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Evaluation of antioxidant sources in fermented cocoa pod husk as animal feed

YUNILAS[1,♥], RIZKA RAMADHANI[2], ALFONSINA MARTHINA TAPOTUBUN[3], ELIZABETH JULENY TAPOTUBUN[4], MUHERI INDRA AJA NASUTION[5], ARMINA FARIANI[6], RAYHAN AKBAR[7]

[1]Department of Animal Science, Faculty of Agriculture, Universitas Sumatera Utara. Jl. Dr. A. Sofian No. 3, Medan 20155, North Sumatra, Indonesia. Tel./fax.: +62-618-213236, ♥email: yunilas@usu.ac.id
[2]Department of Nutrition and Feed Technology, Faculty of Animal Science, Institut Pertanian Bogor. Jl. Agatis, Kampus IPB Darmaga, Bogor 16680, West Java, Indonesia
[3]Program of Fisheries Product Technology, Faculty of Fisheries and Marine Sciences, Universitas Pattimura. Jl. Mr. CHR. Soplanit, Ambon 97233, Maluku, Indonesia
[4]Program of Fisheries Product Technology, Politeknik Perikanan Negeri Tual. Jl. Raya Langgur-Sathean, Southeast Maluku 97611, Maluku, Indonesia
[5]Department of Animal Production and Technology, Faculty of Animal Science, Institut Pertanian Bogor. Jl. Agatis, Kampus IPB Darmaga, Bogor 16680, West Java, Indonesia
[6]Department of Animal Science, Faculty of Agriculture, Universitas Sriwijaya. Jl. Palembang-Prabumulih Km 32, Inderalaya, Ogan Ilir 30662, South Sumatra, Indonesia
[7]Department of Pharmacy, Faculty of Pharmacy, Universitas Indonesia. Jl. Prof. DR. Mahar Mardjono, Depok 16424, West Java, Indonesia

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Abstract. *Yunilas, Ramadhani R, Tapotubun AM, Tapotubun EJ, Nasution MIA, Fariani A, Akbar R. 2025. Evaluation of antioxidant sources in fermented cocoa pod husk as animal feed. Biodiversitas 26: 521-527.* Cocoa has pods that have the potential to be used as feed. However, before being applied to livestock, fermentation using local microorganisms is necessary. This study aimed to evaluate antioxidant sources in fermented cocoa pod husk as feed for animal science and fisheries. The design used in this study was a Complete Randomized Design 3 x 3 factorial pattern with 3 replicates. Factor I was the dose of LMO (DM1 = 2%, DM2 = 4%, DM3 = 6%) and Factor II the fermentation time (LF7 = 7 d, LF14 = 14 d, LF21 = 21 d). Results showed that the dose of LMO and the fermentation time had a very significant effect ($p<0.01$) on phenol content and there was an interaction between the dose of LMO and the fermentation time ($p<0.01$). The dose of LMO had a very significant effect ($p<0.01$) and the fermentation time had no effect ($p>0.05$) on flavonoid content and there was no interaction between the dose of LMO and the fermentation time ($p>0.05$). The antioxidant content of the best fermented CPH in this study is DM1LF14 with a phytochemical screening was alkaloid (+), phenol (++), flavonoid (++), saponin (-), tannin (+), yield percentage 15.20%, flavonoids 0.20% and phenols 0.87%.

Keywords: Animal feed, antioxidant, CPH, fermentation, LMO

Abbreviations: ANOVA: Analysis of Variance; CPH: Cocoa Pod Husk; CRD: Complete Randomized Design; DMRT: Duncan Multiple Range Test; LAB: Lactic Acid Bacteria; LMO: Local Microorganisms; SPSS: Statistical Package for Social Sciences; UMMB: Urea Molasses Multinutrient Block

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is one of the plantation commodities that has high productivity in the world, especially in Indonesia and has been widely studied by researchers on various aspects (Devy et al. 2018; HS et al. 2020; Suparno et al. 2024). Based on data from the BPS (2020), the total cocoa production in Indonesia is 720.66 thousand tons with a total cocoa plantation area of 1.51 million hectares. Cocoa consists of pods, seeds, pulp and placenta (Soares and Oliveira 2022). One of the by-products of cocoa is the Cocoa Pod Husk (CPH) which accounts for 70% (Anoraga et al. 2024). CPH consists of three layers, namely the exocarp, mesocarp and endocarp layers (Grob et al. 2021). However, CPH has not been maximally utilized.

CPH can be utilized and processed into feed for animal science and fisheries, this is because CPH has a nutritional composition comparable to the nutritional composition of grass so CPH has the potential as an alternative feed to replace grass or forage (Sujono et al. 2020). CPH also contains antioxidants in the form of phenolic compounds and flavonoids that are useful in counteracting free radicals that cause stress for animals and fisheries and increase digestibility in the rumen in livestock which can be applied in the form of livestock supplements and pellets (Seravina et al. 2019; Ramos et al. 2023; Sultanayeva et al. 2023). Flavonoids are one of the bioactive components found in plants that are pigmented and consist of polyphenols, have antioxidant properties to alter rumen fermentation and can reduce NH_3 production (Kholif et al. 2023). In addition, phenolic compounds are usually found in the leaves seeds and fruits of plants and the content of phenolic compounds

will increase with the age of the plant (Alara et al. 2021). The concentration of phenol and flavonoid components can be influenced by various factors such as rainfall, temperature, soil composition, ultraviolet radiation and plant varieties (Bibi et al. 2022).

CPH if given directly to ruminants, nonruminants and fish without processing (fermentation) is not optimal because of the low content of phenolic and flavonoid compounds, some ingredients to be used as feed must be processed first because they can inhibit the digestive process (Yunilas et al. 2023; Lima et al. 2024). One process that can be done with fermentation technology is using Local Microorganisms (LMO) derived from the CPH itself. LMOs are derived from easily available natural ingredients and contain sources of microorganisms, carbohydrates, and glucose (Kabatia et al. 2023). LMO in fermentation technology can be used as a starter because the microbes in LMO come from their substrate and their ability to degrade the substrate is high (Yunilas et al. 2013). LMO contains a group of Lactic Acid Bacteria (LAB) that can degrade the substrate (Hudha et al. 2022). Adebo and Medina-Meza (2020) stated that fermentation using LAB groups can increase the levels of soluble and bound phenolic compounds caused by acidification, and the production of hydrolytic enzymes that damage the structure of the main cell wall so that various bioactive compounds in it can come out. Ordoñez-Araque et al. (2020) added that Bacillus and LAB including indigenous microbes in CPH are generally *Lactobacillus plantarum, Lactobacillus fermentum* and various species of *Bacillus* with diverse populations of $1.2x10^7$ CFU/g and $6.4x10^7$ CFU/g. So, the fermentation in this study used LMO derived from the CPH themselves.

Research that is currently developing utilizes CPH as a feedstuff for ruminants, poultry and fisheries (Mael 2024), Suryani et al. (2024) researched that giving 5% CPH flour gave the best results on the growth of native chickens, Irsyad et al. (2023) researched that giving Urea Molasses Multi-Nutrient Block (UMMB) based on fermented CPH with LMO as much as 10% gave the best growth in sheep, Olugosi et al. (2021) conducted a study that feeding 12.5% fermented CPH flour gave the best performance and health conditions in rabbits and Mustaqim and Zulkifli (2022) conducted a study that feeding 45% fermented CPH in pellets increased the growth and survival of carp (*Cyprinus carpio* Linnaeus, 1758). Based on this, the authors conducted a study evaluating antioxidant sources in fermented CPH as feed for animal science and fisheries.

MATERIALS AND METHODS

Materials

The materials used in the study were fresh CPH, rice washing water, coconut water, brown sugar, molasses, urea, quercetin, HCL 2 N, concentrated HCL, dragendorf reagent, $FeCl_3$ 5%, NaCl 10%, gelatin 2%, Na_2CO_3, folin ciocalteu reagent, Mg powder, amyl alcohol, $AlCl_3$ 5%, and ethanol 96%.

The chopper, tarpaulin, grinder, jerry cans with a capacity of 5 L, polyethylene plastic with a capacity of 2 kg, analytical scales, measuring cup 50 mL, beaker 200 mL, ph meter, thermometer, erlenmeyer 250 mL, UV-Vis spectrophotometer (Shimadzu® UV-1800) with a wavelength of 730 nm, micropipettes 100µ and 1000µ, mesh 80, upright cooler, microplate desiccator, vortex, rotary evaporator (Buchi rotavapor R-200), Soxhlet and Whatman paper No. 1 are tools used during this study.

Method

The design used in this study is a complete randomized design (CRD) 3 × 3 factorial pattern with 3 replications. Factor I is the dose of LMO (DM1 = 2%, DM2 = 4%, DM3 = 6%) and Factor II is the fermentation time (LF7 = 7 d, LF14 = 14 d, LF21 = 21 d).

Research procedure

CPH-based LMO and fermented CPH production

The production of CPH-based LMO and the production of fermented CPH refer to Yunilas et al. (2022) which was modified. Provide a fermenter container with a capacity of 5 L, then put 800 g of chopped CPH, add 120 g of rice bran, 60 g of brown sugar and 60 g of molasses, 1.480 mL of rice washing water and 1.480 mL of coconut water and homogenize and then cover anaerobically for 14 d. While making LMO fermented cocoa pods, provide a 2 kg polyethylene plastic, weigh as much as 950 g of chopped cocoa pods, 20 g of rice bran, 20 g of molasses and 10 g of urea and then homogenize and then add LMO as much as 20 mL, 40 mL, 60 mL and homogenize again. Then put into polyethylene plastic and fermented anaerobically for 7, 14 and 21 d.

Preparation of fermented CPH flour

The preparation of fermented CPH flour refers to Tamrin et al. (2020). The fermented CPH were placed in an oven at 60°C for 24 h to stop the fermentation process and dry the pods. The dried CPH were then pulverized into powder using a grinder. Then the fermented CPH powder was sieved using an 80 mesh sifter until a fine powder of fermented CPH was obtained.

Preparation of fermented CPH extract

Preparation of fermented CPH extract refers to Andriana and Jura (2022). Total 30 g of fine powder of fermented CPH was weighed, then put into a 350 mL glass bottle container and added 96% ethanol as much as 300 mL and stirred for 5 minutes. Maceration was carried out for 3 days with every day stirring for 10 minutes and extracted until thick using a rotary evaporator then calculated the yield.

Variables

Parameters of this research were yield percentage, phytochemical screening, flavonoid and phenol content in fermented CPH. CPH before fermentation is shown in Table 1.

Table 1. CPH before fermentation

Parameters	Results
Yield (%)	11.40
Flavonoid content (%)	0.35
Phenol content (%)	2.45
Phytochemical screening	
Alkaloid	++
Phenol	+
Flavonoid	+
Saponin	+
Tannin	++

Yield test is the residue of the extraction that has been filtered and dried from the solvent and weighed. The weight obtained was used to calculate the extraction yield against the mass of the simplisia. The calculation of yield is % yield = mass of simplisia - mass of residue/mass of simplisia × 100% (Adiwibowo et al. 2020). Flavonoid testing is to weigh a sample of 5 g and then dissolve it in 100 mL of ethanol, filter (centrifuge) the sample solution, take as much as 1 mL of clear solution, add 3 mL of 5% $AlCl_3$ solution, add ethanol solution until the volume becomes 10 mL, read the absorbance using a spectrophotometer with a wavelength of 420 nm, make a standard curve using Quercetin and weight 15 mgr Quercetin dilute to 100 mL = 0.15 mgr/mL (Rao et al. 2023). Phenol testing is to weigh 5 g of CPH powder sample and then put it in a 100 mL erlenmayer, dilute the sample using distilled water to a volume of 100 mL using a volumetric flask, filter/centrifuge the solution until a clear filtrate solution is obtained, take as much as 1 mL of solution (clear filtrate) into a test tube, then add as much as 0.5 mL of follin denis (follin 1:1), then added as much as 1 mL of saturated Na_2CO_3 solution and let stand for 10 minutes, add distilled water to a volume of 10 mL, after which the solution is vortexed until homogeneous, read the sample absorbance using a spectrophotometer with a wavelength of 730 nm, record the data obtained then calculate using a phenol standard curve and make a phenol standard curve (Rachmawaty et al. 2019).

Phytochemical Screening testing consists of alkaloids, phenols, flavonoids, saponins and tannins. In the alkaloid test, to 5 mL of CPH extract, 1 mL of HCl 2N and 9 mL of distilled water added, and heated on a water bath for 2 minutes, cooled and filtered, 2 mL of filtrate was then added 2 drops of Dragendorff's reagent solution and form an orange or orange precipitate (Sabdoningrum et al. 2021). In phenol testing, provide as much as 0.5 g of cocoa pod flour samples distilled with 10 mL of distilled water, filtered, then diluted with distilled water until it is no longer colored, then take as much as 2 mL of filtrate solution and add as much as 1 or 2 drops of 5% $FeCl_3$ solution, so that the presence of polyphenols shows the blackish green color formed (Indriaty et al. 2023). In the flavonoid test, take 5 mL of filtrate and add 0.1 g of mg powder, 2 mL of amyl alcohol and 1 mL of concentrated HCl, stirred and allowed to separate, so that positive flavonoids are indicated if there is a change in red, yellow, orange color in the amyl alcohol layer (Dubale et al. 2023). In the saponin test, provide 5 mL of extract in a test tube and mix with 10 mL of hot distilled water, after cooling, vortex for 10 seconds, a strong foam appears for 10 seconds, 1-10 cm high and 1 drop of 2 N HCl is added, so if the foam does not disappear, it indicates the presence of saponins (Shalihin et al. 2022). The test for tannin is to provide 0.5 g of CPH flour sample distilled with 10 mL of distilled water, filtered and then the filtrate is diluted with distilled water until colorless, then take 2 mL of filtrate solution and add 1 to 2 drops of 10% NaCl and 2% gelatin, so that if a white precipitate forms, it indicates the presence of tannin (Rizki et al. 2024).

Data analysis

Data on flavonoids and phenols of fermented CPH were analyzed statistically using Analysis of Variance (ANOVA) and phytochemical screening and percentage yield of fermented CPH were analyzed descriptively. If a significant or very significant result was obtained, Duncan Multiple Range Test (DMRT) was conducted. Data processing used the Statistical Package for Social Sciences (SPSS) program.

RESULTS AND DISCUSSION

Yield of fermented CPH

Yield is one of the parameters to be able to see how much extract is produced from a sample during the extraction process. The yield value can also be used to assess and determine the efficiency of a processing process. The amount of yield is directly proportional to the water content in a sample. The higher the water content, the more yield is produced. This is because the CPH sample applies the fermentation process, where the fermentation process produces H_2O compounds. Thus, the longer the fermentation process runs, it will result in an increase in moisture content in a material. The percentage yield of fermented CPH extract can be seen in Figure 1.

Based on Figure 1, the highest percentage yield was recorded in DM3LF21 at 18.40% followed by DM3LF14 at 18.23%, DM2LF21 at 17.03%, DM3LF7 at 17.00%, DM2LF7 at 16.73%, DM2LF14 at 16.60%, DM1LF14 at 15.20%, DM1LF21 at 13.27% and DM1LF7 at 13.10%. Low or high yields are influenced by the level of mesh size and in this study using an 80 mesh sieve produced high yields. The use of 96% ethanol solvent also affects the yield value, the ability of ethanol as a solvent to bind compounds that have polar or nonpolar properties causes the attraction of many compounds in cocoa pods, not only secondary metabolite compounds but other compounds such as fats are also attracted. So the yield of CPH in this study indicates that it does not only contain secondary metabolite compounds but other compounds as well. Fermentation with its substrate origin can accelerate the fermentation process and improve the degradation efficiency of the substrate, thereby increasing the yield. In addition, bioactive components will also increase and produce high yields. De Souza Vandenberghe et al. (2022) stated that fermentation with its substrate origin can increase yield. Shodehinde et al. (2021) added that fermentation affects bioactive compounds in cocoa which have the potential as a source of antioxidants.

Phytochemical screening of fermented CPH

A phytochemical screening test on CPH is used to identify the content of secondary metabolite compounds found in fermented CPH. Table 2 shows that qualitatively the fermented CPH are indicated positive for alkaloid, phenol, flavonoid, and tannin compounds with different intensities and no indication of saponin compounds. During the fermentation process, the structure of the compounds is broken down, resulting in changes in the presence of compounds.

Based on Table 2, the presence of alkaloid compounds was found in all treatments with various intensities. This shows that fermentation with various doses and fermentation duration gives different responses. Treatment with various doses and fermentation time resulted in moderate intensity (+). In the treatment of DM2LF7, DM3LF7, DM3LF14 and DM3LF21 did not show an increase in phenol levels qualitatively by showing moderate intensity (+), this is in line with the phenol content in treatments DM2LF7, DM3LF7, DM3LF14 and DM3LF21 (Table 4) showing that these treatments have low phenol content so that the phytochemical screening (Table 2) produces moderate intensity (+). Alkaloids work by destroying part of the peptidoglycan in the bacterial cell wall, stopping the energy metabolism of bacterial cells. This is in accordance with research conducted by Savira et al. (2024) that CPH are positive (+) containing alkaloid compounds in phytochemical screening tests. Zhang et al. (2020) stated that alkaloids inhibit bacterial growth through various mechanisms, including inhibition of bacterial nucleic acid and protein synthesis, modification of bacterial cell membrane permeability, damage to cell membranes and cell walls, inhibition of bacterial metabolism. Wang et al. (2021) stated that LAB activity in LMO breaks down glucose bonds into primary metabolites (lactic acid) and secondary metabolites (phenols), resulting in an overall increase in phenol content.

Table 2 flavonoid content of CPH in treatments DM1LF7, DM1LF14, DM2LF14, DM2LF21, DM3LF7, DM3LF14 and DM3LF21 is strong intensity (++) compared to before fermentation (+). This shows that the fermentation process can increase flavonoid compounds because during fermentation there is a breakdown of complex compounds. All treatments did not show the presence of saponin compounds (-) compared to before fermentation (+). This shows that the fermentation process can eliminate saponin compounds with the absence of foam appearance in phytochemical screening analysis. Fermentation can also reduce the tannin content evident in all treatments (Table 2) compared to without fermentation (Table 1). The longer the fermentation time, the more tannins were degraded. In addition, fermentation with LMO can hydrolyze sugars and carbohydrates causing tannins to hydrolyze due to carbohydrate groups contained in the molecular structure of tannins. Yang et al. (2023) stated that flavonoids are bound to complex polymer compounds in the form of glycosides before fermentation, with fermentation breaking these bonds into simpler compounds. Faradilla and Rizal (2023) stated that the appearance of foam indicates the presence of saponins that appear to form circular bubbles (+). Elihasridas et al. (2023) stated that fermentation can reduce the amount of tannin with the length of fermentation from 21.21% to 16.03-19.36%. Villacrés et al. (2020) added that fermentation can hydrolyze and remove tannin compounds from 920.34-975.74 mg/100 g to 144.48-185.86 mg/100 g.

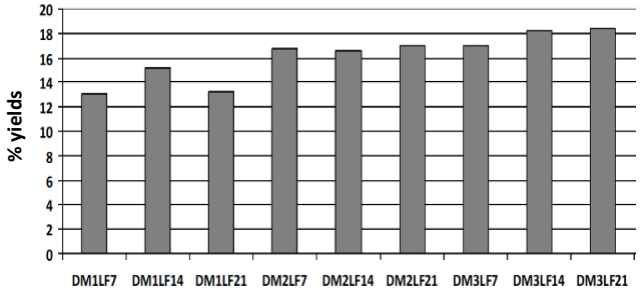

Figure 1. Yield of fermented CPH

Table 2. Phytochemical screening of fermented CPH

Treatment		Phytochemical compounds				
Doses LMO	**Fermentation time**	**Alkaloid**	**Phenol**	**Flavonoid**	**Saponin**	**Tannin**
DM1 (2%)	LF7 (7 d)	++	++	++	-	+
	LF14 (14 d)	+	++	++	-	+
	LF21 (21 d)	+	++	+	-	+
DM2 (4%)	LF7 (7 d)	+	+	+	-	+
	LF14 (14 d)	+	++	++	-	+
	LF21 (21 d)	++	++	++	-	+
DM3 (6%)	LF7 (7 d)	+	+	++	-	+
	LF14 (14 d)	+	+	++	-	+
	LF21 (21 d)	+	+	++	-	+

Notes: +: Active compound with moderate intensity; + +: Active compound with strong intensity; -: No active compound

Flavonoid content of fermented CPH

Flavonoid levels in this study before fermentation compared to after fermentation fluctuated. The results of the total flavonoid analysis of fermented CPH are presented in Table 3. Based on the data presented in this table, it can be seen that the flavonoid content of fermented CPH ranged from 0.18%-0.45%. Analysis of variance showed that various doses of LMO had a very significant effect ($p<0.01$) and the fermentation time had no effect ($p>0.05$) on flavonoid levels and there was no interaction between the dose of LMO and the fermentation time ($p>0.05$). In this study, between doses of LMO had a very significant effect because the higher the dose given to fermentation, the flavonoid content increased due to microbial activity in breaking down substrates that would form new flavonoid compounds. In addition, the fermentation time had no effect because the microbes were still in the stationary phase (constant). Chen et al. (2023) stated that the concentration of starter culture affects bioactive compounds such as flavonoids in a substrate due to increased microbial activity during fermentation and depends on fermentation conditions. Urcan et al. (2024) added that during fermentation, bacteria experience relatively constant growth (stable) and will be in the stationary phase, namely the ability of bacteria to degrade the substrate slowly decreases.

Table 3 shows that the lowest flavonoid content was obtained in the DM1LF7 treatment at 0.18% and the highest flavonoid content in the DM2LF14 treatment at 0.45%. The increase in flavonoid levels occurred due to the biodegradation process of polyphenolic compounds contained in fermented CPH into simpler molecules by enzymes secreted by bacteria. The decrease in flavonoid levels in fermented CPH is thought to be due to flavonoid degradation and oxidation during the oven and extraction process which involves heat. Fermented flavonoid levels are higher than without fermentation because during fermentation there is a release of flavonoids with glycoside complex bonds. In addition, the higher the dose of LMO, the higher the microbial population which will result in higher microbial activity and ability to break down the substrate, so that secondary metabolite compounds in fermented cocoa pods are hydrolyzed which results in flavonoid levels fluctuating. De Montijo-Prieto et al. (2023) stated that the activity of lactic acid bacteria, which can produce enzymes to break down sugars and destroy complex phenolic compounds and release phenol compounds from the substrate, can cause an increase in total flavonoid levels during fermentation. Polania-Hincapié et al. (2023) stated that fermented CPH produces flavonoid levels that very fluctuate. Marlyati et al. (2024) added that the high dose of inoculum in fermentation will affect flavonoid levels and this can occur in all substrates containing antioxidants such as CPH which is hydrolyzed by bacterial activity so that flavonoids fluctuate.

Phenol content of fermented CPH

Results of the total phenol analysis of fermented CPH are presented in Table 4. Based on the data presented in Table 4, it can be seen that the phenol content of fermented CPH ranged from 0.12%-0.87%. Analysis of variance showed that various doses of LMO (2%, 4% and 6%) and fermentation time (7 d, 14 d, and 21 d) in the fermentation process had a very significant effect ($p<0.01$) on the phenol content of CPH and there was an interaction between LMO dose and fermentation time ($p<0.01$). Phenolic levels in this study had an interaction, this indicates that the increasing dose of LMO and the longer fermentation time can result in a decrease in phenolic levels due to microbial activity breaking the bonds of phenolic compounds and transforming into new compounds so that the phenolic levels obtained are not optimal. However, the DM1LF14 treatment resulted in an insignificant decrease in phenolic levels from the other treatments because the dose of LMO was 2% (DM1) and the fermentation time for 14 days (LF14) the microbes were still in the stationary phase. Kiai et al. (2020) stated that fermentation reduces phenolic levels due to depolymerization of phenolic compounds from high molecular weight to low molecules and degradation of complex phenolic compounds.

Treatment of various doses of LMO and the fermentation time resulted in fluctuating phenolic levels in fermented CPH. The higher the dose of LMO given, the lower the phenolic content. This occurs due to microbial activity that results in the transformation of phenolic compounds to form new compounds resulting in a decrease in phenolics. This also proves that during fermentation, the decrease in polyphenol content is due to biochemical modification through enzyme activity produced by microorganisms during the fermentation process. Michalak-Tomczyk et al. (2024) stated that the decrease in phenolic content during fermentation is due to the process of transformation and biochemical modification through enzyme activity by microorganisms such as LAB.

Table 3. Flavonoid content of fermented CPH (%)

Doses LMO (DM)	Fermentation time (LF)			Average
	LF7 (7 d)	LF14 (14 d)	LF21 (21 d)	
DM1 (2%)	0.18	0.20	0.18	0.19^{A}±0.04
DM2 (4%)	0.41	0.45	0.39	0.42^{B}±0.15
DM3 (6%)	0.40	0.44	0.32	0.39^{B}±0.09
Average	0.33±0.12	0.36±0.14	0.30±0.17	

Note: Different superscripts in the same column indicate significantly different ($p<0.01$)

Table 4. Phenol content of fermented CPH (%)

Doses LMO (DM)	Fermentation time (LF)			Average
	LF7 (7 d)	LF14 (14d)	LF21 (21d)	
DM1 (2%)	0.44^{B}	0.87^{B}	0.41^{B}	0.57±0.26
DM2 (4%)	0.16^{A}	0.18^{A}	0.14^{A}	0.16±0.05
DM3 (6%)	0.12^{A}	0.12^{A}	0.12^{A}	0.12±0.01
Average	0.24±0.16	0.39±0.38	0.22±0.15	

Note: Different superscripts in the same row and column indicate significantly different ($p<0.01$)

According to Dibacto et al. (2021) stated that due to the acidic atmosphere, phenolic compounds become more stable and difficult to release protons that can bind to 2,2-diphenyl-1-picrylhydrazyl (DPPH), thus reducing antioxidant activity. According to Tarko et al. (2020), polyphenols diffuse out of the cotyledons and undergo oxidation and condensation after fermentation, which results in a decrease in polyphenol levels. Melini and Melini (2021) added that during fermentation phenol levels can decrease because phenol compounds bind to other molecules, are damaged by microbial enzymes or hydrolyzed by certain microbial strains and the influence of enzyme activity produced by microorganisms such as glucosidase, decarboxylase, esterase, hydrolase and reductase.

Based on this research, it can be concluded that CPH fermentation with various doses of LMO and fermentation time can increase the yield, reduce phenol and be able to maintain flavonoid. The use of LMO as much as 2% and fermentation time for 14 days (DM1LF14) produces phytochemical screening, namely alkaloid (+), phenol (++), flavonoid (++), saponin (-), tannin (+), yield 15.20%, flavonoid 0.20% and phenol 0.87%. CPH processed with fermentation has the optimal quality of antioxidant levels compared to without fermentation to be given to livestock.

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