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The combination of *Bacillus subtilis* with *Lactobacillus fermentum* in Improving the Quality and Nutrient Contents of Fermented Palm Kernel Meal (FPKM)

Pratiwi Chintya Devi[1], Mirnawati*[2] and Yetti Marlida[2]

[1]Postgraduate Study Program Student, Faculty of Animal Science, Andalas University.
[2]Department of Nutrition and Feed Technology, Faculty of Animal Science, Andalas University, Padang 2022, Indonesia.
***Corresponding author:** mirnawati@ansci.unand.ac.id

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ABSTRACT

The purpose of the study was to get the best-mixed ratio of *Bacillus subtilis* and *Lactobacillus fermentum* bacteria in fermented palm kernel meal (FPKM) on the activity of cellulase, mannanase, and protease enzymes as well as the content of crude protein and crude fiber. A combination of two bacteria, *Bacillus subtilis*, and *Lactobacillus fermentum*, were used to make palm kernel meal fermentation products. This study used a completely randomized design (CRD) with five treatments and 4 replications. The treatment consisted of the ratio of *Bacillus subtilis* and *Lactobacillus fermentum*: A=(5: 5), B=(4: 6), C=(6: 4), D=(8: 2), E=(2: 8). The parameters in this study were cellulase activity, mannanase activity, and protease activity, crude protein, and crude fiber content. The analysis of diversity showed that treatments A, B, C, D, and E had a significant ($P<0.05$) effect on increasing cellulase, mannanase, protease activity, and crude protein and decreasing crude fiber. Treatment with a ratio of *Bacillus subtilis* and *Lactobacillus fermentum* (6: 4) gave optimal results. This can be seen from the average results obtained successively activity with cellulase (23.26U/mL), mannanase (26.97U/mL), protease (10.95U/mL), crude protein (26.91%) and crude fiber (17.04%). This study concluded that the ratio (6:4) of *Bacillus subtilis* and *Lactobacillus fermentum* gave the best results.

Key words: *Bacillus Subtilis*, *Lactobacillus fermentum*, Enzyme Activity, Crude Protein, Crude Fiber

INTRODUCTION

Palm oil is a vegetable oil that has a crucial role in the Indonesian economy, which could be due to its ability to produce vegetable oils required by various industries. Indonesia is number one in the world in palm oil production. In 2018, the area of oil palm plantations reached 14,326,350 hectares, and crude palm oil production (CPO) increased by 11.8 million tons in the last four years (Ditjenbun 2019). The processing of fresh fruit bunches (FFB) produces a by-product of palm kernel meal (PKM). This by-product accounts for 3.5% of each FFB, or 45-46% of palm oil production, resulting in an estimated PKM production of 12 million tons/year.

PKM is a feed ingredient with high nutritional values, such as crude protein, crude fiber, calcium, phosphorus and copper (Mirnawati et al. 2018). Even though the nutritional value of PKM is quite high, it was used 10% in broiler ration (Sinurat 2013). Palm kernel meal has high crude fiber in the form of β-mannan, which was 57.8% (Azman et al. 2016). Meanwhile, poultry does not have fiber-breaking enzymes in their digestive organs. The high mannan content in PKM can be degraded by processing it fermentatively using bacteria that are cellulolytic and mananolytic (Meryandini 2008; Mirnawati et al. 2022b).

Fermentation is a process of decomposing complex components such as carbohydrates, proteins, and fats into simpler ones with the help of enzymes produced by microbes, resulting in products that are more easily digested and utilized by livestock (Mirnawati et al. 2022a; Ciptaan et al. 2022a). In accordance with Ciptaan et al. (2022b) opinion that the result of fermented products is easier to digest, it improves the nutritional value of a feed ingredient and can affect poultry production.

Mirnawati et al. (2019a) studied PKM fermentation with cellulolytic and mananolytic bacteria (*Bacillus subtilis*), which resulted in an increase in nutritional quality such as crude protein (24.65%), crude fiber (17.35%), nitrogen retention (68.47%) and digestibility of crude fiber (53%). Furthermore, it was also added by (Mirnawati et al. (2019b) that PKM fermentation with *Bacillus subtilis* had enzyme activity of mannanese

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(24.27U/mL) and cellulase (17.13U/mL). It has even been tested biologically on broilers and can be used for up to 25% (Mirnawati et al. 2020).

Seftiadi et al. (2020) isolated cellulolytic and mananolytic lactic acid bacteria from rotting PKM and obtained *Lactobacillus fermentum*, which has activity of enzymes cellulase (18.84U/mL), manganese (24.86U/mL) and protease (10.45U/mL). *Lactobacillus fermentum* is lactic acid, gram-positive, not spore-formed, facultatively anaerobic, forming (bacilli), and non-pathogenic; it also retains microbes in the gut to increase digestibility and improves broiler performance (Karlyshev et al. 2015).

Bacillus subtilis and *Lactobacillus fermentum* can cooperate in degrading substrates in the fermentation process. This was in accordance with the opinion of Velly et al. (2022), who stated that *Bacillus subtilis* consortium with *Lactobacillus* could mutually utilize coenzymes or exoenzymes expressed by other microbes can decompose substrates that a microbe has previously degraded. For this reason, in this study, a combination of the two microbes, *Bacillus subtilis*, and *Lactobacillus fermentum*, was carried out to obtain the best ratio to process FPKM.

For this reason, there is a need to conduct a study entitled "The combination of *Bacillus subtilis* with *Lactobacillus fermentum* in improving the quality and nutrient content of Fermented Palm Kernel Meal.

MATERIALS AND METHODS

This study used materials such as 1) palm kernel meal from Incasi Raya Padang Company, West Sumatra, 2) *Bacillus subtilis* and *Lactobacillus fermentum* resulting from rejuvenation, 3) Agar Nutrient Media from Sigma Aldrich, 4) Humic Acid 5) Bran, 6) Aquades and standard minerals, 7) Buffer solutions and chemicals for cellulase, mannanase and protease activity analysis, 8) Chemicals for determination of crude protein and crude fiber content 9) Wattman # 1 coarse fiber filter paper. The tools used were autoclaves, ovens, analytical balances, spectrophotometers, centrifuges, incubators, water bath shakers, Erlenmeyer, Eppendorf, Kjeldahl flasks, beakers, porcelain dishes, Petri dishes, desiccators, laminar air flow, test tubes, and incubators.

Research design

The design of this study was a completely randomized design with 5 treatments and 4 replications. The treatment was the ratio between *B. subtilis* and *Lactobacillus fermentum* used, A= (5: 5), B=(4: 6), C=(6: 4), D=(8: 2), E=(2: 8). Parameters measured were cellulose activity, mannanase activity, and protease activity, crude protein (CP) and crude fiber (CF).

Manufacture of Fermented Products

Fermented products were produced in A, B, C, D, and E treatments. Each treatment was added with 70mL of distilled water and sterilized with an autoclave for 45min at 121°C; the sample then cooled at room temperature. Then, dilute the bacteria in the test tube with a mineral solution, inoculate each treatment, and incubate for 3 days.

Enzyme Extract

Ten grams of sample for each treatment were weighed into an Erlenmeyer tube, and about 90mL of 0.05M acetate buffer (crude extract of cellulase and mannanase enzymes) were added to the sample. The filtrate was collected and centrifuged for 15min at 5000rpm at 4°C; the supernatant was taken to measure enzyme activity.

Measurement of Enzyme Activity

Cellulase and mannanase were measured following the Samogy-Nelson method (1994). Briefly, as much as 1mL of mannan substrate was prepared by mixing (0.5g/mL of mannan into 10mL phosphate buffer). After that, the solution was added with 1mL of crude enzyme extract. Then incubate for 30min with a water bath shaker at 40°C for cellulase and 60°C for mannanase. When finished, 1mL of the enzyme was extracted and added to 1mL Nelson AB solution. Then the enzyme mixture was heated for 20min and cooled at room temperature. After that, 1mL of phosphomolybdate solution was added to 7mL of distilled water. Enzyme activity was measured with a Uv-Vis spectrophotometer (575nm).

Protease was measured following the method described by Enggel et al. (2004). Briefly, as much as 0.25mL of enzyme solution was added to 0.25mL phosphate buffer pH 7 and pre-incubated at 37°C for 5min. After pre-incubation, 0.25mL of the substrate (2% casein in phosphate buffer pH 7 was added, and the mixture was incubated at 37°C for 10min. The reaction was stopped by adding 0.5mL of 0.4M trichloroacetic acid (TCA), which was then centrifuged to collect the supernatant. To 0.2mL of the supernatant was added 1mL of 0.5M sodium carbonate, pre-incubated for 10min, then added 0.2mL of Folin-Ciocalteau reagent and incubated for 30min. Take a UV-Vis spectrophotometer reading at 660nm. Each test's sample results were reduced by one blind test with spaces.

Measurement of Crude Protein and Crude Fiber with the AOAC method (1999)

The value of CP was calculated using the Kjeldahl method, which consisted of the stages of destruction, distillation, and titration. One gram of sample was weighed and then put into a Kjehdal flask, and it was then added with 1g of catalyst (selenium), 25mL of concentrated H_2SO_4, then cooled at room temperature. The solution that had been digested was added with 500mL of distilled water. Ten mL of filtrate was added into the distillation flask, then about 25mL of 0.3N NaOH, 75mL of distilled water, and boiling stones were added into it; the distillation was carried out until it showed an explosion. The distillate was collected in 25mL of 0.3N H_2SO_4 which had been given 3 drops of red indicator. After an explosion occurs (the distillation process is complete), titrate the distillate with 0.1N until the color changes. We also used blank titration.

Crude fiber content was decided using the Weende analytical method. One gram increment of the fermented product was added to 300mL of distilled water. Then 50mL of 0.3N H2SO4 was added and boiled for 30min; after that 25mL of 1.5N NaOH was added and heated for 30min. It was then filtered with Watman no. 1 coarse fiber filter paper (A grams). During filtration, the precipitate was washed successively with 50mL hot distilled water, 50mL 0.3N H2SO4, and 2mL acetone.

The filter paper and its contents were placed in a porcelain beaker and dried in an oven at 105-110°C for 1hr. It was then cooled in a desiccator and weighed (B grams). After that, it was baked in an oven (600°C) until it was ashes. It was then cooled in a desiccator and weighed (grams C).

Data analysis

The data obtained in this study were processed with an analysis of diversity according to Steel and Torie (1991). Differences between treatments were tested with the Duncan Multiple Range Test.

RESULTS AND DISCUSSION

Effect of Treatment on Cellulase Activity

Based on data in Table 1 showed that the effect of the combination of *Bacillus subtilis* and *Lactobacillus fermentum* bacteria in palm kernel meal (PKM) fermentation was significant (P<0.05) on cellulose activity. Treatment C had the highest cellulase activity (23.26U/mL) with a joint ratio of *Bacillus subtilis* and *Lactobacillus fermentum*, which was (6: 4). The improvement of enzyme activity was also due to stronger bacterial numbers as shown by colony counts as high as $9.5x10^{10}$ CFU/g. The higher the growth of bacteria, the higher the cellulase activity produced. This agreed with the findings of Mirnawati et al. (2019). The more microorganisms grow, the higher the cellulase enzymatic activity is produced. The growth of bacteria in the fermentation media is influenced by the nutrients present in the substrate and those given to the substrate.

Furthermore, the increased cellulase activity was also due to the combination of 2 bacteria with a ratio of (6: 4) which turned out to be a consortium. They did not compete, but the bacteria interacted, synergized, and shared the same source of nutrients. This agreed with the opinion of Velly et al. (2022) that the *Bacillus subtilis* consortium with *Lactobacillus* could mutually utilize coenzymes or exoenzymes secreted by other microbes, besides that other microbes can decompose substrates that a microbe has previously degraded.

These results differed from those reported by Mirnawati et al. (2019) and Seftiadi et al. (2020). PKM fermentation with *Bacillus subtilis* and *Lactobacillus fermentum* gave cellulase activity of 17.13U/mL and 18.84U/mL. Combining the combination of the 2 bacteria can increase cellulase activity based on these data. This agreed with the findings of Ul-Haq et al. (2005), saying that a mixed culture of 2 types of microbes can increase cellulase enzyme activity compared to monoculture.

Low cellulase activity was found in treatment E (16.07U/mL) with a ratio of bacteria (2: 8). Low cellulase activity is suspected because the respective bacteria do not synergize with each other in this combination ratio. So that the number of growing microbial populations is also low, as seen from the total colonies produced, namely $3.2x10^{-10}$ CFU/g. The low growth of microbes is due to the different abilities of each microbe to divide. This was by the opinion of Syachroni (2019) that bacteria have different adaptation times and abilities in dividing. Under conditions of the combination of bacteria, competitiveness usually occurs in the growth process. This could be due to the different best growth times for *Bacillus subtilis* and *Lactobacillus fermentum*. Puspitasari et al. (2017) said that the optimum time for the growth of *Lactobacillus fermentum* occurred after 12, while *Bacillus subtilis* occurred after 48h (Zhao et al. 2019). It is suspected that at the time of harvest, the fermented product *Lactobacillus fermentum* has already experienced a death phase, so the ability to degrade cellulose decreases which causes cellulase activity to be low.

Effect of Treatment on Mannanase Activities

Based on data in Table 1 showed that the effect of the combination of *Bacillus subtilis* and *Lactobacillus fermentum* bacteria in palm kernel meal fermentation was significant (P<0.05) on mannanase activity. Treatment C had the highest enzyme activity (26.97U/mL) with a joint ratio of *Bacillus subtilis* and *Lactobacillus fermentum* (6: 4). The improvement of enzyme activity was caused by a number of bacteria that grew more, as seen from the high number of colonies, namely $9.5x10^{10}$ CFU/g. The more bacterial colonies that grow, the higher the enzyme activity produced. This result was in line with Krishaditersanto et al. (2017) that bacterial growth with enzyme production has a positive relationship where high bacterial growth will increase the enzymes produced.

The results in Table 1 showed that the two bacteria in treatment C could have worked synergistically to increase mannanase activity. It was suspected that the combination of *Bacillus subtilis* and *Lactobacillus fermentum* in the ratio (6: 4) is the best combination for bacterial growth so that they work together in breaking down mannan into mannose. Bacteria that work together will form a consortium to produce the best product. This result was In line with the opinion of Asri et al. (2016) that a consortium would produce products that can be used together to support the growth of single and other isolates. The result obtained in this study is higher than Mirnawati et al. (2019), who got that PKM fermentation with *Bacillus subtilis* gave a mannanase enzyme activity of 24.27U/mL. The result in this study was also higher than Seftiadi et al. (2020), who fermented PKM with *Lactobacillus fermentum* and got 24.86U/mL of mannanase enzyme activity.

Table 1: The average PKMF enzyme activity, crude protein, and crude fiber with *Bacillus subtilis* and *Lactobacillus fermentum*

Treatments	Cellulase Activity (U/mL)	Manganese Activity (U/mL)	Protease Activity (U/mL)	Colony Total (CFU/g)
A (5: 5)	19.46±2.78b	20.43±1.49b	8.86±1.39a	$7.7x10^{10}$
B (4: 6)	17.89±2.36b	17.21±2.79b	7.99±1.29ab	$5.2x10^{10}$
C (6: 4)	23.26±1.62a	26.97±2.39a	10.95±1.44a	$9.5x10^{10}$
D (8: 2)	17.12±1.38b	24.27±1.77a	7.51±1.21ab	$4.8x10^{10}$
E (2: 8)	16.07±1.53b	15.87±2.05bc	7.31±2.22ab	$3.5x10^{10}$

Values (Mean±SD) bearing different alphabets in a column differ significantly (P<0.05).

Low mannanase activity in treatment E (15.87U/mL), presumably because the ratio of bacteria used cannot synergize well, so the number of bacterial populations produced is also low. The low number of bacterial populations results in the low ability to degrade mannan in palm kernel meal. This was supported by the findings of Kassim et al. (1985), who said that microbial growth and enzyme production had a positive correlation. In addition, the low activity of enzymes is also due to the optimum growth time for each bacterium. The optimum time for the growth of *Lactobacillus fermentum* (Noviardi et al. 2020) and *Bacillus subtilis* (Zhao et al. 2019) has been reported after 12 and 48h, respectively.

Effect of Treatment on Protease Activity

Based on data in Table 1 showed that the effect of the combination of *Bacillus subtilis* and *Lactobacillus fermentum* bacteria in palm kernel meal fermentation on protease activity was significant (P<0.05). Treatment C had the highest enzyme activity (10.97U/mL) with a combined ratio of *Bacillus subtilis* and *Lactobacillus fermentum* (6: 4). This could happen because the bacteria are consortium so that they can cause more microbial growth on the substrate. The activity of the resulting protease enzymes also increases. Increased enzyme activity can also be seen from the high number of colonies, namely $9.5x10^{10}$ CFU/g. This improvement is due to the availability of large amounts of nutrients needed by bacteria to carry out cell metabolism. The protein content in PKM is quite high, about 17.31% (Mirnawati et al. 2018), which can be used as a metabolic process.

The increased protease activity was also due to the combination of 2 bacteria with a ratio of 6: 4 which turned out to be a consortium and did not compete with each other. However, these bacteria interact and synergize and share the same source of nutrients. Velly et al. (2021) stated that the *Bacillus subtilis* consortium with *Lactobacillus* could mutually utilize coenzymes or exoenzymes secreted by other microbes, besides that other microbes can decompose substrates that a microbe has previously degraded. This result was better than Mirnawati et al. (2019), who found that PKM fermentation with *Bacillus subtilis* gave a protease enzyme activity of 10.27U/mL. The result in this study was also higher than Seftiadi et al. (2020), who fermented PKM with *Lactobacillus fermentum* and got 10.45U/mL of protease enzyme activity.

Low protease activity in treatment E (7.31U/mL) due to the use of a combination of bacteria. The combination of bacteria with an incorrect ratio causes the two bacteria to be unable to grow in utilizing food nutrients properly. Each bacterium has a different optimum time to grow and divide. According to Effendi (2017) best protease activity in *Bacillus* subtilis was achieved at 46hrs. Meanwhile, Noviardi et al. (2020) stated that the optimum time for the growth of *Lactobacillus fermentum* occurs after 12hr. This causes the two bacteria to not synergize with each other in hydrolyzing proteins into simpler compounds such as peptide bonds and amino acids.

The data in Fig. 1 showed that the effect of the combination of *Bacillus subtilis* and *Lactobacillus fermentum* bacteria in PKM fermentation on crude protein content was significant (P<0.05). The increase in CP was due to the high growth of bacteria. The higher bacterial growth produces the more the cell mass of bacteria, increasing protein content. This result was in line with the stated results of Krisnan et al. (2005) that the increase of protein was contributed by microbial cells that recognize as single cell protein (SCP) or cell biomass containing about 40-65% protein, and it increased protein content.

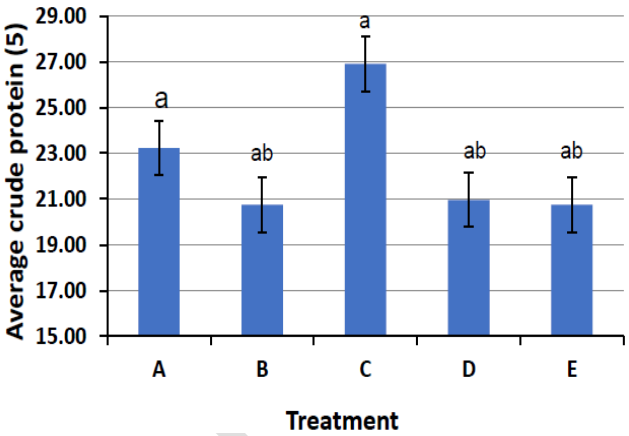

Fig. 1: The average PKMF crude protein with *Bacillus subtilis* and *Lactobacillus fermentum*. Bars (Mean±SD) bearing different alphabets differ significantly (P<0.05).

The increase in CP in treatment C was also caused by high enzyme activity that resulted in high enzyme production, while the enzyme itself is a protein. Mirnawati et al. (2012) stated that microbes in fermentation would produce enzymes that are a kind of protein and microbes are contributors to single-cell protein resulting in an increase in crude protein at the end of fermentation. The results obtained in this study were higher than Mirnawati et al. (2019), who mentioned that the crude protein content in PKM after being fermented with *Bacillus subtilis* was 24.65. The result in this study was also higher than Seftiadi et al. (2020), who fermented PKM with *Lactobacillus fermentum* and got 25.81% of crude protein.

The low crude protein content in treatment D (20.75%) was due to the low activity of the protease enzyme. Low enzyme activity will reduce the performance of bacteria in hydrolyzing proteins into peptide bonds and amino acids so that the resulting crude protein content is also small.

Data in Fig. 2 showed that the effect of the combination of *Bacillus subtilis* and *Lactobacillus fermentum* bacteria in palm kernel meal fermentation on crude fiber content was significant (P<0.05). The growing number of bacteria caused the decrease in crude fiber. The increase in bacterial growth will certainly increase cellulase enzyme activity in altering cellulose into glucose, resulting in decreased CF. This result was consistent with Sudarmono et al. (2016), who explained that high microbial growth would result in high cellulose enzymes produced to alter cellulose into simple sugars, resulting in decreased crude fiber content. This result was lower than the findings of Mirnawati et al. (2019), where palm kernel meal fermentation with *Bacillus subtilis* can

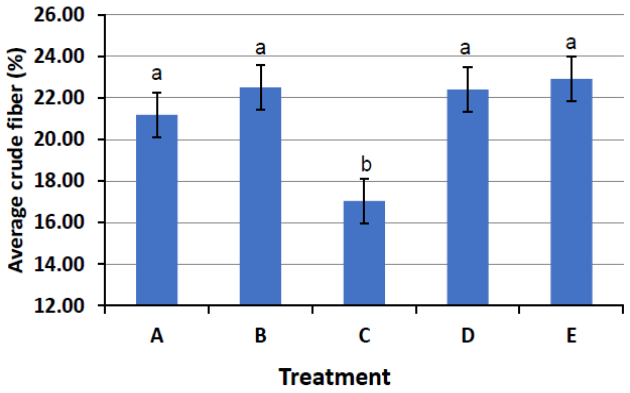

Fig. 2: The average PKMF crude fiber with *Bacillus subtilis* and *Lactobacillus fermentum*. Bars (Mean±SD) bearing different alphabets differ significantly (P<0.05).

reduce crude fiber by 17.35% and cellulase enzyme activity by 17.13U/mL. These results are also lower than those of Seftiadi et al. (2020), where palm kernel meal fermentation with *Lactobacillus fermentum* reduced crude fiber by 18.66%, and the resulting cellulase activity was 17.63U/mL.

The decrease in crude fiber by 17.04% was also supported by the increased activity of the resulting cellulase enzyme, which was 23.26U/mL. Increased cellulase enzyme activity can reduce the crude fiber content. Mirnawati et al. (2012) also expressed that cellulase can alter cellulose into a simple form like glucose to produce energy, making it a lower crude fiber.

The increase in crude fiber occurred in treatment E, namely 22.92%. This is presumably because the combination of bacteria with this ratio causes the bacteria not to work synergistically so that the cellulase enzymes produced are also small. Cellulase enzymes are affected by the number of growing microbial populations (Mirnawati et al. 2017). According to Mirnawati et al. (2017), the number of microbes affects the activity of enzymes produced, especially cellulase and mannanase enzymes, to alter cellulose into glucose, which is used as energy by mold so that crude fiber after fermentation decreases.

Conclusion

Based on this study, it can be concluded that the ratio of mixed bacteria *Bacillus subtilis* and *Lactobacillus fermentum* in the best PKM fermentation is (6: 4). This can be seen from the average results obtained from activity of cellulase (23.26U/mL), mannanase (26.97U/mL), protease (10.95U/mL), crude protein (26.91%), and crude fiber (17.04%) successively.

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