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LEUKOCYTE PROFILE OF BROILER CHICKENS (*Gallus domesticus*) AFTER CONSUMPTION OF FEED WITH *SPIRULINA* (*Spirulina* sp.) FEED ADDITIVES AND LIQUID NANO CHITOSAN

PROFIL LEUKOSIT AYAM BROILER (*Gallus domesticus*) SETELAH KONSUMSI PAKAN DENGAN ADITIF PAKAN *SPIRULINA* (*Spirulina* sp.) DAN NANOKITOSAN CAIR

Rida Arwanda Gunawan, Sunarno Sunarno*, Muhammad Anwar Djaelani, Kasiyati
Diponegoro University, Prof. Soedarto, SH, No.13, Tembalang, Semarang, Central Java 50275
**Corresponding author:* sunarno@lecturer.undip.ac.id

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Abstract

Broilers (*Gallus domesticus*) are prone to stress and immune suppression due to inadequate environmental conditions and feed quality. *Spirulina*, known for its antioxidant and immunostimulant properties, and liquid nano chitosan, which has antibacterial activity, are considered promising feed additives for broiler health. This study aimed to evaluate the effects of *Spirulina* flour, liquid nano chitosan, and their interaction on broiler leukocyte profiles. A factorial completely randomized design was used, with six treatment groups and four replications. Treatments included a control and diets supplemented with 0; 3; or 6% *Spirulina* powder, combined with 0 or 5% liquid nanochitosan. The treatments were applied for 26 days. Leukocyte observations were performed using Giemsa-stained blood smears at 400× magnification. Data were analyzed using Two-Way ANOVA (α= 0.05) and the Friedman Test. Results showed normal leukocyte morphology. *Spirulina* supplementation had no significant effect (P >0.05) on leukocyte, heterophil, or lymphocyte counts. In contrast, 5% liquid nano chitosan significantly affected (P <0.05) leukocyte and lymphocyte numbers. No interaction between the two additives was observed. In conclusion, *Spirulina* (3–6%) showed potential as a feed additive, while 5% liquid nano chitosan reduced leukocyte and lymphocyte counts. These natural additives may support broiler health without altering leukocyte profiles.

Keywords*:* Antioxidant; Broilers; Feed additive; Leukocyte; Liquid nanochitosan; *Spirulina*

Abstrak

Ayam broiler (*Gallus domesticus*) *rentan terhadap stres dan gangguan sistem imun akibat kondisi lingkungan dan kualitas pakan yang tidak optimal. Spirulina, yang dikenal memiliki sifat antioksidan dan imunostimulan, serta nanokitosan cair yang bersifat antibakteri, memiliki potensi sebagai aditif pakan yang menjanjikan untuk mendukung kesehatan broiler. Penelitian ini bertujuan menganalisis pengaruh tepung Spirulina, nanokitosan cair, dan interaksinya terhadap profil leukosit broiler. Rancangan percobaan yang digunakan adalah rancangan acak lengkap faktorial dengan enam perlakuan dan empat ulangan. Perlakuan terdiri atas kontrol dan pakan basal yang ditambahkan tepung Spirulina 0; 3; atau 6%, dikombinasikan dengan nanokitosan cair 0 atau 5%. Perlakuan diberikan selama 26 hari. Pengamatan leukosit dilakukan pada sediaan darah yang diwarnai Giemsa dengan perbesaran 400×. Data dianalisis menggunakan Anova dua arah (α= 0,05) dan uji Friedman. Hasil menunjukkan morfologi leukosit yang normal. Spirulina tidak berpengaruh nyata (P >0,05) terhadap jumlah leukosit, heterofil, maupun limfosit. Sebaliknya, nanokitosan cair 5% berpengaruh nyata (P <0,05) terhadap jumlah leukosit dan limfosit. Tidak terdapat interaksi antara keduanya. Kesimpulannya, Spirulina 3–6% berpotensi sebagai aditif pakan, sedangkan nanokitosan cair 5% menurunkan jumlah leukosit dan limfosit tanpa mengubah profil leukosit secara keseluruhan. Aditif alami ini berpotensi mendukung kesehatan broiler tanpa mengubah profil leukosit*.

Kata kunci*: Aditif pakan; Antioksidan; Broiler; Leukosit; Nanokitosan cair; Spirulina*

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INTRODUCTION

The increasing demand for broiler meat in Indonesia, with an 8.73% rise in per capita consumption in 2022, has driven higher poultry production (Ministry of Agriculture Directorate General of Livestock and Animal Health, 2023). However, broilers are highly susceptible to stress and immune-related health issues, particularly due to heat and oxidative stress (Sarawit, 2022). Broiler health can be assessed through leukocyte profiles, including white blood cell count (WBC) and differentials such as basophils, eosinophils, heterophils, lymphocytes, and monocytes (Sugiharto, 2016; Hendrawan et al., 2019; Kasiyati et al., 2021). Abnormal leukocyte levels indicate health problems, with elevated counts linked to disease or stress and decreased levels associated with heat stress and nutritional deficiencies (Djaelani et al., 2020; Maulidya et al., 2022). The heterophil-to-lymphocyte (H/L) ratio serves as a stress index, where higher values indicate increased stress (Hendrawan et al., 2019). Antibiotics were previously used to address these issues, but their use as growth promoters has been banned since 2018 as stipulated in the Ministry of Agriculture Regulation No. 14 of 2017, creating the need for safe alternative feed additives (Yuanita, 2023). While plant-based additives such as ginger, turmeric, and teak leaf extract have shown limited effectiveness, algae-based alternatives, particularly *Spirulina*, have demonstrated potential due to their phycocyanin content, which has antioxidant and anti-inflammatory properties (Firdiyani et al., 2015).

Spirulina is a species of *algae, that* contains various bioactive compounds, including phycocyanin, flavonoids, and phenolic acids, which function as antioxidants, antimicrobials, and immunostimulants (Elbaz et al., 2022). While some studies indicate that *Spirulina* supplementation enhances immune response (Hassan et al., 2022), others report no significant effects on leukocyte profiles (Situmeang et al., 2017), highlighting the need for further research. To enhance its effectiveness, liquid nano chitosan, derived from chitin in shrimp, lobster, and crab, can be added to drinking water, as it supports immunity by promoting beneficial microbes and neutralizing oxidative stress (Sahara et al., 2019; Dinana et al., 2019; Gu et al., 2022). Chitosan additives have been found to positively influence leukocyte counts in broilers (Rakhimovna et al., 2023), and previous studies on *Spirulina* with selenium nanoparticles have shown improved immune responses (Al-Khalaifah et al., 2022). However, the combination of *Spirulina* with other nanoparticles remains underexplored. This study aims to investigate the interaction of *Spirulina* powder as a feed additive and liquid nanochitosan in drinking water, analyzing their effects on broiler health through hematological analysis, and leukocyte morphology. The findings are expected to provide a solution for broiler health management, particularly in addressing stress and immune-related challenges. Researchers expect that the results of this study will provide solutions to health issues or environmental stress commonly experienced by broiler chickens through the utilization of the combination of *Spirulina* powder and liquid nano chitosan as feed additives.

MATERIALS AND METHODS

The research was conducted in an experimental chicken coop at Perintis Kemerdekaan Street No. 30, Semarang with 14 days of acclimation, 26 days of treatment, and blood samples were taken when the chickens were 42 days old. The preparation and observation of blood smear preparations were carried out at the Biologi Struktur dan Fungsi Hewan (BSFH) Laboratory, Diponegoro University. Total leukocyte and differential leukocyte analysis were conducted at the Animal Health Laboratory in Semarang.

The equipment used included chicken coops, feed and water containers, a lamp, spray bottles, cable ties, digital scales, measuring cups, plastic containers, stirring spoons, labels, a thermohygrometer, 1 mL disposable syringes, Ethylene-Diamine-Tetraacetic-Acid (EDTA) tubes, cooler boxes, a hematology analyzer, glass slides, and pipette droppers. The materials used included unsexed day-old chicken (DOC) broilers, BR1 broiler feed, 511 broiler feed, *Spirulina* powder (*Spirulina* sp.), liquid nano chitosan, water, rodalon solution, Vita Chick, Vita Stress, broiler blood samples, ice gel, Giemsa stain, methanol, and aquadest.

Experimental Design

The study used a 3 × 2 Factorial Completely Randomized Design (CRD), consisting of two treatment factors: *Spirulina* powder at 0; 3; and 6%, and liquid nanochitosan at 0 and 5%. The experimental design included 6 treatments with 4 replications (Table 1). The treatment groups are as follows: S_0N_0 (standard feed with no additives or control), S_3N_0 (standard feed with 3% *Spirulina* powder, no liquid nano chitosan), S_6N_0 (standard feed with 6% *Spirulina* powder, no liquid nano chitosan), S_0N_5 (standard feed with no *Spirulina* powder, 5% liquid nano chitosan), S_3N_5 (standard feed with 3% *Spirulina* powder and 5% liquid nano chitosan), and S_6N_5 (standard feed with 6% *Spirulina* powder and 5% liquid nanochitosan).

Table 1. Experimental design of factorial completely randomized design (CRD) with *Spirulina* flour and liquid nano chitosan treatments

Replication	Treatment					
	S_0N_0	S_3N_0	S_6N_0	S_0N_5	S_3N_5	S_6N_5
1	S_0N_0 1	S_3N_0 1	S_6N_0 1	S_0N_5 1	S_3N_5 1	S_6N_5 1
2	S_0N_0 2	S_3N_0 2	S_6N_0 2	S_0N_5 2	S_3N_5 2	S_6N_5 2
3	S_0N_0 3	S_3N_0 3	S_6N_0 3	S_0N_5 3	S_3N_5 3	S_6N_5 3
4	S_0N_0 4	S_3N_0 4	S_6N_0 4	S_0N_5 4	S_3N_5 4	S_6N_5 4

Note: S= *Spirulina* flour; N= liquid nanochitosan

Preparation of the Coop

The coops are arranged in rows and sprayed with rodalon at a concentration of 15 mL per 10 L of water before use. Each coop is equipped with feed and water containers, as well as light bulbs to provide warmth for the day-old chickens (DOC).

Preparation of Test Animals

A total of 24-day-old chickens (DOC) were allowed to rest for 30 minutes upon arrival, given a 10% sugar water solution, and then acclimated for 14 days to allow for the maturation of blood formation, as leukopoiesis occurs over 7–11 days (Aliviameita & Puspitasari, 2019). The broilers were provided with Vita Chick during the first week and Vita Stress from 2 to 4 weeks of age before the treatments were administered.

Preparation of Feed and Drinking Water

The feed used includes BR1 (for chickens aged 0–3 weeks), 511 (for chickens aged over 3 weeks until harvest), and *Spirulina* powder. The treatments are as follows S_0 (2,000 g of commercial feed with no *Spirulina* powder), S_3 (1,940 g of commercial feed with 60 g of *Spirulina* powder), and S_6 (1,880 g of commercial feed with 120 g of *Spirulina* powder) (Sunarno et al., 2023a). Drinking water preparation involves providing water and liquid nano chitosan according to the treatments. N_0 (tap water without liquid nano chitosan) and N_5 (liquid nano chitosan with a concentration of 5%). The process of making liquid nano chitosan using the ionic gelation method is carried out by dissolving chitosan in acid, and then anionic or polyanion polymers are added, so that nanoparticles are formed spontaneously through mechanical stirring at room temperature (Sunarno et al., 2023b).

Research Treatment

The treatments for the chickens were administered after a 14-day acclimation period so that the chickens could adapt to the cage environment before the treatment was carried out. Another purpose of acclimatization is to allow the blood formation process to mature, as the leukopoiesis process takes place within 7–11 days (Aliviameita & Puspitasari, 2019). Chickens were provided with water and feed ad libitum (Perawati et al., 2021) according to the concentrations specified in the experimental design. Temperature and humidity were recorded every morning and evening, as these external factors could influence the chickens.

Blood Sample Collection

Blood samples were taken when the broilers were 42 days old. Blood was collected via the brachial vein using a 1 mL syringe. The samples were placed in ethylene-diamine-tetraacetic-acid (EDTA) tubes and then stored in an ice box (Bikrisima et al., 2013). The blood samples were transported to the Animal Health Laboratory in Semarang for analysis.

Preparation and Observation of Blood Smear Preparations

Blood smear preparations were made using two glass slides, which were labeled according to the sample. One drop of blood was placed on a glass slide about 2 cm from the edge. The second glass slide was positioned at a 30–40° angle to the first slide with the blood drop. The second slide was then drawn back until it touched the blood drop, allowing capillarity to occur, and then quickly pushed forward (Susilawati et al., 2021). The smear was air-dried, fixed with methanol, and left to dry. Next, the preparation was stained with Giemsa and allowed to air-dry for about 15–30 minutes (Islawati et al., 2021). The blood smear preparations were observed under a microscope with 400× magnification to examine the differential leukocyte profile (Maheshwari et al., 2017).

Total Leukocyte and Differential Leukocyte Analysis

Blood analysis was conducted using a hematology analyzer, which is an automated device designed for hematological testing. The principle of this analyzer is based on the calculation and measurement of cells, which are determined by changes in electrical resistance as blood cells pass through an electrically conductive diluent, which is an isotonic solution containing electrolytes. The electrical impedance is recorded according to the volume or size of the cells, as variations in blood cell impedance are detected when they move through a gap with electrodes positioned on either side (Saputra & Aristoteles, 2022).

To operate the hematology analyzer, the power cable must be connected to a stabilizer, and the "on" button should be pressed on the switch. The display will indicate "Please wait." It must be ensured that the blood sample is properly mixed with the anticoagulant. Subsequently, the "whole blood (WB)" button on the screen should be selected, followed by pressing the "id" button to enter the sample number before confirming with "enter". The top of the sample chamber needs to be opened so that the sample can be placed in the adaptor. After closing the sample chamber, the "run" button should be pressed. The results will then be automatically displayed on the screen and printed out (Dabukke et al., 2023).

Data Analysis

Data analysis was performed using SPSS Version 22. Normality and homogeneity tests were conducted. The data followed a normal distribution and were homogeneous ($P > 0.05$) for leukocyte count and lymphocyte variables. For the heterophil and H/L ratio variables, which did not follow a normal distribution and were not homogeneous ($P < 0.05$), data transformation was applied. A two-way Analysis of Variance (ANOVA) at a 5% significance level was conducted for total leukocytes, heterophils, and lymphocytes. The transformed data still did not follow a normal distribution and were not homogeneous ($P < 0.05$) for the H/L ratio variable. Therefore, the data were converted to ordinal form and analyzed using the non-parametric Friedman test (Gio & Elly, 2016).

RESULTS

The phytochemical contents of *Spirulina* spp. are presented in Table 2. The flavonoid content in *Spirulina* was reported to be 79.6 mg/g. Flavonoids played an important role as antioxidants that neutralized free radicals and exhibited immunostimulatory effects. The phycocyanin content in *Spirulina* spp. was 0.301 mg/g. This pigment possesses anti-inflammatory and antioxidant activities. Another bioactive compound found in *Spirulina* was chlorophyll, ranging from 1,300 to 1,700 mg/100 g. This pigment exhibited detoxifying properties and promoted tissue regeneration, including blood cells. Another pigment, carotenoid, was present in amounts ranging from 400 to 650 mg/100 g in *Spirulina* spp. and functioned to enhance immune function and protect cells through its antioxidant activity. The β-carotene pigment in *Spirulina* spp. Ranged from 150 to 250 mg/100 g and served as a precursor of vitamin A, which was essential for the growth and differentiation of immune cells. The

next pigments were xanthophylls and zeaxanthin. Xanthophylls in *Spirulina* spp. were present in the range of 250 to 470 mg/100 g and acted as potent antioxidants. Zeaxanthin was found at levels ranging from 125 to 200 mg/100 g, and specifically functioned to protect body cells from oxidative stress and enhance immune responses by maintaining the integrity of immune cell membranes.

Table 2. Phytochemical compounds of *Spirulina*

Phytochemical compounds of *Spirulina*	Contents
Flavonoid	79.6 mg/g
Phycocyanin	0.301 mg/g
Chlorophyll	1,300–1,700 mg/100 g
Carotenoid	400–650 mg/100 g
β-carotene	150–250 mg/100 g
Xanthophylls	250–470 mg/100 g
Zeaxanthin	125–200 mg/100 g

The main composition of the nano chitosan formulation is presented in Table 3. This nano chitosan was a modified form of chitosan in nanoscale size, which exhibited more efficient bioactive properties. This composition was essential for understanding its functional potential in enhancing broiler chicken health, particularly through modulation of the immune system. The nano chitosan consisted of chitosan, crosslinking agents, water, ash, nitrogen, and chitin. Chitosan, present at 85–95%, was the primary component of the nano chitosan formulation. The next component was the crosslinking agent (5–15%), such as sodium tripolyphosphate (TPP), which was used during the nanoparticle formation process to stabilize the nano chitosan structure. The subsequent component was water (≤12%), where the low water content indicated the physical stability of the nano chitosan. A water level of ≤12% helped prevent degradation or microbial growth during storage. The ash content was recorded at ≤5%. Ash represented the inorganic residue of non-combustible materials. A value of ≤5% indicated that the formulation was relatively pure and did not contain high levels of heavy metals or undesirable minerals. Nitrogen was present at ≤5%, representing the protein or amino groups derived from chitosan. This element played a role in the biological activity of the nano chitosan, as the amino groups enabled interaction with cell membranes and contributed to its immunostimulatory effects. Chitin was present at <5%. Chitin, a natural precursor of chitosan, is typically found in the exoskeletons of crustaceans. The low chitin content indicated a high degree of deacetylation, meaning that most of the chitin had been successfully converted into active chitosan. The higher the chitosan content, the greater the potential for bioactive efficacy.

Table 3. Contents of nanochitosan

Contents of nanochitosan	Percentage (%)
Water	≤12
Ash	≤5
Nitrogen	≤5
Kitin	<5
Chitosan	85–95
Crosslinking agent	5–15

Table 4 presented data on feed consumption (g/day) and water intake (mL/day) of broiler chickens that received treatments combining *Spirulina* powder (S) and liquid nano chitosan (N). The highest feed consumption was observed in the S_6N_0 group (142.39 g/day), which received 6% *Spirulina* without nano chitosan. This result indicated that *Spirulina* supplementation could enhance feed palatability and intake in broiler chickens, particularly at higher concentrations. Conversely, the lowest feed consumption was recorded in the S_0N_5 group (96.31 g/day), which received nano chitosan without *Spirulina*. This reduction might have been due to the astringent properties of chitosan or a potential decrease in palatability. The combination treatments (S_3N_5 and S_6N_5) showed lower feed intake compared to *Spirulina*-only groups (S_3N_0 and S_6N_0), suggesting a possible additive or antagonistic interaction affecting feed preference.

Table 4 also presents data on drinking water consumption in broiler chickens. Water intake ranged from 573.52 mL/day (S_0N_5) to 595 mL/day (S_6N_0). The increase in water consumption in the S_6N_0 group was consistent with the increase in feed intake, as broiler chickens have been shown to exhibit a linear relationship between feed intake and water requirements. The groups receiving combined *Spirulina* and nano chitosan (S_3N_5 and S_6N_5) demonstrated relatively stable water consumption, indicating that the supplementation did not induce significant changes in fluid homeostasis.

Table 4 Feed and drink consumption data

Treatment	Feed consumption (g/day)	Drink consumption (mL/day)
S_0N_0	138.04	577.60
S_3N_0	129.77	580.01
S_6N_0	142.39	595
S_0N_5	96.31	573.52
S_3N_5	128.89	576.67
S_6N_5	112.65	585.52

The analysis revealed that the addition of *Spirulina* powder did not significantly affect the total leukocyte count, heterophils, or lymphocytes across treatments ($P > 0.05$) are presented in Table 5. The addition of liquid nano chitosan significantly affected the total leukocyte count and lymphocyte count across treatments. However, liquid nano chitosan did not significantly affect the heterophil count across treatments. Two-way ANOVA analysis indicated that there was no interaction between *Spirulina* powder and liquid nano chitosan.

Table 5. Average total leukocyte count and differential leukocytes after treatment

Treatment	Variable ($\bar{x}$ ± SD)			
	Total leukocyte count ($\times 10^9$/L)	Heterophils ($\times 10^9$/L)	Lymphocytes ($\times 10^9$/L)	H/L ratio
Spirulina (S)				
S_0	74.38 ± 12.99	2.16 ± 1.44	72.21 ± 11.85	0.03 ± 0.02
S_3	74.59 ± 14.77	1.89 ± 0.70	72.70 ± 14.20	0.03 ± 0.01
S_6	73.91 ± 73.91	1.73 ± 0.56	72.19 ± 6.75	0.02 ± 0.01
Nanochitosan (N)				
N_0	79.78 ± 11.61[b]	2.27 ± 1.13	77.51 ± 10.79 [b]	0.03 ± 0.01
N_5	68.81 ± 8.80[a]	1.58 ± 0.61	67.23 ± 8.52 [a]	0.02 ± 0.01
Spirulina (S) x Nanochitosan (N)				
S_0N_0	81.35 ± 14.60	2.68 ± 1.83	78.68 ± 13.15	0.03 ± 0.02
S_3N_0	84.95 ± 13.39	2.20 ± 0.93	82.75 ± 12.60	0.03 ± 0.01
S_6N_0	73.03 ± 1.26	1.93 ± 0.32	71.10 ± 1.40	0.03 ± 0.01
S_0N_5	67.40 ± 7.14	1.65 ± 0.90	65.75 ± 6.57	0.03 ± 0.01
S_3N_5	64.23 ± 6.61	1.58 ± 0.17	62.65 ± 6.49	0.03 ± 0.01
S_6N_5	74.80 ± 10.53	1.53 ± 0.72	73.28 ± 10.06	0.02 ± 0.01
Normal Range	0.01–0.04	2.23–9.76	0.06–0.020	<0.20

Note: S_0N_0 (control), S_3N_0 and S_6N_0 (feed with 3% and 6% *Spirulina* powder, respectively, without liquid nano chitosan), S_0N_5 (feed without *Spirulina* powder, with 5% liquid nano chitosan), S_3N_5 and S_6N_5 (feed with 3% and 6% *Spirulina* powder, respectively, with 5% liquid nano chitosan)

The use of **Giemsa staining** in this study allows for the visualization of different types of leukocytes (white blood cells) in the blood smear, which is crucial for understanding the immune response in broiler chickens. **Heterophils** are the avian equivalent of neutrophils and play a key role in the initial immune response, particularly in fighting bacterial infections. **Lymphocytes**, on the

other hand, are involved in adaptive immunity, with roles in antibody production and recognizing foreign pathogens. By examining these differential leukocytes under **400× magnification**, researchers can assess the chicken's immune status and response to various stressors or infections. The **differential leukocyte profile**, shown in **Figure 1**, provides visual evidence of the distribution and abundance of these immune cells, which can be used to evaluate the broiler chickens' health and immune function.

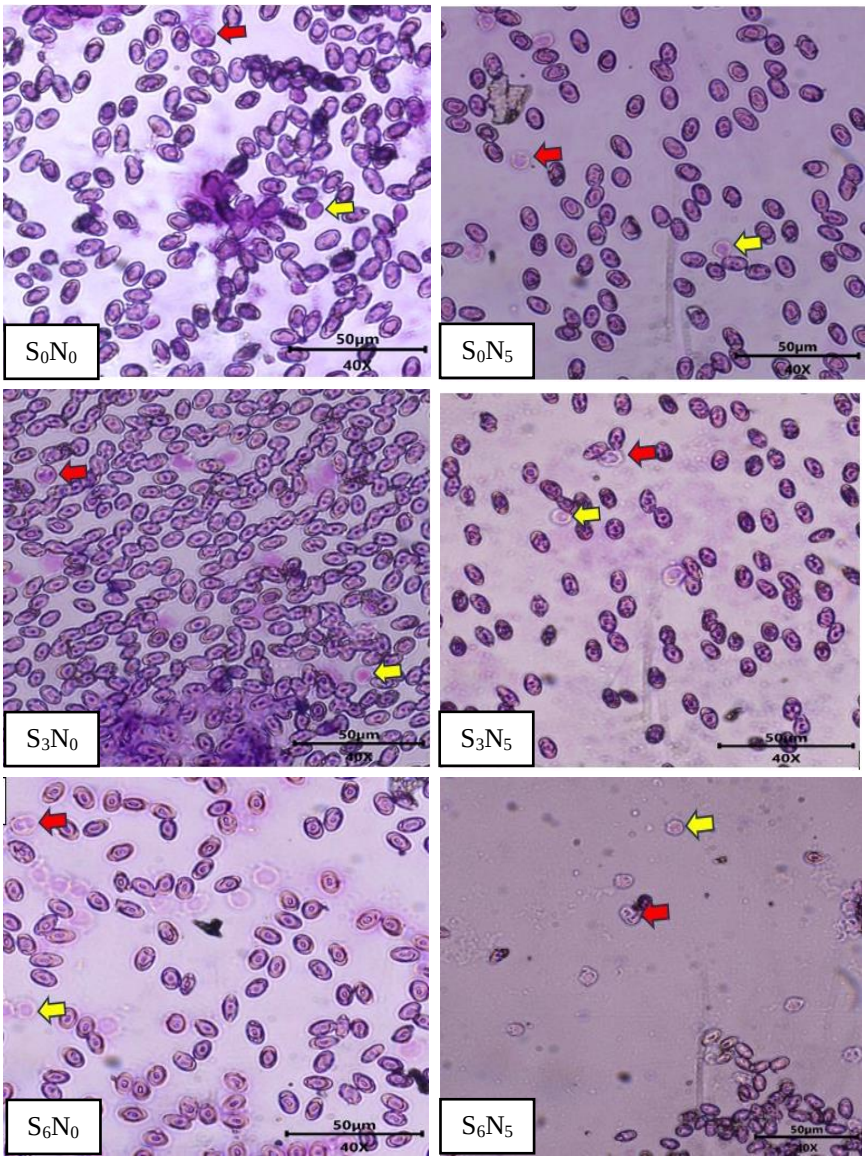

Figure 1. Differential leukocytes in broiler chicken blood smears. S_0N_0 (control), S_3N_0 and S_6N_0 (feed with 3% and 6% *Spirulina* powder without liquid nano chitosan), S_0N_5 (feed without *Spirulina* powder with 5% liquid nano chitosan), S_3N_5 and S_6N_5 (feed with 3% and 6% *Spirulina* powder with 5% liquid nano chitosan). Red arrows indicate heterophils. Yellow arrows indicate lymphocytes.

DISCUSSION

The administration of *Spirulina* powder did not significantly affect the total leukocyte count, heterophils, and lymphocytes among treatments ($P > 0.05$). The lack of significant differences may be because the amount of *Spirulina* provided was adequate for the chicken's needs. Additionally, the lack of significant results could be attributed to the fact that polysaccharides and bioactive compounds, such as polyphenols, in *Spirulina*, may not be fully absorbed by the digestive system. Chen et al. (2018) reported that only about 5–10% of total polyphenols are absorbed by the small intestine, with the remainder accumulating in the large intestine and being excreted with the feces.

The polyphenols that are absorbed are often conjugated in the small intestine and liver, which may not significantly impact the leukocyte profile of broilers.

Polysaccharides and polyphenols that are not digested by the small intestine are used by microbes as substrates for fermentation in the colon, producing short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate (Wang et al., 2020). This fermentation indirectly impacts metabolism and immune function because SCFAs are absorbed into the bloodstream and can affect the leukocyte profile of broilers. Vinolo et al. (2011) explain that SCFAs are involved in gut microbiota effects and immune function through mechanisms such as activating cell surface receptors, namely G Protein-Coupled Receptors (GPCRs), and inhibiting histone deacetylases. SCFAs also play a role in regulating leukocyte function, including cytokine production (TNF-α,

IL-2, IL-6, and IL-10), chemokines (MCP-1 and CINC-2), and influencing leukocyte migration to areas of inflammation, as well as contributing to the destruction of pathogenic microbes. This indirect impact can help maintain the leukocyte profile, preventing significant changes in leukocyte levels.

The administration of liquid nano chitosan significantly affected the total leukocyte count and lymphocyte count among treatments ($P < 0.05$). The significant differences in results are attributed to the differing compositions of the two types of water administered, which caused treatment N_5 to have lower leukocyte and lymphocyte values compared to N_0. Chitosan has antibacterial properties that can minimize the risk of bacterial infection by forming a polymer membrane on the surface of bacterial cells (Arifianingsih et al., 2014). Nanochitosan also influences the leukopoiesis process by inhibiting the proliferation of cells that have exceeded normal numbers, as well as inhibiting degranulation and cytokine production through its antioxidant capacity against superoxide anions. Chitosan can induce apoptosis but may also inhibit apoptosis due to its organic functional groups (Zivarpour et al., 2021). Liquid nanochitosan did not significantly affect heterophil counts among treatments ($P > 0.05$). The heterophil count was not influenced by the differences between the two types of drinking water provided. Jannah et al. (2017) explained that the lack of significant effect on heterophils may occur because the chickens were healthy and not exposed to pathogens that stimulate heterophil formation.

The two-way ANOVA analysis showed no interaction between *Spirulina* powder and liquid nano chitosan on total leukocyte count, heterophil count, and lymphocyte count. The lack of interaction can be attributed to several factors, including differences in mechanisms affecting leukocytes, different chemical properties, and the administration being performed in varying media and concentrations. El-Shall et al. (2023) explained that *Spirulina* contains natural antioxidants, such as polyphenols, carotenoids, and phycocyanin, which have the potential to influence beneficial gut microbiota. Polyphenols have antibacterial properties through mechanisms such as bacterial invasion and inhibition of pathogenic bacteria motility. *Spirulina* also has antiviral potential by inhibiting virus entry into the host and stimulating cytokine production. In the immune system of chickens, *Spirulina* can enhance phagocytosis by macrophages and activation of natural killer (NK) cells.

Liquid nanochitosan contains chitin, which has potential antimicrobial properties against both Gram-positive and Gram-negative bacteria. The antimicrobial mechanism of chitosan is not fully detailed; generally, chitosan inhibits bacterial growth by forming a polymer membrane around bacteria, preventing the delivery of nutrients to them. Chitosan can also affect immune responses by activating innate immune cells and inducing the production of chemokines and cytokines (Menconi et al., 2014). Both *Spirulina* and nanochitosan have prebiotic effects. Situmeang et al. (2017) explained that *Spirulina* contains mannose and rhamnose, which have the potential to stimulate the development of beneficial bacteria in the digestive tract. Nanochitosan, as a prebiotic, facilitates the growth of beneficial bacteria. The similar potential of both substances does not interact to affect the leukocyte profile of the broiler.

The total leukocyte count, as shown in Table 5, ranges from $67.40–84.95 \times 10^9$/L. This result exceeds the normal range for leukocyte count in chickens. Purnomo et al. (2015) state that the normal leukocyte count for broiler chickens is $6–40 \times 10^3$/mL, equivalent to $0.006–0.04 \times 10^9$/L. The elevated leukocyte levels observed in this study did not lead to a decrease in the health condition of the broiler

chickens. A study by Jannah et al. (2017) reported a total leukocyte count ranging from 225.20 to 487.40 × 10^3/mL or 0.2252 × 10^9/L, indicating that the chickens remained healthy despite the elevated leukocyte count. The high leukocyte count observed in this study and the previous research does not necessarily indicate illness. The broilers, based on observations, displayed signs of good health, including proportional body weight for their age, active movement, normal feed and water consumption, and bright, clear eyes. Purnomo et al. (2015) explain that a high leukocyte count does not always signify illness, as an increase in leukocytes may indicate a humoral and cellular immune response to the presence of pathogenic organisms.

Prakoeswa (2020) explains that leukocytes are closely related to both humoral and cellular immune responses. Differential leukocytes, particularly lymphocytes, play a crucial role in the immune response. Lymphocytes, which include B cells and T cells, have different roles in the immune system. B cells, in the humoral immune response, act as precursors to plasma cells and are responsible for producing antibodies. T cells, involved in the cellular immune response, respond to foreign cells within the body through a cytotoxic mechanism. The cytotoxic properties of T cells are due to the presence of perforin proteins, which enable them to create pores in the target cells, induce apoptosis in infected cells, and destroy or kill cells infected by pathogens.

The results of the study indicate that the total leukocyte count did not significantly differ between treatments when *Spirulina* powder was used as a feed additive ($P > 0.05$), meaning that the control and treatment groups had relatively similar total leukocyte counts. The addition of *Spirulina* powder can be recommended for further research, but with some considerations. Although the results showed that total leukocytes did not differ significantly between treatments ($P > 0.05$), previous research by El-Shall et al. (2023) demonstrated that the consumption of feed with *Spirulina* additives resulted in a high leukocyte count, as a form of immune response. *Spirulina* contains bioactive compounds with immunomodulatory properties, which enhance the immune system by producing antibodies to fight off invading viruses. This type of feed additive also contains spirulina polysaccharides, which function to prevent virus replication in vitro and inhibit viruses from entering host cells. *Spirulina* is also known to have antibacterial properties, being effective against both Gram-positive and Gram-negative bacteria. One possibility why in this study the use of *Spirulina* powder as a feed additive did not have a significant effect on total leukocytes is the dosage that is not yet appropriate. Adjustment of the dosage based on body weight or metabolic needs of the test animals needs to be studied further.

The total leukocyte count differed significantly ($P < 0.05$) between the chickens given tap water and those given 5% nanochitosan. Table 5 shows that the total leukocyte count for broiler chickens given 5% nanochitosan (N_5) was 68.81 × 10^9/L. This value is lower compared to broiler chickens given tap water (N_0), which showed a total leukocyte count of 79.78 × 10^9/L. The lower leukocyte count indicates that nano chitosan was effective in reducing the total leukocyte count compared to the control. Sahara et al. (2019) explain that chitosan has antimicrobial properties that help enhance the immune system or function as an immunomodulator, thereby minimizing the potential for inflammation, which results in a lower leukocyte count in the N_5 treatment compared to N_0. The amino groups in Nanochitosan act as immunomodulators. Aranaz et al. (2021) state that the -COO- (ester group) from the microorganism cell membrane can interact with the -NH_2- groups present in chitosan. This interaction helps block bacteria from accessing nutrients, thereby inhibiting bacterial growth. The study by Ivanishcheva and Sizova (2021) also demonstrates that chitosan can reduce the total leukocyte count in broiler chickens by 5.3% compared to the control.

The analyzed levels of heterophils ranged from 1.53–2.68 × 10^9/L. Table 5 shows that the normal heterophil count was only found in the control group (S_0N_0), with a count of 2.68×10^9/L, while the heterophil counts in all other treatments were below normal, ranging from 1.53–2.20×10^9/L. This difference was statistically insignificant ($P > 0.05$), and based on morphological characteristics and behavior, the broiler chickens were in a healthy condition. Widhowati et al. (2015) state that the normal heterophil count in chickens ranges from 2.23–9.76 × 10^3/mm^3, equivalent to 2.23–9.76 × 10^9/L. Jannah et al. (2017) show that a normal percentage of heterophils ranges from

18.8–34.6%. Generally, the normal range of heterophils in broiler chickens is 20–30%. Increases or decreases in heterophil counts are not problematic as long as the variations are not significantly different from the normal range.

The heterophil counts in the other treatments were below normal, whereas the control group was within the normal range. Lower-than-normal heterophil counts indicate a reduced nonspecific response to pathogens. *Spirulina* and nano chitosan have antimicrobial properties that can minimize the likelihood of bacterial infections. Heterophils are not produced in large quantities unless there is an indication of a bacterial attack. Verawati and Heru (2023) explain that heterophil counts below the normal range indicate the absence of bacterial infection within the body.

The lymphocyte counts, according to Table 5, ranged from 62.65–78.68 × 10^9/L. This range exceeds the normal limit for lymphocytes in broilers. Fahreza et al. (2020) state that the normal lymphocyte count for broiler chickens is 5.52–20.36 × 10^3/mL, equivalent to 0.00552–0.02036 × 10^9/L. Sugiharto et al. (2022) also showed that the lymphocyte count with 0.3% *Spirulina* treatment was above the normal range, at 186 × 10^9/L. The higher-than-normal lymphocyte count could be due to a viral infection affecting the chickens. Olivia et al. (2017) explain that elevated lymphocyte levels indicate an immune response in chickens to fight viral infections. The immune response to the presence of pathogens or viruses occurs through both humoral and cellular mechanisms.

Meilani et al. (2023) explain that the humoral immune response involves B lymphocytes, while the cellular immune response involves T lymphocytes.

The *Spirulina* powder had no significant effect on lymphocyte counts across treatments ($P > 0.05$). Both the control and treated groups had relatively similar lymphocyte counts. This result indicates that *Spirulina* powder did not significantly affect lymphocyte levels. This lack of effect could be due to external factors such as temperature and humidity around the cages. Suboptimal environmental conditions can reduce the effectiveness of *Spirulina* powder on lymphocyte counts. Astuti et al. (2019) mention that temperature can affect the phycocyanin pigment in *Spirulina*. Mauliasari et al. (2019) explain that the phycocyanin pigment in *Spirulina* acts as an antioxidant but is sensitive to temperature and humidity, and has low stability. Suboptimal temperature and humidity may have affected the *Spirulina*'s effectiveness on lymphocytes due to degradation or decreased quality, resulting in no significant difference.

The lymphocyte count differed significantly between treatments ($P < 0.05$) when different types of drinking water were provided. The administration of liquid nano chitosan can stimulate both humoral and cellular immune responses in broiler chickens. Liquid nano chitosan appears to be one-factor causing lymphocyte counts to be higher than the normal range. In this study, the treatment with liquid nano chitosan could be considered an antigen since the chickens had not been previously exposed to it. Meilani et al. (2023) state that antigens can be proteins, polysaccharides, lipids, or chemical compounds recognized by B lymphocytes. Antigen recognition by B lymphocytes occurs with the help of dendritic cells, which function as antigen-presenting cells (APCs). B cells activated by the antigen undergo differentiation and develop into memory B cells and plasma cells. Memory B cells express antibodies similar to those of the parent B cells, while antibodies from plasma cells are released to combat the pathogen or target cells.

The Friedman analysis shows that the H/L ratio in the treatments was not significantly different from the control. The lack of significant difference is suspected to be due to the chickens not being in a stressed condition. Table 5 shows that the H/L ratio in this study ranged from 0.02–0.03.

Fahrina et al. (2021) explain that the H/L ratio is an indicator of stress levels in poultry. An H/L ratio of 0.2 indicates low stress, 0.5 indicates moderate stress, and 0.8 indicates high stress. Stress in chickens can be influenced by external factors. This study had a temperature range of 25.65–33.37 °C and humidity of 56.82–79.63%. Masti et al. (2020) mention that the optimal temperature for broiler life is between 18–22 °C. The humidity needed for broilers is 50–60% (Mansyur, 2018). The heat conditions in this study were still tolerable for the broilers, as evidenced by the absence of stress in the test animals. El-Shall et al. (2023) state that the *Spirulina* feed additive provided to broiler chickens was effective in reducing the adverse effects of high environmental temperatures.

The addition of ***Spirulina* flour** and **liquid nanochitosan** can effectively minimize heat stress and counteract the effects of free radicals, which cause oxidative stress, due to their unique bioactive properties. As described by Moustafa et al. (2021), ***Spirulina*** contains a variety of bioactive compounds that act as antioxidants. These antioxidants can inhibit, break, and stop the production of free radicals, which are highly reactive molecules that can damage cells, tissues, and organs, especially under heat-stress conditions. By disrupting the chain reactions of free radicals, *Spirulina* helps reduce the negative impacts of environmental temperature stress. Antioxidants, in general, work by balancing oxidation and reduction reactions, neutralizing the harmful effects of free radicals, and restoring cellular stability. Furthermore, **liquid nano chitosan** plays a critical role in managing oxidative stress. According to Dinana et al. (2019) and Gu et al. (2022), **nano chitosan** can minimize the effects of free radicals in poultry by inhibiting the production of reactive oxygen species (ROS) and enhancing the activity of intracellular antioxidant enzymes. Ivanova and Zvezdelina (2020) emphasize that nano chitosan regulates redox reactions within biological systems by reducing ROS production, thus preventing oxidative damage. Sari et al. (2013) explain that nano chitosan has a unique ability to bind to ROS and stabilize them, preventing further radical formation or reducing their reactivity. For example, the hydroxyl radical (OH^-) produced from lipid oxidation can react with hydrogen ions on the NH^{3+} groups of chitosan, resulting in the formation of stable molecules that reduce oxidative stress. Together, *Spirulina* and nanochitosan complement each other by providing antioxidant protection, reducing oxidative damage, and enhancing the body's resilience to heat stress and free radical-induced damage. This dual action helps maintain cellular integrity and overall health during stressful environmental conditions.

Blood smears are often considered inaccurate when scanned with a microscope with limited magnification; therefore, a hematology analyzer is used as the main method in this study, with blood smear preparations serving as visual support to clarify the results obtained. The leukocyte morphology shown in Figure 1 indicates that each treatment contains differential leukocytes including heterophils and lymphocytes. Heterophils appear to have two to four lobes and their cytoplasm contains faint granules. This finding is consistent with the report by Hadrian et al. (2023), which states that Giemsa staining provides a weaker color to the cytoplasm. Thida et al. (2021) describe that heterophils have round, spindle-shaped, and oval forms with granules covering the nucleus, making the nuclear segmentation less visible. Heterophils generally have two to four segments, with the nucleus appearing dark purple in Giemsa staining. The acidic nature of the nucleus has a strong affinity for the basic Giemsa stain, resulting in a dark purple coloration. Lymphocytes, as shown in Figure 1, have a centrally located nucleus with a dark purple color and cytoplasm that does not contain granules. Thida et al. (2021) state that lymphocytes have a nucleus with coarse chromatin that appears dark purple, with no granules present in the cytoplasm. The size of lymphocytes appears to vary, with small, medium, and large sizes, all of which are normal and functional. Kolesnik et al. (2020) indicate that *Aves* lymphocytes come in various sizes. The observed morphology of broiler blood shows normal cells, as evidenced by cells that do not exhibit degeneration. This leukocyte profile suggests that the addition of *Spirulina* powder and liquid nano chitosan as drinking water additives does not have cytotoxic effects on leukocytes.

CONCLUSION

Spirulina at 3% and 6% in this study has the potential as a feed additive. Liquid nano chitosan at 5% as drinking water has the potential to decrease the number of leukocytes and lymphocytes. The feed and drinking water additives provided do not interact with each other and do not cause significant changes in the broiler leukogram profile. Researchers recommend conducting further research to explore potential oxidative stress by measuring total antioxidant levels, malondialdehyde (an oxidant), and cortisol hormones in the blood.

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