Effects of a Low Crude Protein Diet With and Without Spirulina platensis Inclusion on White Blood Cell Profiles in Broilers

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Cover Page Footnote

Heather Glenn is a May 2021 honors program graduate with a major in Poultry Science. Garrett J. Mullenix is a Ph.D. graduate student in Poultry Science. § Gisela F. Erf, the faculty mentor, is an Immunologist and a Tyson Endowed Professor in Avian Immunology.

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Meet the Student-Author

Heather Glenn

Heather is identifying white blood cells on a Wright-stained blood smear to determine the proportions among different white blood cells. An image of white blood cells she viewed under the microscope is displayed on the image analysis computer screen.

I am a Spring 2021 Magna Cum Laude graduate of the Dale Bumpers College of Agricultural, Food, and Life Sciences with a degree in Poultry Science. Before attending the University of Arkansas, I was an active Future Farmers of America (FFA) member at Fayetteville High School. I participated in the FFA Veterinary Science contest, where my team and I went to the state competition. At the University of Arkansas, I was awarded the Honor's Fellowship, which is an exclusive scholarship awarded over four years to the top incoming freshmen. I also received the Arkansas Distinguished Governor's Scholarship for the four years of my studies. Poultry Science allowed me to explore my different interests and provided numerous opportunities for me as a student and as a graduate. The Poultry Science Department gave me the opportunity to learn and practice different lab techniques through their Honors Current Approaches in Agricultural Laboratory Research class. I also had the chance to raise chickens from hatch through the Poultry Productions class. The Poultry Science Department gave me so many wonderful experiences, including this research project. I'd like to thank my research mentor, Dr. Gisela F. Erf, for all her help and guidance with the project, and my research committee members, Dr. Samuel J. Rochell and Dr. Sara K. Orlowski. I'd also like to thank my lab mates Chrysta Beck, Jossie Santamaria, and Dr. Marites Sales for their help with blood smear preparation and flow cytometry analyses.

Research at a Glance

- *Spirulina* is investigated as an environmentally friendly source of protein for inclusion in low protein broiler diets formulated to sustain growth performance and health.

- *Spirulina* inclusion in a low protein diet had no effect on white blood cell profiles and prevented increased levels of inflammatory cells observed with the low protein diet.

- This study also contributed comprehensive white blood cell population data for modern commercial broilers.

Effects of a Low Crude Protein Diet With and Without *Spirulina platensis* Inclusion on White Blood Cell Profiles in Broilers

Heather Glenn,* Garrett J. Mullenix,[†] and Gisela F. Erf[§]

Abstract

Spirulina microalgae is an alternative protein source under consideration for feed formulation in commercial broiler production. The purpose of this study was to determine the effects of a low crude protein (LCP) diet and a LCP diet formulated with 100 g/kg *Spirulina* (LCP-SP) on blood cell measurements in broilers. One-day-old Ross 708 male broilers were assigned to three dietary treatments: a standard crude protein (SCP), the LCP, or the LCP-SP diet, with five pens/treatment. When the chickens were 37-days old, blood samples were obtained from 2 birds/pen. Each blood sample was used to determine 1) the concentrations of white blood cells (WBC), thrombocytes, red blood cells (RBC), hemoglobin, and hematocrit by automated hematology; 2) the proportions among WBC populations by microscopic evaluation of >300 WBC on Wright-stained blood smears; and 3) the proportions among lymphocyte-subsets by immunofluorescent staining and flow-cytometric cell population analysis. Except for monocytes, none of the blood cell measurements were affected ($P > 0.05$) by diet. The LCP diet resulted in increased ($P \leq 0.05$) monocyte concentration and proportion compared to the SCP diet, indicating heightened inflammatory activity with lower dietary protein content. The LCP-SP diet reversed the effect of the LCP diet, resulting in monocyte concentrations and proportions not different ($P > 0.05$) from those of the SCP diet. The ability of *Spirulina* microalgae to maintain normal WBC profiles in broilers fed the LCP diet is a promising sign for its use as a proteinaceous feed component without compromising the health of the bird.

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* Heather Glenn is a May 2021 honors program graduate with a major in Poultry Science.
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Introduction

Protein is one of the more expensive feed components in commercial broiler production. One risk of cutting down protein content in feed is that it may have damaging effects on the chicken's immune system and natural defenses. Broilers fed diets deficient in amino acids had reduced primary antibody responses compared to broilers fed a typical diet (Kidd, 2004). Protein deficiency also decreased lymphocyte numbers and overall white blood cell numbers (Kidd, 2004). Similarly, altered innate immune system activities were observed with low crude protein diets, even when the reduced crude protein diets were formulated to maintain a normal digestible amino acid content (Mullenix et al., 2021). Specifically, compared to broilers fed a standard protein diet, broilers fed a low crude protein diet had increased bacterial translocation across the gut mucosal epithelium and exhibited systemic inflammation as indicated by upregulated mRNA expression of circulating pro-inflammatory cytokines, chemokines, and the NLRP3 inflammasome. Hence, research is underway to identify alternative nutrient sources for the formulation of low protein diets that sustain growth performance and health of broilers.

One feed additive that poultry researchers are examining is *Spirulina (Arthrospira) platensis. Spirulina* is a blue-green cyanobacterium microalga that can be consumed by humans and animals. It not only is a rich source of high-quality protein but also has antioxidant, immunomodulatory, anti-inflammatory, antiviral, and antimicrobial properties (Park et al., 2018; Mullenix et al., 2021). At up to 5% consumption, *Spirulina* had no long-term toxic side effects (Yang et al., 2011). When administered for the first 21 days of a broiler's life, dietary *Spirulina* supplementation resulted in similar immune system changes to in-feed antibiotics, with a lower number of leukocytes, lymphocytes, and eosinophils (Sugiharto et al., 2018). Moreover, as reported by Mullenix and colleagues, formulation of *Spirulina* into a low protein diet reduced the systemic inflammation and bacterial translocation observed with the low crude protein diet, supporting the use of *Spirulina* as an alternative protein source in poultry diets (Mullenix et al., 2021).

Analysis of blood cell populations provides important insight into immune system development and function (Erf et al., 1996; Wang et al., 2003). The purpose of this study was to examine white blood cell profiles in broilers fed a low crude protein (CP) diet with and without *Spirulina* inclusion. Specifically, blood measurements conducted included determination of concentrations and proportions of various white blood cell (WBC) populations (i.e., lymphocytes, heterophils, monocytes, basophils, and eosinophils) and lymphocyte subsets (i.e., B cells, T helper cells, cytotoxic T cells, and T-cell receptor defined sub-populations). It was hypothesized that the inclusion of *Spirulina* in the low protein diet would return potential alterations in WBC profiles to levels comparable to those in broilers fed the standard diet.

Materials and Methods

The live performance phase of this experiment was described by Mullenix et al. (2021). Briefly, a total of 180 one-day-old male Ross 708 broiler chicks were allotted randomly to one of 15 floor-pens (5 pens/diet; 12 birds/pen) on wood shavings litter. The temperature gradually decreased from 32 °C on the day of placement to 20 °C by day 27. Birds received 23 h of light until day 10, at which time the light duration was decreased to 18 h for the remainder of the trial. Birds were given *ad libitum* access to feed and water throughout the trial. A standard corn-soybean meal basal diet (3250 kcal/kg, 21% CP) was fed to all birds until day 14, at which point experimental diets were introduced until 37 days of age. The experimental diets included an industry standard level protein (~20% CP) corn/soybean meal control (SCP) diet, reduced (~17% CP) corn/soybean meal diet (LCP), and LCP diet where *Spirulina* was formulated into the diet at 100 g/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). The animal study was reviewed and approved by the University of Arkansas Animal Care and Use Committee (protocol # 21002)

On day 37, 3-mL heparinized blood samples, collected from birds selected randomly (10 birds/treatment; 2 birds per pen; *n* = 5), were provided by Mullenix et al. (2021). A portion (1 mL) of each blood sample was used to determine the concentrations of WBC, thrombocytes, red blood cells (RBC), hemoglobin, and hematocrit using an automated hematology analyzer (Cell-Dyn; Abbot Diagnostics, Abbott Park, Illinois) calibrated for chicken blood (French et al., 2020). The remaining blood was used to prepare blood smears and mononuclear cell suspensions.

Blood smears were prepared on glass slides, stained with Wright stain (Lucas and Jamroz, 1961), and examined at 1000×-magnification with oil immersion using a bright field microscope (Olympus BX50, Meyer Instruments, Houston, Texas). At least 300 Wright-stained WBC in the monolayer of blood were evaluated across the slide and the number of observed lymphocytes, heterophils, monocytes, eosinophils, and basophils were recorded (Wang et al., 2003). The percentage of each cell type (% WBC) was calculated by dividing the number

of a cell-type by the total number of WBC evaluated and multiplying by 100. The concentration of each of the WBC populations was calculated using the proportion of each cell type and the total WBC concentration determined by automated hematology. The heterophil/lymphocyte ratio was calculated by dividing the heterophil concentration by the lymphocyte concentration.

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 blood was mixed with 1 mL of room temperature Dulbecco's phosphate-buffered saline (PBS; Sigma, Chemical Company, Saint Louis, Missouri). The diluted blood samples were then carefully layered on top of 2 mL of Ficoll (Sigma). The Ficoll-blood mixture was centrifuged at room temperature at $400 \times g$ for 30 min. After centrifugation, the layer of PBMC at the Ficoll-plasma interphase was collected, mixed with 3 mL of cold PBS, and the cells were washed by centrifugation at 4 °C for 8 minutes at $250 \times g$. After centrifugation, the supernatant fluid was discarded, the cell pellet resuspended in 3 mL of cold PBS, and the cell suspension washed again as before. The final 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 were left on ice until use.

A direct, one- or two-color staining procedure was followed to identify various lymphocyte subsets in the PBMC suspensions (French et al., 2020). For each broiler, PBMC were incubated with fluorescently labeled mouse monoclonal antibodies (mAb) specific for chicken lymphocyte populations and thrombocytes. Four combinations of mouse-anti-chicken (mac-) mAb were used to identify 1) CD4+ T helper cells [CD4-FITC; mac-CD4 mAb conjugated to fluorescein isothiocyanate (FITC)] and CD8+ cytotoxic T cells [CD8-PE, mac-CD8α mAb conjugated to phycoerythrin (PE)], 2) B cells [Bu-1-PE] and T cells [CD3-SPRD, spectral red fluorochrome (SPRD) conjugated mac-CD3 mAb], 3) T cells with αβ T cell receptors [αβ1- & αβ2-TCR-FITC] and T cells with γδ TCR [γδTCR-PE], and 4) thrombocytes [CD41/61-FITC]. All lymphocyte-specific mAb were purchased from Southern Biotech, Birmingham, Alabama. The chicken thrombocyte-specific mAb was purchased from BioRad Life Science, Hercules, California. All mAb used were IgG1 and were prepared at 1:100 dilution in PBS+.

For each PBMC sample, the cells (50 μL) and reagents (50 μL) were added to 4 wells of 96-well round-bottom plates and incubated at 4 °C for 30 min. After the incubation, cells were washed twice in PBS+ by centrifugation of plates at 4 °C for 4 min at $250 \times g$. After the final wash, the pellet was resuspended in 200 μL PBS+ for flow cytometric analysis.

Staining controls included: an isotype control to examine the non-specific binding of antibodies and determine the cut-off between negative and positive fluorescence, and single-stained cells to adjust fluorescence compensation. For controls, 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.

For flow cytometric analysis, each sample was mixed well, transferred into a 1.5-ml microcentrifuge tube, and placed on the sample port of a BD C6 Accuri flow cytometer (Becton Dickinson, San Jose, California) for the 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 software v. 1.05. A region was drawn around the small PBMC population containing lymphocytes and thrombocytes and the percentage of each cell type (B cells and CD4+, CD8+, αβ and γδ TCR+ T cells, thrombocytes, and lymphocytes) determined based on their fluorescent staining. 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). The total T cell population was calculated by adding the proportions of αβ- and γδ-TCR+ 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 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.

All cell population data were analyzed for the effect of diet by one-way analysis of variance using SigmaPlot (Systat Software, Inc, San Jose, California). Differences were considered significant at $P \leq 0.05$. If there was a significant diet 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 and Discussion

The immune system of chickens, like that in mammals, consists of innate and adaptive immunity. Innate immunity includes physical and chemical barriers that prevent

entry of a pathogen, as well as soluble and cellular components working to eliminate or contain the pathogens that infected an individual (Abbas et al., 2018). Cells of innate immunity include phagocytes like heterophils (the avian counterpart to neutrophils) and monocytes/macrophages, as well as granulocytes like eosinophils that fight larger parasites, and basophils that release pro-inflammatory factors. If innate immunity cannot eliminate the pathogen, the more specific adaptive immunity will be called into action. Lymphocytes, specifically B- and T-cells, are the cells of adaptive immunity, with B cells being responsible for antibody production and T cells for cell-mediated immunity. There are several subsets of T cells, such as the CD4+ T helper cells that are critical in the activation of adaptive immunity, CD8+ cytotoxic T cells that eliminate infected host cells, and T cell populations defined by the type of T cell receptor they have to bind antigen (i.e., T cells with αβ TCR that are restricted to recognizing antigen-peptides displayed on MHC-molecules of an antigen-presenting cell, and γδ T cells that do not need antigen presentation and are able to readily respond to frequently encountered microbes) (Abbas et al., 2018). All these WBC circulate in the blood, ready to be called to the tissues to fight infections. Considering the different functions of various WBC in innate and adaptive immunity, analysis of the concentrations and proportions of WBC populations is an important diagnostic tool to determine the health and disease of an individual (Abbas et al., 2018; French et al., 2020). This same approach also provides important insight into the influence of nutrition on immune system development and function (Klasing, 2007; Chandra et al., 2015; French et al., 2020).

The purpose of this study was to examine white blood cell profiles in broilers fed a low crude protein diet with and without *Spirulina* inclusion compared to a standard crude protein diet. The analyses conducted revealed no effect of the three diets on the concentrations of WBC, thrombocytes, RBC, hemoglobin, and hematocrit (Table 1) and on the concentrations and proportions of heterophils, lymphocytes, basophils, eosinophils, and the various lymphocyte populations examined (Tables 2 and 3). Additionally, the ratios between heterophils and lymphocytes (Table 2), as well as the T cell to B cell and the CD4+ T cell to CD8+ T cells ratios (Table 3), were not affected ($P < 0.05$) by dietary treatment. However, the concentration and the proportions of monocytes were greater ($P < 0.05$) in broilers fed the LCP diet. The inclusion of *Spirulina* in the LCP diet returned monocyte levels to those observed with the SCP control diet (Table 2). 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. The addition of 10% *Spirulina* in the LCP diet did not alter the normal blood cell profiles in broilers and prevented the increase in monocytes observed with the LCP diet.

Similar observations were reported by Mullenix et al. (2021) for broilers from the same experiment as the current study. They reported increased ($P < 0.05$) expression levels of pro-inflammatory cytokines, chemokines, and the NLRP3 inflammasome in blood from broilers fed the LCP diet. The increased inflammatory activity was also not observed when *Spirulina* was included in the LCP diet. These findings are in line with our observation of increased proportions and concentrations of monocytes, especially since monocytes are the most likely source of the reported inflammatory activity observed in broilers fed the LCP diet (Abbas et al., 2018).

Conclusions

Analysis of blood cell profiles revealed increased inflammatory activity, i.e., elevated proportions and con-

Table 1. Concentrations of blood cells, hemoglobin, and hematocrit in 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 formulated with 100 g/kg *Spirulina* (LCP-SP).

Blood measurement	SCP	LCP	LCP-SP	*P*-value
WBC (10^3/μL)[†]	11.8 ± 1.84[‡]	13.9 ± 1.41	13.8 ± 1.16	0.561
Thrombocytes (10^3/μL)[†]	2.07 ± 0.13	1.98 ± 0.22	1.78 ± 0.14	0.467
RBC (10^6/μL)[†]	2.63 ± 0.04	2.70 ± 0.06	2.70 ± 0.04	0.517
Hemoglobin (g/dL)[†]	7.71 ± 0.35	7.79 ± 0.10	7.88 ± 0.14	0.658
Hematocrit (g/dL)[†]	61.1 ± 1.91	62.5 ± 1.05	63.2 ± 1.17	0.444

[†] Concentrations measured using an automated hematology analyzer.

[‡] Data are mean ± SEM; n = 5; blood from 2 broilers processed per replicate.

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 formulated with 100 g/kg *Spirulina* (LCP-SP).

WBC Measurements	SCP	LCP	LCP-SP	*P*-value
Heterophils (10^3/μL)[†]	1.39 ± 0.30[¶]	1.84 ± 0.34	1.60 ± 0.19	0.548
Heterophils (% WBC)[‡]	11.5 ± 1.33	12.9 ± 1.22	11.60 ± 0.85	0.637
Lymphocytes (10^3/μL)[†]	9.58 ± 1.44	8.70 ± 1.01	11.23 ± 1.03	0.592
Lymphocytes (% WBC)[‡]	81.4 ± 1.97	78.8 ± 1.84	81.29 ± 1.00	0.478
Monocytes (10^3/μL)[†]	0.236 ± 0.027 b	0.503 ± 0.082 a	0.370 ± 0.076 ab	0.046
Monocytes (% WBC)[‡]	2.17 ± 0.35 b	3.64 ± 0.50 a	2.84 ± 0.77 ab	0.079
Basophils (10^3/μL)[†]	0.32 ± 0.074	0.31 ± 0.088	0.26 ± 0.020	0.970
Basophils (% WBC)[‡]	2.60 ± 0.21	2.16 ± 0.43	1.88 ± 0.14	0.185
Eosinophils (10^3/μL)[†]	0.285 ± 0.075	0.311 ± 0.058	0.290 ± 0.045	0.949
Eosinophils (% WBC)[‡]	2.31 ± 0.40	2.33 ± 0.44	2.18 ± 0.45	0.965
Heterophil/Lymphocyte Ratio[§]	0.143 ± 0.019	0.165 ± 0.043	0.143 ± 0.012	0.573

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

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

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

[¶] Data are 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.

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 formulated with 100 g/kg *Spirulina* (LCP-SP).

Lymphocyte population	SCP	LCP	LCP-SP	*P*-value
B cells (10^3/μL)[†]	1.63 ± 0.30[#]	2.00 ± 0.40	1.76 ± 0.22	0.713
B cells (%)[‡]	16.8 ± 1.81	17.8 ± 1.99	15.5 ± 1.04	0.646
T cells (10^3/μL)[†]	7.95 ± 1.20	8.88 ± 0.66	9.47 ± 0.85	0.523
T cells (%)[‡]	83.2 ± 1.81	82.2 ± 1.99	84.5 ± 1.04	0.646
CD4+ T cells (10^3/μL)[†]	3.66 ± 0.51	4.12 ± 0.35	4.18 ± 0.22	0.585
CD4 + T cells (%)[‡]	39.0 ± 2.65	38.2 ± 1.73	38.1 ± 2.80	0.756
CD8+ T cells (10^3/μL)[†]	1.82 ± 0.19	2.17 ± 0.23	2.40 ± 0.19	0.166
CD8+ T cells (%)[‡]	19.7 ± 1.65	20.1 ± 1.58	21.5 ± 0.58	0.340
αβTCR+ T cells (10^3/μL)[†]	5.86 ± 0.95	6.73 ± 0.57	6.79 ± 0.69	0.627
αβTCR+ T cells (%)[‡]	60.9 ± 1.46	62.3 ± 2.61	60.5 ± 2.08	0.814
γδTCR+ T cells 10^3/μL)[†]	2.47 ± 0.44	2.46 ± 0.19	2.98 ± 0.34	0.491
γδTCR+ T cells (%)[‡]	25.4 ± 1.66	22.9 ± 1.05	26.4 ± 1.38	0.224
T cell/B cell Ratio[§]	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

[†] 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.

[‡] The % of various lymphocyte populations is based on immunofluorescent staining of peripheral blood mononuclear cells and cell population analysis by flow cytometry.

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

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

[#] Data are mean ± SEM; n = 5; blood from 2 broilers processed per replicate.

centrations of monocytes, with the LCP diet that agreed with more complex measurements made by others. This underlines the usefulness of blood cell profile analyses as a window into normal immune system development and function in broilers. Formulation of *Spirulina* into the LCP diet did not alter blood cell profiles compared to the SCP diet and maintained normal monocyte levels. Taken together, these observations support further investigation of *Spirulina* microalgae as a proteinaceous feed component that does not compromise the health of broilers. Lastly, using automated hematology, manual differential WBC counting, and immunofluorescent staining to identify various lymphocytes subsets, this study contributed comprehensive WBC data for modern commercial broilers.

Acknowledgments

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