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LEUKOCYTE PROFILE OF BROILER CHICKENS (*Gallus domesticus*) AFTER CONSUMPTION OF FEED WITH SPIRULINA (*Spirulina* sp.) FEED ADDITIVES AND LIQUID NANOCHITOSAN (14pt)

PROFIL LEUKOSIT AYAM BROILER (*Gallus domesticus*) SETELAH KONSUMSI PAKAN DENGAN ADITIF PAKAN SPIRULINA (*Spirulina* sp.) DAN NANOKITOSAN CAIR (12 pt)

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***Abstract** (12 pt)*

Single feed additives in broiler (Gallus domesticus) cultivation have not been effective in increasing immunity and reducing metabolic stress. Spirulina and liquid nanochitosan can be used as feed additives because they are antioxidants and immunostimulants, thereby minimizing metabolic stress and modulating broiler immunity. This study aims to analyze the effect of Spirulina flour, liquid nanochitosan, and their interactions on broiler leukocyte profiles. The research design used factorial RAL, 6 treatments and 4 replications, namely control and basal feed groups with 0%, 3% and 6% Spirulina, and 0% and liquid nanochitosan. Giemsa stained blood smears were observed at a magnification of 40x10. Data were analyzed using Two Way ANOVA with a significance level of 5% and the Friedman Test. The results showed normal leukocyte morphology, Spirulina had no significant effect (P>0.05) on the number of leukocytes, heterophils and lymphocytes, liquid nanochitosan had a significant effect (P<0.05) on the number of leukocytes and lymphocytes, and there was no interaction between the two. The conclusion of this study, Spirulina maintains the leukocyte profile, 5% liquid nanochitosan reduces the number of leukocytes and lymphocytes, Spirulina and liquid nanochitosan do not interact with each other.

***Keywords**: Feed Additives; White Blood Cell; Heterophiles; Lymphocytes; H/L Ratio (11 pt)*

Abstract (12 pt)

Aditif pakan tunggal pada budi daya broiler (*Gallus domesticus*) belum efektif meningkatkan imun dan mengurangi stres metabolik. *Spirulina* dan Nanochitosan cair dapat digunakan sebagai aditif pakan karena bersifat antioksidan dan imunostimulan, sehingga meminimalkan stres metabolik dan memodulasi imun broiler. Penelitian ini bertujuan menganalisis pengaruh tepung *Spirulina*, Nanochitosan cair, serta interaksinya terhadap profil leukosit broiler. Desain penelitian menggunakan RAL Faktorial, 6 perlakuan dan 4 ulangan, yaitu kontrol dan kelompok pakan basal dengan *Spirulina* 0%, 3%, dan 6%, serta Nanochitosan cair 0% dan 5%. Preparat apus darah pewarnaan *Giemsa* diamati dengan perbesaran 40x10. Data dianalisis menggunakan *Two Way ANOVA* taraf signifikansi 5% dan Uji *Friedman*. Hasil menunjukkan morfologi leukosit normal, *Spirulina* berpengaruh tidak nyata (P>0,05) terhadap jumlah leukosit, heterofil, dan limfosit, Nanochitosan cair berpengaruh nyata (P<0,05) terhadap jumlah leukosit dan limfosit, serta tidak terdapat interaksi antara keduanya. Kesimpulan penelitian ini, *Spirulina* mempertahankan profil leukosit, Nanochitosan cair 5% menurunkan jumlah leukosit dan limfosit, *Spirulina* dan Nanochitosan cair tidak saling berinteraksi.

Kata kunci*:* Imbuhan Pakan; Sel Darah Putih; Heterofil; Limfosit; Rasio H/L (11 pt)

INTRODUCTION (12 pt)

The increasing population of Indonesia has led to a rise in the demand for food, including animal-based food products such as broiler meat. Data from the Ministry of Agriculture Directorate General of Livestock and Animal Health (2023) shows that the average per capita consumption of broiler meat increased by 8,73% in 2022. The high consumption of broiler meat has driven an increase in broiler production in recent years. Broilers are generally raised for their meat. These birds have several characteristics, including rapid body growth, large body frames, and high feed efficiency, as most of the feed is converted into meat. However, broilers are very susceptible to stress, frequently infected with diseases, and do not adapt easily (Sarawit, 2022). Broilers are prone to heat stress due to unsuitable environmental temperatures or oxidative stress due to high metabolism, making broiler health management crucial in the poultry industry.

The physiological health of chickens can be assessed through their immune system, one of which is via blood profile (Kasiyati *et al*., 2021). Leukocytes and leukocyte differentials as blood components can serve as indicators of chicken health (Sugiharto, 2016). The leukocyte profile, consisting of White Blood Cell Count (WBC) and leukocyte differentials including basophils, eosinophils, heterophils, lymphocytes, and monocytes, can be analyzed through a complete blood count. Abnormal leukocyte levels are associated with animal health conditions. Elevated leukocyte profiles can be caused by disease or environmental stress but can also indicate an immune response (Djaelani *et al*., 2020). Decreased or below-normal leukocytes are due to heat stress and nutritional deficiencies (Maulidya *et al*., 2022). The leukocyte profile can be used to determine the stress index in chickens based on the heterophil (H) to lymphocyte (L) ratio. A high H/L ratio index indicates high stress levels in poultry (Hendrawan *et al*., 2019).

Health issues related to immun and stress in chickens can be addressed with antibiotics; however, Indonesia has implemented a ban on the use of antibiotic growth promoters for poultry as of January 2018, as stated in the Minister of Agriculture Regulation Number 14 of 2017. Therefore, safe feed additives for livestock need to be found as alternatives to antibiotics. Previous research has used plant-based additives, such as ginger powder, turmeric powder, and teak leaf extract, but these have not been effective. Alternative feed additives can also come from algae, such as *Spirulina*. A unique compound in *Spirulina*, called phycocyanin, is not found in plants because it is only present in Cyanobacteria. Phycocyanin is known to boost the immune system due to its antioxidant and anti-inflammatory properties (Firdiyani *et al*., 2015).

Spirulina sp. is a natural substance that contains bioactive components such as phycocyanin, steroids, saponins, chlorophyll, β-carotene, flavonoids, triterpenoids, and phenolic acids, which function as antioxidants, antivirals, antimicrobials, anti-inflammatories, and immunostimulants (Elbaz *et al*., 2022). Research by Hassan *et al*. (2022) demonstrated that broilers given 0.25%, 0.5%, and 1% Spirulina for 56 days showed that 1% *Spirulina* can improve immunity and enhance the immune response. Research by Situmeang *et al*. (2017) proved that administering 1% *Spirulina* for 7, 21, and 35 days to broilers did not significantly affect the leukocyte and leukocyte differential counts. These differing results indicate that further research on *Spirulina* supplementation is necessary, such as through modifications supported by other substances in drinking water.

Liquid nanochitosan can be added to drinking water to support and maintain chicken immunity because it is natural, safe, and provides many benefits. Nanochitosan is a nano-sized form of chitosan derived from the synthesis of shrimp, lobster, and crab, which contain chitin (Sunarno *et al*., 2023). Nanochitosan added to feed can accelerate the growth rate of beneficial microbes and has antimicrobial properties against harmful microorganisms (Sahara *et al*., 2019). Nanochitosan administration can minimize the negative impact of free radicals, which are molecules or ions with one or more unpaired electrons that can cau chain reactions, leading to the disruption or damage of surrounding molecules. Nanochitosan has amino and hydroxyl groups that can interact with free radicals by donating electrons. The donated electrons from nanochitosan result in free radicals having paired electrons, thus preventing chain reactions (Dinana *et al*., 2019). Nanochitosan can also reduce oxidative stress in chickens (Gu *et al*., 2022). Several researchers have proven that nanochitosan has immune-boosting properties and acts as an immunological stimulant in animals (Yoon *et al*., 2008;

Kong *et al*., 2014). Research by Rakhimovna *et al*. (2023) shows that chitosan bioadditives effectively influence the number of leukocytes.

The combination of *Spirulina* with selenium nanoparticles has been previously studied, and the results show that chickens treated with this combination have a better immune response (Al-Khalaifah *et al*., 2022). However, research on the combination of *Spirulina* with other nanoparticles as feed additives for chickens has been limited. Considering the potential of *Spirulina* and nanochitosan, the combination of *Spirulina* powder as a feed additive and liquid nanochitosan as drinking water, and their interaction in broiler chickens, presents an opportunity for further research, as it has not been conducted before. This study involves blood sample analysis using a hematology analyzer and morphological observation of leukocytes based on Giemsa-stained preparations. The results of this study are expected to provide solutions to health issues or environmental stress commonly experienced by broiler chickens through the utilization of the combination of *Spirulina* powder and liquid nanochitosan as feed additives. (12 pt)

MATERIALS AND METHODS (12 pt)

The research was conducted in an experimental chicken coop at Jl. Perintis Kemerdekaan No. 30, Semarang, for 10 months. The preparation and observation of blood smear preparations were carried out at the 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, light bulbs, spray bottles, cable ties, digital scales, measuring cups, plastic containers, stirring spoons, labels, thermohygrometers, 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 nanochitosan, water, rodalon solution, Vita Chick, Vita Stress, broiler blood samples, ice gel, Giemsa stain, methanol, and distilled water. (12 pt).

Experimental Design

The study used a 3×2 factorial Randomized Block Design (RAL), 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, as shown in 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 nanochitosan), S6N0 (standard feed with 6% *Spirulina* powder, no liquid nanochitosan), S_0N_5 (standard feed with no *Spirulina* powder, 5% liquid nanochitosan), S_3N_5 (standard feed with 3% *Spirulina* powder and 5% liquid nanochitosan), and S_6N_5 (standard feed with 6% *Spirulina* powder and 5% liquid nanochitosan).

Table 1. Experimental Design

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_0N_5 1	S_6N_5 1
2	S_0N_0 2	S_3N_0 2	S_6N_0 2	S_0N_5 2	S_0N_5 2	S_6N_5 2
3	S_0N_0 3	S_3N_0 3	S_6N_0 3	S_0N_5 3	S_0N_5 3	S_6N_5 3
4	S_0N_0 4	S_3N_0 4	S_6N_0 4	S_0N_5 4	S_0N_5 4	S_6N_5 4

Note : S = *Spirulina* powder, 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 liters 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 kg 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). Drinking water preparation involves providing water and liquid nanochitosan according to the treatments. N_0 (tap water without liquid nanochitosan) and N_5 (liquid nanochitosan with a concentration of 5%).

Research Treatment

The treatments for the chickens were administered after a 26-day acclimation period. The chickens were provided with water and feed ad libitum and 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 40x10 magnification to examine the differential leukocyte profile (Maheshwari *et al*., 2017).

Total Leukocyte and Differential Leukocyte Analysis

Blood analysis was performed using a hematology analyzer, an automated device for hematological testing. The principle of the hematology analyzer involves the calculation and measurement of cells based on changes in electrical resistance as blood cells pass through an electrically conductive diluent, which is an isotonic solution with electrolytes. The electrical impedance is measured according to the volume or size of the cells due to changes in blood cell impedance as they pass through a gap with electrodes on either side (Saputra & Aristoteles, 2022).

To operate the hematology analyzer, connect the power cable to the stabilizer and press the "on" button on the switch. The display will show "Please Wait." Ensure that the blood sample is well-mixed with the anticoagulant. Next, press the "Whole Blood (WB)" button on the screen, then press the "ID" button and enter the sample number, followed by "Enter." Open the top of the sample chamber, place the sample in the adaptor, close the sample chamber, and press "RUN." The results will automatically appear on the screen and print 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. 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).

RESULT (12 pt)

The average results of the data analysis are shown in Table 2. 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). The addition of liquid nanochitosan significantly affected the total leukocyte count and lymphocyte count across treatments (P<0.05). However, liquid nanochitosan did not significantly affect the heterophil count across treatments (P>0.05). Two-way ANOVA analysis indicated that there was no interaction between *Spirulina* powder and liquid nanochitosan.

Table 2. Average Total Leukocyte Count and Differential Leukocytes After Treatment

Trearment	Variable (x̄±SD)			
	Total Leukocyte Count (x10^9/L)	Heterophils (x10^9/L)	Lymphocytes (x10^9/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^b±11,61	2,27±1,13	77,51^b±10,79	0,03±0,01
N_5	68,81^a±8,80	1,58±0,61	67,23^a±8,52	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 : The data in the table are presented as mean ± standard deviation. Superscripts that differ within the same column indicate significant differences between treatments (P<0.05). S_0N_0 (control), S_3N_0 and S_6N_0 (feed with 3% and 6% *Spirulina* powder, respectively, without liquid nanochitosan), S_0N_5 (feed without *Spirulina* powder, with 5% liquid nanochitosan), S_3N_5 and S_6N_5 (feed with 3% and 6% *Spirulina* powder, respectively, with 5% liquid nanochitosan).

The observation of blood smear preparations of broiler chickens at 40x10 magnification with Giemsa staining revealed differential leukocytes, specifically heterophils and lymphocytes. The differential leukocyte profile can be seen in Figure 1.

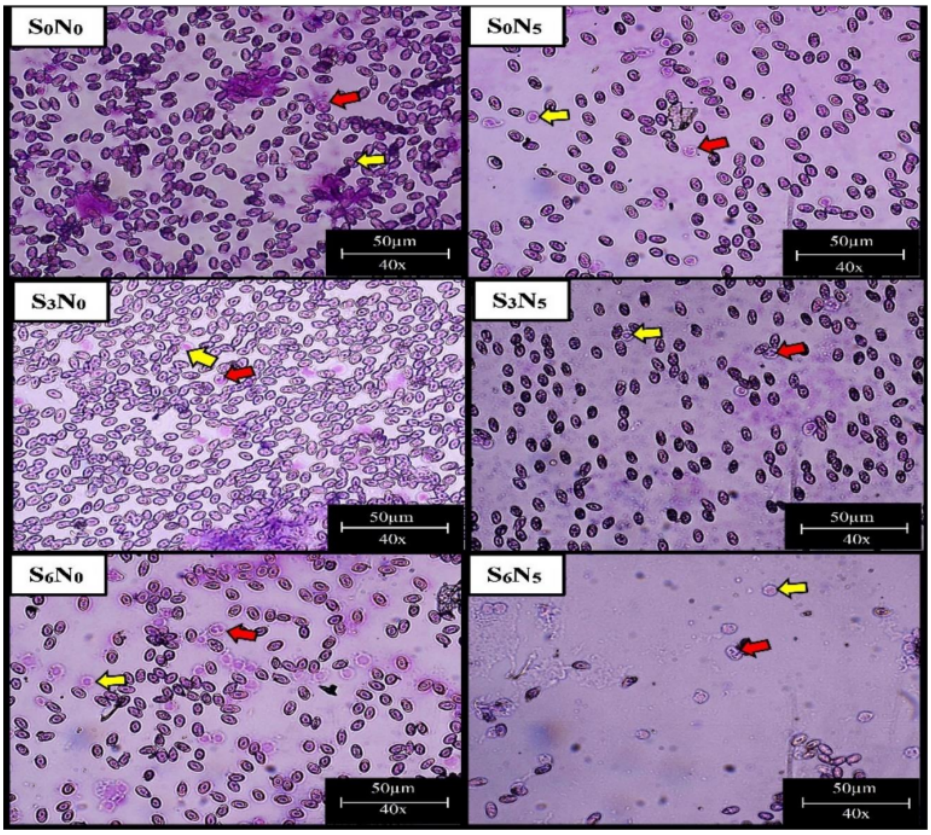

Figure 1. Differential Leukocytes in Broiler Chicken Blood Smears. S_0N_0 (control), S_3N_0 and S_6N_0 (feed with 3% and 6% *Spirulina* powder without liquid nanochitosan), S_0N_5 (feed without *Spirulina* powder with 5% liquid nanochitosan), S_3N_5 and S_6N_5 (feed with 3% and 6% *Spirulina* powder with 5% liquid nanochitosan). Red arrows indicate heterophils. Yellow arrows indicate lymphocytes. (12pt)

DISCUSSION (12pt)

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 due to the fact that the amount of *Spirulina* provided was adequate for the chickens'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 nanochitosan 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 nanochitosan 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 broiler.

The total leukocyte count, as shown in Table 2, ranges from 67,40-84,95×10^9/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 6–40×10^3/ml equivalent to 0,006-0,04×10^9/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. 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 spirulan 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.

The total leukocyte count differed significantly ($P<0.05$) between the chickens given tap water and those given 5% Nanochitosan. Table 2 shows that the total leukocyte count for broiler chickens given 5% Nanochitosan (N_5) was $68{,}81\times10^9$/L. This value is lower compared to broiler chickens given tap water (N_0), which showed a total leukocyte count of $79{,}78\times10^9$/L. The lower leukocyte count indicates that nanochitosan 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 N0. 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 -NH2- groups present in chitosan. This interaction helps block bacteria from accessing nutrients, thereby inhibiting bacterial growth. The study by Ivanishcheva & 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 $1{,}53\text{-}2{,}68\times10^9$/L. Table 2 shows that the normal heterophil count was only found in the control group (S0N0), with a count of $2{,}68\times10^9$/L, while the heterophil counts in all other treatments were below normal, ranging from $1{,}53\text{-}2{,}20\times10^9$/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 $2{,}23\text{-}9{,}76\times10^3$/mm^3, equivalent to $2{,}23\text{-}9{,}76\times10^9$/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 nanochitosan 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 & 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 2, ranged from $62{,}65\text{-}78.68\times10^9$/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 $5{,}52\text{–}20{,}36\times10^3$/ml, equivalent to $0{,}00552\text{–}0.02036\times10^9$/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 viru occurs through both humoral and cellular mechanisms. Meilani *et al*. (2019) 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 indicat 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 nanochitosan can stimulate both humoral and cellular immune responses in broiler chickens. Liquid nanochitosan appears to be one factor causing lymphocyte counts to be higher than the normal range. In this study, the treatment with liquid nanochitosan 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. Arif & Talista (2019) explain that 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 2 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 treatments with *Spirulina* powder and liquid nanochitosan were able to minimize heat stress and combat free radicals that cause oxidative stress. Moustafa *et al*. (2021) explain that *Spirulina* contains bioactive compounds that function as antioxidants. The potential of these bioactive compounds can inhibit, break, and stop the production and chain reactions of free radicals, thereby reducing the negative impacts of environmental temperature stress. Antioxidants work by balancing oxidation and reduction reactions. Dinana *et al*. (2019) and Gu *et al*. (2022) explain that the effects of free radicals and oxidative stress in poultry can be minimized with the administration of nanochitosan. Ivanova & Zvezdelina (2020) state that nanochitosan plays an important role in regulating redox reactions by inhibiting ROS production and enhancing intracellular antioxidant enzymes within biological systems. Sari *et al*. (2013) describe that nanochitosan has the ability to inhibit and bind ROS production, preventing the formation of free radicals or reducing their reactivity. The hydroxyl radical (OH-) formed from lipid oxidation will react with hydrogen ions on the chitosan's NH3+ groups. The result of this process is stable molecules.

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 Hardian *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 nanochitosan as drinking water additives does not have cytotoxic effects on leukocytes. (12pt)

CONCLUSION AND SUGGESTION (12pt)

Spirulina at 3% and 6% in this study has potential as a feed additive because it can maintain the leukocyte profile of broilers. Liquid nanochitosan 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. It is recommended that further research be conducted to explore potential oxidative stress by measuring total antioxidant levels, malondialdehyde (an oxidant), and cortisol hormones in the blood.

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S/V This subject and verb may not agree. Proofread the sentence to make sure the subject agrees with the verb.

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Confused You have used **through** in this sentence. You may need to use **though** instead.

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Article Error You may need to use an article before this word. Consider using the article **the**.

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Garbled Grammatical or spelling errors make the meaning of this sentence unclear. Proofread the sentence to correct the mistakes.

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