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ORIGINAL ARTICLE

Effect of two-step fermentation by *Chrysonilia crassa* and *Bacillus subtilis* on nutritional values and antioxidative properties of agro-industrial by-products as poultry feed ingredients

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ABSTRACT

Objective: This current study was subjected to investigate the influence of two-stage fermentation by *Chrysonilia crassa* and *Bacillus subtilis* on nutritional values and antioxidative properties of agro-industrial by-products.

Materials and methods: Two-stage fermentation with *Ch. crassa* (inoculated in advance; single-step fermentation) and *B. subtilis* (inoculated later; two-step fermentation) was conducted on agro-industrial by-products, i.e., banana peel meals, cassava pulp, and rice bran. The pH measurement, microbial enumeration, proximate, and antioxidant analyses were conducted following 4- and 2-days aerobic incubation with *Ch. crassa* and *B. subtilis*, respectively.

Results: The pH of banana peels and cassava pulp increased with *Ch. crassa*-fermentation, but then decreased following *B. subtilis*-fermentation. *Chrysonilia crassa*-fermentation did not change, but *B. subtilis*-fermentation decreased pH of rice bran. The number of lactic acid bacteria was higher in two-stage than in single-stage fermented by-products. Crude protein and fat were higher in fermented than in unfermented banana peels. Crude protein was higher in single- and two-stage fermented, while fat higher in single-stage fermented than in unfermented cassava pulp. Crude fat and ash contents increased with fermentation in rice bran. Single-stage fermentation increased some of amino acids contents in banana peels and cassava pulp. The concentration of polyphenols, tannins, and antioxidant potential of banana peels reduced with fermentation. Total polyphenols and tannins were higher, whereas antioxidant activity was lower in fermented than in unfermented cassava pulp. Total polyphenols, tannins, and antioxidant activity were lower in two-stage than in single-stage fermented and unfermented rice bran.

Conclusion: Single-stage fermentation with *Ch. crassa* improved nutritional characteristics of agro-industrial by-products.

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Introduction

In response to the increase in feed price, nutritionists are now searching for alternative feedstuffs for poultry. Agro-industrial by-products have long been used as alternative feed ingredients in poultry ration as they are abundantly available throughout the year. However, the inclusion of such by-products into poultry ration is often limited by the high levels of undegradable fiber and the presence of antinutritional factors [1,2]. It has widely been known that fermentation could be an easy technique to improve the nutritive values of agro-industrial by-products. Fermentation may lower the fiber and increase protein contents of agro-industrial waste, so that can be incorporated in poultry ration at higher proportions [2,3]. In addition to the improved nutritional characteristics, fermentation may produce functional properties, such as antioxidants, which are essential for poultry health [2].

Fermentation has commonly been carried out either with single- or two-stage fermentation method, depending on the purpose of fermentation [4,5]. With respect to

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nutritional improvement, two-stage fermentation may, however, favor better nutritional characteristics in the fermented products, relative to single-stage fermentation [4,6,7]. Indeed, two-phase fermentation with *Rhizopus* spp. and *B. subtilis* resulted in higher protein content, when compared with fermentation using *B. subtilis* alone [7]. In another work, two-step fermentation with *B. subtilis* and *Enterococcus faecium* could increase crude protein content, amino acids, ash and total phosphorus. Such fermentation also decreased neutral detergent fiber, hemicellulose and phytate in maize-soybean meal based feed [8]. Likewise, recent study showed that two-stage fermentation resulted in higher level of polyphenols in the vinegars generated from cornelian cherry, when compared with that produced through single-stage fermentation [5]. Owing to the aforementioned studies, two-stage fermentation could be the preferred method to increase the nutritive and functional values of by-products derived from agro-industries.

Application of two-stage fermentation is generally subjected to gain the merits of both microorganisms used as starter cultures [7]. Shi et al. [8] used *B. subtilis* as a first fermentation starter to decrease antinutritional factors, while *E. faecium* was used in the second step for producing acids as well as lowering the pH values of feed. In the earlier preliminary study, we did fermentation on rice bran using *Ch. crassa*, a fungus isolated from intestine of the Indonesian indigenous chicken. The fungus lowered crude fiber, but had a little impact on crude protein and fat contents of rice bran [9]. In another studies, fermentation with *B. subtilis* increased the content of solvable sugars, solvable proteins, crude protein, and crude fat in soya bean hull [7,10]. Taking these into consideration, fermenting in advance with *Ch. crassa* followed with *B. subtilis* was, therefore, expected to decrease and increase the fiber and protein contents, respectively, of the agro-industrial waste. It has been shown from the previous study that some agro-industrial by-products possess antioxidative properties, which may promote the healthy growth of poultry [2]. Considering the antioxidative features of *Ch. crassa* [9] and the antioxidant-enhancing effect of *B. subtilis* [11,12], two-step fermentation with *Ch. crassa* and *B. subtilis* was, therefore, subjected not only to improve the nutritional qualities, but also to increase the antioxidant potentials of the agro-industrial waste. The objectives of the current work were to evaluate the impact of two-stage fermentation by the fungus *Ch. crassa* and later by *B. subtilis* on nutritional values and antioxidative properties of agro-industrial by-products as poultry feed ingredients.

Materials and Methods

Fermentation procedures

The isolate of *Ch. crassa* was obtained from the stock culture of fungi kept at 4°C. The isolate was rejuvenated on potato dextrose agar (PDA; Merck KGaA, Darmstadt, Germany) containing chloramphenicol. After 48 h aerobic incubation (at 38°C), the mycelia of fungi were dislodged and diluted in sterile distilled water (100 ml). The aforementioned suspension (inoculum) was subsequently inoculated to 500 gm of sterilized (using autoclave at 121°C for 15 min) agro-industrial by-products (i.e., cassava pulp, banana peel meals, and rice bran). For each inoculation, the inoculum was standardized to contain *ca*. 1×10^{12} cfu/ml of *Ch. crassa*. To obtain the water content of *ca*. 40% in the substrates during solid-state fermentation, a 400, 300, and 100 ml of sterilized water was incorporated into the cassava pulp, banana peel meals, and rice bran, respectively. Following the 4 days of aerobic incubation at room temperature, sample (100 gm, "as is") from each culture was collected for pH measurement, microbial enumeration, proximate, and antioxidant analysis. The rest of the fermented agro-industrial by-products were then inoculated with *B. subtilis* (1 mg/gm inoculum containing minimum 10^{10} spores/gm; PT. Bayer Indonesia, Jakarta, Indonesia) and aerobically fermented for 2 days at room temperature. Samples from every culture were eventually obtained for the analysis. The experiment was conducted using triplicate replications.

Measurement of parameters and data analysis

The value of pH of each sample was determined with pH meter (Eutech EcoTestr pH 1, Thermo Fisher, Singapore). The population of coliform in every sample was counted on MacConkey agar (Merck KGaA) as red colonies. The coliform enumeration was conducted after 24 h aerobic incubation at 38°C. The number of lactic acid bacteria (LAB) was determined on deMan, Rogosa, and Sharpe (MRS; Merck KGaA) agar. The bacteria were enumerated after 48 h anaerobic incubation at 38°C. The number of yeast was counted on PDA (Merck KGaA) containing chloramphenicol. The enumeration was performed after 48 h aerobic incubation at 38°C. The lowest dilution applied for the microbial counting was 1:10,000.

The chemical characteristics of each by-product were assessed following the procedures/proximate analysis [13]. The content of amino acid in each product was measured according to a standard ultra-performance liquid chromatography procedure [14]. The antioxidant activities of the agro-industrial by-products were determined by the 2, 2-diphenylpicrylhydrazyl (DPPH) test according to Wu et al. [15]. The assay was preceded by the preparation of the sample extracts by dissolving the sample of each by-product (10 gm) in methanol (100 ml). Ultrasonication for 30 min and maceration for 3 days were then conducted. Homogenization for 30 min was conducted every day (during the period of maceration) with magnetic stirrer. Subsequently, evaporation of the homogenate was done using rotary vacuum evaporator (50°C, 100 rpm, Sigma-Aldrich, St. Louis, MO) until

the volume of the homogenate was 25 ml. To test the free radical scavenging activity, the homogenate (0.5 ml) was diluted in DPPH solution (3 ml) and incubated at room temperature for 30 min. The absorbances of the solution were then measured at 515 nm using spectrophotometer. The DPPH assays were performed in triplicates. Total polyphenols in the agro-industrial by-products were determined according to Folin-Ciocalteu method [16]. The same homogenate (0.5 ml) as prepared above was mixed with distilled water (8 ml), Folin-Ciocalteu reagent (0.5 ml; Merck KGaA) and sodium carbonate (1 ml; Merck KGaA). After 30 min incubation at room temperature, the spectrophotometer was employed to measure (at 765 nm) the absorbance of the mixture. Gallic acid was employed to plot the standard curve. The test was run in triplicate. The content of tannins in the agro-industrial by-products was assayed colorimetrically, and Folin-Denis reagent was used to estimate the tannins content in the samples [17]. Along with distilled water (8 ml) and sodium carbonate (1 ml, Na_2CO_3, Merck KGaA), Folin-Denis reagent (0.5 ml, Merck KGaA) was added to the homogenate (0.5 ml). The solution was subsequently incubated for 30 min at room temperature, and eventually the measurement of the absorbance was conducted at 760 nm. Tannic acid solution (Sigma-Aldrich) was used to prepare the standard curve. Each sample was assayed triplically.

Data on pH, proximate compositions, amino acids, and antioxidant properties of each by-product were subjected to analysis of variance. Duncan's multiple range test was further conducted to evaluate the variance among group means. Data on microbial populations were analyzed by *t*-test to contrast the group means between single- and two-stage fermented by-products. A substantial level of $p < 0.05$ was implemented.

Results

The pH values and populations of microbes in the agro-industrial by-products are presented in Table 1. The pH values of banana peels and cassava pulp increased ($p < 0.05$) after fermentation with *Ch. crassa* (first-stage), but then decreased to the values as of the unfermented by-products when followed by *B. subtilis*-fermentation. Different from these two by-products, the pH value of rice bran did not change ($p > 0.05$) after *Ch. crassa*-fermentation, but decreased following the fermentation with *B. subtilis*. The numbers of coliform, yeast, and LAB were not detected in all by-products (sterilized by-products) prior to fermentation. No substantial difference in coliform bacteria populations was observed in the by-products between the first- and second-fermentation. In all by-products, the number of LAB was higher ($p < 0.05$) in two-stage than in single-stage fermented by-products.

The data on chemical compositions of the fermented agro-industrial by-products are shown in Table 2. The crude protein and fat contents were higher ($p < 0.05$) in single- and two-stage fermented than in unfermented banana peel meals. In cassava pulp, crude protein was

Table 1. pH and microbial populations in the fermented agro-industrial by-products.

Items	Unfermented by-product	Single-stage fermented by-product	Two-stage fermented by-product	SE	*p* value
Banana peels					
pH	6.60[b]	7.73[a]	7.10[b]	0.15	0.01
Coliform (log cfu/gm)	ND	5.91	5.94	0.26	0.93
Yeast (log cfu/gm)	ND	7.92	>8.26	0.34	0.42
LAB (log cfu/gm)	ND	7.96[b]	>10.3[a]	0.30	0.02
Cassava pulp					
pH	4.93[b]	6.67[a]	5.20[b]	0.23	<0.01
Coliform (log cfu/gm)	ND	4.36	5.32	0.48	0.93
Yeast (log cfu/gm)	ND	7.49	>8.26	0.39	0.42
LAB (log cfu/gm)	ND	5.33[b]	>10.2[a]	0.44	0.02
Rice bran					
pH	6.37[a]	6.10[a]	5.33[b]	0.25	0.02
Coliform (log cfu/gm)	ND	5.33	4.81	0.38	0.93
Yeast (log cfu/gm)	ND	7.38	>8.26	0.13	0.42
LAB (log cfu/gm)	ND	7.52[b]	>10.2[a]	0.18	0.02

[a,b]Values with different letters within the same row and type of agro-industrial by-products are significantly different ($p < 0.05$).
The symbol ">" indicates that some observations from which the mean was calculated had values above detection levels. When the colonies could not be enumerated on the plates, the detection level was applied and used to make the calculations. Hence, the real mean value is above than that reported.
ND (not detected) indicates that some observations had values below detection levels.
LAB = lactic acid bacteria.
SE = standard error.

Table 2. Chemical compositions (as-dry basis) of the fermented agro-industrial by-products.

Items (%)	Unfermented by-product	Single-stage fermented by-product	Two-stage fermented by-product	SE	*p* value
Banana peels					
Crude protein	7.99[b]	8.94[a]	9.06[a]	0.09	<0.01
Crude fat	4.23[b]	5.96[a]	6.72[a]	0.47	0.02
Crude fiber	18.0	15.9	15.0	0.93	0.15
Ash	12.9	13.1	12.6	0.57	0.80
Cassava pulp					
Crude protein	2.12[b]	2.33[a]	2.32[a]	0.04	0.01
Crude fat	0.33[b]	1.10[a]	0.88[ab]	0.17	0.04
Crude fiber	11.2	8.59	10.6	0.80	0.11
Ash	3.29[b]	3.75[a]	3.46[b]	0.05	<0.01
Rice bran					
Crude protein	10.9	11.9	12.2	0.35	0.08
Crude fat	1.92[b]	9.04[a]	8.73[a]	0.62	<0.01
Crude fiber	10.3	8.67	9.05	0.44	0.08
Ash	9.14[b]	10.2[a]	10.5[a]	0.28	0.03

[a,b]Values with different letters within the same row and type of agro-industrial by-products are significantly different ($p < 0.05$).SE = standard error.

substantially higher ($p < 0.05$) in single- and two-stage fermented than that in unfermented product. The single-stage fermented cassava pulp contained higher ($p < 0.05$) crude fat than unfermented cassava pulp, but such variation was not significant as compared with two-stage fermented cassava pulp. Single-stage fermented cassava pulp had higher ($p < 0.05$) ash content than two-stage fermented and unfermented cassava pulp. There was a tendency ($p = 0.08$) that both single- and two-stage fermentation increased the content of crude protein in rice bran, while crude fat and ash contents notably increased ($p < 0.05$) with the fermentation. Crude fiber tended ($p = 0.08$) to decrease in the fermented as compared with unfermented rice bran. In general, proximate compositions did not significantly differ between single- and two-stage fermented by-products, except for the ash content of cassava pulp.

The data of amino acid contents in the fermented agro-industrial by-products are presented in Table 3. First-stage fermentation of banana peel meals using *Ch. crassa* increased ($p < 0.05$) the contents of L-alanine, L-aspartate acid, L-isoleucine, L-leucine, L-lysine HCl, L-threonine, L-tyrosine, and L-valine in banana peel meals. However, the subsequent fermentation (second-stage fermentation) with *B. subtilis* tended to lower the amino acid contents in banana peels. Compared with unfermented cassava pulp, single-stage fermented cassava pulp contained higher ($p < 0.05$) levels of glycine and L-threonine, but the concentrations of these amino acids were not different as compared with two-stage fermented cassava pulp. L-serine content was greater ($p < 0.05$) in single-stage fermented cassava pulp than in unfermented and two-stage fermented cassava pulp. With regard to rice bran, single- or two-stage fermentation did not influence ($p > 0.05$) the amino acid contents of the product.

Data on total polyphenols, total tannins, and free radical (DPPH) removing activity are presented in Table 4. Total polyphenols, total tannins, and antioxidant potential of banana peels decreased ($p < 0.05$) following the fermentation. The concentrations of total polyphenols and total tannins were greater ($p < 0.05$) in single- and two-stage fermented than in unfermented cassava pulp. Yet, the antioxidant activity was weaker ($p < 0.05$) in the fermented than in unfermented cassava pulp. In rice bran, total polyphenols, tannins, and free radical scavenging capacity were less ($p < 0.05$) in two-stage fermented rice bran, when compared with single-stage fermented and unfermented rice bran.

Discussion

Data in our present study showed that first-stage fermentation with *Ch. crassa* increased the pH values of banana peels and cassava pulp. The reason for these increased pH values was not clear, but such increase in pH was also previously reported in soybean koji fermented with *Aspergillus oryzae* S. [18]. The latter authors further reported that the increase in enzyme production seemed to be responsible for the increased pH value in the fungal fermented products. Moreover, Liang et al. [19] revealed that the increased production of extracellular protein in the fermentation substrate (due to metabolic activity of fungi) may also lead to the increased pH value in the fermented products.

Table 3. Amino acid contents in the fermented agro-industrial by-products.

Items (mg/kg)	Unfermented by-product	Single-stage fermented by-product	Two-stage fermented by-product	SE	*p* value
Banana peels					
Glycine	2.61	5.19	4.06	0.79	0.09
L-Alanine	2.5[b]	5.89[a]	3.99[ab]	0.79	0.03
L-Arginine	2.3	5.15	3.05	0.89	0.09
L-Aspartate acid	3.32[b]	8.13[a]	6.28[ab]	1.21	0.04
L-Glutamic acid	3.56	11.7	7.12	2.21	0.06
L-Phenylalanine	2.44	4.95	3.47	0.76	0.09
L-Histidine	0.71	1.91	1.26	0.33	0.07
L-Isoleucine	1.63[b]	3.75[a]	2.64[ab]	0.51	0.03
L-Leucine	2.78[b]	6.67[a]	4.62[ab]	0.99	0.04
L-Lysine HCl	0.98[b]	4.69[a]	2.19[ab]	0.94	0.04
L-Proline	2.47	4.23	3.15	0.46	0.05
L-Serine	1.93	4.46	3.58	0.73	0.07
L-Threonine	1.94[b]	4.01[a]	3.34[ab]	0.51	0.03
L-Tyrosine	1.1[b]	2.33[a]	1.83[ab]	0.31	0.04
L-Valine	2.42[b]	5.13[a]	3.25[ab]	0.69	0.04
Cassava pulp					
Glycine	0.82[b]	1.07[a]	0.92[ab]	0.06	0.03
L-Alanine	1.00	1.19	1.03	0.08	0.20
L-Arginine	0.77	0.92	0.87	0.05	0.18
L-Aspartate acid	1.39	1.63	1.45	0.10	0.24
L-Glutamic acid	1.52	1.80	1.71	0.12	0.25
L-Phenylalanine	0.86	0.99	0.86	0.06	0.22
L-Histidine	0.33	0.40	0.38	0.02	0.07
L-Isoleucine	0.56	0.63	0.56	0.04	0.41
L-Leucine	1.00	1.09	1.04	0.12	0.88
L-Lysine HCl	1.21	1.36	1.20	0.09	0.38
L-Proline	0.91	1.11	1.03	0.10	0.43
L-Serine	1.10[b]	1.40[a]	1.10[b]	0.07	0.01
L-Threonine	0.89[b]	1.12[a]	1.03[ab]	0.06	0.04
L-Tyrosine	0.30	0.24	0.24	0.02	0.06
L-Valine	0.90	1.01	0.89	0.06	0.35
Rice bran					
Glycine	5.97	6.45	5.33	0.45	0.26
L-Alanine	5.7	6.09	5.01	0.71	0.58
L-Arginine	7.35	6.64	5.15	0.65	0.08
L-Aspartate acid	8.29	8.33	7.38	0.55	0.41
L-Glutamic acid	15	13.5	11.6	1.17	0.14
L-Phenylalanine	4.96	5.95	4.77	0.49	0.23
L-Histidine	2.58	2.66	2.04	0.22	0.13
L-Isoleucine	3.62	4.31	3.51	0.33	0.21
L-Leucine	7.34	7.87	6.31	0.63	0.24
L-Lysine HCl	6.26	5.85	4.8	0.57	0.21
L-Proline	4.47	4.51	3.79	0.33	0.25
L-Serine	5.02	5.54	4.26	0.39	0.09
L-Threonine	4.17	4.65	3.8	0.3	0.16
L-Tyrosine	2.91	2.88	2.28	0.21	0.09
L-Valine	5.39	5.87	4.7	0.45	0.22

[a,b]Values with different letters within the same row and type of agro-industrial by-products are significantly different ($p < 0.05$).
SE = standard error.

Our inference should, however, be noted with caution as *Ch. crassa*-fermentation did not change the pH value of rice bran in the present study. In this regard, the nature of substrates used for fermentation seemed to determine the change in pH value of the *Ch. crassa*-fermented products. Note that each substrate may have divergent buffering capacities [8]. In all agro-industrial by-products, pH values were lower in two-stage fermented than in single-stage fermented by-products. The greater number of LAB in two-stage fermented than in single-stage fermented by-products was most likely to induce the decreased pH in two-stage fermented in the present study. Concomitant to

Table 4. Total polyphenols, total tannins, and DPPH radical scavenging activity (IC_{50}) of the fermented agro-industrial by-products.

Items	Unfermented by-product	Single-stage fermented by-product	Two-stage fermented by-product	SE	*p* value
Banana peels					
Total polyphenols (mg/gm)	5.14[a]	1.56[b]	1.64[b]	0.09	<0.01
Total tannins (mg/gm)	4.34[a]	1.32[b]	1.16[b]	0.09	<0.01
IC_{50} (ppm)[1]	479.2[b]	3964[a]	3793[a]	448	<0.01
Cassava pulp					
Total polyphenols (mg/gm)	0.39[b]	1.21[a]	1.94[a]	0.23	0.01
Total tannins (mg/gm)	0.15[b]	1.09[a]	1.83[a]	0.22	0.01
IC_{50} (ppm)[1]	3110[c]	5571[a]	4361[b]	237	<0.01
Rice bran					
Total polyphenols (mg/gm)	2.91[a]	3.37[a]	1.80[b]	0.18	<0.01
Total tannins (mg/gm)	2.53[a]	3.12[a]	1.60[b]	0.18	<0.01
IC_{50} (ppm)[1]	1139[b]	875.7[b]	1869[a]	138	0.01

[a,b,c]Values with different letters within the same row and type of agro-industrial by-products are significantly different ($p < 0.05$).
[1]IC_{50} is considered as the concentration of the DPPH radicals were scavenged by 50%. A lower IC_{50} value implies a higher of DPPH radical scavenging activity.
SE = standard error.

our finding, Shi et al. [8] showed that fermentation using *B. subtilis* and *E. faecium* produced higher population of LAB as well as lactic acid resulting in lower pH value in the fermented products. With regard to coliform bacteria, the presence of such pathogenic bacteria in the fermented products seemed due to be the presence of free sugars in the by-products that may promote the proliferation of the bacteria [20].

It was apparent in our present study that fermentation especially using *Ch. crassa* elevated the contents of raw protein and fat in the substrates. Earlier work showed that fermentations using *Aspergillus niger* and *Trichoderma pseudokoningii* were capable of increasing the protein content in cassava root [21] and cassava residue [22], respectively. According to Liang et al. [19], the increased production of extracellular protein by the fungus may be responsible for the increased protein content of the fermented products. Bayitse et al. [22] further suggested that the capability of the fungus to produce enzymes to degrade amylum/starch and non-starch polysaccharides to monosaccharides that are conveniently processed to protein may also be the reason for the protein-enhancing capacity of the fungal starters. With regard to fat, fermentation increased the content of fat in the by-products. Sukma et al. [23] previously showed that the fermentation with the fungus *R. oryzae* increased fat content of rice bran. The latter authors suggested that the increased fat content in the fermented products may be associated with the increased biomass of the fungi. Note that cell wall and plasma membrane of the fungi are generally composed of fat (phospolipid and lipoprotein). In cassava pulp and rice bran, ash content was increased by the fungal fermentation. In accordance with this, fermentation with *R. oryzae* increased ash content of rice bran in the study of Sukma et al. [23]. This increase seemed to be associated with the increased fungal populations in the substrates, as cell wall of fungi was rich in minerals [24]. In this current study, there was a tendency that the fiber content in rice bran decreased with the fungal fermentation. Although not significant, such decrease was also seen in the fiber contents of banana peel meals and cassava pulp following the fungal fermentation. Concomitant with our present finding, the decrease in fiber content was also seen in rice bran after being fermented with *R. oryzae* [23] and *Ch. crassa* [9]. The mechanism by which the fungus decreased fiber content of the by-products was not exactly known, but the fungus seemed to produce enzymes to degrade complex fiber to simpler carbohydrates [23].

In general, the proximate compositions did not differ between the single- and two-step fermented by-products, except for the lower ash content in two- than in single-step fermented by-products. This circumstance seemed to indicate the incapability of *B. subtilis* in improving the nutrient composition of the by-products. Our present result was in concomitant with Kanghae et al. [25] showing the absent influence of fermentation with *B. subtilis* on the proximate composition of soybean. In contrast, Wongputtisin et al. [10] revealed an improvement effect of *B. subtilis* on the proximate compositions in soybean hulls. The rationale for such divergent results was not definitely known, but the differences in nature of substrates and strains of *B. subtilis* used as a fermentation starter as well as the conditions during fermentation may affect the nutritional compositions of the fermented products.

Data in our present study showed that fermentation with *Ch. crassa* was capable of increasing the contents of both

essential (L-leucine, L-isoleucine, L-lysine HCl, L-threonine, and L-valine) and non-essential amino acids (L-alanine, L-aspartate acid, and L-tyrosine) in banana peel meals. In accordance with our finding, Wronkowska et al. [26] showed that fermentation with the fungus *Rhizopus oligosporus* resulted in higher amino acids in buckwheat. Bujang and Taib [27] further documented that *R. oligosporus*-fermentation increased the contents of amino acids (both essential and non-essential) in soybean, garbanzo bean, as well as groundnut. The proteolytic activity of protease enzyme in the filamentous fungi seemed to be responsible for the decomposition of protein to amino acids resulting in higher free amino acids in the fermented products [27]. Former study showed the capacity of filamentous fungi in hydrolyzing the long-chain carbohydrates to produce protein-rich biomass [28]. Owing that the fungal biomass is rich in amino acids [29], the production of biomass during fungal fermentation may also be attributable to the elevated amino acid levels in the fermented stuffs. Study by Sarkar et al. [30] as well as Song et al. [31] noticed that the fermentation using *Bacillus* sp., *L. plantarum*, and *B. lactis* were able to enhance free amino acids in soybean, respectively. Different from the above studies, further (two-stage) fermentation using *B. subtilis* did not increase the content of amino acids in the fermented banana peels in the current study. The reason for the absent impact of *B. subtilis*-fermentation on amino acid contents was not exactly known, but the increased population of yeast in the substrates during the fermentation process may attenuate the potential of *Bacillus* to increase amino acid content in the substrates. Indeed, Song et al. [31] reported that fermentation using *Saccharomyces cerevisae* resulted in reduced amino acid contents in soybean meal. In the very earlier study by Majumdar and Bose [32], it was shown that amino acids may be utilized by *B. subtilis* during the growth and the production of secondary metabolites (such as antibiotic). In this regard, certain amino acids may be used by *B. subtilis* resulting in lack-increased amino acids contents in the two-stage fermented banana peel meals. In cassava pulp, the first fermentation using *Ch. crassa* resulted in increased amino acids glycine, L-serine, and L-threonine. Following the second-stage fermentation, the concentrations of glycine and L-threonine did not significantly change, while the concentration of L-serine decreased from that of first-stage fermentation. In the earlier study, Weng and Chen [7] noticed that the changes in amino acids contents of substrates were not constant during the fermentation process. The duration of fermentation seems to be one of the factors affecting the content of amino acids. Bujang and Taib [27] revealed that *R. oligosporus*-fermentation for 24 h elevated, whereas extended fermentation for 30 h decreased amino acid contents in soybean, garbanzo bean, and groundnut. Such prolonged fermentation may deplete nutrients, increase yeast population, and change the fermentation conditions, which in turn reduce the amino acids production [27]. With regard particularly to the decreased L-serine level in cassava pulp following the second stage fermentation, the exact reason for such condition remains unclear. It has been reported by Zhang et al. [33] that *Escherichia coli* was capable of utilizing L-serine to produce pyruvate. In this regard, the numerical increased coliform bacteria population after second stage fermentation seemed, therefore, to be responsible for the reduced L-serine content in the two-stage fermented cassava pulp. Unlike the other by-products, there were no substantial changes in amino acids in rice bran following the fermentations. No definite reason could be presented regarding the latter condition, but the different substrates of fermentation may affect the nature of protein that can be decomposed to free amino acids.

It was shown in this work that the fermentation resulted in reduction in total polyphenols, tannins, and antioxidant activity of banana peel meals. In most studies, fermentation was attributed to the increased antioxidant components and antioxidant potential of the substrates [34]. The contrasting data between our finding and other workers may not be elucidated clearly, but one possibility could be that the prolonged period of fermentation decreased antioxidant compounds and antioxidant activity of the substrates. Adetuyi and Ibrahim [35] reported that fermentation (for 24 h) of okra (*Abelmoschus esculentus*) seeds increased the contents of total phenols, vitamin C, flavonoids, and antioxidant activity. However, the antioxidant compounds and antioxidant activity of okra seeds decreased after fermentation for 72 and 120 h. In the study of Hunaefi et al. [36], phenolic compounds in red cabbages increased until day 7 of fermentation using *L. plantarum* and *L. acidophilus*, but gradually decreased with the time of fermentation. Amarasinghe et al. [37] suggested that the reduced antioxidant components may be associated with the utilization of such compounds by the microorganisms during fermentation. In our case, fermentation of banana peel meals for 4 days by the fungus *Ch. crassa* and then 2 days by *B. subtilis* seemed to promote the use of polyphenols and tannins by the microbes resulting in decreased concentration of the anti-oxidative compounds and hence antioxidant activity of banana peel meals. The extended fermentation may also prolong the exposure of antioxidant components to oxidation [37] and increase the diffusion of phenolic compounds out of the substrates [35], resulting in lower antioxidant properties of the substrates. In this context, it is crucial to investigate the most appropriate fermentation time to improve the nutritional values without inducing deleterious effect on the antioxidant properties of agro-industrial by-products.

Unlike banana peels, fermentation with *Ch. crassa* and then *B. subtilis* increased total polyphenols and tannins in cassava pulp. This was in accordance with that of noticed

by Hur et al. [34]. They suggested that fermentation increases the release of antioxidant components (from their complex bindings) and the production/synthesis of antioxidant compounds. In this study, the increased polyphenols and tannins was, however, not accompanied by the increased antioxidant activity in cassava pulp. In the study using kombucha, Amarasinghe et al. [37] pointed out that the antioxidant capacity of the substrate was not always determined by the concentration of phenolic compounds, but several metabolites produced during fermentation may have substantial effect on the antioxidant activity instead. In our study, other antioxidative compounds (not investigated in the current study) may be decreased by fermentation and hence, reduced the antioxidant activity of cassava pulp. With regard to rice bran, our current data showed that single-stage fermentation did not change the antioxidant compounds and antioxidant activity of the substrate. Unexpectedly, the two-step fermentation using *B. subtilis* reduced phenols and tannins contents, and antioxidant potential of rice bran. In accordance with this present finding, our previous study also showed the reduction of total polyphenols, tannins, and antioxidant activity of herbal medicine waste following the fermentation using *B. subtilis* for 4 days [38]. Concomitant result was also noted by Yoon et al. [39], in which *B. subtilis* KU3- fermentation alleviated antioxidative activity of rice bran. The reason for the reduced antioxidant properties in the rice bran was not clearly known, but *B. subtilis* seemed to degrade phenolic compounds and tannins in the substrate during fermentation resulting in reduced antioxidant activity. Our inference was supported by the facts regarding the capability of *B. subtilis* in degrading phenols [40] and tannins [41].

Conclusion

It can be concluded that the single-stage fermentation using *Ch. crassa* produced better nutritional characteristics of agro-industrial by-products, when compared with the two-stage fermentation (*Ch. crassa* inoculated in advance and *B. subtilis* inoculated later). Single-stage fermentation with *Ch. crassa* seems, therefore, to be more practical to produce poultry feed from the agro-industrial by-products.

Conflict of Interest

We declare that we do not have any conflicts of interest.

Authors' Contribution

Sugiharto Sugiharto planned, carried out the experiment, and prepared the article. Turrini Yudiarti, Endang Widiastuti, Hanny Indrat Wahyuni, and Tri Agus Sartono carried out the *in vivo* experiment and corrected the article and II conducted the statistical analysis and revised the article.

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