Research Article

Comparative Evaluation of Nutrient Balance, Rumen Fermentation, and Production Performance in Dairy Systems Producing Milk With High and Low CLA Levels

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Abstract | This study evaluated differences in dietary structure, nutrient balance, rumen fermentation, digestibility, and production performance between dairy systems producing milk with high and low conjugated linoleic acid (CLA) under field conditions. A total of 269 milk samples were collected, and 15 highest and 15 lowest CLA samples were selected along with their corresponding diets for *In vitro* evaluation. Dietary structure did not differ significantly between groups. However, the high-CLA group showed lower dry matter (DM) and total digestible nutrients (TDN) supply, resulting in negative nutrient balance ($p < 0.05$). Rumen fermentation parameters (NH_3, total VFA, protozoa) were similar, although rumen pH (6.84 vs. 6.79) and organic matter digestibility were higher in the high-CLA group ($p < 0.05$). Milk CLA concentration was markedly higher in the high-CLA group (2.91 vs. 0.71% of milk fat; p < 0.05), accompanied by greater fat and CLA yields ($p < 0.05$) without differences in milk composition. These findings indicate that CLA variation could not be explained by general rumen fermentation or dietary composition, but is associated with nutrient balance and digestibility. Negative nutrient balance was associated with higher CLA concentration and may reflect metabolic conditions linked to altered fatty acid metabolism. However, the physiological mechanisms underlying this association require further investigation. Therefore, strategies to enhance milk CLA should aim to optimize rumen lipid metabolism while maintaining adequate nutrient balance and animal health.

Keywords | Conjugated linoleic acid, Nutrient balance, Rumen biohydrogenation, Digestibility, Dairy cow

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INTRODUCTION

Milk fatty acid composition has gained increasing attention because of its importance for human health, particularly conjugated linoleic acid (CLA). CLA has been associated with anti-atherogenic (Silanikove *et al*., 2010), anti-carcinogenic (Toral *et al*., 2024), and metabolic regulatory effects, including body weight control and potential roles in diabetes management (Jose and Joseph, 2020; Park *et al.*, 2007). Among CLA isomers, cis-9, trans-11 CLA is the predominant form in ruminant-derived products (Jensen, 2002). This isomer is mainly formed through rumen biohydrogenation of unsaturated fatty acids and can also be synthesized endogenously in the mammary gland from vaccenic acid (Badawy *et al.*, 2023). Therefore, increasing milk CLA concentration has become an important objective in dairy nutrition.

Most studies aiming to enhance milk CLA have focused on dietary lipid manipulation. Oilseed supplementation has been widely investigated to increase CLA content (Martha *et al.*, 2025). Protected fat supplementation has also been reported to influence rumen biohydrogenation pathways (Rojas-González *et al.*, 2023). Subsequent studies confirmed similar effects under different conditions (Behan *et al.*, 2024) and further explored their role in fatty acid metabolism (Albarki et al., 2025). These approaches are based on the assumption that CLA formation is primarily governed by the availability and transformation of dietary lipids within the rumen.

However, under practical farm conditions, milk CLA concentration often varies among farms despite similar feeding strategies (Anzhany *et al.*, 2022). This suggests that CLA variation may not depend solely on specific dietary lipid sources (Albarki et al., 2025), but may also reflect differences in feeding systems, nutrient supply, and production conditions (Despal *et al.*, 2021). Such inconsistencies indicate a gap between controlled experimental findings and practical production systems. In addition to nutritional and production-related factors, individual animal variation, including possible genetic influences on milk fat metabolism, may also contribute to differences in milk CLA concentration under field conditions (Guimarães *et al.*, 2023).

Nutrient balance is a key factor influencing dairy cow performance because milk production depends on nutrient intake relative to animal requirements (Uchida *et al.*, 2001). Imbalances between nutrient intake and requirements may affect metabolic status and production efficiency (Urban-Chmiel and Sobiech, 2025), while variations in feeding practices may contribute to differences in milk production and composition (Anzhany *et al.*, 2022). Rumen fermentation also plays an essential role in nutrient availability and fatty acid intermediate formation (Maia *et al.*, 2007). *In vitro* techniques are commonly used to evaluate rumen fermentability and digestibility (Sheikhalipour *et al.*, 2026).

Despite these advances, it remains unclear whether differences in milk CLA under field conditions are associated with general rumen fermentation parameters such as volatile fatty acids (VFA), ammonia (NH_3), protozoal population, and digestibility, or are more closely linked to broader production system characteristics including dietary structure, nutrient balance, and production performance. Although variations in milk CLA concentration, dietary structure, and nutrient balance have been reported (Anzhany *et al.*, 2022), their interactions within dairy systems producing contrasting CLA levels remain poorly understood.

Therefore, it is hypothesized that variation in milk CLA under field conditions is more strongly associated with feeding- and production-related variables, including dietary structure, nutrient balance, digestibility, and milk production performance, rather than with general rumen fermentation intensity. This study aimed to compare dietary structure, nutrient balance, rumen fermentation, and production performance between dairy systems producing high- and low-CLA milk, with emphasis on feeding practices, nutrient adequacy, *In vitro* rumen fermentability, and milk production characteristics.

MATERIALS AND METHODS

Milk and feeds sample collections

A total of 269 milk samples were collected from 30 dairy farmers affiliated with KPS Bogor (Bogor Dairy Farmers Cooperative) in Kunak Cibungbulang and Kebon Pedes (lowland; <200 m above sea level; THI 72–84) and 31 farmers affiliated with KPBS (South Bandung Dairy Farmers Cooperative) in Pangalengan (highland; >1000 m above sea level; THI 64–72), West Java Province, Indonesia. Sampling was conducted during the dry, rainy, and transition seasons. Approximately 500 mL of milk was collected from each cow and cooled prior to analysis.

Milk composition, including fat, protein, SNF, and lactose, was analyzed using a Milkotronic milk analyzer (serial I-17-817, Bulgaria). Milk fatty acid (FA) composition, including conjugated linoleic acid (CLA), was determined using an Agilent GC-7820A/G4350B gas chromatograph equipped with a flame ionization detector (FID) and an HP-5 capillary column (30 m × 0.320 mm × 0.25 μm; Agilent Technologies, Palo Alto, CA, USA) following Despal *et al.* (2021).

Based on CLA concentration, 15 samples with the highest and 15 with the lowest CLA levels were selected. Each selected sample represented a different individual cow, and no cow was included more than once in the final high-CLA or low-CLA dataset. The associated feeding practices were identified, and corresponding feed samples were collected for nutrient composition, *In vitro* fermentation, and digestibility analyses. Feed offerings were classified into forage, concentrate, soybean waste, and by-product categories to characterize dietary structure.

Measurement of feed composition and nutrient balance

Feed nutrient composition, including dry matter (DM) and crude protein (CP), was analyzed using a Buchi NIRFlex N-500 Solids Cell spectrometer (Buchi, Switzerland) with calibration based on a locally developed feed database. Total digestible nutrients (TDN) were estimated using the equation proposed by Indah *et al.* (2020): TDN (%) = 49.87 – (3.49 × ash) + (0.112 × ash × CP) + (0.056 × ash × NFE), where CP denotes crude protein and NFE denotes nitrogen-free extract.

Nutrient balance was calculated as the difference between nutrient supply and animal requirements according to the National Academies of Sciences, Engineering, and Medicine (NASEM, 2021). Nutrient requirements were calculated individually for each cow based on NASEM (2021) equations using body weight, lactation stage, milk production, milk fat percentage, and growth condition as calculation inputs, while nutrient supply was estimated from the actual ration consumed by each cow.

Feed fermentability and digestibility

Feed fermentability and digestibility were evaluated using the two-stage *In vitro* method of Tilley and Terry (1963), consisting of rumen fermentation followed by pepsin-HCl digestion. Rumen fluid was collected from three fistulated Friesian Holstein cattle fed a standardized diet of 50% Napier grass and 50% commercial concentrate, composited prior to incubation, and used consistently across all incubation runs. For fermentability measurements, samples were incubated with rumen fluid for 4 hours. After incubation, 1 mL was collected for protozoal enumeration, while the remaining contents were used for pH measurement. Samples were centrifuged at 3000 rpm for 15 minutes, and the supernatant was preserved with two drops of saturated $HgCl_2$ for ammonia and total volatile fatty acid (VFA) analyses. Rumen fluid pH was measured after 4 hours using a calibrated pH meter (standard buffers pH 4 and 7) prior to $HgCl_2$ addition.

Fermentability was evaluated based on ammonia and total VFA concentrations. Ammonia was determined using the Conway microdiffusion method, while total VFA was measured by steam distillation following (Anzhany *et al.*, 2024).

Dry matter digestibility (DMD) and organic matter digestibility (OMD) were determined according to Tilley and Terry (1963). Samples were incubated anaerobically for 48 hours, centrifuged at 3000 rpm for 15 minutes after $HgCl_2$ addition, and the supernatant was discarded. Residues were then digested with 50 mL of 0.2% pepsin-HCl and incubated aerobically for another 48 hours. The residues were filtered and oven-dried at 105 °C for 24 hours to determine dry matter, followed by ashing at 650 °C for 4 hours to determine organic matter residues.

Total protozoal population was determined using the staining method of Ogimoto and Imai (1981). One milliliter of rumen fluid was mixed with 1 mL of trypan blue formal saline (TBFS), placed on a Fuchs-Rosenthal counting chamber, covered with a cover glass, and observed under a microscope at 40× magnification.

Research design and data analysis

This study combined field-based exploratory research and laboratory experiments. The field study used a survey approach with cluster sampling, while the laboratory study employed an *In vitro* technique using a randomized block design. Data were analyzed using independent samples t-tests to compare high- and low-CLA milk groups in SPSS version 27. Principal component analysis (PCA) was conducted after variable standardization using the prcomp function in R version 4.4.2 (RStudio). The first two principal components (PC1 and PC2) were retained and visualized using grouped biplots with 95% confidence ellipses. Pearson correlation analysis was also performed and visualized using the corrplot package, with only statistically significant correlations (95% confidence level) presented.

RESULTS

Dietary structure of high- and low-CLA producing diets

The dietary structure of high-CLA and low-CLA producing systems is presented in Table 1. The proportion of forage was numerically lower in the high-CLA group, while concentrate proportion showed the opposite pattern, although differences were not statistically significant ($p > 0.05$). Forage proportion did not differ significantly between groups; however, wide ranges were observed within both the high-CLA (12.82–82.64%) and low-CLA (16.80–83.34%) systems. Soybean waste inclusion was also numerically higher in the high-CLA group (p= 0.082), whereas by-product proportion did not differ between groups ($p > 0.05$). Overall, the dietary composition was generally comparable between high-CLA and low-CLA producing systems.

Nutrient balance of high-CLA and low-CLA diets

Nutrient offers, requirements, and balances of high-CLA and low-CLA diets are presented in Table 2. Dry matter (DM) and total digestible nutrients (TDN) offers were significantly lower in the high-CLA group than in the low-CLA group ($p < 0.05$), whereas protein offer did not differ between groups ($p > 0.05$). Dry matter requirements were similar between groups ($p > 0.05$). Protein and TDN requirements were numerically higher in the high-CLA group, although differences were not statistically significant ($p > 0.05$). Nutrient balance differed significantly between groups. The high-CLA group showed negative balances for DM, protein, and TDN, whereas the low-CLA group showed positive balances for all parameters ($p < 0.05$).

Table 1: Dietary structure of high- and low-CLA producing diets.

Parameter	High CLA (n=15)	Low CLA (n=15)	SEM	p value
Diet composition (% DM)		-		
Forage	50.64	61.92	6.26	0.214
Concentrate	37.38	29.58	4.20	0.200
Soybean waste	(27.14)	(14.18)	(5.08)	(0.082)
By-product feeds	11.97	8.50	4.85	0.618

Note: CLA = conjugated linoleic acid; DM = dry matter, SEM = standard error mean; Soybean waste was included within the concentrate/by-product category.

Table 2: Nutrient balance of high-CLA and low-CLA diets.

Parameter	High CLA (n=15)	Low CLA (n=15)	SEM	p value
Nutrient supply, kg/d				
Dry matter	11.79	15.70	1.04	0.047
Protein	1.52	1.91	0.16	0.167
Total digestible nutrient	7.84	10.42	0.69	0.049
Nutrient requirement, kg/d				
Dry matter	13.89	13.62	0.31	0.696
Protein	1.83	1.64	0.05	0.056
Total digestible nutrient	8.42	7.67	0.22	0.057
Nutrient balance				
Dry matter	-2.10	2.08	1.91	0.037
Protein	-0.31	0.27	0.27	0.039
Total digestible nutrient	-0.59	2.76	1.31	0.016

Note: Values are presented as means ± SEM. Differences between high-CLA and low-CLA groups were analyzed using independent samples t-test. Significance was declared at $p < 0.05$.

In vitro fermentation and digestibility parameters

The *in vitro* fermentation and digestibility parameters of high-CLA and low-CLA diets are presented in Table 3. Rumen pH was significantly higher in the high-CLA group than in the low-CLA group ($p < 0.05$). In contrast, protozoa population, ammonia (NH_3), and total volatile fatty acid (VFA) concentrations did not differ between groups ($p > 0.05$).

Table 3: *In vitro* fermentation and digestibility parameters of high-CLA and low-CLA diets.

Parameter	High CLA	Low CLA	SEM	p value
pH	6.84	6.79	0.010	0.03
Protozoa (log cell/mL)	6.26	6.24	0.020	0.60
NH_3 (mM)	7.96	8.41	0.373	0.30
Total VFA (mM)	117.20	117.01	1.753	0.94
Dry matter digestibility (%)	65.08	62.41	1.010	0.06
Organic matter digestibility (%)	64.57	61.31	1.034	0.03

Note: *In vitro* data were analyzed using a randomized block design with CLA group as the fixed effect and incubation run as the blocking factor. Values are means ± SEM. Significance was declared at $p < 0.05$.

Dry matter digestibility was numerically higher in the high-CLA group but did not reach statistical significance (65.08% vs. 62.41%; $p = 0.06$). Organic matter digestibility was higher in the high-CLA group (64.57% vs. 61.31%; $p = 0.03$), although this finding should be interpreted cautiously because multiple comparisons were performed. Overall, fermentation characteristics were largely similar between groups, except for rumen pH and organic matter digestibility.

Milk production, composition, and component yield

Milk production, milk composition, and component yields of high-CLA and low-CLA groups are presented in Table 4. Milk production was numerically higher in the high-CLA group, although the difference was not statistically significant ($p > 0.05$). Milk composition parameters, including fat, solid non-fat (SNF), lactose, and protein percentages, did not differ between groups ($p > 0.05$). In contrast, CLA concentration (% of milk fat) was significantly higher in the high-CLA group ($p < 0.001$).

Fat yield was significantly higher in the high-CLA group ($p < 0.05$), while CLA yield was markedly higher ($p < 0.001$). The higher CLA yield reflected both greater milk fat yield and substantially higher CLA concentration (% of milk fat), indicating that the increase was not solely attributable to greater fat production. Other component yields, including SNF, lactose, and protein were numerically higher in the high-CLA group, although differences were not statistically significant ($p > 0.05$).

Principal component analysis and correlation of variables associated with CLA variation

Principal component analysis (PCA) showed partial differentiation between high-CLA and low-CLA groups along PC1 and PC2 (Figure 1). PC1 and PC2 explained 26.2% and 21.5% of the total variance, respectively. The high-CLA group was associated with milk production variables, particularly fat and CLA yields, as well as higher organic matter digestibility (OMD). In contrast, the low-CLA group was more closely associated with nutrient supply variables, including dry matter (DM) and total digestible nutrients (TDN). General rumen fermentation parameters, including ammonia (NH_3), total volatile fatty acids (VFA), and protozoa population, were located near the center of the plot, indicating limited contribution to group separation. Rumen pH was numerically higher in the high-CLA group, but the magnitude of difference was small (6.84 vs. 6.79; $p = 0.03$).

Table 4: Milk production and composition of high-CLA and low-CLA diets.

Parameter	High CLA (n=15)	Low CLA (n=15)	SEM	p value
Milk production (kg/d)	15.67	13.93	1.07	0.116
Milk component				
Fat, % milk	3.93	3.66	0.33	0.409
SNF, % milk	7.55	7.55	0.15	0.993
Lactose, % milk	4.17	4.14	0.08	0.797
Protein,% milk	2.78	2.76	0.05	0.763
CLA, % fat	2.91	0.71	0.13	<0.001
Milk component production				
Fat yield (g/d)	606.42	499.88	50.17	0.043
SNF yield (g/d)	1177.23	1047.47	73.55	0.089
Lactose yield (g/d)	649.62	575.35	40.14	0.075
Protein yield (g/d)	433.59	383.63	26.68	0.072
CLA yield (g/d)	17.74	3.50	1.57	<0.001

Note: Values are presented as means ± SEM. Differences between high-CLA and low-CLA groups were analyzed using independent samples t-test. Significance was declared at $p < 0.05$.

Correlation analysis showed that CLA concentration was positively correlated with CLA and fat yields (Figure 2). CLA yield was also positively correlated with milk component yields, including protein, lactose, and solid non-fat (SNF). In contrast, CLA concentration and yield were negatively correlated with nutrient balance parameters, particularly DM and TDN. No significant correlations were observed between CLA variables and rumen fermentation parameters, including NH_3, VFA, and protozoa population. Rumen pH showed a positive but relatively weak correlation with CLA.

DISCUSSION

The results of Table 1 indicate that overall dietary structure was broadly comparable between high- and low-CLA systems, as shown by the absence of significant differences in forage, concentrate, and by-product proportions. However, forage proportion varied widely within both groups, indicating substantial heterogeneity in practical feeding systems that may not be fully reflected by group averages. These findings suggest that milk CLA variation under field conditions was not determined solely by forage proportion or major differences in diet composition. Similar observations were reported by Despal *et al*. (2021), who found that fiber feed quality did not directly determine milk fatty acid composition.

Despite the lack of statistical significance, the high-CLA group received numerically less forage and more concentrate, consistent with Anzhany *et al*. (2022), who reported that rations containing 40% forage and 60% concentrate produced the highest milk CLA. The tendency for greater soybean waste inclusion in the high-CLA group may also be relevant because soybean by-products contain residual unsaturated fatty acids that serve as substrates for rumen biohydrogenation and CLA formation (Almeida *et al*., 2013; Martha *et al*., 2025; Loor and Herbein, 2003; O'Donnell-Megaro *et al*., 2012). Increased milk CLA following soybean supplementation has been reported previously (Kim *et al*., 2024; Rabiee *et al*., 2012), although excessive unsaturated fatty acid supply may also increase trans-10, cis-12 CLA associated with milk fat depression (Griinari, 2007). Therefore, subtle differences in lipid composition and feed quality may contribute to CLA variation even when overall dietary structure appears similar. Differences in forage fatty acid profile across altitudes have also been reported to influence rumen biohydrogenation pathways (Anzhany *et al*., 2024).

The nutrient balance results (Table 2) revealed a clear contrast between systems. The high-CLA group showed negative balances of dry matter (DM), crude protein (CP), and total digestible nutrients (TDN), whereas the low-CLA group showed positive balances. Similar observations were reported by Hötger *et al*. (2013), although contrasting findings have also been described (Nardone *et al*., 2010; Shingfield *et al*., 2009). Lower DM and TDN supply in the high-CLA group suggests reduced nutrient intake relative to production demands, which may indicate possible body reserve mobilization, although this was not directly confirmed by metabolic indicators. If body reserve mobilization occurred, it may have increased circulating long-chain fatty acids, including vaccenic acid precursors that can be converted to cis-9, trans-11 CLA in the mammary gland via Δ9-desaturase activity (Esposito *et al*., 2014; Guo *et al*., 2026; Griinari *et al*., 2000).

In contrast, the positive nutrient balance observed in the low-CLA group was associated with greater nutrient supply and likely higher concentrate feeding. High-concentrate diets promote rapid fermentation and lower rumen pH

(Li *et al*., 2014; Anzhany *et al*., 2022), altering microbial populations and favoring more complete biohydrogenation of unsaturated fatty acids into stearic acid (Fernando *et al*., 2010; Petri *et al*., 2013; Hassim *et al*., 2010; Jenkins *et al*., 2008). Under such conditions, vaccenic acid accumulation is reduced, limiting endogenous CLA synthesis in the mammary gland (Bauman and Griinari, 2003; Shingfield *et al*., 2013).

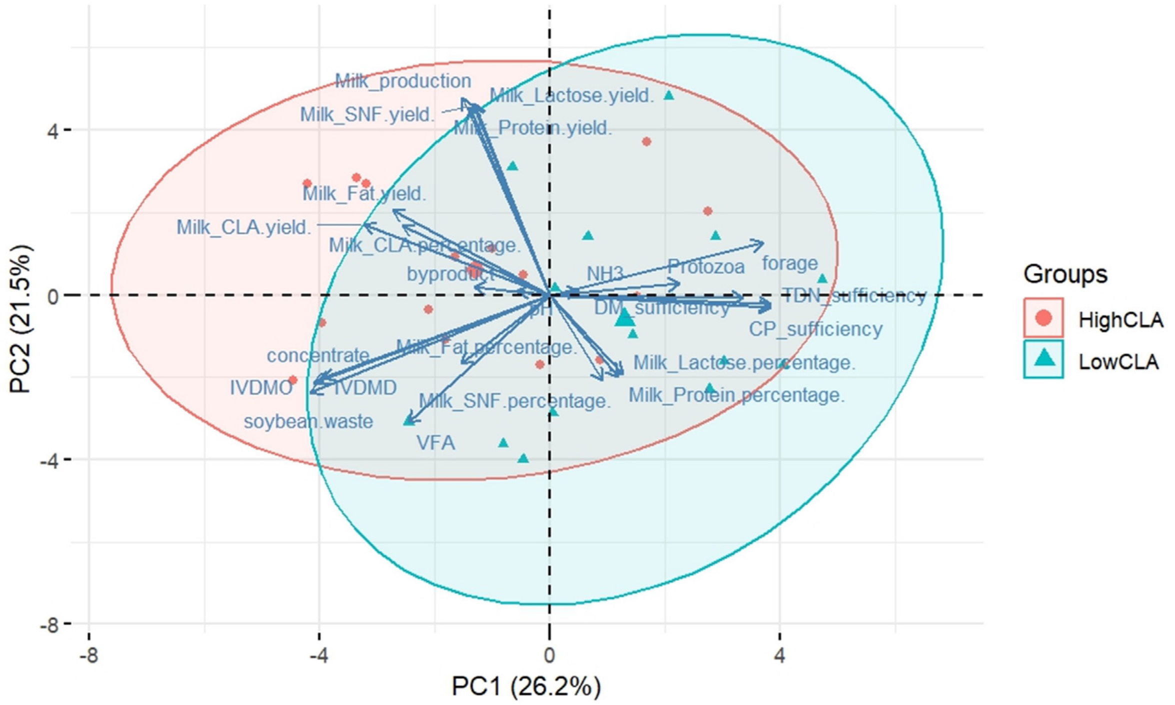

Figure 1: Principal component analysis (PCA) biplot.

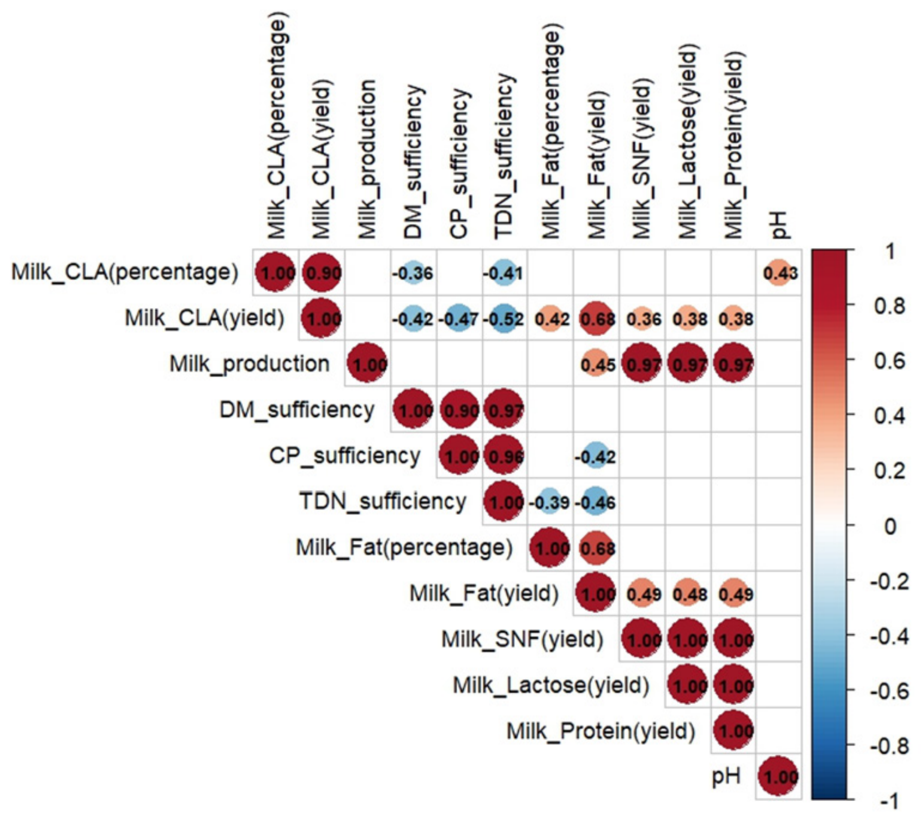

Figure 2: Pearson correlation matrix between CLA (concentration and yield) and selected nutrient, fermentation, and production parameters. Only significant correlations ($p < 0.05$) are displayed, with correlation coefficients (r values) presented within the circles. CLA% represents CLA concentration in milk fat, whereas CLA yield (g/d) represents daily CLA production.

This interpretation is consistent with the fermentation results in Table 3, where the low-CLA group showed slightly lower rumen pH. Although the difference remained within the physiological range, even moderate pH reductions can alter microbial activity and lipid metabolism (Krause and Oetzel, 2006; Fernando *et al*., 2010; Jenkins *et al*., 2008). However, most fermentation parameters, including NH_3, total VFA, and protozoa population, did not differ between groups, indicating that overall fermentation intensity was broadly similar. Similar findings have been reported previously (Jenkins *et al*., 2008; Lourenço *et al*., 2005; Valizadeh *et al*., 2021; Anzhany *et al*., 2022). These results suggest that CLA variation is more closely related to specific lipid biohydrogenation pathways than to general fermentation output. However, specific biohydrogenation intermediates such as vaccenic acid and stearic acid were not directly measured in the present study; therefore, the involvement of specific lipid metabolism pathways remains inferential.

Organic matter digestibility (OMD) was higher in the high-CLA group, while dry matter digestibility (DMD) showed a similar trend. Higher digestibility is commonly associated with improved nutrient synchronization and more efficient microbial utilization (Despal *et al*., 2021). This may partly explain why the high-CLA group maintained milk production despite negative nutrient balance, possibly through differences in nutrient utilization efficiency and metabolic status (Roche *et al*., 2009; Huhtanen *et al*., 2007; Dijkstra *et al*., 2012). Reduced heat stress in highland areas may also have contributed to differences in feed efficiency and metabolic status (West, 2003).

The production results (Table 4) showed that milk yield was numerically higher in the high-CLA group without reductions in fat, protein, lactose, or SNF percentages. Normally, higher milk production is associated with dilution of milk components (Bauman and Griinari, 2003), but this pattern was not observed here, suggesting relatively efficient nutrient utilization, consistent with Anzhany *et al*. (2022). Environmental differences may also contribute, as the high-CLA group was predominantly located in highland areas with lower ambient temperatures, whereas cows in lowland systems are more exposed to heat stress and greater maintenance energy expenditure (Despal *et al*., 2021). Seasonal distribution differed between the high- and low-CLA groups, with most high-CLA samples collected during the dry season, indicating that seasonal and environmental conditions may also contribute to the observed variation in milk CLA concentration. Previous studies conducted in the same production areas also demonstrated altitude-related differences in forage fatty acid composition, rumen biohydrogenation characteristics, and milk fatty acid profiles (Anzhany *et al*., 2024), indicating that environmental conditions may interact with nutrient balance and feeding systems in determining milk CLA variation.

The significantly higher fat and CLA yields in the high-CLA group reflected both greater milk production and higher CLA concentration. Similar findings were reported by Riestanti *et al*. (2021). Interestingly, these results occurred despite negative nutrient balance, suggesting compensatory mechanisms such as improved digestibility and enhanced nutrient extraction. Lower feed intake may reduce digesta passage rate and increase rumen retention time, improving microbial attachment and feed degradation (Nichols *et al*., 1998; Rodríguez-Prado *et al*., 2004; Aharoni *et al*., 2004).

However, prolonged negative energy balance has been associated with excessive body reserve mobilization and metabolic disorders in dairy cows according to previous studies (Martens, 2023; Asl *et al*., 2011), however, these responses were not directly evaluated in the present study. Therefore, although negative nutrient balance was associated with higher CLA concentration, intentionally inducing negative nutrient balance cannot be recommended. Nutritional approaches aimed at improving energy supply while maintaining rumen stability may be beneficial (Riestanti *et al*., 2021). The lower dry matter intake combined with maintained milk production may indicate possible mobilization of body reserves; however, body condition score, body weight change, NEFA, and β-hydroxybutyrate were not measured in the present study, and therefore this interpretation remains speculative.

The PCA and correlation analyses further supported these findings. High-CLA systems were associated with milk production variables, CLA yield, and digestibility, whereas low-CLA systems were associated with higher nutrient supply variables. General fermentation parameters contributed little to group separation, suggesting that overall fermentation intensity alone could not explain the observed CLA variation. Similar relationships between dietary lipid supplementation, milk production, and energy efficiency have been reported previously (Bauman and Griinari, 2003; Razzaghi *et al*., 2022; Stoop *et al*., 2009; Huhtanen and Hristov, 2009).

Correlation analysis showed positive associations between CLA and milk component yields, indicating that higher CLA production was aligned with improved milk output rather than reduced productivity. In contrast, CLA concentration and yield were negatively correlated with nutrient balance parameters, supporting the hypothesis that negative nutrient balance may co-occur with increased CLA formation (Bauman and Griinari, 2003). No significant correlations were observed between CLA and NH_3, VFA, or protozoa population, reinforcing the limited role of general fermentation parameters in explaining CLA variation (Jenkins *et al*., 2008).

A positive correlation between CLA and rumen pH was also observed. Although slightly higher pH conditions may theoretically favor incomplete biohydrogenation and greater accumulation of intermediates such as vaccenic acid through altered microbial activity and lipid metabolism (Jenkins *et al*., 2008), the results should be interpreted cautiously because the *In vitro* system used buffered rumen fluid. Therefore, the relatively narrow pH range observed in this study suggests that CLA variation was more strongly associated with integrated production system characteristics than with pH alone.

Collectively, these results indicate that differences in milk CLA among farms were associated more closely with nutrient balance, digestibility, and environmental production conditions than with general rumen fermentation characteristics.

CONCLUSION

Under the field conditions of the present study, CLA variation was more consistently associated with nutrient balance, digestibility, and production system characteristics than with general rumen fermentation parameters alone. High-CLA systems were characterized by negative nutrient balance and higher digestibility, suggesting differences in nutrient utilization and metabolic status. These findings indicate that strategies to increase milk CLA should focus on maintaining adequate nutrient supply and favorable rumen lipid metabolism without inducing prolonged negative energy balance. Further research is needed to better understand rumen biohydrogenation pathways, particularly the roles of specific microbial populations and lipid intermediates involved in CLA formation, to support the development of sustainable nutritional strategies for enhancing milk CLA.

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NOVELTY STATEMENT

This study provides a novel comparison between dairy systems producing high- and low-CLA milk in tropical smallholder farms by evaluating nutrient balance, rumen fermentability, and digestibility of the diets associated with contrasting milk CLA concentrations. The study offers new insights into how feeding and nutrient utilization characteristics are related to natural variation in milk CLA under practical tropical dairy farming conditions.

AUTHOR'S CONTRIBUTION

Despal, Wulansih Dwi Astuti, and Irma Isnafia Arief conceived and supervised the study and secured research funding. Dwitami Anzhany, Rika Zahera, Muhammad Naufal Farras, and Fandini Meilia Anjani conducted sample collection, laboratory analyses, and data acquisition. Dwitami Anzhany, Rika Zahera, Muhammad Naufal Farras, and Fandini Meilia Anjani performed data analysis and visualization. Despal, Dwitami Anzhany, and Rika Zahera prepared the original manuscript draft. Wulansih Dwi Astuti and Irma Isnafia Arief critically reviewed and edited the manuscript. All authors contributed to manuscript revision, validated the results, and approved the final version of the manuscript.

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Ethical approval

Rumen cannulation surgery for animals used as rumen fluid donors in the *In vitro* study was performed by a licensed veterinarian in accordance with animal handling and care protocols approved by the IPB University Animal Ethics Committee (Ethical Clearance No. 395/KEH/SKE/XI/2025). Milk and feed samples were collected by trained students under the supervision of cooperative extension workers in their respective working areas.

Generative AI and AI-assisted technology statement

The authors declare that generative AI and AI-assisted technologies were used solely for language improvement and grammar checking. All scientific content, data analysis, interpretation of results, and conclusions were developed and verified by the authors, who take full responsibility for the content of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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