Efforts to Control Salmonellosis Through the Supplementation of *Zingiber zerumbet* (L.) Smith Extract in Feed for Broiler Chicken Health

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Abstract. The study aims to control Salmonellosis through the addition of *Zingiber zerumbet* (L.) Smith extract as a feed additive to maintain the health of broilers' visceral organs and blood serum. An experimental method was employed using Completely Randomized Design (CRD), which included five treatments: T0 (basal diet), T1 (basal diet + *S. enteridis* infection), T2 (basal diet + *S. enteridis* infection + 0.33 % *Z. zerumbet* extract), T3 (basal diet + *S. enteridis* infection + 0.67 % *Z. zerumbet* extract), and T4 (basal diet + *S. enteridis* infection + 1 % *Z. zerumbet* extract). Each treatment was repeated five times. As a result, the treatments affected the percentage of spleen and bursa weight. The highest percentage of spleen weight was found in T1, and the highest percentage of bursa weight was indicated in T3. The treatment did not affect the serum biochemistry variables, except for globulins, whose highest level was found in T4. It is concluded that the ethanolic extract of *Z. zerumbet* is a potentially safe feed additive for the control of Salmonellosis in broiler chickens.

Keywords: Antibiotic growth promoters, feed additive, foodborne disease, herbal medicine, phytobiotic.

1 Introduction

Regulation of the Minister of Agriculture of the Republic of Indonesia Number 14/PERMENTAN/PK.350/5/2017 has been officially issued to stop the use of antibiotic growth promoters (AGP) as a feed additive in animal feed as it may cause negative impacts in the form of bacterial resistance, antibiotic residues in livestock products, and foodborne diseases that interfere peoples' health as the consumers. The cause of foodborne disease that is well-known today is *Salmonella enteritidis* (Gaertner 1888) Castellani & Chalmers 1919, which is commonly found in chicken farms. It causes contamination of poultry products, such

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as eggs or meat. Reported that pathogenic bacteria affect the humoral livestock's immune system leading to the decrease in the weight of the bursa of fabricius and immunosuppression [1].

Research has shown that AGP as a feed additive can cause growth inhibition and colonization of beneficial gut bacteria, including Lactobacillus, Bifidobacteria, Boeteroides, and Enterococci [2]. Bacterial resistance to several antibiotics, including erythromycin, penicillin, and vancomycin, with a resistance rate of 100 % occurred in the isolates of *S. enteridis* and *Salmonella typhimurium*, separated from broiler chicken meat [3]. Sulfadiazine and oxytetracycline antibiotic residues were found in broiler meat [4], while tetracycline, ampicillin, streptomycin, and aminoglycoside residues were found in chicken kidneys and liver [5]. *Salmonella* sp. was isolated from the liver and intestinal contents, namely *S. enteridis* (33.32 %) and *S. typhimurium* (11.10 %) [6].

The solution to replace the role of AGP as a feed additive is to develop a feed formulation containing functional components in the form of phytogenics as they contain antibacterial agents that can be extracted and used as a feed additive [7]. The most dominant compound in the extract of *Zingiber zerumbet* (L.) Smith with various concentrations of ethanol is zerumbon, in each ethanol 45 %, 70 %, and 95 %, respectively, the content of zerumbon was 91.01 %, 75.42 %, and 43.14 %. Other compounds are also found in the 95 % ethanol extract, like alpha-humulene (15.61 %), linalool (10.76 %), and humaladienone (10.42 %); 95 % ethanol extract is the most complete extract containing active compounds [8]

Phytobiotics have hepatoprotective and hepatogenic properties in improving liver function, nutritional utilization, and livestock performance. They also protect the intestinal mucosa from pathogenic bacteria colonization, increase the growth of beneficial bacteria, such as Lactobacilli and Bifidobacteria, and reduce the number of coliforms and clostridium perfringens, [9–12].

A colony of *S. enteritidis* was not found in the faeces of broiler chickens, which were given *Z. zerumbet* extract of 0.67 % to 1 %. This fact proves the high efficacy of the antimicrobial agent in the extract of *Z. zerumbet* against *S. enteritidis*. In other words, it can maintain its activity up to the back of the digestive tract, namely the cecum, as a site of the *S. enteritidis* colonization [13].

This study examines the effect of *Z. zerumbet* extract supplementation on broiler chicken feed to treat Salmonellosis, specifically the response of lymphoid organs, intestines, digestive glands, and blood serum biochemistry. In the long term, phytobiotics as feed additives are expected to replace AGP and increase broiler productivity. Therefore, the supply of Salmonella-free broiler meat can be maintained to meet the domestic demand. Another significant target of the use of phytobiotics is to reduce the level of bacterial resistance to antibiotics and suppress the cases of foodborne disease and antibiotic residues in meat and visceral. Eventually, food security and safety in Indonesia can be ensured.

2 Material and methods

2.1 Plant materials and preparation

The *Z. zerumbet* rhizome was obtained from Technical Implementation Unit (*Unit Pelaksana Teknis* – UPT) Materia Medica Batu, East Java, Indonesia. This rhizome was initially sorted out in order to separate it from any foreign material; then it was washed, drained, sliced, and dried in an oven (UF260plus, Germany) with a temperature of 45 °C until the water concentration reached ≤ 10 %. Next, the dried rhizome was ground into powder, soaked with 95 % ethanol in a jar and stirred (Argo LAB. Tipe: M2-D PRO, Italy) at a speed of 50 rad s^{-1} for 24 h. The liquid extract was then filtered, vaporized with a rotary evaporator

(RE-5250, China) for 1 h and above a water bath (TC-150, USA) for 12 h. At the last step, the thick liquid extract was supplemented with aquadest so that it became a 10 % extract. Finally, the extract was dried in an oven at 40 °C for 2 d and added with 5 % amylum starch until it became powder [8].

2.2 Diet and care management

The feed formulation of the broiler chickens is shown in Table 1.

Table 1. Feed formulation of the treated broiler chickens.

Feed formulation		
Feedstuff	**Starter**	**Finisher**
	Amount (%)	**Amount (%)**
Rice bran	0.6	0.4
Yellow corn	59.22	65.11
Fish flour	2.8	2.31
Bone flour	1	2
Meat flour	4.42	-
Soybean pulp	29	27
Coconut oil	1.22	1.12
Calcium	0.35	0.5
Salt	0.5	0.5
Lysine	0.7	0.9
Methionine	0.19	0.16
Total (%)	**100.00**	**100.00**
Feed composition		
Metabolic Energy (kkal kg^{-1})	3 000.00	3 000.00
Crude Protein (CP) (%)	23	20
Crude Fat (CF) (%)	4.00	4.00
Crude Fiber (CFB) (%)	3.55	3.47
Ca (%)	0.89	0.76
P (%)	0.42	0.36
Na (%)	0.15	0.15
AMINO ACIDS:		
Arginine (%)	0.88	0.98
Histidine (%)	0.35	0.38
Isoleucine (%)	0.77	0.82
Leucine (%)	0.98	1.02
Lysine (%)	0.99	1.00
Methionine (%)	0.46	0.40
Phenylalanine (%)	0.76	0.82
Threonine (%)	0.66	0.69
Tryptophan (%)	0.31	0.26
Valine (%)	1.23	1.09

Source: Rahayu *et al.* [8].

The research material consisted of 125 One-Day-Old-Chick (DOC) broilers, which the initial body weight ranged from 34 g to 47 g, with an average of 39.536 g ± 2.481 g and a raising period of 35 d. Then, the ND vaccination was performed on chickens aged 4 d, 17 d, and 24 d. Meanwhile, artificial infections using *S. enteritidis* ATCC 31194 orally were given on 10 d with Optical Density (OD) 1×10^{10} CFU, while the *Z. zerumbet* extract powder was given when the chickens were 7 d to 35 d old [8].

2.3 Research design

The study used an experimental method, Completely Randomized Design (CRD), consisting of five treatments, namely: T0 (basal diet/negative control), T1 (basal diet + *S. enteritidis* infection 10^{10} CFU/positive control), T2 (basal diet + *S. enteritidis* infection 10^{10} CFU + 0.33 % *Z. zerumbet* extract of the total feed), T3 (basal diet + *S. enteritidis* infection 10^{10} CFU + 0.67 % *Z. zerumbet* extract of the total feed), and T4 (basal diet + *S. enteritidis* infection 10^{10} CFU + 1 % *Z. zerumbet* extract of the total feed). Each treatment was repeated five times, and each replication consisted of five chickens [8].

This research was carried out with the recommendations stated in the Description of Ethical Approval, No.5.a/048.a/KEPK-UMM/III/2022, issued by the Health Research Ethics Committee (*Komite Etik Penelitian Kesehatan* - KEPK), Faculty of Medicine, University of Muhammadiyah Malang.

2.4 Measured variable

The variables measured in this study were the weight percentage of visceral organs and serum biochemistry, including protein and serum lipid profiles.

2.5 Measurement

The research sampling was carried out when the broilers reached 35 d old. The blood samples were taken before the chickens were slaughtered; meanwhile, the pancreatic and small intestine samples for measuring digestive enzyme activities were taken when the chickens were 36 d old.

2.5.1 Weight percentage of visceral organs

Five broilers in each treatment were slaughtered on day 35. After the post-mortem examination, the visceral organs, including thymus, heart, spleen, pancreas, small intestine, and bursa of Fabricius were taken and weighed. The weight percentage of each organ was calculated according to the formula as described in Equation (1).

$$\frac{\text{organ weight (g)}}{\text{body weight (kg)}} \times 100\ \% \quad (1)$$

2.5.2 Serum biochemistry

The observed variables were protein profiles (total protein, albumin, globulin), fat profiles (triglycerides, cholesterol, LDL and HDL). Blood samples were taken when the chickens were 35 d old, with five broilers per treatment, and 3 mL were taken from each using a syringe containing the anticoagulant EDTA through the axillary vein; the plasma was then separated for further analysis. The measurement of serum biochemistry was carried out manually using a microplate reader ELISA assay, with the brand Ez Read 400 Elisa, 80-4001-40, serial number 131022, produced by Biochrom, US, Molliston, MA 01746.

2.6 Data analysis

The data were tabulated in the MS Excel program. The percentage of visceral organ weight and serum biochemistry was analyzed using Variance of Analysis (ANOVA). When an effect

difference in between treatments was found, the Duncan Multiple Range Test (DMRT) was applied [14, 15].

3 Results and discussion

3.1 Weight percentage of visceral organs

Based on the variance of analysis, the *S. enteritidis* infection at a dose of 10^{10} CPU and the addition of *Z. zerumbet* extract 0.33 % to 1 % did not affect the weight percentage of thymus, liver, pancreas, and intestines. Thus, these organs were still normal. The treatments affected the bursa of Fabricius weight percentage. The lowest percentage was found with the addition of 0.33 % *Z. zerumbet* extract; as it could not overcome *S. enteritidis* infection, the bacteria reached and replicated themselves in the bursa through the lymph system [16]. Pathogenic bacteria invasion affecting the humoral system of lymphoid organs, including the bursa of fabricius, can cause immunosuppression, which is characterized by a decrease in the weight of the bursa of fabricius [1]. The decrease in the bursa weight also occurred in ducks with Zn deficiency, and the effect on the bursa was indicated earlier with the biggest impact found in the thymus, then the spleen. The data of the visceral organ weight percentage are shown in Table 2.

Table 2. The effect of *S. entertiidis* infection and addition of *Z. zerumbet* ethanol extract on the weight percentage of visceral organs (%).

Treatment	Weight percentage of visceral organs ($\frac{\text{organ weight}}{\text{body weight}} \times 100$ %)					
	Thymus	Liver	Spleen	Pancreas	Intestines	Bursa of fabricius
T0	0.42 ± 0.15^{a}	2.47 ± 0.28^{a}	0.16 ± 0.02^{ab}	0.30 ± 0.05^{a}	4.27 ± 0.64^{a}	0.09 ± 0.03^{ab}
T1	0.51 ± 0.05^{a}	2.43 ± 0.25^{a}	0.19 ± 0.07^{b}	0.29 ± 0.05^{a}	4.68 ± 0.40^{a}	0.07 ± 0.01^{ab}
T2	0.54 ± 0.19^{a}	2.37 ± 0.07^{a}	0.13 ± 0.03^{a}	0.31 ± 0.07^{a}	4.58 ± 0.33^{a}	0.05 ± 0.01^{a}
T3	0.50 ± 0.16^{a}	2.46 ± 0.11^{a}	0.12 ± 0.02^{a}	0.29 ± 0.04^{a}	4.65 ± 0.80^{a}	0.12 ± 0.08^{b}
T4	0.51 ± 0.21^{a}	2.60 ± 0.23^{a}	0.15 ± 0.04^{ab}	0.28 ± 0.03^{a}	4.51 ± 0.67^{a}	0.06 ± 0.02^{ab}

Note: Numbers followed by the same letter in the same column indicate no difference in Duncan 5 % test. The letter ab means that there is no difference in spleen weight between T0, T4 and T2, T3; also, there is no difference in the weight of the bursa between T0, T1 and T4, T2.

Description: T0 (standard feed/negative control), T1 (standard feed + *S. enteritidis* infection/positive control), T2 (standard feed + *S. enteritidis* infection + *Z. zerumbet* extract 0.33 %), T3 (standard feed + *S. enteritidis* infection + *Z. zerumbet* extract 0.67 %), T4 (standard feed + *S. enteritiidis* infection + *Z. zerumbet* extract 1 %).

Bursa of Fabricius is a primary lymphoid organ having an essential role in the production of immune, IgM, IgG and IgA, T and B lymphocyte cells in poultry [17, 18]. The highest percentage of bursa weight was achieved with the addition of 0.67 % *Z. zerumbet* extract (T3). It indicates that the bursa is the most vigorous organ in producing immune as compared to other treatments and controls. Herbal medicine works can increase immune production as they contain antiviral, antibacterial, antioxidant, and immunomodulatory properties that can improve the immune system [19]. The highest percentage of spleen weight was achieved in T1 (positive control), that was the group of broilers infected with *S. enteritidis* at a dose of 10^{10} CPU mL^{-1}, which actually boosted the immune system. It is in accordance with the statement that Bursa of Fabricius and the thymus serve as the primary lymphoid organs of the immune system. B cells use surface immunoglobulins as antigen receptors and

differentiate into plasma cells to secrete antibodies. Three classes of antibodies are produced: IgM, IgG (also called IgY), and IgA [20].

3.2 Serum biochemistry

The results showed that the treatments did not affect the level of total protein and albumin serum. However, it affects the globulin levels, with the lowest levels occurring in T0, T1, and T2 groups. The addition of 0.67 % to 1 % *Z. zerumbet* extracts (T3 and T4) significantly increased globulin levels in broilers infected with bacteria; this indicated a higher level of inflammation. The researchers suspect that in the light infection of *S. enteritidis* with a dose of 10^{10} CFU, 0.33 % or a low level of *Z. zerumbet* extract is needed as an antibacterial agent (T2). The addition of a higher-level extract may cause damage to the intestinal wall; this triggers pathogenic bacteria to move to the epithelial wall and ultimately worsens inflammation. The administration of herbal ingredients above a 2.5 mL L^{-1} of drinking water shows different organ damage levels in the liver, pancreas, kidneys, duodenum, jejunum, and ileum [21]. This condition may be due to the excess of bioactive compounds in herbal ingredients in the form of essential oils, curcumin, methyl chavicol, gingerol, eugenol, citral A, citral B, flavonoids, and allicin. The complete profile of serum protein can be seen in Table 3.

Table 3. Serum protein profile.

Treatment	Level of serum protein (g dL^{-1})		
	Total protein	**Albumin**	**Globulin**
T0	5.87 ± 0.81[a]	1.77 ± 0.53[a]	3.65 ± 0.95[a]
T1	5.74 ± 0.30[a]	2.09 ± 0.37[a]	3.83 ± 0.28[a]
T2	5.81 ± 0.28[a]	1.98 ± 0.29[a]	4.09 ± 0.46[ab]
T3	6.10 ±1.05[a]	1.84 ± 0.64[a]	4.26 ± 1.05[b]
T4	6.59 ±1.06[a]	1.54 ± 0.32[a]	5.05 ± 0.99[b]

Note: Numbers followed by the same letter in the same column indicate no difference in Duncan's 5 % test. The letter ab means no difference in the globulin level between T2 and T0, T1.

Description: T0 (standard feed/negative control), T1 (standard feed + *S. enteritidis* infection/positive control), T2 (standard feed + *S. enteritidis* infection + *Z. zerumbet* extract 0.33 %), T3 (standard feed + *S. enteridis* infection + *Z. zerumbet* extract 0.67 %), T4 (standard feed + *S. enteritidis* infection + *Z. zerumbet* extract 1 %).

S. enteritidis infection, either followed by or without *Z. zerumbet* extract, did not affect the serum triglycerides of broilers; this showed that the body did not need any triglycerides as an energy provider in all treatments the energy in the feed has been sufficient. The energy needs in the body are fulfilled by utilizing the energy reserves in the form of triglycerides in fat tissue [22]. The serum triglycerides from the study were 37.57 mg dL^{-1} to 55.51 mg dL^{-1}; these were much lower than the results of the study by Hasanudin *et al.* [23], with the level between 87.50 mg dL^{-1} to 129.17 mg dL^{-1} and 156 mg dL^{-1} to 174 mg dL^{-1} [24]. The complete data on serum lipid profiles are shown in Table 4.

Table 4. Serum lipid profile.

Treatment	Level of serum lipid (mg dL^{-1})			
	Triglyceride	**Cholesterol**	**LDL**	**HDL**
T0	55.51 ± 11.30[a]	158.67 ± 18.68[a]	150.04 ± 18.86[a]	32.70 ± 4.17[a]
T1	40.75 ± 3.94[a]	149.05 ± 36.99[a]	136.27 ± 33.93[a]	35.19 ± 9.78[a]
T2	45.80 ± 10.82[a]	138.09 ± 26.77[a]	126.97 ± 26.95[a]	34.19 ± 7.48[a]
T3	41.12 ± 19.40[a]	139.25 ± 43.33[a]	125.15 ± 41.00[a]	32.70 ± 4.49[a]
T4	37.57 ± 16.58[a]	149.71 ± 14.49[a]	141.91 ± 15.46[a]	30.54 ± 6.85[a]

Note: Numbers followed by the same letter in the same column indicate no difference in Duncan's 5 % test. Description: T0 (standard feed/negative control), T1 (standard feed + *S. enteritidis* infection/positive control), T2 (standard feed + *S. enteridis* infection + *Z. zerumbet* extract 0.33 %), T3 (standard feed + *S. enteritidis* infection + *Z. zerumbet* extract 0.67 %), T4 (standard feed + *S. enteritidis* infection + *Z. zerumbet* extract 1 %).

The addition of *Z. zerumbet* extract and *S. enteritidis* infection did not affect cholesterol, LDL, and HDL levels. The cholesterol levels strongly influence the levels of LDL and HDL as lipoproteins transporting cholesterol. In this study, the cholesterol level was 138.09 mg dL^{-1} to 158.67 mg dL^{-1}, which was still considered normal. The normal level of cholesterol is around 125 mg dL^{-1} to 200 mg dL^{-1}. It is lower than that of Toghyani and Faghan [24], which showed 177 mg dL^{-1} to 185 mg dL^{-1}. The results indicate that the cholesterol requirements of broiler chickens for various purposes, such as the formation of cell membranes, central nervous system, and vitamin D, have been fulfilled from the feed. Neither did the *S. enteritidis* infection at a dose of 10^{10} CFU mL^{-1} nor the addition of 0.33 % to 1 % *Z. zerumbet* extract affect *de novo* cholesterol biosynthesis in the liver, small intestine, adrenal glands, or reproductive organs. The low cholesterol level can be caused by the phenolic compounds in *Z. zerumbet*, which perform as antioxidants, eliminating superoxide anions and hydroxyl free radicals and increasing the activity of the enzyme Glutathione-S-transferase. Thus, it can increase the conversion of cholesterol into bile acids resulting in the decrease of the cholesterol level [25]. Also, limonene compounds, as monoterpenes and polyphenols, can reduce cholesterol by suppressing cholesterol absorption and increasing the bile acid excretion [26, 24].

The HDL level (30.54 mg dL^{-1} to 35.19 mg dL^{-1}) in this study was relatively high as compared to Toghyani and Faghan [24], with 24.20 mg dL^{-1} to 46.20 mg dL^{-1}. HDL is a lipoprotein with a high density; it functions to carry cholesterol from various tissues to the liver to produce bile and hormones. Therefore, a high level of HDL can lower cholesterol. On the other hand, LDL is responsible for providing cholesterol in the body tissues as the cholesterol carrier from the liver to body tissues. The LDL level of this study was 125.15 mg dL^{-1} to 150.04 mg dL^{-1}, which was in the normal range. This result is in accordance with Toghyani and Faghan [24] stating that the LDL level in broilers given lime juice as an acidifier was 119.19 mg dL^{-1} to 232.65 mg dL^{-1}.

4 Conclusion

It can be concluded that the extract of *Z. zerumbet* has a safe potential as a feed additive to control Salmonellosis in broiler chickens. These potentials include: (i) Increasing the weight percentage of bursa, but not affecting the weight percentage of liver, thymus, pancreas, and intestines. It indicates an increase of antibody production in the bursa of Fabricius, while other visceral organs remain normal. (ii) Not affecting the level of total protein and serum albumin but increasing the level of globulin at the extract supplementation by more than 0.33 % as the body's response to the infection of *S. enteritidis.* (iii) Not affecting the cholesterol level, LDL, and HDL as the HDL level is high and the LDL level is at the normal range.

References

1. A.V.S. Gomes, W.M. Quinteiro-Filho, A. Ribeiro, V. Ferraz-de-Paula, M.L. Pinheiro, E. Baskeville, et al., Avian Pathol., **43**,1: 82–90 (2014) https://doi.org/10.1080/03079457.2013.874006

2. P. Gousia, V. Economou, P. Bozidis, C. Papadopoulou, Foodborne Pathog. Dis., **12**,3: 214–220 (2015) https://doi.org/10.1089/fpd.2014.1832
3. T.Y. Thung, N.A. Mahyudin, D.F. Basri, C.W.J.W.M. Radzi, Y. Nakaguchi, M. Nishibuchi, et al., Poult. Sci., **95**,8: 1888–1893 (2016) https://doi.org/10.3382/ps/pew144
4. R. Khatun, M.A.J. Howlader, M.N. Islam, M.K. Alam, M.S. Mahmud, M.H. Rahman, Am. J. Food Sci. Health, **1**,2: 57–62 (2015) https://www.researchgate.net/publication/299441741_Exploration_the_Causes_of_Infectious_Illness_and_Detection_of_Antibiotic_Residues_in_Warehouse_Poultry
5. A. Sajid, N. Kashif, N. Kifayat, S. Ahmad, Pak. J. Pharm. Sci., **29**,5: 1691–1694 (2016) https://pubmed.ncbi.nlm.nih.gov/27731830/
6. W.A. Ibrahim, W.A.A. El-Ghany, S.A. Nasef, M.E. Hatem, Int. J. Vet. Sci. Med., **2**,1: 67–71 (2014) https://doi.org/10.1016/j.ijvsm.2013.11.001
7. G. Ganapathy, A.R. Nair, J. Herbs Spices Med. Plants, **23**,2: 169–181 (2017) https://doi.org/10.1080/10496475.2017.1283555
8. I.D. Rahayu, W. Widodo, I. Prihartini, A. Winaya, Biodiversitas, **20**,11: 3322–3327 (2019) https://doi.org/10.13057/biodiv/d201127
9. S. Diaz-Sanchez, D. D'Souza, D. Biswas, I. Hanning, Poult. Sci., **94**,6: 1419–1430 (2015) https://doi.org/10.3382/ps/pev014
10. W. Widodo, I.D. Rahayu, A. Sutanto, A.D. Anggraini, H. Sahara, S. Safitri, et al., Sarhad J. Agric., **37**,Special issue 1: 41–47 (2021) https://dx.doi.org/10.17582/journal.sja/2021/37.s1.41.47
11. W. Widodo, I.D. Rahayu, A. Sutanto, R.H. Setyobudi, M. Mel, Proc. Pak. Acad. Sci.: B. Life Environ. Sci., **56**,4: 39–46 (2019) https://ppaspk.org/index.php/PPAS-B/article/view/70
12. W. Widodo, A. Sutanto, I.D. Rahayu, A.D. Anggraini, T. Handayani, R.H. Setyobudi, et al., E3S Web Conf., **374**,00037: 1–6 (2023) https://doi.org/10.1051/e3sconf/202337400037
13. I.D. Rahayu, W. Widodo, A. Sutanto, A.D. Anggraini, Adv. Soc. Sci. Educ. Humanit. Res., **477**: 343–347 (2020) https://www.atlantis-press.com/article/125945332.pdf
14. P.G. Adinurani, Perancangan dan Analisis Data Percobaan Agro: Manual and SPSS [Design and Analysis of Agrotrial Data: Manual and SPSS] (Plantaxia, Yogyakarta, 2016) [in Bahasa Indonesia] https://opac.perpusnas.go.id/DetailOpac.aspx?id=1159798#
15. P.G. Adinurani, Statistik Terapan Agroteknologi (disusun sesuai rencana pembelajaran semester) [Agrotechnology Applied Statistics (compiled according to the semester learning plan)] (Deepublish, Yogyakarta, 2022) [in Bahasa Indonesia] https://deepublishstore.com/shop/buku-statistika-terapan-3/
16. K.M. Burkholder, K.L. Thompson, M.E. Einstein, T.J. Applegate, J.A. Patterson, Poult. Sci., **87**,9: 1734–1741 (2008) https://doi.org/10.3382/ps.2008-00107
17. A. Akbarian, A. Golian, H. Kermanshahi, R. Farhoosh, A.R. Raji, S. De Smet, et al., Span. J. Agric. Res., **11**,1: 109–119 (2013) https://doi.org/10.5424/sjar/2013111-3392
18. K.G. Baratawidjaja, I Rengganis, *Imunologi dasar* [Basic Immunology]. 11th Ed. Jakarta, Balai Penerbit Fakultas Kedokteran Universitas Indonesia (2014). p.860 [in Bahasa Indonesia] https://lib.ui.ac.id/detail?id=20417471
19. T. Hartady, M.R.A.A. Syamsunarno, B.P. Priosoeryanto, S. Jasni, R.L. Balia, Vet. World., **14**,11: 2889–2906 (2021) http://www.doi.org/10.14202/vetworld.2021.2889-2906
20. N.S. Rabie, Z.M.S.A. Girh, Bull. Natl. Res. Cent., **44**,15 (2020) https://doi.org/10.1186/s42269-019-0260-1

21. L. Agustina, M. Hatta, S. Purwanti, *Penggunaan ramuan herbal untuk meningkatkan performa dan gambaran histopatologi organ dalam broiler* [Use of herbal medicine to improve performance of broiler] Paper presented in Seminar Nasional Teknologi Peternakan dan Veteriner (Universitas Hasanudin Makassar, Indonesia, 2010). p.732–737 [in Bahasa Indonesia]
22. K. Tornheim, N.B. Ruderman, Intermediary metabolism of carbohydrate, protein and fat. In: Metabolic Basic of Obesity, R. Ahima (Eds). New York, Springer (2011). p.25–51 https://doi.org/10.1007/978-1-4419-1607-5_2
23. S. Hasanuddin, V.D. Yunianto, T. Tristiarti, JITP, **3**,1: 11–17 (2013) [in Bahasa Indonesia] https://journal.unhas.ac.id/index.php/peternakan/article/view/724/521
24. M. Toghyani, N. Faghan, Indian J. Pharm. Educ. Res., **51**,3: S295–S298 (2017) https://doi.org/10.5530/ijper.51.3s.33
25. N. Puvača, L. Kostadinović, D. Ljubojević, D. Lukač, J. Lević, S. Popović, et al., Europ. Poult. Sci., **79**: 1–13 (2015) https://doi.org/10.1399/eps.2015.73
26. S. Waheed, A. Hasnain, A. Ahmad, O.M. Tarar, Z. Yaqeen, T.M. Ali, J. Anim. Plant Sci., **27**,3: 714–724 (2017) http://thejaps.org.pk/docs/v-27-03/03.pdf