the number of broilers raised without antibiotics increased to 60% [2020].

With this decrease in antibiotic use, producers are starting to look for feed additives to use as replacements [Demir et al., 2003]. Phytogenic feed additives (PFAs), additives derived from plants, herbs, and spices, have been shown to be a possible alternative to antibiotics [Abou-Elkhair et al., 2014]. PFAs have been shown to improve innate immunity, host disease resistance, and antioxidative status [Pirgozliev et al., 2019]. There have been studies that showed that diets supplemented with black pepper and coriander seeds enhance the health status of broilers [Abou-Elkhair et al., 2014]. With more data showing the benefits of PFAs, research is

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looking for more possibilities for new and better plants to use as feed additives to help with immunity.

Protein is one of the more expensive components of broiler production, and producers are looking to see if they can cut cost by reducing protein in diets or find alternative protein sources. A risk of cutting down protein content in feed is that it potentially has damaging effects the on the bird's immune system, even if the reduced protein diets are formulated to maintain a normal digestible amino acid content. Studies have shown that broilers fed diets that are deficient in amino acids had reduced primary antibody responses compared to broilers fed a typical diet [Kidd, 2004]. Further research on protein deficiency showed that it decreased lymphocyte numbers and overall white blood cell numbers [Kidd, 2004].

One feed additive that poultry researchers are examining is *Spirulina (Arthrospira) platensis*. *Spirulina* is a blue-green microalgae that not only is a rich source of high-quality protein, but also has antioxidant, immunomodulatory, antiviral, and antimicrobial properties [Park et al., 2018]. Blue-green algae has been shown to have no long-term toxic side-effects at up to 5% consumption [Yang et al., 2011]. A study showed that *Spirulina* administered for the first 21 days of a broiler's life resulted in a similar immune response, with a lower number of leukocytes, lymphocytes, and eosinophils, compared to in-feed antibiotics [Sugiharto et al., 2018].

The purpose of this study was to determine whether a lower crude protein diet had a significant effect on blood leukocytes, specific blood leukocyte populations, and specific blood lymphocyte populations in broilers compared to a normal broiler crude protein diet, and whether adding *Spirulina* to a low protein diet affected blood leukocytes, specific blood leukocyte populations, and specific blood lymphocyte populations in broilers compared to a normal crude protein diet and a lower crude protein diet.

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Literature Review

Blood is a great tool to examine what is happening in an animal's immune system. Blood collection is a minimally invasive procedure, white blood cells (WBC) can be identified in whole blood, based on size, morphology and staining patterns, and WBC can be separated from the rest of the blood and analyzed. More importantly, we can differentiate the different types of blood leukocytes and determine the proportion of each one is in the blood. Each leukocyte subset has a specific function it performs for certain immune responses. Analyzing this information can determine whether there is an abnormality in the concentration and/or proportion of a specific leukocyte. Knowing the leukocyte with the abnormality can also determine a likely source of the change in the animal, since the type of leukocytes can be linked to a specific function.

Blood Leukocytes

In broilers, there are five different leukocyte subsets that are analyzed: heterophils, lymphocytes, monocytes, basophils, and eosinophils. Each of the leukocyte types has an important function in immunity. Heterophils are the predominant leukocyte responsible for the inflammation response in the tissues. They are also important for host defense against microbial pathogens [Stedman et al., 2001]. Eosinophils deal with larger parasitic infections, like worms. Basophils usually are responsible for allergic reactions. Monocytes deal with more stubborn bacterial infections, such as infections by intracellular bacteria. Lymphocytes kill virus-infected cells and tumor cells, help other cells to become fully activated, and produce antibodies.

To determine the proportion of each, they must be distinguished and counted by either the observer or the machine. Heterophils usually range between 4.2 to 9.0 μm in size [Lucas and Jamroz, 1961]. When stained with Wright stain, they typically have a violet, multi-lobed nucleus,

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and colorless cytoplasm. They also have orange-red, rod-shaped granules present in the cytoplasm [Figure 1]. Eosinophils are slightly smaller than heterophils [Lucas and Jamroz, 1961]. When stained with Wright stain, they typically have a violet, multi-lobed nucleus, and blue cytoplasm. They also have red-orange, round granules present in the cytoplasm [Figure 1]. Basophils stained with Wright stain have pale blue nucleus, which is usually covered by dark purple cytoplasmic granules [Figure 1]. Monocytes are typically larger than the average heterophil and are often round [Lucas and Jamroz, 1961]. When stained with Wright stain, they typically have a purple nucleus, and an abundant blue-grey cytoplasm [Figure 1]. Lymphocytes are small cells (5-7 µm) consisting mostly of nucleus with a rim of cytoplasm, but antibody producing plasma cells and lymphoblasts may be as large as the average heterophil [Lucas and Jamroz, 1961]. When stained with Wright stain, they have a dark purple, non-lobed nucleus, and blue-grey cytoplasm [Figure 1].

To determine whether an animal has an abnormal leukocyte count, the average proportion and concentration of each leukocyte subset must be known. Once the sample data is calculated, it can be compared to the average data [Lucas and Jamroz, 1961], to determine if the sample data is above or below average. By comparing these data points, the cause of illness in the sample can be narrowed down or determined.

When analyzing leukocyte cell populations, the concentration and proportion are calculated to get the most accurate result. The concentration of each type of leukocyte is often more informative than its proportion [Blumenreich, 1990]. The proportion is a good basic start in order to find more obvious abnormalities. However, the blood could have normal proportions of leukocytes but have an abnormal concentration (number of leukocytes/µL). Finding the concentration of each type of leukocyte requires the proportion of the leukocyte and the total

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number of leukocytes per volume unit [Blumenreich, 1990]. Further testing is required to obtain the leukocyte concentration, but it allows for a more informative result than only the proportion.

The heterophil to lymphocyte ratio has been increasingly used by researchers as a measurement of stress in chickens. Lymphocytes tend to decrease and the number of heterophils increase in response to stressors [Gross and Siegel, 1983]. The heterophil/lymphocyte ratio was also less variable than the number of heterophils and the number of lymphocytes alone [Gross and Siegel, 1983]. The heterophil to lymphocyte ratio, since it measures a direct physiological change, is a better measure of long-term changes in stressors [Gross and Siegel, 1983]. The heterophil to lymphocyte ratio is used by researchers as a stress indicator more than a disease indicator.

Blood Lymphocytes

Blood lymphocytes, the cells of adaptive immunity, can be further separated into two main categories: B-cells and T-cells. B-cells main function in an immune response is antibody production [LeBien and Tedder, 2008]. T-cells can be further divided into two functional subsets: killer T-cells (CD8+ cells) and helper T-cells (CD4+ cells). Killer (cytotoxic) T-cells kill cells infected with intracellular pathogens before the pathogen can proliferate and infect more cells [Alberts et al., 2002a]. Helper T-cells help activate B cells to secrete antibodies, activate macrophages to better kill phagocytosed pathogens, and they also help activate killer T-cells to kill infected target cells [Alberts et al., 2002b]. Since CD4+ and CD8+ are both types of T-cells, they cannot be distinguished morphologically. Immunofluorescent staining for expression of the cell-specific surface molecules CD4 and CD8 is the only method of identifying and distinguishing between CD4+ and CD8+ T-cells, respectively.

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Immunofluorescent staining involves the use of molecule-specific antibodies, e.g., chicken CD4- or CD8-specific mouse monoclonal antibody that is conjugated with fluorescent molecules like green fluorescing fluorescein isothiocyanate (FITC) or orange-fluorescing phycoerythrin (PE). During incubation of chicken lymphocytes with these antibodies, the CD4-specific antibody will bind to the CD4 molecules and the CD8-specific antibody to the CD8 molecules present on lymphocytes. The binding of these antibodies can then be detected based on their fluorescent label, i.e., green fluorescent cells have the CD4 molecule and hence are CD4+ T cells, whereas orange fluorescing cells have the CD8 molecules and are CD8+ T cells [Figure 2]. For each cell, the fluorescent signal can be detected by fluorescence microscopy or by fluorescence-based cell-population analysis using a flow cytometer. For chicken immunology studies, a large panel of chicken lymphocyte marker-specific mouse monoclonal antibodies is commercially available, and there are many choices of attached fluorochromes..

T-cells can also be separated by the type of the T-cell's antigen receptor (TCR) present on the surface. The two main types of TCRs are heterodimers consisting of either and alpha- and a beta-protein (αβTCR) or a gamma- and a delta- protein (γδTCR). The function of αβTCR is to bind antigen-peptides displayed on presentation molecules of antigen-presenting cells. This specific binding of TCR to Ag-peptide then activates the T cells to carry out its immune functional activities [Leichne and Kambayasi, 2014]. CD4+ and CD8+ cells are the main T-cells that express αβTCR [Eberl and Hayday, 2000]. In chickens, there are two types of αβ TCR, αβ1 and αβ2 TCR that have different β-chains. T-cells with γδ TCRs also can recognize distinct antigens but do not require antigen-presentation. Compared to T cells with αβ TCR, T cells with γδ TCR have specificity that is limited to frequently encountered pathogens and hence, are generally considered as the T cell with more innate, less sophisticated functional activities than αβ T cells.

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However, they make important contributions to immune responses with distinct kinetics [Vantourout and Hayday, 2013], especially in barrier tissues.

The CD4+/CD8+ ratio can help determine how strong the immune system is and can also help predict how likely the subject is to develop an infection [Haldeman-Englert et al., 2021]. An abnormal T/B cell ratio can help determine what type of pathogen is potentially causing disease, since T cells can only recognize viral antigens inside infected cells while B cells can recognize the surface antigens of bacteria and viruses in the extracellular environment [Panawala, 2017].

By analyzing both the CD4+/CD8+ and the T/B cell ratio, the entire spectrum of a bird's health can be analyzed. Not only can the current health of the bird be evaluated, but the future health of the bird as well. With this information, researchers and chicken producers can adjust management practices to better accommodate the needs of the birds.

Materials and Methods

Animals and Treatment

The live animal phase of this experiment was previously described by Mullenix et al. (2021). Briefly, a total of 180 one-day-old male Ross 708 broiler chicks were obtained from a commercial hatchery and randomly allotted to one of 15 floor pens (5 pens/diet; 12 birds/pen) in an environmentally controlled pilot house. Chicks were reared in pens top-dressed with approximately 4 cm of fresh wood shavings and temperature gradually decreased from 32 °C at day one to 20 °C at day 27. The trial was conducted from February to March 2020. Birds received 23 hours of light until day 10, then light duration was decreased to 18 hours for the remainder of the trial. Birds were given *ad libitum* access to feed and water throughout the trial. A standard corn-SBM basal diet (3250 kcal/kg^{-1}, 21% CP) was fed to all birds until day 14, at

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which point experimental diets were introduced until 37 days of age. The experimental diets included an industry standard level protein (~20% CP) corn/SBM control (SCP), reduced (~17% CP) corn/SBM diet (LCP) and LCP diet where *Spirulina* was included at the level of 100g/kg (LCP-SP). All experimental diets were isocaloric and met all essential amino acid needs set forth by the primary breeder. Both low crude protein diets were formulated to be isonitrogenous [Mullenix et al., 2021].

Blood Sampling and Analysis

On day 37, 3 mL heparinized blood samples were collected from randomly selected birds (10 birds/treatment; 2 birds per pen; n = 5) by Mullenix et al. (2021). A portion (1 mL) of each blood sample was run through a Cell-Dyn automated hematology analyzer calibrated for chicken blood. Data collected from the test included white blood cell concentration (10^3/µL), thrombocyte concentration (10^3/µL), red blood cell concentration(10^6/µL), hemoglobin concentration (g/dL), and hematocrit concentration (g/dL).

Part of the remaining sample was used to perform a blood smear on slides. A small drop (3-5 µL) of the blood was placed onto a microscope slide. With another slide, the sample was spread evenly across the first slide and left to dry. Afterwards, the blood smears were stained with Wright stain (Lucas and Jamroz, 1961). For this, the entire smear was covered with Wright stain and left for 6 minutes. After that, distilled water was added to make a 50:50 ratio on the slide and left for another 6 minutes. Then the water-stain mixture was removed from the slide and more distilled water was added on top of the slide. The slides were left for another minute. The water was removed, and the wash procedure was repeated twice more. After the final wash, the slide was placed in a distilled water bath and waved back and forth until no more dye comes off. The back of the slide was wiped off and placed on a paper towel to dry. For each sample two blood

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smears were prepared and stained as described above, to ensure that each one had an acceptable slide.

For evaluation, the best slide with the most uniform monolayer of blood cells from each sample was examined under a bright field microscope. For the manual differential leukocyte count, cells were observed under oil at 1000x magnification. Using the "across slide" rather than the margin counting methods, a minimum of 300 leukocytes (WBC) were identified and counted while making at least two complete passes across the narrow side of each slide. The heterophils counted had a purple stained nucleus with pink/orange, oblong-shaped granules [Figure 1]. The lymphocytes were smaller than RBC and had a small amount of blue-stained cytoplasm surrounding a purple-stained nucleus [Figure 1]. The monocytes were larger than the RBCs and had a large amount of blue-stained cytoplasm surrounding a purple-stained nucleus [Figure 1]. The eosinophils had a purple-stained nucleus, with blue-stained cytoplasm and pink-stained circular granules [Figure 1]. The basophils had dark-purple granules [Figure 1]. For each sample, the percentage of each cell type identified (heterophils, lymphocytes, monocytes, basophils, and eosinophils) was calculated by dividing the number of a cell-type by the total number of WBC and multiplying by 100 [e.g., (# lymphocytes/ # total WBC evaluated) x 100 = % lymphocytes]. Once the percentages were calculated, the concentration ($10^3/\mu L$) of each of the leukocyte populations was calculated by multiplying the percentage of each leukocyte population by the WBC concentration determined by automated hematology analysis and dividing by 100. The heterophil/lymphocyte ratio was calculated by dividing the heterophil concentration by lymphocyte concentration.

Lymphocyte Subpopulation Analysis by Immunofluorescent Staining and Flow Cytometry

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Isolation of peripheral blood mononuclear cells: Peripheral blood mononuclear cells (PBMC), consisting of lymphocytes, monocytes, and thrombocytes, were isolated from 1 mL of each blood sample by density gradient separation over Ficoll 1.077. For this, 1 mL of RT-PBS (room temperature-Dulbecco's phosphate-buffered saline) and mixed using a Pasteur pipette. Using a Pasteur pipette, the diluted blood samples were then carefully layered on top of 2 mL of Ficoll. The Ficoll-blood mixture was centrifuged at room temperature at 400 x *g* for 30 minutes. After centrifugation, a layer of cells was visible on top of the denser Ficoll layer, at the interphase of the Ficoll layer and the plasma layer on top. A Pasteur pipette was used to carefully remove the cell layer and placed into a tube containing cold PBS. To wash the cell suspension, the cells and PBS were mixed using a Pasteur pipette, and the mixture centrifuged at 4℃ for 8 minutes at 250 x *g*. Afterwards, the supernatant fluid was poured off, leaving a cell pellet at the bottom of the tube. The cells were then resuspended in 3 mL of cold PBS and washed again by centrifugation. After the second wash, the cell pellet was resuspended in 1 mL of cold PBS+ (PBS containing 1% bovine serum albumin and 0.1% sodium azide) staining buffer and the cell suspensions left on ice until use.

Immunofluorescent staining: Once the cells were isolated, 50 µL of each PBMC suspension was placed into 4 wells in a 96-well round-bottom microtiter plate for direct 1- or 2- color immunostaining. The cell suspensions were immunofluorescently stained using fluorescently labeled mouse monoclonal antibodies (mAb) specific for chicken leukocyte markers. All mAb were mouse IgG1 and were diluted 1:100 in PBS+ staining buffer.

For each sample, PBMC in the first well were incubated with 50 µL of a cocktail containing mouse-anti-chicken (mac-) CD4 mAb conjugated to fluorescein isothiocyanate (FITC) and mac-CD8α mAb conjugated to phycoerythrin (PE). PBMC in the second well were incubated with 50

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μL of mac Bu-1-PE mAb specific for the chicken B cell marker and spectral red fluorochrome (SPRD) conjugated mac mAb specific for the chicken T cell marker CD3 (CD3-SPRD). PBMC in the third well were incubated with 50 μL of a cocktail of mac mAb αβ1TCR-FITC and αβ2 TCR-FITC to identify αβ T cells and γδ TCR-PE to identify γδ T cells. PBMC in the fourth well were incubated with 50 μL of mac-mAb specific for thrombocytes (Thromb-FITC) to determine the proportions of thrombocytes (Thromb-FITC-positive) and lymphocytes (Thromb-FITC-negative) in the small PBMC population which contains thrombocytes and lymphocytes.

The cells and reagents were mixed, and the cells were incubated at 4°C for 30 minutes. After the incubation, a wash was done by adding 150 μL of PBS+ to each well containing cells and centrifuging the plates at 4°C for 4 minutes at 250 x *g*. After that, the supernatant fluid was discarded, resuspended in 200 μL PBS+, and washed again. After the second wash, the supernatant fluid was discarded and 200 μL of PBS+ was added to each well containing cells.

Staining controls included: isotype control to examine non-specific binding of antibodies and determine cut-off between negative and positive fluorescence, and single stained cells to set fluorescence compensation during calibration of the flow cytometer. For this, a pooled sample of PBMC was used and incubated with a cocktail of mIgG1 isotype control antibodies (no specificity for chicken molecules) labeled with FITC, PE, or SPRD, or for the single staining controls, with leukocyte-specific mAb CD45-FITC, or CD45-PE, or CD45-SPRD.

Lymphocyte population analysis by flow cytometry: For flow cytometric analysis, each sample was mixed well, transferred into a 1.5 ml microcentrifuge tubes, and placed on the sample port of a BD C6 Accuri flow cytometer for acquisition of the percentage of each cell type based on light scatter characteristics (FSC-size, SSC-granularity) and FL-1 (FITC fluorescence), FL-2 (PE-fluorescence), and FL-3 (SPRD fluorescence). The data were analyzed using FlowJo

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software v1.05. A region was drawn around the small PBMC population and the percentage of each cell type (B cells and CD4+, CD8+, αβ and γδ TCR+ T cells, thrombocytes, and lymphocytes) determined. For each sample, the proportion of the various lymphocyte subsets were then expressed as a percentage of lymphocytes by calculation [i.e., dividing the proportion of each lymphocyte population by the proportion of total lymphocytes in the small PBMC population and multiplying by 100]. Total T cell population was calculated by adding the proportions of αβ and γδ TCR-positive T cells. The T/B cell ratio was calculated by dividing the T cell population by the B cell population estimates for each sample. Similarly, the CD4+/CD8+ T cell ratio was calculated by dividing the CD4+ T cell population by the CD8+ T cell estimates for each sample. The concentrations ($10^3/\mu L$) of each lymphocyte population were calculated by multiplying the percentage of each lymphocyte subpopulation by the total blood lymphocyte concentration determined by Cell-Dyn as described above and dividing by 100.

Statistical Analysis

All cell population data were analyzed for the effect of diet by one way ANOVA using SigmaPlot statistical software. Differences were considered significant at $P \leq 0.05$. If there was a significant difference, means were compared using a Student's t-test and a Welch's t-test. Differences were considered significant at $P \leq 0.05$.

Results

Cell-Dyn automated hematology

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Compared to the control diet (CP), the LCP diet had no significant ($P > 0.05$) effect on the concentration of white blood cells, thrombocytes, red blood cells, hemoglobin, and hematocrit. Adding *Spirulina* to the LCP (LCP-SP) diet also had no significant effect on the concentration of white blood cells, thrombocytes, red blood cells, hemoglobin, and hematocrit [Table 1].

Concentration and proportions among WBC populations

The LCP diet had no significant ($P > 0.05$) effect on the concentrations and proportions of heterophils, lymphocytes, basophils, eosinophils, and on the heterophil/lymphocyte ratio. The LCP diet increased ($P < 0.05$) the concentration and proportion of monocytes compared to the control diet [Table 2].

The concentration and proportion of heterophils, lymphocytes, monocytes, basophils, and eosinophils in broilers fed the LCP-SP diet were not different from the CP diet and the LCP diet [Table 2].

Concentrations and proportions of lymphocyte populations

There was no effect of dietary treatment ($P > 0.05$) on the proportions and concentrations of any of the lymphocyte populations (B cells, T cells, CD4 + T cells, CD8 + T cells, αβTCR + T cells, and γδTCR + T cells) and on the T/B cell ratio and the CD4+/CD8+ ratio [Table 3].

Discussion

Blood leukocyte information can be an important tool to help determine whether an animal is in good condition or whether it is undergoing disease or stress. Changes in the heterophil/lymphocyte ratio have been shown to be related to different forms of stress in avian

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species [Post et al., 2003]. Increased stress can also cause the release of monocytes and lymphocytes in broilers [Rosales, 1994]. Examination of blood leukocyte profiles during the acute inflammatory response initiated in skin tissue by injection of lipopolysaccharide (LPS), also demonstrated the blood to be an important diagnostic tool to identify the presence of inflammation [French et al., 2020]. As more information comes out of studies on blood leukocyte profiles broilers, examination of blood alongside other methods provides important insights into immune system development and function in these commercial important production animals.

The relationship between protein provided in the diet and immune system development and function is another aspect of immunity that is increasingly evaluated, especially because of the high cost of crude protein content in the diet. It has been shown that protein or amino acid deficiency in chickens alters their immune response [Konashi et al., 2000]. Certain amino acids, such as arginine and tryptophan, have been shown to positively influence the systemic immune response in chickens [Emadi et al., 2011]. One study showed that a dietary crude protein (CP) increase caused an increase in the proportion of lymphocytes and a lower heterophil to lymphocyte ratio [Jahanian, 2009]. Another study showed that reducing protein in birds injected with an abdominal inflammatory substance could enhance the broiler's ability to recruit leukocytes upon infection [Kamely et al., 2020]. However, one study showed that protein level had no significant effect on immunity [Houshmand et al., 2012]. These types of studies tend to focus on multiple aspects of immunity or study other factors alongside low protein. There are limited studies that focus on how blood leukocyte population profiles are affected by solely low crude protein diets.

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Studies done in other animals have shown that protein deficiency increased an animal's susceptibility to diseases. One study done in mice showed that the mice on the low protein diet were more susceptible to infection with influenza and had more severe symptoms [Taylor et al., 2013]. In lambs, the animals fed a lower protein diet were more susceptible to parasitic infections [Kambara et al., 1993]. However, information on broilers is limited. Examination of broilers by Mullenix et al. (2021) from the same experiment as the current study, recently reported increased ($P < 0.05$) expression levels of pro-inflammatory cytokines, chemokines, and the MLRP3 inflammasome in blood from broilers fed the LCP diet, which was not observed when *Spirulina* was added to the LCP diet. These findings are in line with our observation of increased proportions and concentrations of monocytes, the likely source of the reported inflammatory activity in broilers fed the LCP diet. Similarly, with *Spirulina* supplementation, this effect of the LCP diet on monocytes was no longer observed. Hence it appears that low crude protein content in the broiler diet stimulates inflammatory activity in healthy, fast growing broilers that can be observed in the peripheral blood circulation. While no other effects of the LCP diet on blood cell measurements were observed, including lymphocyte profiles, one would expect that under more challenging situations, like infection and environmental stress, the LCP diet may have more substantial effects on immune system activities.

Controlling disease is one of the most important factors in broiler production. A study conducted by Zachar et al. showed that the broiler chicken industry in Saskatchewan loses 3.9 million kilograms of meat production a year solely due to various strains of the infectious bursal disease virus (IBDV) [Zachar et al., 2016]. Diseases, like IBDV, lead to higher mortality, poorer feed conversion ratio (FCR), and decreased meat production [Zachar et al., 2016]. With broiler sales of meat raised without antibiotics increasing by 25% [Mainstream, 2017], it is becoming more

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important than ever to find alternatives to help with the control of disease in broilers. While IBDV cannot be directly treated with antibiotics, since antibiotics cannot treat viral infection, boosting the immune system before infection can allow the prevention of IBDV infection. Feed supplements other than antibiotics may prove to have immunomodulatory activities that provide better immune responses to viral infections and naturally improve the broiler's defenses against infections with pathogens. However more research would be needed in this area.

Most studies done on *Spirulina* supplementation and broilers focus on the growth and nutritional aspects of this dietary treatment. There are few studies that evaluate *Spirulina* as a prebiotic substitute and certain immune responses. *Spirulina* has been shown to stimulate the phagocytic potential of macrophages and increase the level of natural killer cells [Quershi et al., 1996]. *Spirulina* has also been shown to lower the level of heterophils and raise the level of lymphocytes present in the blood [Khan et al., 2020]. These immunity studies, however, have used *Spirulina* as a supplement in the diet. There are limited studies that evaluate the effects of *Spirulina* on immunity when it is part of the balanced diet. In addition, there is limited information about the impact *Spirulina* has on all blood leukocytes, not just the heterophil/lymphocyte ratio.

In conclusion, this is the first report, to our knowledge, addressing the effects of a low protein diet and a low protein diet supplemented with *Spirulina* on blood cell concentrations, blood leukocyte proportions and concentrations, and blood lymphocyte proportions and concentrations.

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Table 1. Concentrations of blood cells, hemoglobin, and hematocrit in blood of 37-day old male Ross 708 broilers reared on either a standard corn/soy (SCP) diet, a low crude protein (LCP) diet, or the LCP diet supplemented with 100g/kg *Spirulina* (SP-LCP).

Blood measurement	SCP	LCP	SP-LCP	*P*-value
WBC ($10^3/\mu L$)[1]	11.80 ± 1.84	13.85 ± 1.41	13.78 ± 1.16	0.561
Thrombocytes ($10^3/\mu L$)[1]	2.07 ± 0.13	1.98 ± 0.22	1.78 ± 0.14	0.467
RBC ($10^6/\mu L$)[1]	2.63 ± 0.04	2.70 ± 0.06	2.70 ± 0.04	0.517
Hemoglobin (g/dL)[1]	7.71 ± 0.35	7.79 ± 0.10	7.88 ± 0.14	0.658
Hematocrit (g/dL)[1]	61.13 ± 1.91	62.46 ± 1.05	63.23 ± 1.17	0.444

Mean ± SEM; n = 5; blood from 2 broilers processed per replicate

[1] Concentrations measured using an automated hematology analyzer (Cell-Dyn)

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Table 2. White blood cell (WBC) concentrations and proportions of 37-day old male Ross 708 broilers reared on either a standard corn/soy (SCP) diet, a low crude protein (LCP) diet, or the LCP diet supplemented with 100 g/kg *Spirulina* (SP-LCP).

WBC Measurements	SCP	LCP	SP-LCP	*P*-value
Heterophils (10^3/µL)[1]	1.39 ± 0.30	1.84 ± 0.34	1.60 ± 0.19	0.548
Heterophils (% WBC)[2]	11.47 ± 1.33	12.89 ± 1.22	11.60 ± 0.85	0.637
Lymphocytes (10^3/µL)[1]	9.58 ± 1.44	8.70 ± 1.01	11.23 ± 1.03	0.592
Lymphocytes (% WBC)[2]	81.427 ± 1.97	78.82 ± 1.84	81.29 ± 1.00	0.478
Monocytes (10^3/µL)[1]	0.236 ± 0.027**b**	0.503 ± 0.082**a**	0.370 ± 0.076**ab**	0.046
Monocytes (% WBC)[2]	2.17 ± 0.35**b**	3.64 ± 0.50**a**	2.84 ± 0.77**ab**	0.079
Basophils (10^3/µL)[1]	0.32 ± 0.074	0.31 ± 0.088	0.26 ± 0.020	0.970
Basophils (% WBC)[2]	2.60 ± 0.21	2.16 ± 0.43	1.88 ± 0.14	0.185
Eosinophils (10^3/µL)[1]	0.285 ± 0.075	0.311 ± 0.058	0.290 ± 0.045	0.949
Eosinophils (% WBC)[2]	2.31 ± 0.40	2.33 ± 0.44	2.18 ± 0.45	0.965
Heterophil/Lymphocyte[3]	0.143 ± 0.019	0.165 ± 0.043	0.143 ± 0.012	0.573

Mean ± SEM; n = 5; blood from 2 broilers processed per replicate, **a, b:** means within a row without a common letter are different P ≤ 0.05 based on multiple means comparisons.

[1]The concentration of individual leukocyte populations was calculated using WBC concentration (Table 1) times the proportion of each leukocyte population (% WBC) divided by 100.

[2]Manual differential leukocyte count to determine proportions (% WBC) of individual leukocytes populations based on evaluation of ≥ 300 WBC.

[3]Heterophil/Lymphocyte ratio was calculated by dividing the heterophil concentration by the lymphocyte concentration.

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Table 3. Concentrations and proportions of various lymphocyte populations in blood from 37-day old male Ross 708 broilers reared on either a standard corn/soy (SCP) diet, a low crude protein (LCP) diet, or the LCP diet supplemented with *Spirulina* (SP-LCP).

Lymphocyte population	SCP	LCP	SP-LCP	*P*-value
B cells ($10^3/\mu L$)[1]	1.63 ± 0.30	2.00 ± 0.40	1.76 ± 0.22	0.713
B cells (%)[2]	16.81 ± 1.81	17.77 ± 1.99	15.53 ± 1.04	0.646
T cells ($10^3/\mu L$)[1]	7.95 ± 1.20	8.88 ± 0.66	9.47 ± 0.85	0.523
T cells (%)[2]	83.19 ± 1.81	82.23 ± 1.99	84.47 ± 1.04	0.646
CD4+ T cells ($10^3/\mu L$)[1]	3.66 ± 0.51	4.12 ± 0.35	4.18 ± 0.22	0.585
CD4 + T cells (%)[2]	38.99 ± 2.65	38.17 ± 1.73	38.06 ± 2.80	0.756
CD8+ T cells ($10^3/\mu L$)[1]	1.82 ± 0.19	2.17 ± 0.23	2.40 ± 0.19	0.166
CD8+ T cells (%)[2]	19.72 ± 1.65	20.08 ± 1.58	21.49 ± 0.58	0.340
αβTCR+ T cells ($10^3/\mu L$)[1]	5.86 ± 0.95	6.73 ± 0.57	6.79 ± 0.69	0.627
αβTCR+ T cells (%)[2]	60.89 ± 1.46	62.30 ± 2.61	60.46 ± 2.08	0.814
γδTCR+ T cells $10^3/\mu L$)[1]	2.47 ± 0.44	2.46 ± 0.19	2.98 ± 0.34	0.491
γδTCR+ T cells (%)[2]	25.39 ± 1.66	22.88 ± 1.05	26.37 ± 1.38	0.224
T/B cell Ratio[3]	5.58 ± 0.68	5.02 ± 0.63	5.72 ± 0.40	0.679
CD4+/CD8+ T cell Ratio	0.143 ± 0.019	0.165 ± 0.043	0.143 ± 0.012	0.548

Mean ± SEM; n = 5; blood from 2 broilers processed per replicate

[1]The concentration of individual lymphocyte populations was calculated by multiplying the lymphocyte concentration (Table 2) by the proportion (%) of each lymphocyte population and dividing by 100.

[2]The % of various lymphocyte populations is based on immunofluorescent staining with and cell population analysis by flow cytometry.

[3]The T/B cell ratio was calculated for each sample using the T cell concentration and the B cell concentration.

[4]The CD4+/CD8+ cell ratio was calculated for each sample using the CD4+ concentration and the CD8+ concentration.

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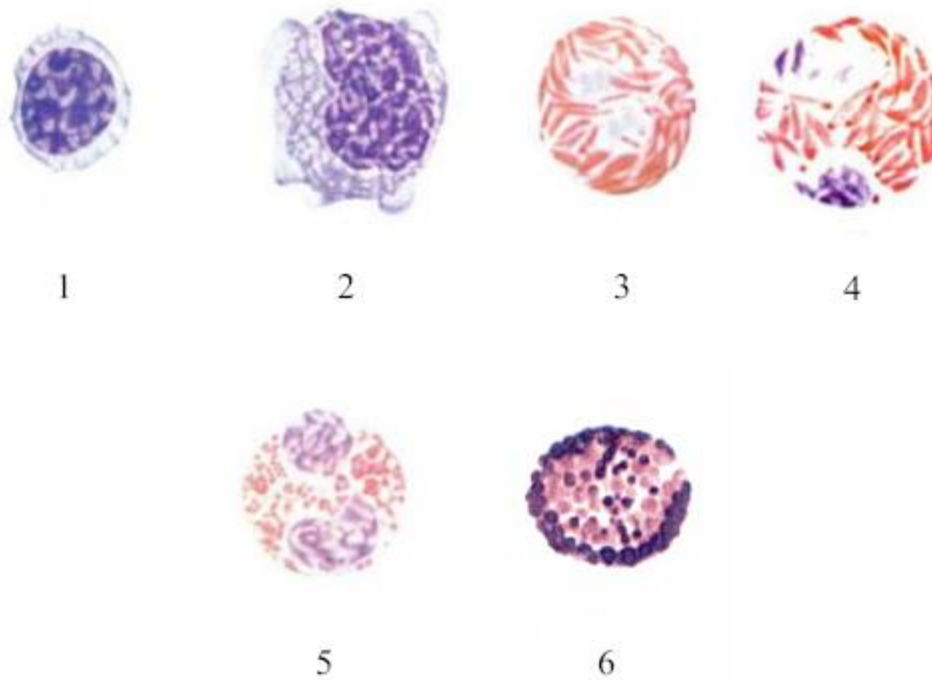

Figure 1: Typical avian blood leukocytes stained with Wright stain adapted from Lucas and Jamroz [1961]. Cell 1 is a typical lymphocyte. Cell 2 is a typical monocyte. Cell 3 is a heterophil that has poorly stained nuclear lobes. Cell 4 is a heterophil with variable staining of the nuclear lobes. Cell 5 is a typical eosinophil. Cell 6 is a typical basophil.

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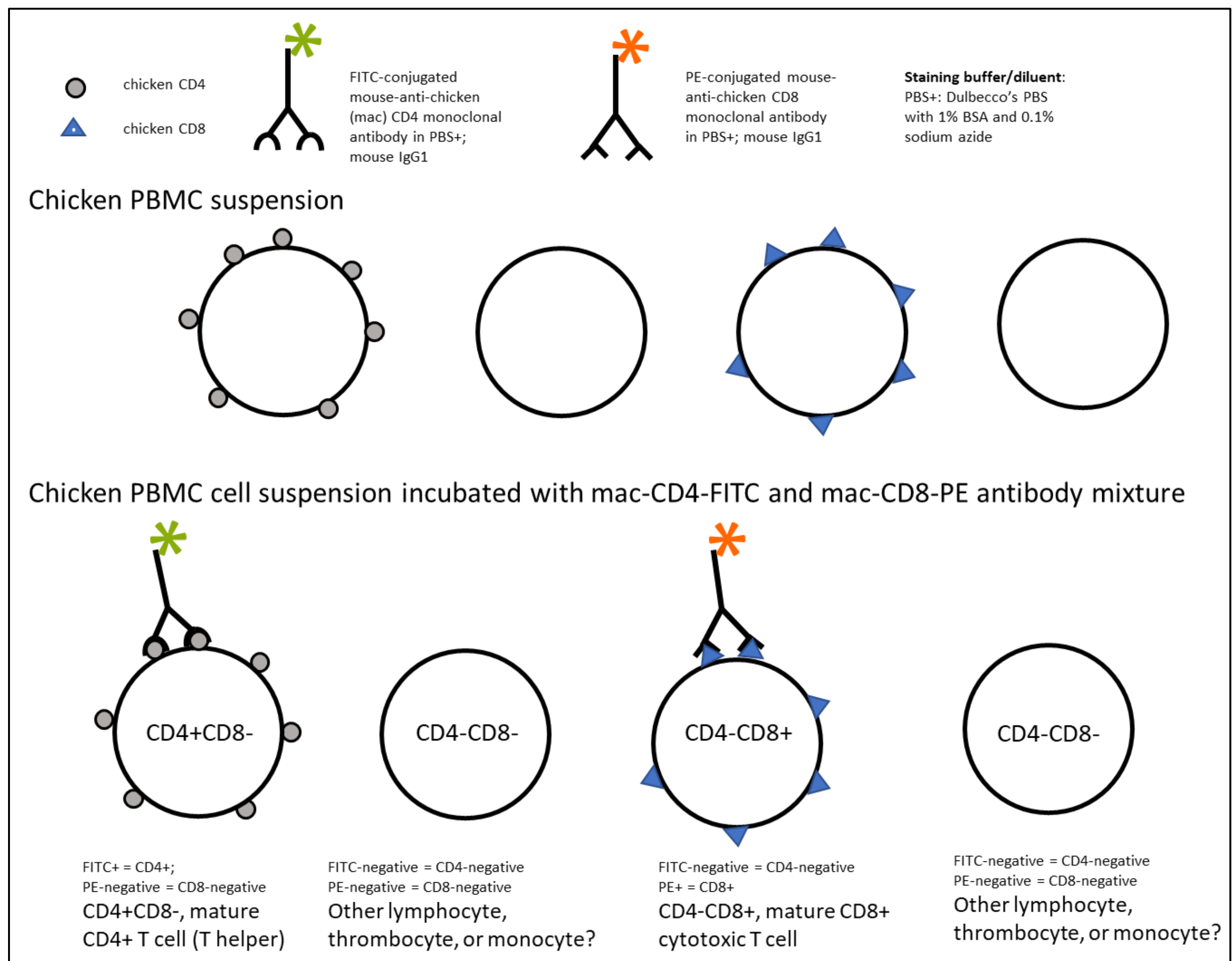

Figure 2. Direct immunofluorescent staining steps for a blood cell suspension. Top half explains the antibodies with the stains and the cell suspension. Bottom half explains how the staining reagents attach to CD4+ and CD8+ cells in the cell suspension. Provided by G. F. Erf.

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