Livestock Research for Rural Development 28 (4) 2016 | Guide for preparation of papers | LRRD Newsletter | Citation of this paper

Haematological and biochemical parameters of broilers fed cassava pulp fermented with filamentous fungi isolated from the Indonesian fermented dried cassava

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Abstract

The study aimed to evaluate the effect of feeding cassava pulp (CP) fermented with *Acremonium charticola* and/or *Rhizopus oryzae* (isolated from the Indonesian fermented dried cassava) on haematological and biochemical parameters and immune organs of broilers. A total of 440 day-old male chicks were allotted to 11 groups as follows: control diet, 80-CP (diet with 80 g/kg unfermented CP), 80-CP-AC (diet with 80 g/kg *A. charticola*-fermented CP), 160-CP-AC (diet with 160 g/kg CP-AC), 240-CP-AC (diet with 240 g/kg CP-AC), 80-CP-RO (diet with 80 g/kg *R. oryzae*-fermented CP), 160-CP-RO (diet with 160 g/kg CP-RO), 240-CP-RO (diet with 240 g/kg CP-RO), 80-CP-ACRO (diet with 80 g/kg *A. charticola* + *R. oryzae*-fermented CP), 160-CP-ACRO (diet with 160 g/kg CP-ACRO), 240-CP-ACRO (diet with 240 g/kg CP-ACRO). Blood and internal organs were sampled on day 21 and 34.

The 80-CP-AC and 240-CP-AC chickens had lower ($P<0.01$) heterophils to lymphocytes ratio (H/L ratio), while control chickens had lower ($P<0.05$) thymus weight than others at day 21. At day 34, H/L ratio was higher ($P<0.01$) in control and 80-CP than in other chickens. The control, 80-CP and 80-CP-ACRO chickens had higher ($P<0.01$) albumin to globulin ratio (A/G ratio) than others. The weight of Bursa of Fabricius was lower ($P<0.01$) in 80-CP-ACRO chickens than others, but the difference was not significant compared to the control and 80-CP chickens. Overall, dietary inclusion of *A. charticola*-fermented CP up to 24% did not impair the physiological and health conditions of broilers as indicated by the haematological and biochemical parameters.

Keywords: *A. charticola, blood profiles, broiler, cassava pulp, fermentation, R. oryzae*

Introduction

Due to global rise in the price of feed ingredients, there is now increasing trends in poultry industry to use agro-industrial residues as feed ingredients. Cassava pulp (CP), which is a by-product of tapioca processing industry, is one of agro-industrial wastes that have attracted the interest of poultry nutritionists. This product contains a large quantity of starch, and therefore can be used as an alternative energy source in poultry diets (Khempaka et al 2009). The low protein and high fibre content in CP, however, may limit its utilization in poultry rations. Khempaka et al (2009) reported that although dried CP can be included in boiler diet to supply part of the dietary energy requirement, it should be limited to 8% or less as the higher levels may impair growth performance and nutrient digestibility. From these considerations, attempts to lower the fibre and enhance the protein content of CP become essential.

Fungal fermentation is one of the oldest technologies to improve the nutritional quality of food and feedstuffs. During fermentation, fungi break down the fibre into simple sugars, and protein biosynthesis take places (Czajkowska and Ilnicka-Olejniczak 1988). Owing to this fact, fungal fermentation has been exploited as an inexpensive tool for improving the nutritional properties of CP in order to be used in poultry rations at high inclusion levels (Khempaka et al 2014). Our previous study showed that fermentation with *Acremonium charticola* and/or *Rhizopus oryzae*, which are filamentous fungi isolated from the Indonesian fermented dried cassava (*gathot*), was able to decrease the fibre content of CP. The fermentation could also increase the crude protein content of CP by using urea as a fermentation supplement (Sugiharto et al 2015). Apart from the nutrition-improving effects, fermentation has widely been acknowledged to produce functional feed that can improve the physiological conditions and health of animals. In our previous work, the fungi isolated from *gathot* exhibited probiotic properties (Sugiharto et al 2015). Hence, fermentation of CP with these respective fungi was expected not only to improve the nutritional characteristic of CP, but also to produce fermented CP with health-promoting effects (functional feed) for broiler chickens.

Haematological parameters have commonly been used as indicators of physiological conditions and nutritional deficiency in chickens. The changes in haemoglobin concentration, erythrocyte count, hematocrit level and differential leukocytes may indicate stress (Borges et al 2004; Hrabčáková et al 2014), while the changes in erythrocyte, haemoglobin and pack cell volume may reflect an alteration of energy status in chickens (Karadeniz et al 2008; Alabi et al 2015). Aside from the physiological and nutritional aspects, haematological variables can also be used as an indicator of health in birds (Hrabčáková et al 2014). Abdel-Fattah et al (2008) reported that high globulin level and low albumin to globulin ratio (A/G ratio) implicate a better disease resistance and immune response of chickens. In addition, high serum total protein could be beneficial for antibody production in chickens. Indeed, several factors have been shown to influence the haematological variables including species, age, sex, environment, nutrition, infection and physiological conditions (Borges et al 2004; Hrabčáková et al 2014; Bańbura et al 2007). With regard to nutrition, this factor seems to be a determinant factor in the production of blood constituents (such as haemoglobin, erythrocyte and leukocyte) (Karadeniz et al 2008; Alabi et al 2015). Moreover, nutrition and its components affect the development of immune organs of chickens (Abdel-Raheem and Abd-Allah 2011; Zhang et al 2013). The present study aimed to investigate the effect of feeding CP fermented with filamentous fungi isolated from the Indonesian fermented dried cassava on haematological and biochemical parameters and immune organs of broilers.

Materials and Methods

Fungi and inoculation preparation

The pure cultures of *A. charticola* and *R. oryzae* isolated from the Indonesian fermented dried cassava (Yudiarti and Sugiharto 2015) were maintained on a Potato-Dextrose-Agar (PDA) medium (Merck KGaA, Darmstadt, Germany). The fungi were retrieved on PDA and grown at 37ºC for 3 days. The fungal mycelium was then dislodged from the PDA under sterile conditions by scraping with the aid of a spatula. The mycelium was diluted in 200 mL of sterilized-distilled water and further used in preparation of the fungal starter.

About 200 g of dry CP (87.5% dry matter, DM) was put in a plastic bag, autoclaved at 121ºC for 15 min and allowed to cool. The CP was inoculated with the mycelial (either *A. charticola*, *R. oryzae* or *A. charticola* and *R. oryzae*) suspensions and then mixed thoroughly. After incubation at room temperature for 14 days, the inoculation starter was enumerated by the colony counting method. The fungal starter produced was subsequently used to ferment the CP for the in vivo trials.

Fermented cassava pulp preparation

The fermented CP was prepared according to Sugiharto et al (2015). In brief, 10 kg of dry CP was put into a plastic bag and autoclaved at 121ºC for 15 min and then allowed to cool in a plastic fermentation bucket. The fermentation started with soaking the CP with sterile water in a ratio 1:1 (one part CP to one part water). The CP was inoculated with 55 g/kg fungal starter (*ca* 4×10^{10} cfu/g) and 41 g/kg urea and then thoroughly mixed. The calculations of fungal starter and urea concentrations were based on DM basis (Khempaka et al 2014). The mixture was incubated for 14 days and turned every 2 days. The fermented CP was produced for several buckets to fulfil the need of in vivo experiments, and that the procedures and conditions were similar for the entire fermentations. The fermentation products were eventually sun dried for 2 days. Prior to diet formulation, samples from different fermentation buckets were obtained and pooled for proximate analysis. The chemical compositions of the dried CP and fermented CP are presented in Table 1.

Table 1. The nutrient composition of fermented cassava pulp (as-dry basis)

Component (%, unless otherwise noted)	Dried cassava pulp	Fermented cassava pulp		
		CP-AC	CP-RO	CP-ACRO
Crude protein	2.14	11.3	12.8	14.8
Ether extract	1.98	1.18	0.97	1.34
Crude fibre	25.6	20.8	22.7	21.4
Ash	3.31	2.23	2.42	1.89
Metabolizable energy[1] (kcal/kg)	2,714	2,886	2,804	2,886

[1] *Metabolizable energy was calculated according to Balton formula = 40.81 (0.87 (Crude protein + 2.25 Ether extract + Nitrogen-free extract) + 2.5). CP-AC, cassava pulp fermented with A. charticola; CP-RO, cassava pulp fermented with R. oryzae; CP-ACRO, cassava pulp fermented with A. charticola + R. oryzae.*

In vivo experiment

A total of 440 Lohman MB-202 day old chicks (body weight = 41.87 ± 0.23 g; mean ± SD) purchased from a local hatchery were placed in an open-sided naturally ventilated broiler house and randomly allotted to 11 groups of treatment diets, each consisting of 4 replicates of 10 birds. The treatment diets were as follows: (1) diet without dried CP or fermented CP (control diet), (2) diet with 80 g/kg (as-fed basis) dried CP (80-CP), (3) diet with 80 g/kg CP fermented with *A. charticola* (80-CP-AC), (4) diet with 160 g/kg CP fermented with *A. charticola* (160-CP-AC), (5) diet with 240 g/kg CP fermented with *A. charticola* (240-CP-AC), (6) diet with 80 g/kg CP fermented with *R. oryzae* (80-CP-RO), (7) diet with 160 g/kg CP fermented with *R. oryzae* (160-CP-RO), (8) diet with 240 g/kg CP fermented with *R. oryzae* (240-CP-RO), (9) diet with 80 g/kg CP fermented with *A. charticola* and *R. oryzae* (80-CP-ACRO), (10) diet with 160 g/kg CP fermented with *A. charticola* and *R. oryzae* (160-CP-ACRO), (11) diet with 240 g/kg CP fermented with *A. charticola* and *R. oryzae* (240-CP-ACRO). The diets were formulated to similar levels of calculated metabolizable energy and crude protein and meet or exceed the minimum NRC (1994) requirements for broilers (Table 2). The experimental diets were provided *ad libitum* in mash form, and for the entire experimental period the birds were reared in concrete floor pens with rice husk as bedding material equipped with round bottom feeder and manual drinker.

Table 2. Ingredients and composition (as-dry basis) of experimental diets

Items (%, unless otherwise noted)	Experimental diets										
	Control diet	80-CP	80-CP-AC	160-CP-AC	240-CP-AC	80-CP-RO	160-CP-RO	240-CP-RO	80-CP-ACRO	160-CP-ACRO	240-CP-ACRO
Corn	50.0	48.0	45.0	36.0	32.5	44.0	39.0	36.0	41.5	36.5	39.0
Rice bran	18.0	10.0	17.0	19.0	16.0	19.0	17.5	13.0	22.0	21.0	10.0
Soybean meal	26.0	26.0	22.0	19.0	18.0	17.0	17.5	17.5	18.5	17.0	23.0
Fish meal	2.00	1.00	2.00	4.00	3.00	3.50	3.00	4.00	2.50	2.00	2.00
Poultry meat meal	4.00	7.00	6.00	6.00	6.50	8.50	7.00	5.50	7.50	7.50	2.00
CP	-	8.00	-	-	-	-	-	-	-	-	-
CP-AC	-	-	8.00	16.0	24.0	-	-	-	-	-	-
CP-RO	-	-	-	-	-	8.00	16.0	24.0	-	-	-
CP-ACRO	-	-	-	-	-	-	-	-	8.00	16.0	24.0
Calculated composition											
Metabolizable energy (kcal/kg)	2,924	2,925	2,936	2,917	2,917	2,961	2,943	2,932	2,943	2,939	2,914
Crude protein	22.8	22.7	22.4	22.7	22.2	22.5	22.2	22.3	22.5	22.2	22.2
Analyzed composition											
Metabolizable energy[1] (kcal/kg)	3,051	3,114	2,993	3,000	2,821	2,918	3,007	3,007	2,943	2,906	3,183
Crude protein	22.6	22.8	23.3	22.9	22.9	23.7	23.9	23.9	23.4	24.3	24.9

[1] *Metabolizable energy was calculated according to Balton formula = 40.81 (0.87 (Crude protein + 2.25 Ether extract + Nitrogen-free extract) + 2.5).*
Control diet, diet without dried CP or fermented CP; 80-CP, diet with 80 g/kg (as-fed basis) unfermented CP; 80-CP-AC, diet with 80 g/kg CP fermented with A. charticola; 160-CP-AC, diet with 160 g/kg CP fermented with A. charticola; 240-CP-AC, diet with 240 g/kg CP fermented with A. charticola; 80-CP-RO, diet with 80 g/kg CP fermented with R. oryzae; 160-CP-RO, diet with 160 g/kg CP fermented with R. oryzae; 240-CP-RO, diet with 240 g/kg CP fermented with R. oryzae; 80-CP-ACRO, diet with 80 g/kg CP fermented with A. charticola and R. oryzae; 160-CP-ACRO, diet with 160 g/kg CP fermented with A. charticola and R. oryzae; 240-CP-ACRO, diet with 240 g/kg CP fermented with A. charticola and R. oryzae. CP, dried cassava pulp; CP-AC, cassava pulp fermented with A. charticola; CP-RO, cassava pulp fermented with R. oryzae; CP-ACRO, cassava pulp fermented with A. charticola + R. oryzae.

On 21 and 34 days of age, one bird per replicate was removed from pen for sampling (4 birds/treatment, n=4), and the chickens were deprived from feed for 8 h. Blood was collected from the birds' wing veins and placed either in EDTA-containing vacutainers (for haematological analysis) or vacutainer tubes containing no anticoagulant (for serum biochemical analysis). To produce serum, the blood was allowed to clot for 2 h at room temperature. After centrifugation at 2,000 rpm for 15 min the serum was obtained and stored at −20ºC until serum biochemistry analysis. The same birds as blood sampled were sacrificed after being weight and, immediately the immune and visceral organs were removed and the weights of these organs were determined. The relative weight was expressed as wet organ weight/live body weight. The rest of the birds were raised until 42 days of age to evaluate the effect of treatments on the performances and meat quality of broilers which were published elsewhere.

Haematological analysis

The numbers of erythrocytes and leukocytes were determined using the dilution flask method and a Bürker chamber was used to count corpuscles. The level of haemoglobin was estimated by Sahli's method and haematocrit values were determined by means of the microhematocrit technique. The

differential leukocytes of chickens were determined using a light microscope with an immersion lens. Coverslip technique was applied when preparing blood smears. Heterophils to lymphocytes ratio (H/L ratio) was calculated by dividing the numbers of heterophils and lymphocytes.

Serum biochemical analysis

A total serum protein was estimated by photometric test according to biuret method using the kit (total protein kit, DiaSys Diagnostic System GmbH, Holzheim, Germany) according to the manufacturer's instructions. Albumin was determined by photometric test using bromocresol green (DiaSys Diagnostic System GmbH, Holzheim, Germany). Globulin was obtained by subtracting albumin values from total serum protein. Albumin to globulin ratio (A/G ratio) was calculated by dividing albumin values and globulin values.

Statistical analysis

The data were analyzed based on a completely randomized design by ANOVA using the General Linear Models Procedure in SAS (SAS Inst. Inc., Cary, NC, USA). Pen was treated as the experimental unit and the results were presented as least square means (LSMEANS) and standard error of the mean (SEM). Significant differences among treatment groups were further analyzed with Duncan's multiple-range test. A significant level of $P<0.05$ was applied.

Results

Haematological parameters

The haematological parameters of chickens are presented in Table 3. At day 21, chickens in 80-CP-AC and 240-CP-AC groups had lower ($P<0.01$) H/L ratio as compared to other chickens. The rest of the haematological variables of chickens measured at day 21 did not show any significant difference. Data from day 34 showed that chickens in 80-CP group had the lowest ($P<0.01$) concentration of haemoglobin in the blood compared to other chickens. However, the difference was not significant when compared with chickens in 160-CP-AC and 80-CP-RO groups. Compared to other chickens, the level of hematocrit was lower ($P<0.01$) in 80-CP chickens. Yet, the difference was not significant when compared with 160-CP-AC and 80-CP-RO chickens. The numbers of leukocytes differed ($P<0.05$) among the chickens, in which chickens in 240-CP-RO and 160-CP-ACRO groups had higher leukocytes than chickens in the other groups. With regard to H/L ratio, chickens in control and 80-CP groups had higher ($P<0.01$) H/L ratio than other chickens.

Table 3. Haematological parameters of broilers fed fermented cassava pulp

Items	Experimental diets											Pooled SEM	*P*-value
	Control diet	80-CP	80-CP-AC	160-CP-AC	240-CP-AC	80-CP-RO	160-CP-RO	240-CP-RO	80-CP-ACRO	160-CP-ACRO	240-CP-ACRO		
Day 21 of age													
Hemoglobin (g/dL)	11.2	10.1	11.2	10.7	9.58	10.9	11.0	11.0	11.4	10.1	10.3	0.52	0.28
Erythrocytes (10^{12}/L)	2.98	2.53	2.15	2.75	2.81	2.68	2.84	2.38	2.77	2.81	2.77	0.21	0.29
Hematocrit (%)	42.1	38.8	43.1	41.8	39.8	41.3	43.1	42.3	45.4	38.5	40.1	1.64	0.07
MCV (fl)	135	142	165	162	129	157	165	174	166	151	156	10.1	0.08
MCH (pg)	39.4	39.8	42.1	40.9	32.3	41.3	41.2	43.2	42.0	40.7	40.1	3.12	0.60
MCHC (g/dL)	26.4	25.8	26.0	25.5	25.1	26.7	25.4	26.0	25.8	26.8	25.8	0.50	0.36
Leukocytes (10^9/L)	15.7	15.1	15.3	16.5	9.25	20.8	18.4	16.7	16.4	16.8	19.9	2.22	0.10
Eosinophil (10^9/L)	3.91	3.24	2.60	3.54	1.96	4.57	2.77	4.74	2.29	2.37	2.80	0.78	0.22
Lymphocytes (10^9/L)	6.77	7.61	6.88	5.58	4.56	7.60	7.64	5.15	7.65	7.75	8.48	1.57	0.77
Monosit (10^9/L)	0.77	0.95	0.86	0.86	1.15	1.37	1.24	1.05	0.85	1.72	2.52	0.72	0.48
H/L ratio	4.99^{a}	3.98^{a}	1.66^{bc}	4.83^{a}	1.10^{c}	4.11^{a}	4.27^{a}	4.87^{a}	4.71^{a}	3.21^{ab}	4.77^{a}	0.65	<0.01
Day 34 of age													
Hemoglobin (g/dL)	12.0^{a}	8.45^{d}	10.9^{ab}	8.68^{cd}	11.0^{ab}	9.43^{bcd}	10.3^{bc}	10.3^{bc}	10.1^{bc}	10.0^{bc}	10.3^{bc}	0.50	<0.01
Erythrocytes (10^{12}/L)	3.65	2.28	2.97	2.35	3.02	2.69	3.05	3.22	2.66	2.95	2.95	0.29	0.09
Hematocrit (%)	35.0^{a}	24.8^{d}	31.8^{ab}	25.5^{cd}	32.5^{ab}	27.8^{bcd}	30.5^{ab}	30.3^{b}	29.5^{bc}	29.5^{bc}	30.0^{bc}	1.45	<0.01
MCV (fl)	98.3	95.2	109	116	143	115	108	120	116	121	125	15.2	0.67
MCH (pg)	33.5	32.4	36.1	35.7	35.3	36.0	36.7	37.9	36.3	33.6	34.4	4.03	0.99
MCHC (g/dL)	34.3	33.9	34.2	34.0	33.8	34.1	33.8	33.9	34.4	34.0	34.3	0.15	0.09
Leukocytes (10^9/L)	17.6^{ab}	12.4^{b}	19.8^{ab}	13.8^{b}	12.9^{b}	16.0^{b}	17.5^{ab}	28.1^{a}	13.2^{b}	29.4^{a}	12.1^{b}	3.78	0.02
Eosinophil (10^9/L)	6.37	2.82	4.07	5.64	4.26	5.60	4.97	7.15	5.10	7.11	4.96	1.62	0.76
Lymphocytes (10^9/L)	6.24	4.46	5.29	5.65	3.87	4.66	4.25	8.40	4.26	8.00	3.93	1.19	0.11
Monosit (10^9/L)	1.73	1.40	1.79	0.44	0.65	1.15	1.38	1.91	0.77	1.48	0.54	0.62	0.37
H/L ratio	6.04^{a}	5.77^{a}	4.92^{ab}	0.62^{d}	0.41^{d}	2.58^{bcd}	4.86^{ab}	4.43^{abc}	0.89^{d}	0.42^{d}	1.65^{cd}	1.09	<0.01

^{a,b,c,d,e} Means in a row with different superscripts are significantly different (P<0.05). Control diet, diet without dried CP or fermented CP; 80-CP, diet with 80 g/kg (as-fed basis) unfermented CP; 80-CP-AC, diet with 80 g/kg CP fermented with A. charticola; 160-CP-AC, diet with 160 g/kg CP fermented with A. charticola; 240-CP-AC, diet with 240 g/kg CP fermented with A. charticola; 80-CP-RO, diet with 80 g/kg CP fermented with R. oryzae; 160-CP-RO, diet with 160 g/kg CP fermented with R. oryzae; 240-CP-RO, diet with 240 g/kg CP fermented with R. oryzae; 80-CP-ACRO, diet with 80 g/kg CP fermented with A. charticola and R. oryzae; 160-CP-ACRO, diet with 160 g/kg CP fermented with A. charticola and R. oryzae; 240-CP-ACRO, diet with 240 g/kg CP fermented with A. charticola and R. oryzae. SEM, standard error of the mean (n=4). MCV, mean corpuscular volume. MCH, mean corpuscular haemoglobin. MCHC, mean corpuscular haemoglobin concentration. H/L ratio, heterophils to lymphocytes ratio.

Biochemical parameters

The blood biochemical profiles of broiler chickens are presented in Table 4. There was no significant difference in the blood biochemical profiles among chickens at day 21. At day 34, serum albumin was lower ($P<0.01$) in chickens at 240-CP-AC group especially when compared with chickens at 80-CP group. Compared to other groups, chickens at groups of control, 80-CP and 80-CP-ACRO had higher ($P<0.01$) A/G ratio. No significant difference was observed with regard to serum globulin and total protein among groups of chickens.

Table 4. Biochemical parameters of broilers fed fermented cassava pulp

Items	Experimental diets											Pooled SEM	*P*-value
	Control diet	80-CP	80-CP-AC	160-CP-AC	240-CP-AC	80-CP-RO	160-CP-RO	240-CP-RO	80-CP-ACRO	160-CP-ACRO	240-CP-ACRO		
Day 21 of age													
Total protein (g/dL)	3.66	2.87	3.54	2.87	3.74	3.33	2.88	3.51	3.01	3.64	4.18	0.37	0.24
Albumin (g/dL)	1.41	1.04	1.27	0.95	1.29	1.21	0.97	1.32	1.02	1.37	1.51	0.19	0.46
Globulin (g/dL)	2.26	1.83	2.27	1.91	2.46	2.13	1.92	2.18	1.99	2.27	2.66	0.19	0.11
A/G ratio	0.62	0.54	0.56	0.48	0.51	0.55	0.50	0.60	0.52	0.60	0.57	0.05	0.73
Day 34 of age													

Total protein (g/dL)	3.77	4.02	3.36	3.06	3.99	3.06	3.63	3.33	3.66	3.83	3.60	0.33	0.44
Albumin (g/dL)	1.24^{abc}	1.43^{a}	0.96^{bcd}	0.83^{cd}	0.63^{d}	0.82^{cd}	1.12^{abc}	0.89^{bcd}	1.26^{ab}	1.16^{abc}	1.04^{abcd}	0.13	<0.01
Globulin (g/dL)	2.28	2.59	2.40	2.23	3.36	2.23	2.51	2.44	2.40	2.67	2.56	0.26	0.20
A/G ratio	0.54^{a}	0.56^{a}	0.40^{b}	0.37^{b}	0.23^{c}	0.37^{b}	0.45^{ab}	0.36^{b}	0.53^{a}	0.44^{ab}	0.40^{b}	0.04	<0.01

^{a,b,c,d,e} Means in a row with different superscripts are significantly different (P<0.05). Control diet, diet without dried CP or fermented CP; 80-CP, diet with 80 g/kg (as-fed basis) unfermented CP; 80-CP-AC, diet with 80 g/kg CP fermented with A. charticola; 160-CP-AC, diet with 160 g/kg CP fermented with A. charticola; 240-CP-AC, diet with 240 g/kg CP fermented with A. charticola; 80-CP-RO, diet with 80 g/kg CP fermented with R. oryzae; 160-CP-RO, diet with 160 g/kg CP fermented with R. oryzae; 240-CP-RO, diet with 240 g/kg CP fermented with R. oryzae; 80-CP-ACRO, diet with 80 g/kg CP fermented with A. charticola and R. oryzae; 160-CP-ACRO, diet with 160 g/kg CP fermented with A. charticola and R. oryzae; 240-CP-ACRO, diet with 240 g/kg CP fermented with A. charticola and R. oryzae. SEM, standard error of the mean (n=4). A/G ratio, albumin to globulin ratio

Immune and visceral organs

The data of immune and visceral organs of broilers are presented in Table 5. At day 21, chickens in the control group had a lower (*P<0.05*) weight of thymus as compared to that in other chickens. The weights of other immune and visceral organs were not significantly different among chickens at day 21. Chickens in 80-CP-ACRO group had lower (*P<0.01*) weight of Bursa of Fabricius compared to other chickens. However, the difference was not significant when compared with chickens in control, 80-CP and 80-CP-RO groups. Chickens in the control and 240-CP-RO groups had higher (*P<0.01*) weight of liver than that in other chickens, but the significant difference was not observed compared to 80-CP, 80-CP-RO and 160-CP-RO chickens. The weight of gizzard was higher (*P<0.05*) in control chickens than in other chickens. Chickens in control and 80-CP groups had lower (*P<0.01*) weight of eviscerated carcass compared to chickens in 80-CP-AC, 160-CP-AC, 240-CP-AC80-CP-ACRO and 160-CP-ACRO groups.

Table 5. Immune and visceral organs of broilers fed fermented cassava pulp

Items (g/100 g body weight)	Experimental diets										Pooled SEM	*P*-value
	Control diet	80-CP	80-CP-AC	160-CP-AC	240-CP-AC	80-CP-RO	160-CP-RO	240-CP-RO	80-CP-ACRO	160-CP-ACRO	240-CP-ACRO	
Day 21 of age												
Spleen	0.10	0.13	0.14	0.15	0.14	0.14	0.15	0.15	0.14	0.15	0.15	0.01	0.10
Thymus	0.11^{a}	0.19^{abc}	0.26^{bcd}	0.14^{ab}	0.18^{abc}	0.30^{cd}	0.23^{abcd}	0.28^{bcd}	0.22^{abcd}	0.21^{abcd}	0.35^{d}	0.05	0.03
Bursa of Fabricius	0.17	0.24	0.25	0.18	0.25	0.17	0.26	0.30	0.35	0.27	0.23	0.04	0.05
Liver	3.01	2.77	2.91	2.73	2.80	2.74	2.90	2.88	2.88	2.90	2.81	0.23	0.99
Heart	0.74	0.63	0.66	0.60	0.74	0.67	0.74	0.74	0.63	0.65	0.61	0.06	0.67
Gizzard	4.60	5.04	4.69	4.29	4.95	3.86	4.08	4.16	4.04	4.24	4.09	0.33	0.22
Proventriculus	0.69	0.69	0.67	0.65	0.67	0.65	0.64	0.68	0.65	0.63	0.60	0.04	0.81
Duodenum	2.20	2.24	2.14	2.07	2.03	2.06	2.12	2.07	2.13	2.13	2.10	0.19	0.99
Jejunum	2.44	2.57	2.54	2.95	2.58	2.56	2.31	2.43	2.58	2.57	2.56	0.31	0.99
Ileum	1.50	1.68	1.74	2.41	1.81	1.99	1.92	1.87	1.94	2.19	2.43	0.30	0.49
Eviscerated carcass	57.9	58.8	59.9	63.3	62.9	63.5	66.8	62.3	62.6	61.9	61.4	1.82	0.09
Day 34 of age													
Spleen	0.14	0.13	0.14	0.12	0.13	0.13	0.12	0.18	0.21	0.13	0.16	0.02	0.15
Thymus	0.10	0.14	0.19	0.27	0.14	0.16	0.16	0.26	0.20	0.17	0.17	0.04	0.22
Bursa of Fabricius	0.13^{ab}	0.16^{abcd}	0.19^{bcde}	0.20^{cde}	0.22^{de}	0.16^{abc}	0.18^{bcd}	0.18^{bcd}	0.11^{a}	0.26^{e}	0.19^{bcde}	0.02	<0.01
Liver	2.92^{a}	2.64^{abc}	2.05^{d}	2.41^{bcd}	1.94^{d}	2.72^{abc}	2.76^{ab}	2.95^{a}	2.26^{cd}	2.00^{d}	2.42^{bcd}	0.15	<0.01
Heart	0.59	0.49	0.53	0.56	0.60	0.53	0.59	0.58	0.63	0.54	0.55	0.04	0.43
Gizzard	4.17^{a}	3.57^{abcd}	3.46^{bcd}	3.34^{cd}	4.09^{ab}	2.97^{d}	3.24^{cd}	3.62^{abcd}	3.43^{bcd}	3.65^{abcd}	3.90^{abc}	0.21	0.01
Proventriculus	0.74	0.64	0.61	0.58	0.59	0.69	0.67	0.68	0.58	0.59	0.55	0.04	0.07
Duodenum	2.12	1.95	2.04	1.82	1.94	2.05	1.77	1.90	1.74	1.87	2.13	0.14	0.50
Jejunum	2.83	2.74	2.28	2.66	2.30	2.63	2.51	2.33	2.13	2.05	2.73	0.23	0.23
Ileum	1.96	1.74	1.72	2.01	1.92	1.81	1.97	1.83	1.70	2.12	2.06	0.22	0.92
Eviscerated carcass	57.4^{a}	58.2^{a}	63.8^{b}	65.3^{b}	66.3^{b}	61.9^{ab}	61.3^{ab}	62.5^{ab}	66.0^{b}	65.7^{b}	61.9^{ab}	1.69	<0.01

^{a,b,c,d,e} Means in a row with different superscripts are significantly different (P<0.05). Control diet, diet without dried CP or fermented CP; 80-CP, diet with 80 g/kg (as-fed basis) unfermented CP; 80-CP-AC, diet with 80 g/kg CP fermented with A. charticola; 160-CP-AC, diet with 160 g/kg CP fermented with A. charticola; 240-CP-AC, diet with 240 g/kg CP fermented with A. charticola; 80-CP-RO, diet with 80 g/kg CP fermented with R. oryzae; 160-CP-RO, diet with 160 g/kg CP fermented with R. oryzae; 240-CP-RO, diet with 240 g/kg CP fermented with R. oryzae; 80-CP-ACRO, diet with 80 g/kg CP fermented with A. charticola and R. oryzae; 160-CP-ACRO, diet with 160 g/kg CP fermented with A. charticola and R. oryzae; 240-CP-ACRO, diet with 240 g/kg CP fermented with A. charticola and R. oryzae. SEM, standard error of the mean (n=4).

Discussion

It has been acknowledged that fermentation can improve the nutritional properties of agricultural waste products such as CP, so that they can be an alternative to conventional feed ingredients for broilers (Khempaka et al 2014). Similar with the previous study, fermentation either with *A. charticola*, *R. oryzae* or *A. charticola* + *R. oryzae* could lower fibre content of CP in the present study. Moreover, the use of urea as a fermentation supplement could increase the crude protein content of CP, which is in accordance with Khempaka et al (2014) and Sugiharto et al (2015). Earlier work has demonstrated that feeding 16% *Aspergillus oryzae*-fermented CP in the diet produced no detrimental impacts on growth performance of broilers (Khempaka et al 2014). Concomitantly, in the current study feeding *A. charticola*-, *R. oryzae*- or *A. charticola* + *R. oryzae*-fermented CP up to 24% had no adverse effects on growth performance of broiler chickens when compared with feeding the control diet or diet containing 8% of unfermented CP (data not presented).

Dietary inclusion of CP was expected to minimize the cost of feed without impairing the physiological conditions and health of chickens. With regard to the physiological conditions of chickens, haematological parameters have long been used as representative indicators. In this present work, the levels of haemoglobin and hematocrit were higher in chickens fed control diet or diet containing 8% and 24% of *A. charticola*-fermented CP as compared to those in chickens fed 8% unfermented CP. Alabi et al (2015) and Bańbura et al (2007) suggested that the levels of haemoglobin and haematocrit indicate nutritional state and metabolic performance of birds. Results in the present study may therefore suggest that chickens received control diet or diets containing *A. charticola*-fermented CP had better nutritional state and metabolic performance than chickens received unfermented CP, although these parameters did not significantly affect the growth performance of chickens. Leukocytes are cells of the immune system that protect the body against infections. High number of leukocytes seems therefore to imply in a superior ability of chickens to respond to infections. However, this assumption may be ambiguous as high number of leukocytes may also indicate stress or illness in animals (Sugiharto et al 2014). In the current study, there was no definite pattern in the number of leukocytes among the chickens. Therefore, it is difficult to withdraw any conclusion based on the number of leukocytes. The H/L ratio has recently been used for measurement of distress conditions in chickens, in which the increased H/L ratio may indicate that chickens suffer from infections, inflammation or stress (Davis et al 2008). In the present study, H/L ratio of chickens fed fermented CP was lower when compared with that of chickens fed control diet or diet containing 8% unfermented CP at day 34. At day 21, the H/L ratio of chickens was also lower in chickens fed diets containing 8% or 24% of CP fermented with *A. charticola* compared to that in chickens fed control or diet containing 8% unfermented CP. This finding suggests that feeding fermented CP, especially *A. charticola*-fermented CP, may preclude the chickens from infections, inflammation or stress. Our previous study revealed that filamentous fungi isolated from *gathot* contained substantial amount of phenolic compounds and exhibited high antioxidant activity (Sugiharto et al 2015). These phenolic compounds could act as antimicrobial agents, and that prevent the chickens from infections and subsequent

inflammation (Alves et al 2013). In addition, the high antioxidant activity in the filamentous fungi may cope the oxidative stress that can compromise the immune system (Lobo et al 2010) of broiler chickens.

The high and low serum globulin content and A/G ratio, respectively, may implicate a better disease resistance and immune response of chickens (Abdel-Fattah et al 2008). Whereas no difference was observed in serum globulin content, A/G ratio was found to be lower especially in the serum of chickens fed *A. charticola*-fermented CP as compared with chickens fed control or unfermented CP at day 34 in the current study. Concomitant with the H/L ratio (as discussed earlier), the low A/G ratio may evidence that chickens fed *A. charticola*-fermented CP had improved immune responses in this study.

In some circumstances, relative weight of immune organs of chickens can reflect the immune responses and functionality (El-Katcha et al 2014). At day 21, the relative weight of thymus was higher in chickens fed fermented CP than that in chickens provided the control diet. Except for chickens in 80-CP-ACRO group, chickens received fermented CP had higher weight of Bursa of Fabricius than chickens received control diet at day 34. The fungi *A. charticola* and *R. oryzae* used to prepare fermented CP in this present study have been suggested to possess probiotic properties in our earlier work (Sugiharto et al 2015). These probiotic properties may therefore be expected to increase the relative weight of immune tissues as reported by Abdel-Raheem and Abd-Allah (2011) when feeding yeast culture probiotics and Zhang et al (2013) when feeding *Bacillus subtilis* UBT-MO_2 to broilers chickens. However, this inference should be interpreted with caution as the relative weights of thymus and Bursa of Fabricius in chickens received fermented CP were not different with those in chickens received unfermented CP. Osundahunsi et al (2012) reported that cassava fibre may act as prebiotic with nutraceutic effects on rat. According to this, unfermented CP which has high content of fibre was most likely inducing the development of immune tissues of chickens in the present study (Sugiharto et al 2014).

Inclusion of urea in the diets of monogastric animals has been a concern due to the toxic effect of urea that may implicate in an abnormal (increased) liver weight (Khempaka et al 2014). In the present study, the relative weights of liver in chickens fed fermented CP were similar or lower than those in chickens fed control or diet containing 8% unfermented CP. This result may suggest that urea used as a nitrogen source during the fermentation of CP did not induce detrimental (toxic) effect. Our result differed from Khempaka et al (2014) who reported that feeding diet containing 20% *A. oryzae*-fermented CP (treated with urea during fungal fermentation) increased the relative weight of liver of broiler chickens when compared with control. Feeding diet with high fibre such as CP has been demonstrated to increase the gizzard weight of broilers (Khempaka et al 2009). In this study, the weight of gizzard in chickens fed fermented CP was similar or lower than that in chickens fed control diet, suggesting that feeding fermented CP up to 24% did not affect the function of the gizzard grinding fibre which may result in increased weight of gizzard. In the earlier study, the use of fermented CP up to 20% of the diets did not affect the eviscerated carcasses of broilers (Khempaka et al 2014). Interestingly, our present finding showed that feeding fermented CP could increase the eviscerated carcasses of broilers. This perhaps associated with the probiotic properties of fungi used to ferment CP, since Abdel-Raheem and Abd-Allah (2011) reported that feeding yeast culture probiotics increased eviscerated carcasses of broiler chickens.

From the present data, it could be concluded that inclusion of *A. charticola*-fermented CP up to 24% into diet did not impair the nutritional state and metabolic performance of chickens as indicated by the level of haemoglobin and hematocrit that were comparable to those in control chickens. Feeding *A. charticola*-fermented CP resulted in improved immune responses of chickens, indicated by the low H/L and A/G ratio.

Acknowledgement

The study was fully funded by Diponegoro University through *PNBP DIPA UNDIP*, *DIPA* No. 042.04.2.400049/2015, 15 April 2015.

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Received 9 January 2016; Accepted 3 March 2016; Published 1 April 2016

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