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OPEN ACCESS Pakistan Journal of Nutrition ISSN 1680-5194 DOI: 10.3923/pjn.2017.538.543 Research Article Role of Humic Acid in Improving the Nutrient Content and Quality of Fermented Palm Oil Sludge Mirnawati, Ade Djulardi and Gita Ciptaan Department of Animal Feed and Nutrition, Faculty of Animal Science, Andalas University, 25163 Padang, Indonesia Abstract Objective: An experiment was conducted to understand the effects of different microbes and doses of humic acid on the quality and nutrient content of Fermented Palm Oil Sludge (FPOS). Materials and Methods: The experiment was conducted using a 2 ×3 factorial Completely Randomized Design (CRD) with 3 replications. The first factor was two species of microbe, Neurospora sitophila and Neurospora crassa and the second was different doses of humic acid: (1) 100 ppm, (2) 200 ppm and (3) 300 ppm. The study parameters were the crude protein content, crude fiber content, nitrogen retention and digestible crude fiber content of FPOS. Results: The study parameters were more significantly affected by the interaction between the type of microbe and the dose of humic acid ($p<0.01$) than the humic acid dose alone. FPOS treated with Neurospora crassa and humic acid at 200 ppm showed better values for crude protein (23.74%), crude fiber (20.14%), crude lipid (2.70%), nitrogen retention (60.97%) and digestible crude fiber (55.63%) compared to FPOS treated with Neurospora sitophila. Conclusion: It is concluded that POS fermented with Neurospora crassa and 200 ppm humic acid provides the best food content and quality of FPOS. Key words: Fermentation, microbes, humic acid, palm oil sludge, quality, nutrient Received: November 22, 2016 Accepted: May 10, 2017 Published: June 15, 2017 Citation: Mirnawati, Ade Djulardi and Gita Ciptaan, 2017. Role of humic acid in improving the nutrient content and quality of fermented palm oil sludge. Pak. J. Nutr., 16: 538-543. Corresponding Author: Mirnawati, Department of Animal Feed and Nutrition, Faculty of Animal Science, Andalas University, 25163 Padang, Indonesia Competing Interest: The authors have declared that no competing interest exists. Data Availability: All relevant data are within the paper and its supporting information files. INTRODUCTION Indonesia is the largest producer of palm oil in the world and 70% of the oil produced in this country comes from the island of Sumatra. Globally, the province of West Sumatra is the fourth largest palm oil-producing region, with a total annual production of 30,948,931 t of crude palm oil1. The continued development of palm plantations produce high amounts of waste in the form of Palm Oil Sludge (POS), which accounts for as much as 2% of the total production2. However, POS can potentially be used as feed material, especially for poultry. Palm oil sludge is similar to bran in its nutrient content, but it has more fibrous material and a lower availability of amino acids, which is a limiting factor in the production of poultry and other monogastric animals3. The nutritional content of POS is as follows: 11.1% crude protein, 17% crude fiber, 12% crude lipid, 50.4% nitrogen-free extracts, 48.04 ppm Cu and 61.10 ppm Zn4. Even with its fairly high crude protein content, the use of POS in poultry rations is still limited; it can only make up 5% of broiler rations2. To be used in poultry rations, POS must be pre -processed because of its low quality2, including its high fiber and low amino acid contents3,5 and the lack of fiber-digesting enzymes in the poultry digestive system. Another disadvantage of POS is its high crude lipid content, which is a limiting factor in poultry rations. Therefore, to improve its quality, POS must be pre-processed via biotechnological fermentation with cellulolytic and lipolytic fungi3,4, which can decrease the contents of crude fiber and crude lipids and increase the availability of amino acids so that the POS can ultimately replace soybean meal in poultry rations. The cellulolytic and lipolytic fungi that can be used to pre-process FPOS are Neurospora crassa and Neurospora sitophila, which also have carotenolytic properties and thus produce β-carotene, which can reduce the amount of cholesterol in chicken eggs and meat as well as provide the yellow pigment that gives the egg yolk and skin as well as the beak and feet of chicken carcasses their yellow color. β-Carotene also serves as a pro-vitamin A carotenoid and thus promotes growth3. Fenita et al.3 stated that POS fermented with Neurospora crassa in the diet of laying hens can reduce eggs contents of cholesterol and fat, which are feared by consumers, because

Neurospora crassa produces large amounts of β-carotene. Mirnawati et al.6 subsequently added that fermenting waste POS with Neurospora crassa can increase the contents of crude protein (20.42%), crude fiber (20.59%) and crude lipid (2.08%) as well as nitrogen retention (56.16%) and digestible crude fiber (50.88%). Despite an increase in the nutrient content of POS after fermentation, the amount that can be used in broiler rations is still low, at 13%7. This is due to the high concentration of heavy metals, such as Cu, after fermentation or to the lack of a significant reduction in heavy metals compared to before fermentation; therefore, limiting factors, such as Cu and Zn, remain high in POS. This is consistent with the opinion of Vidal et al.8 that Cu becomes the limiting factor in the fermentation process. In this study, humic acid was incorporated into POS processing to obtain the optimal conditions for improving the quality of the product. It is necessary to find substances/compounds that can lower the amount of Cu in POS and humic acid can effectively bind micronutrients such as Cu, Zn and Mn9. The humic acid fraction can interact with metals through the formation of chelate compounds10 and it can provide nutrients such as N, P and S into the soil to provide energy for the activities of microorganisms11. Added in the form of EnviromateTM12, humic acid is also used as a source of mineral and organic substances that play important roles in the lives of microorganisms, which also require nutrients, such as N, S and P, for growth during the fermentation process. Kucukersan et al.13 stated that the use of humic acid in animal feed provides several advantages for the health and growth of livestock, including the ability to metabolize carbohydrates and proteins through catalytic processes. Additionally, some researchers have studied the use of humic acid in broiler rations to stimulate growth14-16 because these substances can stimulate the growth of gut microbes13,17. Mirnawati et al.18 stated that the addition of 100 ppm of humic acid to Palm Kernel Cake (PKC) fermented by Aspergillus. niger (A. niger) for 7 days increased protein by 23.20% and reduced crude fiber by 10.59%. Furthermore, the addition of 0.2% humic acid in a diet containing 15% PKC fermented by A. niger increased egg production by 60.79%, egg weight by 66.71 g eggG1 and eggshell thickness by 0.12 mm compared to other treatments19. Mirnawati et al. 20 also stated that the addition of 100 ppm humic acid in drinking water in combination with a diet containing 15% fermented PKC can improve the performance of broilers. Based on the findings described above, further research is necessary to determine the type of fungus and the dose of humic acid that can best improve the nutrient content and quality of FPOS. This experiment aimed to determine the type of fungus and the optimum fermentation time needed to improve the quality of FPOS. MATERIALS AND METHODS The materials used in this experiment were 1) POS, which was obtained from the palm kernel processing and manufacturing facility of Andalas Agro Industry in Pasaman, West Sumatra; 2) The fungi Neurospora sitophila and Neurospora crassa, which were obtained from the Research Center of Applied Chemistry of LIPI, Bogor; 3) Potato Dextrose Agar (PDA); 4) Smooth bran; 5) Aqua Dest and a mineral standard consisting of 0.14%, (NH4)2SO4, 0. 2% KH2PO4, 0. 03% MgSO4.7H2O, 0.03% urea, 0.03% CaCl2.7H2O, 0.0005% FeSO4, 0.00016% MnSO4.H2O, 0.00014% $ZnSO_4.7H_2O$, 0.0002% $CoCl_2$ and 0.075% peptone21. The experiment was conducted using a 2 ×3 factorial Completely Randomized Design (CRD) with 3 replications22. Factor A was the type of fungus: (A1) Neurospora sitophila and (A2) Neurospora crassa. Factor B was the humic acid dose, which varied as follows: (B1) 100 ppm, (B2) 200 ppm and (B3) 300 ppm. The measured variables were included the crude protein content, nitrogen retention, crude fiber content, digestible crude fiber content and crude lipid content of FPOS. The obtained data were statistically analyzed according to Steel and Torrie22 and the differences between treatments were tested by using Duncan's Multiple Range Test (DMRT). Statistical significance was set at p<0.01 RESULTS AND DISCUSSION The effects of treatments on the crude protein, crude fiber, crude lipid, nitrogen retention and digestible crude fiber content of Fermented Palm Oil Sludge (FPOS) were illustrated in Table 1. Crude protein content of FPOS: The analysis of variance showed that there was an interaction (p<0. 01) between the type of fungi and the humic acid dose. Factors A and B had a highly significant effect (p<0.01) on the crude protein content of FPOS. The results showed that the crude protein content was highest with treatment A2B2 (POS fermented with Neurospora crassa and 200 ppm humic acid) because the crude protein content increases with fermentation time. The increase in the crude protein content is due to the addition of protein from the growth of microbial cells, which produced single-cell proteins or biomass cells composed of approximately 40-65% protein23-25. The number of microbial colonies, which are a source of single-cell proteins, increased during the fermentation process26. The high crude protein content in the A2B2 treatment was due to the addition of 200 ppm of humic acid, which produced the optimal conditions for the growth of the fungus Neurospora crassa; furthermore, the addition of humic acid can activate the growth of microorganisms. This is consistent with the results of Kompiang16, who found that humic acid can activate the growth of microorganisms by supplying N, P and S of Stevenson11, who found that humic acid can provide these nutrients. Added as EnviromateTM12, humic acid can increase the population of microorganisms by supplying constituent substances and energy sources such as essential minerals and organic matter. Furthermore, the fungus Neurospora crassa requires nutrients such as N, S and P for growth and as the fungi proliferated, more protein was produced27. Crude fiber content of FPOS: The analysis of variance revealed an interaction (p<0.01) between the type of fungus and the Table 1: Average contents of crude protein, crude fiber, crude lipid, nitrogen retention and digestible crude fiber content of FPOS Factor A Factor B (Humic acid dose) -------------------------- ------------------ --

Parameter (Neurospora species) B1 (100 ppm) B2 (200 ppm) B3 (300 ppm) Average Crude Protein (CP) N. sitophila (A1) N. crassa (A2) Average 17.88bB 21.71cA 19.80 22.47aB 23.74aA 23.11 21.85aB 22.77bA 22.31 Crude Fiber (CF) (%) N. sitophila (A1) N. crassa (A2) Average 25.67aA 23.50aB 24.58 23.21cA 20.14cB 21.68 24.53bA 22.49bB 23.51 Crude Lipid (%) N. sitophila (A1) N. crassa (A2) Average 4.85aA 3.61Ab 4.23 3.64bA 2.70bB 3.17 3.90bA 3.54aB 3.72

Nitrogen Retention (NR) (%) N. sitophila (A1) N. crassa (A2) Average 41.60cB 54.91cA 48.25 55.59aB 60.97aA 58.28 54.49bB 58.42bA 56.45 Digestible Crude Fiber (DCF) (%) N. sitophila (A1) N. crassa (A2) Average 48.97bB 52.46cA 50.72 52.56aB 55.63aA 54.10 51.84aB 53.71bA 52.77 20.56 22.85 24.47 22.04 4.13 3.29 50.56 58.10 51.12 53.93 Different superscripts in the same column or line indicate highly significant differences (p<0. 01) dose of humic acid. Both the factors A and B had a highly significant effect (p<0.01) on the crude fiber content of FPOS. From the above data, it can be seen that in treatment A2B2, fermentation of POS with Neurospora crassa and 200 ppm humic acid decreased the crude fiber content to 20.14%, lower than in the other treatment combinations. The decrease in crude fiber content was caused by the action of cellulose-modifying enzymes, which remodeled the crude fiber substrate and by the increased growth and fertility of the fungi. The greater the growth of the fungi was, the more the fungi produced cellulose-modifying enzymes to break the cellulose down into glucose; thus, the crude fiber content was lower by the end of fermentation. Glucose was used as an energy source for cell growth, as demonstrated by the increased fungal growth19. Li et al.28 reported activity by the following enzymes in Neurospora crassa: peptidase (protease), endoglucanase, exoglucanase, β-glucosidase and cellobiose dehydrogenase, which is an extracellular enzyme that is involved in the hydrolysis of cellulose and hemicellulose. Fermentation using cellulolytic Neurospora can break the cellulose bonds, causing a decrease in the crude fiber content of the substrate. The increased growth of N. crassa in treatment A2B2 is due to the addition of humic acid at 200 ppm, which created the optimal growth conditions, as indicated by the appropriate pH (5.5) for fungal growth. In addition, humic acid can activate the growth of microorganisms18 because it provides nutrients such as N, S and P in the soil that provide energy for their activities11. Added as EnviromateTM12, humic acid is a source of minerals and organic matter that play important roles in the life of microorganisms. As fungi proliferate, more cellulose- related enzymes are produced that can degrade the crude fiber in the fermentation products. Crude lipid content of FPOS: The analysis of variance showed an interaction (p<0.01) between the type of fungi and the dose of humic acid. Both the factors A and B had a highly significant effect (p<0.01) on the crude lipid content of FPOS. From the above data, the A2B2 treatment, which is POS fermented with the fungus Neurospora crassa and humic acid at a dose of 200 ppm, decreased the crude lipid content to 2.70%, which is less than the other treatment combinations. The decline in crude lipid under this type of fermentation showed that Neurospora crassa combined with 200 ppm humic acid has the ability to decrease the lipid content by using lipids as an energy source. This is consistent with the finding of Rizal et al.29, who stated that Neurospora crassa has advantages over other fungi due to its total enzyme activity, which includes amylase, protease and lipase. Lipase contributes to the hydrolysis of lipids, which are broken down into glycerol and free fatty acids, as well as to the production of small amounts of alcohol and various aromatic and fragrant esters30. During fermentation, the fatty acids that form would partly evaporate, causing a decline in crude lipid content. Digestible crude fiber of FPOS: The analysis of variance showed an interaction (p<0.01) between the type of fungus and the dose of humic acid. Both the factors A and B had a highly significant effect (p<0.01) on the digestible crude fiber content of FPOS. From the above data, it can be seen that treatment A2B2, which is POS fermented with the fungus Neurospora crassa and 200 ppm humic acid, increased digestible crude fiber to 55.63%, which is higher than in the other treatment combinations. This is consistent with the findings of Sukaryana et al.24, who stated that fermented food has higher digestibility because the fermentation process breaks down materials that cannot be digested by enzymes, such as cellulose, hemicellulose and other polymers, into simple sugars. The high digestibility of the crude fiber in treatment A2B2 was due to the low content of crude fiber, which was consumed, causing the release of many stored nutrients. In accordance with Walugembe et al.31, the decrease in crude fiber content caused the digestibility of other substances to increase. The increase in crude fiber digestibility was caused by the action of the cellulose-modifying enzymes on the crude fiber substrate. The high digestible crude fiber content under treatment A1B2 was due to the long duration of the fermentation process, which caused a decrease in crude fiber content and an increase in digestible crude fiber. This result is consistent with the findings of Sukaryana et al.24, who reported a positive relationship between fungal growth and the production of cellulose-modifying enzymes. The more fungi grow, the more they produce cellulose enzymes that can convert cellulose into glucose, consequently increasing the amount of digestible crude fiber by the end of fermentation. The high amount of digestible crude fiber in POS fermented by N. crassa is also due to the addition of humic acid at 200 ppm during the fermentation process. This is because humic acid can stabilize the gut flora and improve the usability of nutrients from forage as well as the digestibility of nutrients for livestock29.

CONCLUSION Based on the results of this study, POS fermented with Neurospora crassa and 200 ppm humic acid provides the optimal food content and quality of FPOS: 23.74% crude protein, 20.14% crude fiber, 2.70% crude lipid, 60.97% nitrogen retention and 55.63% digestible crude fiber. SIGNIFICANCE STATEMENTS This study discovers the alternative feed ingredients derived from palm oil processing. This study improves the quality and nutrient content of fermented palm oil sludge so that it can be used as feed material for poultry.

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