P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2023.121

The Role of *Lactobacillus fermentum* in Increasing the Quality and Nutritional Value of Palm Kernel Cake

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Article History: 23-273 Received: 17-Sep-23 Revised: 09-Nov-23 Accepted: 11-Nov-23

ABSTRACT

The aim of this study was to obtain the inoculum doses of *Lactobacillus fermentum* with optimal fermentation time which has the highest protease activity in increasing the protein contents and excellence of fermented palm kernel cake (FPKC). In this study, Palm kernel cake (PKC) and *L. fermentum* were fed to 30 broiler chicken Cobb strain CP-707 of 4-weeks age (body weight of 1.5kg) under completely randomized block design with a 3x3 factorial pattern with 3 replications in each group. The first factor (A) was the inoculum dose consisting of 5, 7.5 and 10% (A1, A2, and A3, respectively). The second factor (B) was fermentation length, consisting of 2, 4 and 6 days (B1, B2 and B3). The observed variables were protease activity, cellulase activity, mannanase activity, crude protein (CP), crude fiber (CF), crude lipid (CL), nitrogen retention (NR), crude fiber digestibility (CFD), and metabolizable energy (ME). The results showed a very significant interaction ($P<0.01$) between inoculum dose and fermentation length on protease activity, crude protein content, crude fat, and nitrogen retention. Each factor also showed a highly significant ($P<0.01$) effect on protease activity, content of CP, CL, and NR. It can be concluded that PKC fermented with an 10% inoculum dosage and 4 days' fermentation gave the best results in terms of cellulase activity 18.01U/mL, manannase 24.95 U/mL, protease 10.55U/mL, CP 26.31%, CF 15.71%, CL 1.45%, NR 63.92%, CFD 55.91% and ME 2752.69kcal/kg.

Key words: Inoculum, *L. fermentum*, Fermentation, Palm Kernel Cake, Fermentation time.

INTRODUCTION

Palm Kernel Cake (PKC) is an alternative feed ingredient as it has good nutritional contents, low price, abundant availability, and does not compete with human food needs. It was produced from a by-product of the processing of palm kernel into crude palm oil (CPO). By 2021, oil palm plantations area in Indonesia reached 15,081,021 hectares with CPO production of 42.9 million tons, an increase of 6.8 million in the last 4 years (Ditjenbun 2021). The increase in CPO production was accompanied by an increase in the by-products of palm oil processing, one of which was PKC.

Research on the nutrient contents of PKC indicated quite high nutritive values, i.e., 17.31% CP, 27.62% CF, 7.14% CL, 0.27% Ca, 0.94% P and 48.4ppm Cu (Mirnawati et al. 2018). The main concerned of PKC is the low quality of its biological nutritional value such as low digestibility of the protein (53%) in poultry as a consequence of high CF contents, thus causing the low availability of nutrients in PKC. Crude fiber molecules inhibit the absorption of protein molecules, making it difficult to be broken down by poultry proteases, thus reducing the protein content of poultry. From its composition, as much as 57.8% of total PKC hemicellulose is β-mannan (Cerveró et al. 2010; Azman et al. 2016). β-mannan is a polysaccharide, it is difficult for poultry to digest and absorb (Poulsen et al. 2022). As an effort to increase PKC quality in poultry rations, a processing technology such as fermentation with the help of bacteria is required so that cellulolytic and mannanolytic of PKC can be used with a larger quantity in poultry ration.

Fermentation of PKC with mannanolytic bacteria, namely *Bacillus subtilis*, was carried out by Mirnawati et al. (2019a) with 7% inoculum dose with 6 days fermentation time that increased CP contents to 24.65%,

Cite This Article as: Mirnawati, Ciptaan G, Martaguri I, Ferawati and Srifani A, 2024. The role of *Lactobacillus fermentum* in increasing the quality and nutritional value of palm kernel cake. International Journal of Veterinary Science 13(4): 484-489. https://doi.org/10.47278/journal.ijvs/2023.121

CF to 17.35%, NR to 68.47% and crude fiber digestibility to 53.25%. It was further explained that fermented PKC with *B. subtilis* was able to provide mannanase activity of 24.27U/mL, cellulase 17.13U/mL and protease 10.27U/mL (Mirnawati et al. 2019b). Even after biological testing on broiler chickens, the fermented PKC utilized up to 25% in ration (Mirnawati et al. 2020). Furthermore, according to Casula and Cutting (2002) that *B. subtilis* can act as a probiotic as well. Imam et al. (2012) stated that *B. subtilis* usually used as a probiotic to increase good bacteria in the digestive tract of livestock to enhance intestinal morphology (Oladokun et al. 2020), regulate gut flora, improve growth performance, and enhance immunity and gut health in poultry under various rearing environments and infectious immune challenges (Dela et al. 2019; Ramlucken et al. 2020).

This study used cellulolytic and mannanolytic bacteria, namely *Lactobacillus fermentum*, of which is also called lactic acid bacteria (LAB) that belongs to probiotic bacteria. These bacteria are useful for lowering pH levels so they can kill pathogenic bacteria in the intestine. The presence of these bacteria can support the digestive system in the body. Besides that, the role of these bacteria is very important for the growth (Aoudia et al. 2016; Okonkwo and Igwilo 2022). *L. fermentum* is a gram-positive bacterium, that does not form spores, a facultative anaerobic, bacillus-shaped, and non-pathogenic lactic acid bacterium. These bacteria also maintain the microbial balance in the digestive tract and increase digestibility which improves the performance of broiler chickens (Guo et al. 2021; Li et al. 2022).

Seftiadi et al. (2021) has isolated bacteria from rotting palm kernel cake. The bacteria obtained were *Lactobacillus spp*. which had the following enzyme activities: cellulase activity 17.63 U/mL; mananase 24.31 U/mL; protease 10.34 U/mL and was able to give good results in increasing crude protein to 25.81%; crude fiber to 16.90%; crude lipid to 1.83%; nitrogen retention to 62.84%; crude fiber digestibility to 54.37%. The weakness in this study was that the doses of inoculum given were only based on one dose, namely 7%. Therefore, further research is needed by combining inoculum doses with fermentation time, because these two factors are very influential in the fermentation process.

The right doses of inoculum will facilitate microbes to grow and mature quickly (Duan et al. 2020). The more doses used, the more ingredients are overhauled. The duration of fermentation provides an opportunity for microbes to decompose nutrients in the substrate, thus by combining the dosage of inoculum and duration of fermentation, the nutritional value of the product will be increased (Mirnawati et al. 2019a; Mirnawati et al. 2022a; Coniglio et al. 2023). Thus, this study was required to obtain the optimum dosage of inoculum and fermentation length, thereby increasing the content and quality of PKC with *L. fermentum.*

MATERIALS AND METHODS

Ethical Approval

The present study was approved by the Animal Ethics Committee of Andalas University, West Sumatera, Indonesia.

Place and Time

This research was conducted from July-October 2022 in Laboratory of Non-Ruminants, Animal Nutrition and Feed Technology Department, Animal Science Faculty, University of Andalas.

Research Materials

The research materials were palm kernel cake (PKC) obtained from Payakumbuh. Rice bran, *Lactobacillus fermentum* inoculum obtained from Indonesia Institute of Science (LIPI), NA media (Nutrient Agar), distilled water, alcohol, Brook et al solution, casein, buffer solution. phosphate pH 7, NaOH, H_2SO_4 and chemicals for proximate analysis, 30 broiler chickens with and average weight of 1.5kg.

This research used an incubator, autoclave, plastic, beaker, measuring cup, test tube, test tube rack, hot plate, micropipette, tip, spectrophotometer, Eppendorf, centrifuge, erlenmeyer, analytical balance, polypropylene plastic bag (15x25cm), oven, filter paper, aluminum foil, grinder, tissue, and metabolic cage equipped with a drinker.

Research Design

The experimental research in the laboratory using a Completely Randomized Design (CRD) with Factorial Design 3 X 3 with 3 replications. The first factor (factor A) was inoculum doses, consisting of: A1=5%, A2=7.5%, A3=10%. Factor B was fermentation time consisting of B1=2 days, B2=4 days, B3=6 days. The observed variables were activities of cellulase, manannase, protease, crude of protein, crude of fiber, nitrogen retention, crude fat, digestibility of crude fiber and metabolizable energy.

Research Procedure

Bacterial Rejuvenation

Bacterial rejuvenation was carried out by growing *Lactobacillus Fermentum* on agar media slanting by mixing 20g of Nutrien Agar (NA)/100mL of distilled water. Then NA and distilled water were cooked until the color changes to yellowish (clear). The cooked NA was then put into test tubes, covered with cotton and aluminum foil and put into the autoclave for 15min at 121°C. The test tubes were then tilted and cooled to a solid state. Then the oblique media was inoculated for 24 hours. After that, it was scratched with *L. fermentum*, then put into an incubator and incubated for 2 days.

Making Inoculums

Inoculum of *L. fermentum* were prepared using 100g of rice bran as a substrate and then added 70mL of distilled water, and it was then being sterilized (15min at 121°C at 1 atm pressure). Then the bacteria were diluted in a test tube with Brook et al's solution and inoculated on the rice bran substrate and then incubated for 3 days. After incubation, the inoculum was ready for use in fresh form.

Substrate Preparation

The substrate consists of PKC and rice bran with ratio of 80% PKC + 20% rice bran. Each substrate component was weighed and put in a 15 x 25 cm polypropylene plastic bag, then 70mL of distilled water was added, then autoclave sterilized at 121ºC for 15min with 1 atm

pressure, then removed and cooled to room temperature, then fermented.

Fermentation Implementation

The sterilized substrate was inoculated with *L. fermentum* inoculum with the inoculum dose according to the treatment, namely A1 5%, A2 7.5% and A3 10%, after which it was reaped and then dried in an oven at 50–60ºC until the weight constant, then the fermented product was milled, and it was ready to be analyzed for its nutritional content.

Enzyme Crude Extract

Enzyme activity determination begins with carrying out the crude extract of the enzyme. Weighed 10g of sample for each treatment then put it into an Erlenmeyer tube, added 90mL of 0.05M buffer solution pH 7 of phosphate (crude extract of protease enzymes) then covered with aluminum foil, then placed in a shaker for 2 hours. Then removed from the shaker, filtered and centrifuged for 15min at 5000rpm at 40ºC, and took the supernatant and measured the enzyme activity.

Parameters Measurement

Measurement of cellulase activity using Nelson method (1944), mannanase activity (Bintang 2010), protease Activity (Cupp-Enyard 2008). Proximate analysis used for analysis of the crude protein (CP), crude of fiber (CF) and crude lipid (CL) and nitrogen retention (NR), crude fiber digestibility (CFD), metabolizable energy (ME) using Sibbald method (Sibbald 1975).

Data Analysis

Data obtained were analyzed by variance analysis (Anova), while differences between treatments were tested by Duncan's Multiple of Range Test (DMRT) according to Steel and Torrie (2002).

RESULTS AND DISCUSSION

Average activity of cellulase, mannanase, protease, CP, CF, CL, NR, CFD and ME of fermented palm kernel cake (FPKC) with *L. fermentum* were presented in Table 1.

Cellulase Activity

Variance analysis result showed that there was a significant interaction (P<0.01) between factor A (inoculum dose) and factor B (length fermentation), each of factor A and factor B also revealed a highly significant (P<0.01) different effect on FPKC cellulase activity. From the data Table 1 showed that the more doses given, the higher the cellulase activity in the 2, 4 and 6 day treatments. Meanwhile, during the fermentation time, there was an increase in cellulase activity on day 4, however, on day 6 cellulase activity was decreased. The increase in cellulase activity could have been caused by the bacteria growing and developing properly. The higher the microbial growth, the more enzymes are produced, especially the cellulase enzymes so that there is an increase in cellulase activity. This is in accordance with the results of Mirnawati et al. (2019a) that the high number of microbes is proportional to the enzyme production produced. Meanwhile, the decrease in cellulase activity could be due to the too long

Table 1: Various enzymatic activities and metabolizable energy of fermented palm kernel cake treated with *Lactobacillus fermentum* with various fermentation length

Parameters	Factor A (Inoculum Doses)	Factor B (Length of Fermentation) B1 (2 days)	B2 (4 days)	B3 (6 days)
Celulase activity (%)	A1	7.32^{bC}	9.29^{aC}	8.88^{aC}
	A2	11.42^{bB}	13.30^{aB}	12.15^{aB}
	A3	14.16^{bA}	18.01^{aA}	15.01^{bA}
Manannase activity (%)	A1	9.29^{bC}	12.05^{aC}	10.97^{aC}
	A2	15.53^{bB}	18.46^{aB}	17.13^{aB}
	A3	20.03^{cA}	24.95^{aA}	21.60^{bA}
Protease activity (%)	A1	6.15^{bC}	7.30^{aC}	7.19^{aC}
	A2	7.59^{bB}	8.51^{aB}	7.80^{bB}
	A3	8.88^{cA}	10.55^{aA}	9.48^{bA}
Crude Protein (%)	A1	17.03^{bC}	18.55^{aC}	17.97^{aC}
	A2	18.55^{cB}	21.23^{aB}	20.18^{bB}
	A3	21.70^{cA}	26.31^{aA}	23.92^{bA}
Crude Fiber (%)	A1	23.40^{aA}	21.13^{bA}	20.83^{bA}
	A2	20.22^{aB}	18.04^{bB}	18.27^{Bb}
	A3	22.23^{aA}	15.71^{cC}	20.04^{Ba}
Crude Fat (%)	A1	5.95^{aA}	3.61^{cB}	4.21^{bA}
	A2	4.52^{aB}	4.27^{aA}	3.52^{bB}
	A3	2.63^{aC}	1.45^{bC}	2.17^{aC}
Nitrogen Retention (%)	A1	48.34^{bC}	51.23^{aC}	50.59^{aC}
	A2	51.33^{bB}	54.42^{aB}	51.93^{bB}
	A3	60.31^{cA}	63.92^{aA}	61.33^{bA}
Crude Fiber Digestibility (%)	A1	44.55^{aB}	48.69^{aB}	47.71^{Ab}
	A2	45.99^{bA}	49.59^{aB}	46.02^{Bc}
	A3	47.47^{cA}	55.91^{aA}	50.77^{Ba}
Metabolizable Energy (kcal/kg)	A1	1499.36^{bB}	1900.60^{aB}	1765.92^{aB}
	A2	1555.37^{bB}	1915.95^{aB}	1608.07^{bB}
	A3	1915.18^{cA}	2752.69^{aA}	2371.40^{bA}

Different lowercase (same row) and uppercase (same column) letters in a respective parameter indicate a very significant (P<0.01) different effect on parameter measurements.

fermentation time so that the available nutrients in the substrate were reduced, thus, microbial growth decreased and eventually died so that cellulase activity also decreased, the above findings were in accord with opinion of Agustina et al. (2015) that the fermentation time that exceeds the optimum limit causes the limited availability of nutr ients for microbial growth so that the microbes will die.

High cellulase activity was produced in the A3B2 treatment with 10% inoculum dose and 4 days of fermentation, namely 18.01U/mL. This result was slightly higher than research result from Mirnawati et al. (2019b) that got cellulase activity in PKC fermentation with *B. subtilis* of 17.35U/mL.

Mannanase Activity

Analysis of variance using DMRT revealed that there was a very significant (P<0.01) interaction between factor A (inoculum dose) and factor B (fermentation time), each of factor A and factor B also disclose a highly significant (P<0.01) different effect on manannase activity.

The data in Table 1 showed that the more doses given, the higher the cellulase activity in the 2-, 4- and 6-day treatments. Meanwhile, during the fermentation time, there was an increase in cellulase activity on the 4th day, then on the 6th day there was a decrease. The increased cellulase activity is caused by the fact that bacteria can grow and develop properly. This results are in accord with Mirnawati et al. (2022a) which states that high doses of inoculum will lead to the high microbial growth and the more enzymes will be established, which will ultimately increase their

activity, especially the manannase enzyme. Meanwhile, at 6 days of fermentation, there was a decrease in manannase activity because the fermentation time had exceeded the optimum limit of 4 days. According to the opinion of Irwansyah et al. (2019), the length of fermentation that exceeds the optimum limits causes the limited availability of nutrients for microbial growth so that the microbes will die. High manannase activity was produced in the A3B2 treatment, namely 24.95U/mL and it was higher than results obtained by Mirnawati et al (2019a) where fermented PKC with *B. subtilis* produced 24.27U/mL manannase activity.

Protease Activity

Analysis of variance in Table 1 revealed that there was a highly significant (P<0.01) interaction between factor A (inoculum dose) and factor B (length fermentation), each of factor A and factor B also revealed a highly significant different (P<0.01) on PKC protease activity. Based on Table 1, it is known that the more doses given, the protease activity increased at doses of 5, 7.5 and 10%. Likewise, with the addition of fermentation time, there was an increase in protease activity and on day 4 is the optimal time and if the fermentation time is increased to 6 days then there was a decrease in enzyme activity.

The high protease activity in the A3B2 treatment (PKC fermentation with 10% inoculum dose with 4 days of fermentation) was caused by increased microbial growth as seen from the total number of colonies. The more the number of microbes that grow, the more protease enzymes will be produced. Mirnawati et al. (2019a) stated that the correct dose of inoculum would provide opportunities for microbes to grow and develop quickly. The more doses used, the more ingredients will be broken down, so that the compound of inoculum dose and duration of fermentation will enhance the nutritional value of the product (Mirnawati et al 2019b; Ciptaan et al. 2022a).

The increase in protease activity in the A3B2 treatment was also influenced by the fermentation time. This is in accord with Mirnawati et al. (2022b) who stated that duration of fermentation is one of the determining factors in fermentation, where the longer of the length of fermentation will cause more and more substrates to be broken down by microbial enzymes so that the enzyme activity will increase. However, the fermentation time that is too long causes the nutrients in the substrate to decrease so that the microbial population will decrease. This is in assent by the opinion of Irwansyah et al. (2019) who stated that the longer the fermentation time will cause a decrease in the growth rate of bacteria because the amount of nutrients in the media begins to decrease. The results of this study Table 1 about protease activity, are better than those of Seftiadi et al. (2021) who obtained a protease activity of 10.34%.

Crude Protein (CP)

Analysis of variance result in Table 1 above revealed that a high significant (P<0.01) interaction between factor A (inoculum dose) and factor B (fermentation time) was found, each of factor A and factor B also revealed a high significant (P<0.01) different effect to crude protein content. From Table 1, it can be seen that the enhance of CP in the A3B2 (10% dose of inoculum and 4 days of length fermentation) was significantly (P<0.01) different higher than the others. The high increase of CP in the A3B2 treatment was due to the large number of inoculum doses given so that the growth of bacteria was better and more even and the bacteria contributed a fairly high protein compared to other treatments. This causes the crude protein content of products of fermentation to increase. Another result obtained is that the more inoculum doses obtained, the more material is broken down, so that the combination of dose of inoculum and fermented substrate will enhance the nutritional value of the product (Mirnawati et al. 2019b). The rise of CP in the A3B2 was also a result by the high enzyme activity where the higher the enzyme activity will lead to the higher production of enzyme, while the enzyme itself is a protein. In accordance with the results of Mirnawati et al. (2012) that the enzymes produced by microbes are proteins and the microbes themselves are single cell proteins resulting in an enhance in CP at the end of the fermentation.

The lower percentage increase of CP in the A3B3 compared to the A3B2 was caused by the longer fermentation time, in accord with the opinion of Irwansyah et al. (2019) that if bacterial growth has extended the stationary phase, the growth rate will exsiccate, the result is reduced nutrient supplies and accumulation of substances metabolic that inhibit of growth and the rate of growth will continue to decrease until the value is equal to zero (the number of cells that grow equals dead cells).

From the data in Table 1, it can also be seen that the accrue in CP of PKC in the A3B2 (10% inoculum dose and 4 days of fermentation) showed the best accrue in CP, namely 26.31%. This result is lower than result obtain by Mirnawati et al. (2022b) who claimed the protein content obtained using *B. subtilis* was 28.78%.

Crude Fiber (CF)

Analysis of variance results (Table 1) showed that a high significant (P<0.01) interaction between factor A (dose of inoculum) and factor B (length fermentation) was found, respectively. Factor A and factor B also showed a highly significant different effect (P<0.01) to CF. From the data in Table 1 revealed that there was an alleviate in CF with a dose of 10% inoculum with a 4 days of fermentation and an increase in crude fiber content on day 6. The decrease in crude fiber at a doses of 10% inoculum was due to the large number of bacteria growing. Increasing the number of bacterial growth will certainly increase the activity of cellulase enzymes in breaking down cellulose from PKC into glucose and the end of the fermentation there is an exsiccate in CF. In consent with a study of Sudarmono et al. (2016) who stated that the more microbial growth, the more cellulase enzymes to decay cellulose into simple sugars which causes the crude content of fiber to decrease at the quell of fermentation.

Crude Lipid (CL)

Based on analysis of variance data in Table 1 above, a high significant (P<0.01) interaction between factor A (inoculum dose) and factor B (length fermentation) was found, each of factor A and factor B also showed a high significant different effect (P <0.01) to crude fat content.

Based on data (Table 1), it revealed that A2B3 and A3B3 treatment (10% inoculum dose with a 4-day fermentation period) result in the alleviate of CL and then

an enhance in CF, namely in the 6-day fermentation, which was 2.17%. The alleviate in CL was due to the large number of bacteria that grow during fermentation which can be seen in the number of colonies that grow. The more bacteria that grow, the more lipase enzymes are produced to decay fats into fatty acids and glycerol. Furthermore, the fatty acids produced will be applied by microbes as an source of energy for growth, resulting in an alleviate in CL at the end of fermentation. In accordance on the opinion of Cuevas-Rodriguez et al. (2004) that the alleviate of CL during fermentation is due to oxidation and the use of fatty acids by bacteria as an energy source.

The increase in crude fat in the B3 treatment was due to the too long fermentation time where the longer the length fermentation is given, the availability of nutrients of substrate will decrease which causes microbial growth to decrease, so that at the end of the fermentation there is an enhance in crude fat. The result of research is suitable with the opinion of Agustina et al. (2015) that the availability of nutrients and nutrients in the fermentation media will decrease with increasing fermentation time which can cause bacteria to die thereby increasing the fat content.

Nitrogen Retention

Results of analysis of variance (Table 1) showed that a high significant (P<0.01) interaction between factor A (dose of inoculum) and factor B (fermentation time) was found, each of factor A and factor B also showed a highly significant (P<0.01) effect to nitrogen retention. The high NR in treatment of the A3B2 and A3B3 was caused by the fermentation process using *L. fermentum* to produce several enzymes which resulted in better quality of protein of PKC. In addition, *L fermentum* bacteria produce protease enzymes and also function to break down proteins into amino acids. The higher the enzymes activity, the greater the work of enzymes breaking down proteins into amino acids so that the quality of the product increases. In according to the opinion of Mahfud et al. (2004) that producing of enzymes by bacteria can change the protein structure o into amino acids so as to enhance nitrogen retention. This enhance in NR is directly proportional to protein quality (Ciptaan et al 2022b).

The low nitrogen retention in the A1B1 and A1B3 treatments was due to the low protein quality in these treatments. In accordance with Corzo et al. (2005) stated that the factors that determine the size of NR are ration consumption, especially protein consumption, protein digestibility, the balance of nitrogen consumption and metabolic energy contained in the ration. The lower nitrogen retention in the A1B2 and A3B3 treatments compared to other treatments, because in these treatments the protein digestibility was not maximized, so that the process of decomposing nutrients also did not occur much and there was no balance of nitrogen consumption. From the data above it revealed that the A3B2 gave a high increase in nitrogen retention of 63.92%. The results of the research are lower than the results obtained by Mirnawati et al. (2019b) where PKC fermentation with Bacillus subtilis resulted in nitrogen retention of 68.47%.

Crude Fiber Digestibility (CFD)

Analysis of variance results (Table 1) showed that a high significant (P<0.01) interaction between factor A (dose of inoculum dose) and factor B (fermentation) was found, respectively, factor A and factor B also showed a high significant different effect (P<0.01) on CFD. It can be concluded that there was an enhancement in CFD with an inoculum dose of 10% and 4 days of fermentation. The high CFD in the A3B2 treatment was associated to the slight CF in the A3B2. The low of CF in feed will certainly enhance CFD, which is in associated with Mirnawati et al. (2019b) and Mirnawati et al. (2022b) who told that CFD depends on the CF content in feed, the higher CF of the feed, the lower CFD of the feed ingredients. High CFD was found in the A3B2 with 10% dose inoculum and 4 days of fermentation, namely 55.91%. This result are slightly higher than the results by Mirnawati et al. (2019b), namely fermentation using *Lactobacillus sp*, namely 53.25%.

Conclusion

It can be concluded that there is a very significant interaction between the dosage of inoculum and the duration of palm kernel cake fermentation using *Lactobacillus fermentum*. 10% inoculum dose and 4 days of fermentation time were the best results which were able to increase cellulase activity (18.01U/mL), manannase activity (24.95U/mL), protease (10.55U/mL), increase protein content crude fiber (26.31%), nitrogen retention (63.92%), digestibility of crude fiber (55.91%) and metabolic energy (2752.69 Kcal/Kg) and reduce the content of crude fiber (17.71%) and fat crude 1.45% fermented palm kernel cake.

Acknowledgment

The authors would like to thank the Institution for Research and Community Service Andalas University which has provided funds for this research through the Hibah Klaster Riset Publikasi Guru Besar with contract No. T/12/UN.16.17/PP.Pangan-PTU-KRP1GB-Unand/2022, date April 11, 2022.

Author's Contribution

Mirnawati was in charge to supervise the experiment and writing the original script. Gita Ciptaan, Imana Martaguri and Ferawati established the experiment and analyzed the data. Anifah Srifani finalize the manuscript.

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