EFFECTS OF FERMENTATION LENGTH ON CHEMICAL COMPOSITIONS, ORGANIC ACIDS, FATTY ACID PROFILES, AND RUMINAL DEGRADATION OF FERMENTED CONCENTRATE USING TAMANU KERNEL CAKE

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ABSTRACT

This study investigated the effects of fermentation length on the chemical compositions, fatty acid profiles, organic acid production, and aerobic stability of fermented concentrate. Concentrate used tamanu kernel cake (TKC), serving as a source of protein and unsaturated fatty acids, combined with dried cassava, wheat pollard, soybean meal, and molasses. The concentrate before fermentation contained 19% crude protein (CP), and 36% neutral detergent fiber. Concentrate was fermented for 3, 7, 14, 21, and 60 days in quadruplicate. Longer fermentation time led to a linear increase in the loss of dry matter (DM) (P=0.004), organic matter (P=0.033), and CP (P=0.002) of fermented concentrate. Longer fermentation time caused a linear decrease in pH (P<0.010), but increased ammonia-N (P=0.006), lactate (P<0.001), acetate (P<0.045), and lactic acid bacteria (LAB) count (P<0.001). The 60 days treatment led to higher ammonia-N (P=0.003), lactate (P<0.001), acetate (P<0.001), and LAB count (P<0.001) compared to 0 to 7 days. Yeast (P<0.001) and bacillus (P<0.001) counts showed a quadratic pattern, where their counts at 60 days decreased lower than 3 days (P<0.001). Total fatty acid decreased linearly (P<0.001) with longer fermentation time, but each fatty acid profile remained unaffected. Aerobic stability was higher (P<0.001) at 21 and 60 days of fermentation compared to other durations. Fermentation at 3 and 7 days increased (P<0.010) the total degradable fraction in the rumen. In conclusion, fermenting concentrate based on TKC for 21 days increased aerobic stability and fermentation quality with less losses of nutrients and fatty acids, while fermenting for 3-7 days increased *in vitro* fermentability.

Keywords: Fermented concentrate, Fatty acid profile, Fermentation length, Ruminal degradation, Tamanu kernel cake

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INTRODUCTION

Fermentation is an essential process in food production used to improve the palatability and digestibility of animal feed (Adebo *et al.*, 2022). In recent years, it has been applied for concentrate feed as a method to improve the nutritional value and the digestibility of diet. This process has been widely adopted by many local farmers, especially those in tropical countries, because feed is often produced from by-products of agriculture or the plantation industry. To ensure improvement, the complex of inoculant consisting of lactic acid bacteria (LAB) and fibrolytic microbes is applied as an additive. The use of lactic acid bacteria in feed additives is able to show beneficial effects on ruminant performance (Kholif *et al*., 2024). Inoculation of LAB produces organic acids that inhibit aerobic microbes and change the physical appearance of fermentation products, such as aroma, texture, and colour (Fitriani *et al.*, 2024). In addition, applications of cellulolytic microbes can help to provide simpler substances with better degradability. Despite its potential, fermentation has been reported to significantly cause nutrient loss (Adebo *et al.*, 2022; Xue *et al.*, 2024). This is because water-soluble carbohydrate (WSC), protein, and fatty acid obtained from the process are used by microbes during fermentation (Adebo *et al.*, 2022; Xue *et al.*, 2024).

According to previous studies, fermentation length is a major factor affecting nutrient loss in the fermented concentrate. Longer length can increase nutrient loss due to higher consumption by microorganisms (Miyaji *et al.*, 2017). Moreover, the change in chemical composition due to fermentation can also affect the digestibility of the diet. Several studies have shown that fermentation is influenced by the change of unsaturated fatty acid (UFA) to saturated fatty acid (SFA) due to the activity of lipolytic microbes (Ke *et al.*, 2022).

A previous study reported that the supplementation of oil-rich UFA in ruminant increased the immune system, health, calving performance, reproduction, and concentration in meat and milk (Moallem, 2018; Chiofalo *et al*., 2020). A high concentration of UFA in animal products has a positive correlation with human health. Dietary UFA for beef cattle also has a positive effect on performance, reproduction, and health. This indicates that its addition to diet is an alternative method to improve animal performance and their product. Unsaturated fatty acids can be digested and absorbed easier (Xu *et al*., 2021). Exploration of local feed containing high UFA concentration can reduce the price of basal diet by eliminating the need for an oil source as a supplement.

Tamanu kernel cake (TKC) is a new alternative animal feed produced from the by-product of tamanu crude oil (Paradhipta *et al.*, 2023a). The cultivation of tamanu trees (*Calophyllum inophyllum*) is expanding to meet the growing market demand for tamanu oil. These trees are widely distributed across Indonesia, exhibit high adaptability to tropical climates, and require minimal maintenance. As a novel feed ingredient, conducting studies on TKC is essential. TKC offers potential benefits as animal feed due to its high crude protein (CP) and ether extract (EE) content, ranging from 19.9% to 23.5% and 13.9% to 15.6%, respectively (Paradhipta *et al.*, 2023a; Umroni *et al.*, 2024). The crude fat content in TKC is higher compared to other oil-press by-products, such as palm kernel cake and copra cake. The high fat content is rich in UFA, which can enhance animal performance and improve the quality of animal products. However, TKC also contains significant levels of plant secondary metabolites, including total phenols, flavonoids, tannins, and saponin approximately 53.3%, 6.47%, 1.70%, and 0.93%, respectively (Paradhipta *et al.*, 2023a). These compounds may reduce palatability and pose toxicity risks, indicating the need for fermentation. However, fermentation may also lead to a reduction in UFA and CP content in the feed.

In Indonesia, a fermented concentrate is often applied in low moisture levels of 30%-40% to enhance handling and storage. In practical, high moisture combined with warm temperatures can promote mold contamination in fermented concentrates. Moreover, excessive moisture makes the fermented concentrate difficult to handle, reducing its acceptance in the market. However, the potential loss of nutrients could increase along with longer storing. Despite the limitation, scientific information on nutrition loss and fatty acid profile during fermentation of concentrate with low moisture level is limited. Moreover, the concentrate used in this study was high in protein and fat, fractions with high potential for loss during fermentation, which could subsequently affect digestibility. Therefore, this study aims to determine the effects of fermentation length on the chemical compositions, fatty acid profiles, organic acid production, aerobic stability, and rumen degradation kinetics of fermented concentrate using TKC.

MATERIALS AND METHODS

Preparation of dietary treatment: This research was conducted in Laboratory of Feed Technology in 2023. Ingredients of dietary treatment consisted of 40% TKC, 26.4% dried cassava, 25% wheat pollard, 7.60% soybean meal, and 1.00% molasses. The proportion was formulated based on % of dry matter (DM), and the dietary treatment was formulated to contain approximately 18%-19% CP and 35%-36% NDF with TKC as the main ingredient for protein and UFA source. The TKC was sub-sampled around 20 g for fatty acid profile analyses using Gas Chromatography-Mass Spectrometry (GC-MS, Agilent 8890 and 5977B) with a column (DB-FastFAME, 30 m x 250 µm x 0.25 µm) according to the procedure of Christie (1998).

All of the ingredients were mixed and applied with 1 x 10^5 colony forming unit (cfu)/mL of commercial inoculant (Saus Berger Pakan, SBP; CV Agromix Lestari, Indonesia). Dried cassava and TCK were ground with a 3 mm screen. All ingredients were mixed using a horizontal mixer for 10 min. Molasses was added as the last ingredient. Then, a total 1 mL of inoculant was applied for 1 kg of diet. The moisture level of the concentrate was adjusted at 35% using sterile water, as well as the commercial inoculant containing the mixture of *Lactobacillus* sp, and fibrolytic and proteolytic microbes from yeast and bacillus. Diet was fermented using mini-silo (4 kg), all silo was maintained for anaerobic conditions and incubated for 3, 7, 14, 21, and 60 days, and each fermentation time consisted of 4 replications, where there were a total of 20 kg in each treatment. The mini silo uses a plastic bag with a thickness of 1mm which is placed in a 20 L bucket. Anaerobic conditions were ensured by applying a vacuum. On the assigned day, each fermented concentrate was compressed at 200 g for analyses of chemical composition. In addition, other fermented concentrates were compressed approximately at 20 g for analyses of fermentation characteristics, microbial counts, and fatty acid profiles, and the determination of aerobic stability used approximately 3 kg of remained sample.

Chemical compositions and fatty acid profiles: In chemical composition analyses, a total of 10 g of sample were dried at 105°C for 24 h to measure dry matter (DM) concentration (method number 934.01, AOAC 2005). A sample of diet was prepared by drying at 60°C for 48 h, and the size of the dried sample was homogenized and grounded through a Wiley mill with a 1 mm screen. A total of 1 g of grounded sample was burned into a muffle furnace at 550°C for 5 h to determine the concentration of OM (method number 942.05; AOAC 2005). In addition, the measurement of CP was

conducted based on the procedure of Kjeldahl, which consisted of destruction, distillation, and titration (method number 984.13, AOAC 2005). The soxhlet method was used to determine the concentration of EE (method number 920.39, AOAC 2005). Furthermore, the fiber fractions consisting of NDF and ADF were determined according to the procedure of Van Soest (method number 2002.04 and 973.13, respectively, AOAC 2005) using ANKOM Fiber Digestion (ANKOM 200 Fiber Analyzer, USA).

The sample of fermented concentrate also analysed fatty acid (FA) profile analyses using Gas Chromatography-Mass Spectrometry. Total of 7 g of feed from each treatment were analysed, with results expressed as mg/g of total FA or mg/100 g of feed. Fatty acid methyl esters (FAME) were prepared and analysed using gas chromatography with flame ionization detection (GC-FID; Agilent 7890B) equipped with a capillary column (30 m × 250 µm i.d., 0.25 µm film thickness; Supelco). FAME were identified by comparison of retention times with a commercial standard mixture (38-Component FAME Mix; Supelco), and further confirmed using gas chromatography–mass spectrometry (GC-MS) under identical chromatographic conditions. The GC-FID injector and detector temperatures were set at 200°C and 250°C, respectively. The oven temperature was programmed from 60°C to 150°C at 30°C/min (1 min hold), then to 200°C at 2°C/min, and finally to 225°C at 3°C/min (5 min hold). Helium was used as the carrier gas at 1 mL/min, with a 10:1 split injection of 1 µL. For GC-MS analysis, the ion source and interface temperatures were set at 200°C and 250°C, respectively.

Fermentation characteristics: A total of 180 mL ultra-pure distilled water was blended with 20 g of sample for 30 sec to conduct the extraction. This was continued to analyse pH, ammonia-N, and organic acid profiles. A pH meter (Mettler Toledo LE438, USA) was used to measure pH. Determination of ammonia-N was conducted using the principle of colorimetric according to Chaney & Marbach (1962). Furthermore, the concentrations of lactate and volatile fatty acid (VFA) were determined using HPLC equipped with a diode array detector (DAD) (LC-2030C, Shimadzu, Japan) and a column (Shim-pack GIST C18, Shimadzu, Japan) according to Vargas *et al.* (2020). The concentrations of fermentation variables consisting of ammonia-N, lactate, and each VFA profile were obtained as mg/L and then were converted as % of DM (%, DM). Fermentation variables with concentrations below 100 mg/L were not detectable.

Microbial count and aerobic stability: The extraction of fermented concentrate was used to analyse microbial counts. The dilution series of extraction was applied until 10^{-7}. Generally, dilution series of 10^{-3} to 10^{-7} was injected into growth mediums. The LAB, yeast, mold, bacillus, and clostridia were counted in different solid mediums. The de Man, Rogosa, and Sharpe (MRS; Sigma Aldrich, USA) agar was used for cultivation of LAB. Yeast and mold were cultivated into Potato Dextrose Agar (PDA; Sigma Aldrich, USA), while bacillus was cultivated into Luria-Bertani (LB) agar (Sigma Aldrich, USA) according to the procedure of studies (Paradhipta *et al.*, 2020; Fitriani *et al.*, 2024). All plates of MRS agar were stored in anaerobic incubator. Whereas, all plates of PDA, and LB agar were stored in aerobe incubator. Both anaerobic and aerobic incubators were applied at 30°C and all plates were stored for 48 h. Colony forming unit per gram was used to express count of each microbial, and then it was calculated to log10. In addition, aerobic stability was applied according to the procedure of Fitraini *et al.* (2024). The temperature of fermented concentrate was measured every hour until its temperature was higher approximately at 2°C than ambient temperature.

***In vitro* rumen incubation:** The protocol for handling cannulated Balinese cattle received approval from the Animal Ethics Committee, Integrated Laboratory for Research and Testing, Universitas Gadjah Mada (No. 00056/04/LPPT/XII/2023). Prior to the morning feeding routine, rumen fluid was obtained from two Balinese cattle equipped with rumen cannulas. These animals were maintained on a diet composed of *Pennisetum purpureum* and a commercial concentrate mixed at a ratio of 7:3 containing 12% of CP and 10 kcal/kg of metabolizable energy. The collected rumen contents were filtered through double-layered cheesecloth to remove large particles (Paradhipta *et al.*, 2023b). To prepare the rumen buffer, the filtered fluid was blended with an anaerobic culture medium in a 1:2 ratio as outlined by Adesogan *et al.* (2005). A 0.5 g portion of the ground feed sample was weighed and transferred into each fermentation bottle. Subsequently, 40 mL of the rumen buffer was added. To ensure anaerobic conditions, CO_2 gas was introduced into each bottle before sealing them securely. The bottle was sealing with rubber cap. Each treatment was carried out in quadruplicate, along with two blank controls. The bottles were incubated at 39°C using a laboratory incubator. Gas production was monitored at 0, 3, 6, 9, 12, 18, 24 and 48 h using 5 mL of syringe. The syringe was injected into rubber cap, and then the production of gas was monitored as mL. The fermentation kinetics were analysed using the PROC NLIN procedure in SAS software (Version 9, Cary, NC, USA), applying the McDonald model (McDonald, 1981) defined by the following equation:

$$Y = a + b\ (1 - e^{-c\,(t-L)}) \text{ for } t > L$$

Where: Y = the fermentation kinetics; a = is the immediately degradable fraction; b = the potentially degradable fraction; a + b = total degradable fraction; C = the fractional degradation rate; L = the lag phase; and t = time of incubation (h).

Statistical analysis: The experimental design in the present study was used a completely randomized design, while all collected data were analysed using one-way ANOVA by PROC ANOVA of SAS. The model for this analysis was defined by the following equation:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where Y_{ij} = response variable; μ = overall mean; T_i = effect of fermentation length; and e_{ij} = error term. Linear and quadratic contrast were measured by PROC GLM of SAS (Version 9, Cary, NC, USA) using a model of polynomial contrast based on an orthogonal coefficient to evaluate the effects of fermentation length. Previously, the coefficients of orthogonal were calculated to regulate an uneven spacing of fermentation length as treatment by PROC IML of SAS. In post-hoc analysis, the Tukey test was applied to decide the significant difference (P <0.05) between treatments.

RESULTS AND DISCUSSION

Chemical compositions: The chemical compositions of TKC used in the present study are shown in Table 1. The chemical compositions of TKC in the present study was in a normal range according to our previous studies. The nutrient content of TKC could be varied depending on the oil-pressing method (Umroni *et al.*, 2023). Commonly, the concentrations of CP and EE from TKC was more than 18% and 10%, respectively (Umroni *et al.*, 2023).

Table 1 Chemical composition of tamanu kernel cake in the present study (%, DM)

Item	
Dry matter	91.0 ± 0.28
Organic matter	91.0 ± 0.32
Crude protein	22.5 ± 0.17
Ether extract	14.5 ± 0.29
Neutral detergent fiber	37.0 ± 0.16
Acid detergent fiber	14.9 ± 0.29

Means ± standard error

According to Table 1, TKC still had a high concentration of EE. Thus, the profiling of fatty acid of TKC was conducted. The fatty acid profiles of TKC used in the present study are shown in Table 2. TKC could be used not only as protein, but also UFA source in the diet. TKC contained 73.7% of UFA and 26.3% of SFA. Mainly, TKC was rich of oleic acid (C18:1n-9) and linoleic acid (C18:2n-6), which could result in beneficial effects for ruminants, such as on methane emission (Amanullah *et al.*, 2021), milk production (Moallem, 2018), and meat quality (Chiofalo *et al.*, 2020). The concentration of oleic acid and linoleic acid on TKC could reach 43.3% and 30.4%, respectively.

Table 2 Fatty acid profiles of tamanu kernel cake in the present study

Item	
Total fatty acid, µg/mL	671.3
C16:0, % total FA	12.5
C18:0, % total FA	13.2
C18:1n-9, % total FA	43.3
C18:2n-6, % total FA	30.4
C20:0, % total FA	0.60
SFA, % total FA	26.3
MUFA, % total FA	43.3
PUFA, % total FA	30.4
UFA, % total FA	73.7
UFA : SFA	1.70

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; UFA: unsaturated fatty acid; UFA : SFA: ratio of UFA to SFA

The present study showed that fermentation of concentrate at 60 d had lower concentrations of DM (P=0.037, 63.1% vs. 59.9%) and CP (P=0.016, 19.7% vs. 15.6%) than at 0 d (Table 3). In addition, longer fermentation time decreased linearly the concentrations of DM (P=0.004), OM (P=0.033), CP (P=0.002), and ADF (P=0.018), and the

fermentation length had no effects on EE and NDF concentrations. The observed changes in DM, OM, and CP concentrations over time may reflect the metabolic processes of fermentative microbes, which progressively consume soluble substrates and convert them into organic acids (Table 4 and Table 5). The production of lactate and acetate increased by longer fermentation time, which was caused by the utilization of DM and OM, such as WSC, during fermentation. Therefore, the concentrations of DM and OM decreased by longer fermentation time, and the present study revealed that short-fermentation of less than 21 days had a role in preserving nutrient contents of fermented concentrate, where the concentration of DM and CP were similar.

Table 3 Effects of fermentation length on chemical composition of fermented concentrate (% DM)

Item	Time of fermentation, d					SEM	*P*-value[1]		
	0	3	7	21	60		Time	L	Q
Dry matter	63.1[a]	61.7[ab]	61.8[ab]	61.6[ab]	59.9[b]	1.222	0.037	0.004	0.781
Organic matter	88.9	89.5	88.5	87.8	87.6	1.076	0.134	0.033	0.260
Crude protein	19.7[a]	18.0[ab]	18.2[ab]	18.4[ab]	15.6[b]	1.166	0.016	0.002	0.664
Ether extract	7.75	7.71	7.83	7.68	7.64	0.808	0.998	0.826	0.985
Neutral detergent fiber	36.5	36.7	35.2	35.5	34.7	1.409	0.347	0.118	0.541
Acid detergent fiber	20.4	20.0	20.6	19.1	19.0	1.251	0.069	0.018	0.119

[a,b]Means in the same column with different superscripts differ significantly ($P<0.05$)
[1]Time: effect of fermentation length; L: linear effect by fermentation length; Q: quadratic effect by fermentation length

On the other hand, long fermentation for 60 d caused nutrient loss significantly in the diet, which could be also indicated by the highest concentrations of ammonia-N, lactate, and acetate. The CP concentration decreased significantly after 60 days of fermentation, which was accompanied by a marked increase in ammonia-N concentration. (Table 4), which indicated protein loss during fermentation (McDonald *et al.*, 1991). Generally, CP loss after 60 d of fermentation was around 20.8% relative to 0 d. The presence of yeast and bacillus in the fermented concentrate (Table 4) potentially had protease activity (Rozanov *et al.*, 2021; Mamo & Assefa, 2018), which could be a reason for high protein loss during fermentation. Although the populations of yeast and Bacillus peaked at 21 days and declined thereafter, this temporal pattern does not preclude their contribution to protein degradation. Proteolytic enzymes produced during earlier fermentation stages may persist and remain active even as viable microbial counts decrease under highly acidic conditions. In addition, microbial autolysis and continued activity of extracellular proteases released into the fermentation matrix may further contribute to protein breakdown at later stages of fermentation (Yang *et al*., 2015). Protease broke the protein into several products such as non-protein nitrogen, ammonia-N, and amino acid nitrogen, which were indicators of protein loss during fermentation (McDonald *et al.*, 1991, Li *et al.*, 2018).

Fermentation characteristics and microbial counts: In the fermentation characteristics, the fermentation at 21 and 60 d had a lower pH than at 7, 3, and 0 d ($P<0.001$, 4.57 and 4.49 vs. 5.05 vs. 5.48 vs. 6.44) (Table 4). Ammonia-N concentration was higher in the fermentation for 60 d than 0, 3, 7, and 21 days ($P=0.003$, 0.67% vs. 0.52%, 0.55%, 0.56%, and 0.52%). Lactate concentration was higher in the fermentation at 21 and 60 days than at 7 days, followed by 3 d, then 0 d ($P<0.001$, 4.37%, and 4.61% vs. 3.57% vs. 2.66% vs. 0.65%), and the acetate concentration was higher by the fermentation at 60 days than at 0 d ($P<0.001$, 2.03% vs. 0.02%). Generally, longer fermentation time decreased linearly pH ($P<0.001$) and increased linearly concentrations of ammonia-N ($P=0.006$), lactate ($P<0.001$), and acetate ($P=<0.001$), but propionate and butyrate were not detected during measurement in this study due to very low concentration.

Table 4 Effects of fermentation length on fermentation characteristics of fermented concentrate

Item	Time of fermentation, d					SEM	*P*-value[1]		
	0	3	7	21	60		Time	L	Q
pH	6.44[a]	5.48[a]	5.05[c]	4.57[d]	4.49[d]	0.100	<0.001	<0.001	0.010
Ammonia-N, % DM	0.52[b]	0.56[b]	0.55[b]	0.52[b]	0.67[a]	0.047	0.003	0.006	0.072
Lactate, % DM	0.65[d]	2.66[c]	3.57[b]	4.37[a]	4.61[a]	0.343	<0.001	<0.001	0.060
Acetate, % DM	0.02[c]	0.44[bc]	0.47[bc]	1.31[ab]	2.03[a]	0.482	<0.001	<0.001	0.086

[a,b,c,d]Means in the same column with different superscripts differ significantly ($P<0.05$)
[1]Time: effect of fermentation length; L: linear effect by fermentation length; Q: quadratic effect by fermentation length

In microbial counts, the growth of LAB was higher in 21 and 60 days than in 3 and 7 days of fermentation, while the lowest was by 0 d of fermentation (P<0.001, 5.86 and 6.17 vs. 4.17 and 4.59 vs. 2.00 log10 cfu/g) (Table 5). The growth of bacillus (P<0.001, 5.53 vs. 4.72 vs. 4.02 vs. 1.96 log10 cfu/g) and yeast (P<0.001, 5.73 vs. 5.00 vs. 4.16 vs. 2.76 log10 cfu/g) was the highest in 21 days of fermentation followed by 3 and 60 days, and then 0 day of fermentation. Generally, the growth of LAB (P<0.001) increased linearly by longer fermentation time, and the growth of yeast (P=0.001) and mold (P=0.001) increased quadratically by longer fermentation time.

Table 5 Effects of fermentation length on microbial count of fermented concentrate (log10 cfu/g)

Item	Time of fermentation, d					SEM	*P*-value[1]		
	0	3	7	21	60		Time	L	Q
Lactic acid bacteria	2.00^{c}	4.17^{b}	4.59^{b}	5.86^{a}	6.17^{a}	0.261	<0.001	<0.001	0.056
Bacillus	1.96^{d}	4.72^{b}	5.10^{ab}	5.53^{a}	4.02^{c}	0.251	<0.001	0.021	0.001
Yeast	2.76^{d}	5.00^{b}	5.51^{ab}	5.73^{a}	4.16^{c}	0.196	<0.001	0.726	0.001
Mold	ND	ND	ND	ND	ND	NA	NA	NA	NA

^{a,b,c,d}Means in the same column with different superscripts differ significantly (P<0.05)
[1]Time: effect of fermentation length; L: linear effect by fermentation length; Q: quadratic effect by fermentation length
ND: not detected; NA: not applicable

The decrease in pH was caused by the increase in lactate and acetate production (Kung *et al.*, 2018, McDonald *et al.*, 1991). The use of TKC as a protein source contributed to the high CP concentration of dietary treatment while fermenting a high CP diet had the potential of buffering capacity that inhibited the decrease of pH during fermentation (McDonald *et al.*, 1991). However, the pH of fermented concentrate could reach 4.57, which was good enough for the preservation and inhibition of spoilage microbes (Kung *et al.*, 2018, Paradhipta *et al.*, 2023, Fitriani *et al.*, 2024). The population of LAB increased along with the longer time of fermentation (Table 5), which could be a reason for increased lactate and acetate during fermentation. This study revealed that microbial growth still occurred even though a moisture level was 35%. The result of the present study revealed that a low moisture level for fermenting concentrate could be applied.

Previous studies also reported that longer fermentation time could increase the production of lactate and acetate in fermented diet (Wang & Nishino, 2013). The absence of butyrate indicated that fermentation was conducted in good condition (Kung *et al.*, 2018, McDonald *et al.*, 1991), and this was an indicator of clostridia fermentation (Zheng *et al.*, 2020). In addition, the populations of bacillus and yeast increased during 21 days and then decreased after 60 d of fermentation. Concentrations of lactate and acetate at 60 days of fermentation could be strong enough to create an acid circumstance of concentrate that inhibited the growths of bacillus and yeast effectively (Table 4). This result was also supported by previous studies, which presented similar results (Paradhipta *et al.*, 2020, Fitriani *et al.*, 2024), and this acetate had a role as an antimicrobial that effectively inhibited undesirable microbes consisting of yeast and mold (Danner *et al.*, 2003, Paradhipta *et al.*, 2020).

Aerobic stability: In aerobic stability, the longer fermentation time could increase linearly (P<0.001) the aerobic stability of fermented concentrate (Figure 1). Furthermore, the fermentation at 21 and 60 d had higher aerobic stability than 0, 3, and 7 day(s) (P<0.001, 27.5 and 27.0 h vs. 23.3, 23.8, and 24.5 h). The aerobic stability of fermented feed was affected by the presence of acetate, which had a role in inhibiting mold and yeast (Danner *et al.*, 2003). The result of the present study showed that this increased after fermenting for 21 days of fermentation, and occurred because the concentration of acetate was increased highly after 21 days of fermentation (Table 4) Furthermore, this could help to increase aerobic stability of fermented feed (Wilk *et al.*, 2020, Fitriani *et al.*, 2024), An increase of aerobic stability has a beneficial for industrial application, where it increases shelf life after opening from the silo without any spoilage.

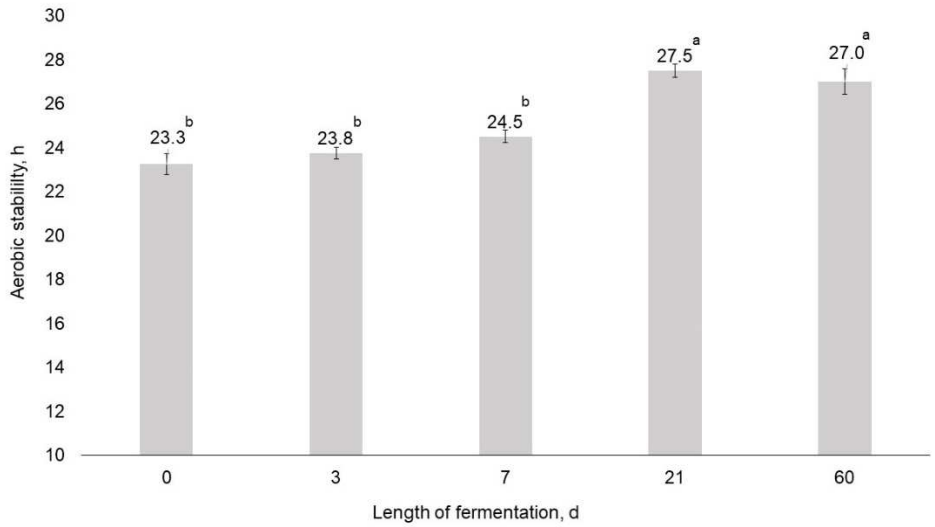


Figure 1 Aerobic stability (hours until a 2ºC temperature rise above ambient) of fermented concentrate across different fermentation lengths. [a,b]Means with different superscripts differ significantly (*P*<0.001). The contrast of linear effect by fermentation length is *P*<0.001 and quadratic effect by fermentation length is *P*<0.010.

Fatty acid profiles: The fermentation length affected the total fatty acid concentration of fermented concentrate (Table 6), and it could be indicated that short-fermentation at 0, 3, and 7 day(s) had a higher total fatty acid compared to long-fermentation at 60 days (*P*<0.001; 95.2, 95.1, and 94.9 vs. 94.5 µg/mL). The concentration of total fatty acid decreased linearly (*P*<0.001) by longer fermentation time, and the profile of fatty acids did not change during fermentation.

Table 6 Effects of fermentation length on fatty acid profiles of fermented concentrate

Item	Time of fermentation, d					SEM	*P*-value[1]		
	0	3	7	21	60		Time	L	Q
Total fatty acid, µg/mL	95.2[a]	95.1[a]	94.9[a]	94.7[ab]	94.5[b]	0.141	<0.001	<0.001	0.053
C14:0, % of FA	0.02	0.04	0.05	0.02	0.06	0.041	0.601	0.307	0.583
C16:0, % of FA	12.8	14.1	13.5	13.8	13.6	0.697	0.214	0.574	0.310
C16:1, % of FA	0.24	0.22	0.33	0.28	0.26	0.075	0.368	0.910	0.265
C17:0, % of FA	0.17	0.17	0.10	0.12	0.20	0.061	0.282	0.255	0.122
C18:0, % of FA	13.3	12.6	12.2	11.6	12.8	0.830	0.104	0.936	0.013
C18:1n9, % of FA	42.3	40.8	41.7	42.3	40.9	1.145	0.206	0.319	0.272
C18:2n6, % of FA	28.5	29.7	30.0	29.6	29.4	1.796	0.833	0.875	0.525
C18:3n3, % of FA	0.54	0.70	0.57	0.64	0.70	0.119	0.296	0.171	0.893
C20:0, % of FA	0.91	0.87	0.73	0.71	0.89	0.102	0.045	0.580	0.048
C20:1n9, % of FA	0.37	0.38	0.33	0.31	0.41	0.062	0.278	0.258	0.066
C22:0, % of FA	0.46	0.42	0.30	0.35	0.42	0.066	0.051	0.811	0.018
C24:0, % of FA	0.32	0.18	0.20	0.22	0.29	0.087	0.053	0.210	0.229
SFA	28.0	28.2	27.1	26.8	28.3	1.461	0.497	0.650	0.116
MUFA	42.9	41.4	42.3	42.9	41.5	1.095	0.189	0.342	0.266
UFA	29.1	30.4	30.6	30.3	30.1	1.891	0.822	0.815	0.525
PUFA	70.9	69.6	69.4	69.7	69.9	1.896	0.818	0.809	0.527
UFA : SFA	2.54	2.47	2.57	2.60	2.47	0.086	0.167	0.394	0.064

[a,b]Means in the same column with different superscripts differ significantly (*P*<0.05)

[1]Time: effect of fermentation length; L: linear effect by fermentation length; Q: quadratic effect by fermentation length.

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; UFA: unsaturated fatty acid; UFA : SFA: ratio of UFA to SFA.

The use of TKC as an ingredient could contribute to a high concentration of UFA in the dietary treatment, which was potentially loss by fermentation (Ke *et al.*, 2020). In the present study, total fatty acid decreased by longer fermentation time, even though the concentration of EE was not affected. However, the decrease of total fatty acid in fermented concentrate after 60 days of fermentation had a minimum loss, only 0.74% relative to the 0 d. The low moisture level could be a reason for less fatty acid loss by fermentation time, which was also supported by Liu *et al.* (2018). Generally, fermentation time did not affect each profile of fatty acid, and the concentration of UFA was not changed to SFA during fermentation. This could indicate that the conversion of UFA to SFA (biohydrogenation) did not occur in the present study. Previous studies reported that biohydrogenation of UFA to SFA occurred in high moisture fermentation such as silage or fermented TMR (Chen *et al.*, 2017, Kim *et al.*, 2018). The biohydrogenation could be affected by microbial activity such as LAB, which was generally conducted in high moisture conditions (Kishino *et al.*, 2009, Chen *et al.*, 2017, Ke *et al.*, 2017). This might be attributed to the low moisture content. The relatively low moisture content likely restricted microbial mobility and enzymatic diffusion, thereby limiting lipase activity. The other, the rapid accumulation of organic acids and the resulting low pH may have inhibited lipolytic enzymes and lipolytic microbial activity, as these processes are known to be sensitive to acidic conditions (Kishino *et al.*, 2009; Kelaita *et al*., 2026). Furthermore, this was conducted during feed fermentation, such as silage (Ranst *et al.*, 2009), and the conversion of UFA to SFA could occur conducted by enzymatic pathways or microbial intervention during fermentation (Ke *et al.*, 2022). There was no direct information that reported the biohydrogenation of UFA to SFA in low-moisture fermentation of concentrate, and the present study discovered that fermenting concentrate with low moisture resulted in less biohydrogenation. In addition, Ke *et al.* (2022) also reported that lower moisture could result in less degradation of UFA during fermentation, and this could increase the concentration of PUFA in animal products such as meat or milk. Less biohydrogenation in the fermented diet had beneficial effects on animal health and also human beings ultimately.

Gas production kinetics: In the rumen, the fermentable fraction decreased linearly (P=0.018) by longer time of fermentation (Table 7). In addition, fermentation at 3 and 7 days resulted in higher the potentially fermentable fraction (P=0.006; 3.74 and 3.37 mL/g DM vs. 3.35 mL/g DM) and the total fermentable fraction (P=0.005; 5.45 mL/g DM and 5.35 vs. 5.02 mL/g DM) than fermentation at 0 day. Both the potentially fermentable fraction (P=0.057) and the total fermentable fraction (P=0.057) tended to present quadratic pattern by longer fermentation period. The fractional fermentation rate and the lag phase were not affected by time of fermentation.

Table 7 Effects of fermentation length on gas production kinetics of fermented concentrate

Item	Time of fermentation, d					SEM	P-value[1]		
	0	3	7	21	60		Time	L	Q
a (mL/g DM)	1.67	1.72	1.62	1.60	1.53	0.093	0.105	0.018	0.506
b (mL/g DM)	3.35^{b}	3.74^{a}	3.73^{a}	3.62^{ab}	3.54^{ab}	0.135	0.006	0.689	0.057
a+b (mL/g DM)	5.02^{b}	5.45^{a}	5.35^{a}	5.22^{ab}	5.07^{b}	0.153	0.005	0.184	0.066
c (%/h)	0.24	0.23	0.24	0.23	0.23	0.021	0.882	0.716	0.578
L (h)	3.95	3.91	3.89	3.91	3.80	0.132	0.588	0.132	0.830

^{a,b}Means in the same column with different superscripts differ significantly (P<0.05)

[1]Time: effect of fermentation length; L: linear effect by fermentation length; Q: quadratic effect by fermentation length

a: the immediately degradable fraction; b: the potentially degradable fraction; a+b: the total degradable fraction; c: the fractional degradation rate; L: the lag phase.

The present study discovered that immediately degradable fraction presented a linear effect by longer fermentation time. This result was supported with the result of DM (Table 3). The soluble fraction could be used by microbial for growth during fermentation (McDonald *et al.*, 1991; Kung *et al.*, 2018) that resulted in the decrease of the immediately degradable fraction. Interestingly, fermentation time presented a quadratic effect on degradable fraction in the rumen. In indicated that short fermentation, such as 3-7 days could increase the digestibility in the rumen. The production of fermented concentrate in the present study used commercial inoculant containing yeast and bacillus that had a fibrolytic activity. The application of fibrolytic microbes could increase *in vitro* fermentability in the rumen of fermented feed (Lee *et al.* 2021; Paradhipta *et al.*, 2023b). In short fermentation, such as 3-7 days, the enzyme of fibrolytic have been produced. This fibrolytic enzyme was produced by commercial inoculant (SBP). The SBP contained not only LAB but also several enzymes, including fibrolytic enzymes. The previous study reported that the use of SBP as inoculant for fermenting rice brand exhibits fibrolytic activity, leading to reductions in cellulose, hemicellulose, and lignin contents (Dilaga et al., 2022). The fibrolytic enzyme was actively helping to increase the digestibility due to the

pH condition of fermented concentrate was still higher than 5.0 (Table 4). It supported by previous study that activity of fibrolytic enzyme is stable in pH 5.0-8.0 (Ju *et al.*, 2012). After the acid condition (pH lower than 5), the activity of fibrolytic enzymes decreased that caused the degradation kinetics of 60 days was similar to 0 day of fermentation.

Although TKC shows promising potential as a protein and unsaturated fatty acid source in ruminant diets, its availability remains limited compared to more established by-products such as palm kernel cake and copra cake. This may affect the scalability and consistency of its use in large-scale feed production. Future studies could explore the long-term impacts of feeding fermented TKC-based concentrate on animal performance, health, and product quality. In addition, evaluating the fermentation process under different environmental conditions and using various inoculant strains may provide a more comprehensive understanding of its stability and nutritional dynamics. Studies involving in vivo trials and economic analysis are also encouraged to validate the practical benefits and feasibility of applying fermented TKC concentrate in commercial model.

Conclusions: The present study concluded that longer fermentation time increased linearly the losses of DM, OM, CP, and total fatty acid of fermented concentrate, which was fermented at a low moisture level. However, it did not affect the biohydrogenation of UFA to SFA. A longer fermentation time also increased linearly fermentation quality, LAB growth, and aerobic stability of fermented concentrate. After 60 days of fermentation, the populations of yeast and bacillus decreased effectively due to the high concentration of lactate and acetate. The present study discovered that fermentation of concentrate for 21 days had less nutrient loss with improvement in aerobic stability, and fermentation quality. However, short-term fermentation (3-7 days) resulted in a modest but significant increase in *in vitro* fermentability.

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