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Original Article

The effect of fungi-origin probiotic *Chrysonilia crassa* in comparison to selected commercially used feed additives on broiler chicken performance, intestinal microbiology, and blood indices

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ABSTRACT

Objective: The objective of the current study was to investigate the influence of probiotic *Chrysonilia crassa* in comparison with zinc bacitracin, commercial probiotic *Bacillus subtilis,* and herbal medicine waste on growth, intestinal microbiology, and blood indices of broilers.

Materials and methods: Three hundreds of Lohmann day-old chicks were allocated to control diet (basal diet; CONT), basal diet with antibiotic zinc bacitracin (AZB), basal diet with *B. subtilis* (PROB), basal diet with *C. crassa* (PROC), and basal diet with herbal medicine waste (HERBW). Sample collections were conducted on day 34 of the experiment.

Results: PROB showed greater ($P<0.05$) body weight than CONT chicks. Leukocytes and lymphocytes numbers were higher ($P<0.05$) in HERBW than in birds of other treatments. Compared to CONT and HERBW, PROC birds showed higher ($P<0.05$) level of vaccine titer to Newcastle disease virus. CONT had lower ($P<0.05$) and higher ($P<0.05$) total protein and globulin, and the ratio of albumin to globulin (A/G ratio) in serum, respectively, compared to other chicks. Higher level ($P<0.05$) of albumin was observed in PROB relative to CONT and PROC. Lower uric acid ($P<0.05$) was seen in PROC when compared with CONT and PROB. PROC had higher ($P<0.05$) aspartate aminotransferase than AGP, PROB, and HERBW. Ileal coliform was decreased ($P<0.05$) in PROB and PROC, relative to CONT and HERBW.

Conclusion: Probiotics were capable of improving the growth, immune responses, and intestinal bacterial populations of broilers. The effects of probiotics *C. crassa* corresponded to that of commercial probiotic *B. subtilis* and antibiotic growth promoters.

KEYWORDS

Antibiotic growth promoters; Broiler; *Chrysonilia crassa*; Growth; Health; Probiotic

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INTRODUCTION

Antibiotics have long been included sub-therapeutically in diets as an antimicrobial agent as well as growth promoters in broiler production. The use of antibiotic growth promoters (AGPs) is, however, no longer permitted in most countries due to the concern about antimicrobial resistance both in birds and humans as consumers (Abudabos et al., 2017a). Indeed, there are some problems related to performances and health upon the removal of in-feed antibiotics in broiler production (Sugiharto, 2016). The health problems in poultry are often the result of the imbalance of microbial community in the gut, which may be aggravated by the withdrawal of antibiotics from feeds (Abid et al., 2016). The change in the balance of bacteria in the gut has been shown to induce immunological disturbances in broiler chickens (Simon et al., 2016; Schokker et al., 2017). This immune stress may lead to physiological disorder and thus negatively affect broiler performances (Feng et al., 2012). Given the interplay between gut microbiota and immune system (Simon et al., 2016), feeding any compounds that can maintain the balanced microbiota in the intestine seems beneficial to support the healthy growth of broiler chickens post in-feed antibiotics removal.

The potentials of probiotics in establishing and maintaining the balance of intestinal microbiota and improving the immunological competences and growth performances of broiler chickens have been documented. However, there is still a hesitation among poultry nutritionists to incorporate probiotics in broiler rations since the results of some *in vivo* experiments with probiotics have been inconsistent (Sugiharto, 2016). Several studies pointed out that the benefits of probiotics for poultry and other farm animals are hosts- and species/strain-specific (Sugiharto et al., 2015a; Sugiharto, 2016; Abudabos et al., 2017b). For the latter reason, attempts are still needed to find novel probiotic microorganisms that can be a satisfactory alternative to in-feed antibiotics for broiler chickens. In our earlier study, we have isolated the fungus *Chrysonilia crassa* from the ileum of the Indonesian indigenous chickens (Yudiarti et al., 2012a) and shown its probiotic activities *in vitro* (Yudiarti et al., 2013). In the *in vivo* experiment, the fungus was capable of controlling bacterial population in the intestine and improving the duodenal villi development of the Indonesian indigenous chicks (Yudiarti et al., 2012b). Our recent study further confirmed the benefits of *C. crassa* in improving the physiological conditions and antioxidant status of broilers under heat stress (Sugiharto et al., 2017a). In the present study, the benefits of *C. crassa* were evaluated specifically for broiler chickens in comparison with commercial probiotics and other feed additives.

Herbal products such as Chinese herbal medicine have long been employed to substitute the use of AGPs for broilers. The use of such products resulted in improved intestinal bacterial community and growth rate in broiler chicks (Guo et al., 2004; Han et al., 2012; Zawqari et al., 2016). However, the application of herbal products in poultry production seems to be impractical as it has to compete with humans for medicine (Elfahmi et al., 2014). In Indonesia, herbal medicine (called *Jamu*) industries have been growing substantially in the recent years. This may consequently increase the waste generated during *Jamu* production (Soetrisnanto et al., 2012). According to Kisworo et al. (2016), most of the *Jamu* waste contains phenols, tannins, flavonoids, alkaloids, saponins, and essential oils that in general possess antibacterial activities. Taking the latter properties into consideration, feeding *Jamu* waste was, therefore, expected to improve the microbial community and thus, the well-being of broiler chickens. To date, the comparative effects among probiotic *C. crassa*, antibiotic zinc bacitracin (AZB), commercial probiotic *Bacillus subtilis,* and herbal medicine waste as the alternatives to AGPs on the production and health performances of broiler chickens have never been documented. Therefore, the objective of the current study was to evaluate the influence of fungi-origin probiotic *C. crassa* in comparison with AZB, commercial probiotic *B. subtilis,* and herbal medicine waste on growth performance, intestinal microbiology, and blood parameters of broilers.

MATERIALS AND METHODS

Ethical approval: The rearing and handling of chicks during the experiment were conducted according to the animal ethics mentioned in the Law of the Republic of Indonesia number 18, 2009.

Probiotics and herbal medicine waste preparations: Probiotic *C. crassa* was prepared based on Sugiharto et al. (2017a). Briefly, *C. crassa* was retrieved from the fungal stock (preserved on a potato dextrose agar [PDA; Merck KGaA, Darmstadt, Germany] and kept at 4°C). The fungal colonies were streaked on PDA and incubated at 38°C for 2 days. After being dislodged from the PDA, the fungal mycelia were diluted in sterile distilled water (100 ml). The sterile rice bran [200 g; 85.9% dry matter (DM)] was then inoculated with the above-mentioned suspension. The inoculum contained 1×10^{12} cfu/ml for each inoculation. Following 4 days of aerobic incubation

at room temperature, the number of fungal colonies grown in rice bran was determined. To do proximate analysis, a sample of fermented rice bran (100 g) was obtained. Standard plate count method showed that the fungi growing in rice bran were 1×10^{11}cfu/gm. Based on proximate analysis (AOAC, 1995), *C. crassa*-fermented rice bran contained (as a dry basis) 2,659 kcal/kg gross energy (GE), 11.6% crude protein (CP), 5.64% crude fat (CF), 23.1% crude fiber, and 12.0% total ash. The commercial probiotic (Baymix Grobig) was obtained from PT. Bayer Indonesia (Jakarta, Indonesia). It contained *B. subtilis* at a minimum 1×10^{10} viable spores/gm. The herbal medicine waste was obtained from PT. Sidomuncul, Tbk. (Semarang, Central Java, Indonesia). The waste was generated from the production of *Jamu* "Tolak Angin" composing of *Foeniculi Fructus, Isorae Fructus, Caryophylli folium, Zingiberis Rhizoma, Menthae arvensis Herba, Phyllanthi Herba, Mel depuratum, Myristicae semen, Amomi Fructus, Centellae Herba, Parkiae semen,* and *Oryza sativa*. Proximate analysis showed that the herbal medicine waste contained (as a dry basis) 2,189 kcal/kg GE, 11.6% CP, 3.28% CF, 40.4% crude fiber, and 4.89% total ash.

Experimental design, broiler management, and analyses: Three hundreds of day-old chicks [body weight (BW) 41.13 ± 0.22 g; mean ± SD] Lohmann meat broilers were placed in an open-sided ventilated broiler house with rice husk as bedding material. They were randomly allocated to five dietary groups, *i.e.*, control diet (basal diet with no additive; CONT), basal diet containing 0.04% of AZB, basal diet containing 0.01% of commercial probiotic *B. subtilis* (PROB), basal diet containing 1% of *C. crassa*-fermented rice bran (PROC), and basal diet with 1% of herbal medicine waste (HERBW). The basal diet was prepared as a single feed throughout the study period (34 days) and formulated to comply the Indonesian National Standards for broiler feed requirement (**Table 1**).

The feeds were prepared in mash form and provided *ad libitum* throughout the trial. Coccidiostat was not included in the basal feed. The feed additives were appended "on top" after feed formulation. The amount of *C. crassa*-fermented rice bran added to the basal feed was based on Sugiharto et al. (2017a) while the other commercial feed additives were according to the manufacturer's instructions. Vaccinations were conducted on chicks using commercial Newcastle disease virus (NDV) vaccine at days 4 and 18 through eye drop and drinking water, respectively. BW and feed consumption were recorded weekly.

On day 34, blood collection was conducted on one bird per pen (six birds per dietary group) from the birds' wing veins. For the analysis of complete blood counts, the blood was put in ethylenediaminetetraacetic acid vacutainers. For the analysis of serum biochemistry, blood was put in vacutainers with no anticoagulant. To make the serum, the blood was permitted to clot for 2 hours at room temperature and then, centrifuged at 5,000×g for 10 min. The same birds as blood sampled were killed by *halal* neck cut after being weighed (Alshelmani et al., 2016). The internal organs and commercial cuts of broilers were obtained and weighed. For the analyses of microbiology and short-chain fatty acids (SCFAs), the contents of ileum and cecum were aseptically collected.

Table 1. Ingredients and chemical composition (as a dry basis) of the basal diet

Items	Composition (%, unless otherwise noted)
Maize	45.5
Soybean meal	17.0
Wheat flour	10.0
Bread flour	5.00
Rice bran	4.45
Crude palm oil	3.50
Corn gluten meal	3.60
Distiller dried grains	3.00
Meat bone meal	2.80
Chicken feather meal	2.00
Bone meal	1.50
Lysine	0.55
Methionine	0.37
L-threonine	0.08
Salt	0.15
Premix[1]	0.50
Chemical composition:	
Metabolizable energy (kcal/kg)[2]	3,300
DM	89.6
Crude protein	21.9
Crude fat	6.40
Crude fiber	5.62
Ash	6.39

[1]*Mineral-vitamin premix provided (per kg of feed) Ca 2.250 gm, P 0.625 gm, Fe 3.570 mg, Cu 0.640 mg, Mn 5.285 mg, Zn 0.003 mg, Co 0.001 mg, Se 0.013 mg, I 0.016 mg, vit A 375 IU, vit D 150 IU, vit E 0.080 mg*

[2]*Values were obtained based on the formula according to Bolton (1967), in which metabolizable energy = 40.81 [0.87 (CP + 2.25 CF + nitrogen-free extract) +2.5]*

The complete blood counts were determined using a hematology analyzer (Prima Fully-auto Hematology Analyzer, PT. Prima Alkesindo Nusantara, Jakarta, Indonesia). The serum antibody titer to NDV vaccine was assayed on the basis of hemagglutination inhibition assay (Villegas, 1987). Total triglyceride was determined in serum according to the enzymatic method of glycerol-

3-phosphate oxidase. Cholesterol and its fractions were assayed based on the enzymatic determination of cholesterol oxidase. The enzymatic colorimetric analysis with glucose oxidase was employed to determine the blood glucose level. The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes in serum were assayed according to the IFCC procedure (www.ifcc.org) with no pyridoxal phosphate. Photometric analyses were conducted to determine the levels of total protein and albumin in serum. The difference between total protein and albumin values was considered as globulin values. Uric acid concentration in serum was determined according to the enzymatic color test. The entire serum biochemical analyses were conducted in duplicate based on the manufacturer's protocols (DiaSys Diagnostic System GmbH, Holzheim, Germany).

The microbiological analysis of the ileal and cecal digesta was conducted according to Sugiharto et al. (2017a) with few modifications. The numbers of coliform and lactose-negative enterobacteria were enumerated on MacConkey agar (Merck KGaA). The inoculated cultures were incubated aerobically at 38°C for 24 h and then, counted as red and colorless colonies, respectively. The sum of coliform and lactose-negative enterobacteria was regarded as enterobacteria. To enumerate the number of lactic acid bacteria (LAB), de Man, Rogosa and Sharpe (MRS; Merck KGaA) agar was used. Bacterial enumeration was conducted after the culture was incubated anaerobically at 38°C for 48 h. The population of *Clostridium perfringens* were enumerated on the tryptose sulphite cycloserine (TSC; Merck KGaA) agar. The black colonies were counted after the plates were incubated anaerobically at 38°C for 48 h. Gas chromatography was used to determine the SCFAs concentration in the cecal digesta of broilers. The analysis was performed according to Sugiharto et al. (2015a).

Results in the present study were analyzed by ANOVA using GLM Procedure (SAS Inst. Inc., Cary, NC, USA). The pen was considered as the unit of the experiment. Duncan's multiple-range test was carried out when significant differences among treatments exist.

RESULTS

Table 2 shows the data on final BW, feed intake, and feed conversion ratio (FCR) of broilers. Birds in PROB group had higher ($P<0.05$) final BW than in CONT, but the significant difference was not observed compared to

Table 2. The effect of fungi-origin probiotic *C. crassa* and other feed additives on performances of broiler chickens

Items	Dietary treatments					SE	*P* value
	CONT	AGP	PROB	PROC	HERBW		
Final BW, gm/bird	1,620[b]	1,663[ab]	1,729[a]	1,663[ab]	1,691[ab]	23.7	0.04
Feed intake, gm/bird	2,906	2,952	3,031	2,970	3,013	30.3	0.05
FCR	1.84	1.82	1.80	1.83	1.83	0.02	0.54

[a,b]Means with different superscripts in each row are significantly different
CONT: basal diet, AGP: basal diet supplemented with zinc bacitracin, PROB: basal diet with B. subtilis, PROC: basal diet with C. crassa, HERBW: basal diet with herbal medicine waste, and SE: standard error

Table 3. The effect of fungi-origin probiotic *C. crassa* and other feed additives on internal organs of broiler chickens

Items (% live BW)	Dietary treatments					SE	*P* value
	CONT	AGP	PROB	PROC	HERBW		
Heart	0.42	0.44	0.43	0.44	0.45	0.03	0.96
Liver	2.16	2.34	2.21	2.14	2.14	0.13	0.78
Bile	0.06	0.05	0.04	0.05	0.05	0.01	0.61
Proventriculus	0.40	0.42	0.37	0.37	0.43	0.02	0.08
Gizzard	1.35[a]	1.18[b]	1.19[b]	1.32[ab]	1.38[a]	0.05	0.03
Duodenum	0.60	0.59	0.55	0.56	0.59	0.04	0.94
Jejunum	1.12	0.99	0.99	1.05	1.17	0.07	0.24
Ileum	0.89	0.89	0.77	0.77	0.78	0.06	0.30
Caeca	0.30	0.26	0.28	0.28	0.35	0.03	0.25
Pancreas	0.25	0.27	0.25	0.26	0.28	0.02	0.81
Spleen	0.07	0.07	0.10	0.09	0.08	0.01	0.14
Thymus	0.28	0.28	0.29	0.36	0.28	0.04	0.59
Bursa of Fabricius	0.10	0.21	0.13	0.19	0.15	0.04	0.37

[a,b]Means with different superscripts in each row are significantly different
CONT: basal diet, AGP: basal diet supplemented with zinc bacitracin, PROB: basal diet with B. subtilis, PROC: basal diet with C. crassa, HERBW: basal diet with herbal medicine waste, and SE: standard error

Table 4. The effect of fungi-origin probiotic *C. crassa* and other feed additives on the hematological parameter of broilers

Items	Dietary treatments					SE	*P* value
	CONT	AGP	PROB	PROC	HERBW		
Complete blood counts							
Hemoglobin (gm/dL)	9.63	9.93	10.3	9.75	9.80	0.35	0.76
Erythrocytes (10^6/µL)	2.23	2.31	2.37	2.26	2.23	0.08	0.72
Hematocrit (%)	30.8	31.3	32.2	30.6	30.7	1.18	0.86
MCV (fl)	138	136	136	135	138	1.36	0.51
MCH (pg)	43.3	43.0	43.3	43.1	44.0	0.54	0.71
MCHC (gm/dL)	31.4	31.7	31.9	31.9	31.9	0.36	0.85
Leukocytes (10^3/µL)	17.5^b	20.2^b	20.6^b	20.9^b	27.2^a	1.74	<0.01
Heterophils (10^3/µL)	0.53	0.45	0.62	0.62	0.63	0.10	0.62
Eosinophils (10^3/µL)	0.85	0.95	1.00	1.05	1.10	0.12	0.60
Lymphocytes (10^3/µL)	16.1^b	18.8^b	19.0^b	19.2^b	25.5^a	1.60	<0.01
Thrombocytes (10^3/µL)	16.7	17.5	17.7	18.3	17.3	1.68	0.97
Biochemical parameters							
Total cholesterol (mg/dL)	142	159	175	164	153	10.9	0.28
HDL (mg/dL)	32.3	27.7	21.7	27.7	31.0	3.14	0.17
LDL (mg/dL)	84.5	94.8	114	95.0	86.4	10.9	0.37
Total triglyceride (mg/dL)	125	181	201	197	177	33.5	0.53
AST (U/l)	260^{ab}	249^b	247^b	317^a	209^b	21.4	0.03
ALT (U/l)	1.02	0.80	1.73	0.70	0.60	0.50	0.46
Total protein (gm/dL)	2.97^b	3.96^a	4.05^a	3.62^a	3.76^a	0.19	<0.01
Albumin (gm/dL)	1.38^b	1.47^{ab}	1.62^a	1.48^{ab}	1.49^{ab}	0.05	0.04
Globulin (gm/dL)	1.59^b	2.49^a	2.44^a	2.18^a	2.27^a	0.17	<0.01
A/G ratio	0.91^a	0.60^b	0.67^b	0.70^b	0.67^b	0.06	0.01
Uric acid (mg/dL)	13.0^{ab}	8.80^{bc}	14.2^a	7.14^c	8.23^{bc}	1.66	0.02
Creatinine (mg/dL)	0.39	0.35	0.37	0.27	0.44	0.05	0.29
Antibody titer against NDV (Log_2 GMT)	4.67^b	5.33^{ab}	5.60^{ab}	6.00^a	4.67^b	0.35	0.04

^{a,b,c}Means with different superscripts in each row are significantly different

CONT: basal diet, AGP: basal diet supplemented with zinc bacitracin, PROB: basal diet with B. subtilis, PROC: basal diet with C. crassa, HERBW: basal diet with herbal medicine waste, MCV: mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, A/G ratio: albumin to globulin ratio, GMT: geometric mean titer, and SE: standard error

that in AGP, PROC, and HERBW birds. There was an obvious tendency (P=0.05) that birds in PROB and HERBW groups had higher feed intake than CONT birds. FCR did not differ (P>0.05) among the birds.

The weight of gizzard relative to BW was lower (P<0.05) in AGP and PROB compared to that in CONT and HERBW birds. No significant divergence was observed with regards to other internal organs among the birds (**Table 3**).

Data on the complete blood count of broiler chicks are shown in Table 4. The numbers of leukocytes and lymphocytes were greater (P<0.05) in HERBW than in other birds. There was no significant difference with regards to the numbers of heterophils, eosinophils, thrombocytes, erythrocytes, hemoglobin, and the percentage of hematocrit. Data on serum antibody titer and biochemical parameters are presented in **Table 4**. Compared to CONT and HERBW, PROC birds had higher (P<0.05) level of vaccine titer to NDV. CONT had lower (P<0.05) and higher (P<0.05) total protein and globulin, and the ratio of albumin to globulin (A/G ratio) in serum, respectively, compared to other chicks. Higher level (P<0.05) of albumin was observed in PROB relative to CONT and PROC. Lower uric acid (P<0.05) was seen in PROC when compared with CONT and PROB. The AST enzyme was higher (P<0.05) in PROC than in AGP, PROB, and HERBW, but not different when compared with CONT birds. The differences in serum creatinine, ALT enzyme, triglycerides, total cholesterol, and low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol were not observed among the birds.

Data on the numbers of microorganisms and pH of ileal and cecal digesta are shown in **Table 5**. Coliform bacteria were lower (P<0.05) in the ileal digesta of PROB and PROC than in CONT and HERBW birds, but the substantial difference was not observed in comparison to AGP. The numbers of *Clostridium perfringens* tended

Table 5. The effect of fungi-origin probiotic *C. crassa* and other feed additives on microbial populations and pH in the ileal and cecal digesta of broilers

Items (log cfu/gm, except pH)	Dietary treatments					SE	*P* value
	CONT	AGP	PROB	PROC	HERBW		
Ileum							
Coliform	6.68[a]	6.21[ab]	5.89[b]	5.94[b]	6.68[a]	0.23	0.04
Lactose-negative enterobacteria	2.88	3.26	2.48	3.69	2.64	1.09	0.93
Enterobacteriaceae	9.56	9.47	8.37	9.63	9.32	0.95	0.88
LAB	8.50	8.45	8.32	8.18	8.15	0.25	0.82
Clostridium perfringens	5.94	3.88	3.46	3.97	5.94	0.77	0.07
pH	5.73	6.02	5.67	5.72	5.87	0.16	0.53
Cecum							
Coliform	6.39	6.18	5.95	6.12	6.64	0.18	0.10
Lactose-negative enterobacteria	5.13	4.46	5.43	5.25	5.00	0.45	0.62
Enterobacteriaceae	11.5	10.7	11.4	11.4	11.7	0.46	0.59
LAB	8.49	8.60	8.57	8.58	8.87	0.11	0.20
Clostridium perfringens	6.39	5.95	6.08	6.67	6.00	0.28	0.35
pH	6.30	6.18	6.17	6.35	6.58	0.16	0.38

[a,b]Means with different superscripts in each row are significantly different
CONT: basal diet, AGP: basal diet supplemented with zinc bacitracin, PROB: basal diet with B. subtilis, PROC: basal diet with C. crassa, HERBW: basal diet with herbal medicine waste, cfu: colony forming units, SE: standard error

(*P*=0.07) to be lower in the ileal digesta of AGP, PROB, and PROC than in that of CONT and HERBW birds. The significant difference was not observed in terms of pH both in the ileal and cecal digesta and microbial populations in the cecal digesta of broilers across the treatment groups.

The significant divergence was not seen with respect to the SCFAs concentrations in the cecal contents and carcass traits of broiler chicks (data not presented).

DISCUSSION

In the present study, commercial probiotic *B. subtilis* showed the most substantial effect on the growth performance of broilers, especially when compared to the control. This result has also been confirmed by other authors (Gao et al., 2017). The latter workers suggested that *B. subtilis* improved the digestibility and metabolic process, and hence increased the growth rate of broilers. Such commercial probiotic has also been documented to improve the intestinal health, and therefore more energy was partitioned into growth rather than maintenance (Gao et al., 2017). Indeed, there was no significant difference in term of final BW among PROB, AGP, PROC, and HERBW birds in this study. With regard particularly to *C. crassa*, the absence of difference in final BW between PROC and AGP birds may, therefore, suggest the opportunity of the fungal probiotic to substitute the use of AGPs in broiler production. With regard to feed intake, PROB and HERBW birds tended to consume more feed than CONT birds in this study. In accordance with our result, feeding *B. subtilis* has been reported to increase feed intake in broiler chickens challenged with *Salmonella Gallinarum* (Park et al., 2017). The mechanism by which probiotic *B. subtilis* increased feed intake in broilers was not definitely known, but probiotics may improve the intestinal microbial ecosystem and increase digestive enzyme activity which aids the nutrient digestion (Sugiharto, 2016). The increased feed intake in HERBW birds was concomitant with Guo et al. (2004) reporting an increased feed intake in female broiler chicks fed Chinese herbal medicine. In such case, herbal preparations may improve the flavor of feeds, increase the secretion of digestive fluids, and improve the bacterial community in the gut of chicks (Wenk, 2003).

Results in the present study showed that dietary supplementation with AGPs or probiotic *B. subtilis* resulted in the lower relative weight of gizzard as compared to other treatment birds. Concomitantly, Barbieri et al. (2015) reported a decreased relative weight of gizzard in 21 days of age broiler chicks fed AGP savilamycin. With regard to probiotic treatment, our present finding was in contrast to most published papers reporting an increase (Khaliq and Ebrahimnezhad, 2016) or no change (Barbieri et al., 2015; Sugiharto et al., 2017a) in the relative weight of gizzard of broilers. At present, the reason for the lower relative weight of gizzard in AGP and PROC birds remains unknown. Perhaps, the relatively higher final BW in AGP and PROC birds (as the denominator in the calculation) resulted in lower values of the relative gizzard weight. Indeed, the significant difference was not observed with regard to the

absolute weight of gizzard across the birds in the present study (data not presented).

Compared to other birds, feeding herbal medicine waste resulted in higher numbers of leukocytes and lymphocytes. The latter data may be attributed to the enhanced immune responses in HERBW birds (Sugiharto et al., 2017b). Considering the lower serum antibody titer against NDV vaccine (and thus lower protection against NDV) in HERBW chicks, the increased numbers of leukocytes and lymphocytes may, therefore, be associated with the acute viral infection or internal stress (Sugiharto et al., 2017b) in the respective birds. In this current study, serum antibody titer against NDV vaccine was significantly higher in PROC than in CONT and HERBW birds. This may suggest that feeding probiotic *C. crassa* was capable of protecting the birds from potential infection by NDV. Corresponding report was revealed by Haghighi et al. (2005) showing an increased level of serum antibody (immunoglobulin M) against sheep red blood cells in broiler chicks treated with a probiotic mixture containing *Lactobacillus acidophilus*, *Bifidobacterium bifidum,* and *Streptococcus faecalis*. The latter authors suggested that probiotics may provoke the immune cells to secrete cytokines, which in turn stimulate the production of antibody.

Relative to CONT, total protein and globulin concentrations in serum increased in the treated chicks. It has widely been known that serum total protein may reflect protein deposition in the body and thus the growth rate of farm animals (Ghazalah and Ali, 2008). Consistent with this, relatively higher final BW was observed in the treated birds as compared to CONT birds. With regard to globulin, the high level of this protein fraction has been associated with the improved immune defense system in broilers (Abdel-Fattah et al., 2008). Hence, the higher concentration of serum globulin may implicate in better disease protection in the treated birds. The latter suggestion was also endorsed by the data that albumin to globulin ratio (A/G ratio) was lower in the treated birds than in the control birds. Note that lower serum A/G ratio was attributed to the better immune competence of broilers (Ghazalah and Ali, 2008; Sugiharto et al., 2016). In the current trial, albumin concentration was higher in the serum of PROB as compared to CONT birds. Given that serum albumin level was positively correlated with growth rate and nitrogen retention in animals (de Ridder et al., 2012), the higher albumin concentration in PROB may, therefore, promote protein deposition and thus growth rate in the respective broilers. In poultry, serum uric acid concentration has been used to indicate muscle proteolysis (Sugiharto et al., 2017a), infections (Lin et al., 2015), and stress conditions (Babacanoğlu et al., 2013). In this study, serum uric acid was lowest in birds supplemented with probiotic *C. crassa*. This finding may, therefore, suggest that the fungus *C. crassa* was capable of protecting the birds from the above detrimental conditions. In this study, *C. crassa*-supplemented birds had a higher level of AST enzyme as compared to other treated birds. This may suggest that feeding *C. crassa* led to the toxicological effect on birds (Sugiharto et al., 2016). It was possible that phytic acid content in rice bran (as a carrier/media for growing the fungus) negatively affected the liver condition (Shibata et al., 2012), resulting in increased level of AST enzyme. This notion should, however, be accounted with caution since the level of ALT enzyme and the liver relative weight were not different among the birds in this current study.

It has previously been revealed that feeding fungal probiotic (*e.g.*, *Acremonium charticola*) (Sugiharto et al., 2017b) and probiotic *B. subtilis* (Jeong and Kim, 2014) was able to reduce the number of coliform in the gastrointestinal tract of broiler chicks. In agreement with this, our present data demonstrated the lower number of coliform bacteria in the ileal content of PROB and PROC as compared to CONT and HERBW birds. In the current study, fungal and bacterial probiotic supplementation was also able to control the population of *C. perfringens* in the ileum of broilers, which was comparable to the effect of AGPs supplementation. The concomitant result has been confirmed by La Ragione and Woodward (2003), in which feeding *B. subtilis* spores reduced the intestinal colonization by *C. perfringens* in broiler chicks. The mechanisms by which the fungal and bacterial probiotics controlled the population of pathogenic bacteria in the intestine of birds are largely unknown. In respect to probiotic *B. subtilis*, this probiotic may, however, decrease the potentially pathogenic bacteria through competitive exclusion (La Ragione and Woodward, 2003). With regard particularly to the fungus *C. crassa*, our present finding was consistent with Yudiarti et al. (2012b) who previously showed the potential of the fungus in controlling the bacterial population (not specific to a certain bacteria) in the intestine of the Indonesian native chickens. According to Sugiharto et al. (2015b; 2017a), one possible mechanism by which the fungal probiotic controls the population of pathogenic microorganism could be that the fungus produced some antibacterial substances that disrupt the biological functions of pathogens. Taken together, dietary inclusion with the fungus *C. crassa* was beneficial in improving the intestinal microbial population of broiler chickens.

In the present study, the influence of treatments was not observed with regard to the concentrations of SCFAs in the cecal digesta of broilers. With regard particularly to probiotic and AZB, Olnood et al. (2015) previously reported no notable effect of probiotic *Lactobacillus* spp. and zinc bacitracin on SCFAs (acetate, propionate, and butyrate) concentration in the contents of ileum and cecum of 21 and 35 days of age broilers. Unlike this, Milián et al. (2013) reported the increased concentrations of acetate, propionate, and total SCFAs in broiler chicks with feeding *B. subtilis*. Moreover, Murugesan et al. (2014) showed a higher concentration of butyrate in broilers fed probiotic *Bacillus licheniformis* strain while the concentrations of acetate, propionate, and total SCFAs did not change with the treatment. The definite reason for these divergent findings remains unclear, but the different species and strains of probiotic microorganisms used as well as the conditions of the study may be responsible. In respect to the effect of herbal medicine waste, corresponding result was reported in pig by Hanczakoska and Swiatkiewicz (2012), at which herbal extract had no impact on the concentrations of acetate, propionate, butyrate, and total SCFAs in the ileum and cecum (Hanczakoska and Swiatkiewicz, 2012).

The lack of influence of dietary treatments on the carcass traits of broilers was observed in the present study. This result was consistent with that of previously reported by Sugiharto et al. (2017a) showing no effects of the probiotic *C. crassa* on carcass characteristics of broiler chickens. With regard to AGPs and probiotic *B. subtilis*, treatment with such additives has also been reported not to affect the carcass traits of broiler chickens (Nunes et al., 2012). In term of treatment with herbal medicine waste, our present data were in accordance with that of reported by Onu (2010) showing no effect of two herbal spices (garlic and ginger) on carcass traits of broilers. However, Puvača et al. (2016) reported conversely, in that, feeding herbal drugs (garlic, black pepper, and hot red pepper) increased carcass weight in broilers. The definite explanation for these divergent results was unknown, but the difference in the composition and nature of herbal products used seemed to be responsible.

Overall, the effect of probiotic *C. crassa* on broiler growth and health performances corresponded to that of commercial probiotic *B. subtilis* and AGPs. On this basis, probiotic *C. crassa* can be a new candidate for AGPs substitute in broiler rations. However, the use of probiotic *C. crassa* as a feed additive for broilers seemed to be less economic especially when compared with probiotic *B. subtilis*. Indeed, the study showed that in general, filamentous fungi had a slow growth rate in the culture media, and therefore need longer time to proliferate (in our case, *C. crassa* was grown in rice bran for 4 days). Moreover, the longer incubation time (for fungal growth) may imply in more accumulation of contaminants in the fungal culture (Miyashira et al., 2010). With regard to *B. subtilis*, these bacteria need a shorter time to proliferate, which is about 24 h (Pant et al., 2015).

CONCLUSION

In conclusion, treatment with probiotics was capable of improving the growth performance, immune responses, and intestinal bacterial populations of broiler chicks. The effects of fungi-origin probiotic *C. crassa* corresponded to that of commercial probiotic *B. subtilis* and AGPs.

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CONFLICT OF INTEREST

The authors have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUTION

SS planed, conducted the experiment, and prepared the article. TY, EW, HIW, and TAS carried out the *in vivo* trial and corrected the article and II conducted the analysis of statistic.

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