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Contrasting Patterns in Rumen Fermentation, Digestibility, and Fatty Acid Biohydrogenation with Increasing Levels of Protected Coconut and Palm Fatty Acid Distillates

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ABSTRACT

Tropical dairy systems face energy deficits from low-quality forages and high rumen fermentation heat, while interest grows in improving milk fatty acid composition, particularly CLA precursors; however, intensive biohydrogenation limits beneficial unsaturated fatty acids. This study evaluated the dose–response effects of coconut (CFAD) and palm (PFAD) fatty acid distillates on rumen fermentation, digestibility, and fatty acid biohydrogenation. An *in vitro* 2 × 5 factorial design was applied, comprising two FAD types and five inclusion levels (0%–4% DM) with 12 replicates. Fermentation characteristics, nutrient digestibility, microbial activity, and biohydrogenation indices were measured, and polynomial regression was used to determine optimal inclusion levels. CFAD exhibited stronger antimicrobial and defaunating effects, resulting in higher total VFA production, improved NH_3 utilization, enhanced microbial protein synthesis, and a more favorable unsaturation pattern at moderate inclusion levels, although it reduced *trans-11* accumulation. In contrast, PFAD maintained more stable fermentation and promoted greater accumulation of *trans-11* and CLA precursors, despite increasing methane-related pathways at higher inclusion levels. Overall, CFAD (2.5%–3% DM) is more suitable for improving fermentation efficiency, whereas PFAD (1.5%–2% DM) is more appropriate when the objective is to enhance CLA-related intermediates.

Keywords: coconut and palm fat supplements; fatty acid biohydrogenation; in vitro fermentation; protected fatty acid distillates; rumen fermentation

INTRODUCTION

Tropical dairy production systems are constrained by the low energy density and limited digestibility of tropical forages, which reduce fermentable energy supply to rumen microbes (Despal *et al.*, 2021a). Although these feeds support milk fat synthesis through acetate and butyrate production (Malekkhahi *et al.*, 2023), they also increase methane formation, leading to energy loss and environmental concerns (Tekin & Kara, 2020; da Cruz *et al.*, 2021). These limitations are exacerbated under tropical conditions, where high ambient temperatures increase maintenance energy requirements and reduce feed intake, widening the gap between nutrient supply and demand (Polsky & von Keyserlingk, 2017; Sammad *et al.*, 2020). At the same time, growing interest in improving milk fat quality, particularly beneficial fatty acids such as conjugated linoleic acid (CLA), highlights the need for strategies that enhance both rumen efficiency and fatty acid composition (Despal *et al.*, 2021b).

Fat supplementation is a practical approach due to its high energy density and low heat increment (Riestanti *et al.*, 2024). Fatty acid distillates (FADs), particularly coconut (CFAD) and palm (PFAD), are abundant and economical lipid sources in tropical regions (Zahera *et al.*, 2024). When protected as calcium salts, these fats are intended to reduce, rather than completely prevent, ruminal metabolism. Consequently, a proportion of the fatty acids may still interact with rumen microorganisms and participate in fermentation and fatty acid metabolism, although at a slower rate than unprotected fats or free fatty acids (Riestanti *et al.*, 2024). Their contrasting fatty acid compositions may lead to different microbial responses, as CFAD is rich in medium-chain fatty acids with strong antimicrobial effects, whereas PFAD contains long-chain fatty acids with generally milder antimicrobial effects on rumen

microorganisms but different effects on hydrogen flow and metabolic pathways (Riestanti *et al.,* 2021; Zahera *et al.,* 2024). These differences are particularly important because rumen fatty acid biohydrogenation determines the formation of CLA and its precursor, vaccenic acid, as intermediates of unsaturated fatty acid metabolism (Anzhany *et al.,* 2024). Therefore, changes in microbial activity and hydrogen utilization induced by different fat sources can directly influence CLA formation and overall milk fatty acid quality.

Despite their potential, direct comparisons between CFAD and PFAD, particularly regarding dose–response effects on rumen fermentation, digestibility, and fatty acid biohydrogenation, remain limited. Therefore, this study aimed to evaluate the effects of CFAD and PFAD supplementation at different inclusion levels on rumen fermentation characteristics, nutrient digestibility, and fatty acid biohydrogenation *in vitro*, and to determine their optimal supplementation levels for tropical dairy diets.

MATERIALS AND METHODS

Ethical Approval

All procedures involving the collection and use of rumen fluid from fistulated cattle were reviewed and approved by the Animal Ethics Committee, School of Veterinary Medicine and Biomedical Sciences, IPB University (Approval No. 395/KEH/SKE/XI/2025).

Sample Preparation

Coconut and palm fatty acid distillates used in this experiment were sourced from the Indonesian refining industry. The fatty acid profiles of CFAD and PFAD differ markedly in chain length distribution (Table 1). CFAD was dominated by medium-chain fatty acids, particularly lauric acid (C12:0; 50.42%) and myristic acid (C14:0; 19.82%), followed by capric acid (C10:0; 5.94%) and palmitic acid (C16:0; 9.29%), with relatively low levels of unsaturated fatty acids such as oleic acid (C18:1 cis; 5.63%) and linoleic acid (C18:2 cis; 1.55%). In contrast, PFAD was primarily composed of long-chain fatty acids, mainly palmitic acid (C16:0; 40.43%) and oleic acid (C18:1 cis; 33.77%), followed by linoleic acid (C18:2 cis; 19.30%) and stearic acid (C18:0; 3.20%), indicating a higher proportion of long-chain and unsaturated fatty acids compared to CFAD. Saponification of CFAD and PFAD with $CaCO_3$ was carried out using a double-decomposition approach (Zahera *et al.,* 2024). Fatty acid distillate was heated until fully melted and saponified with NaOH. Subsequently, $CaCO_3$ dispersed in distilled water was added under continuous heating (70–90 °C) and stirring to facilitate the exchange of Na^+ with Ca^{2+}, forming calcium soap. The mixture was heated until most of the water evaporated, then dried, ground, and stored for further analysis. A molar ratio of 2:1 between $CaCO_3$ and Free Fatty Acid (FFA) was applied to ensure the final product reached an acid value below 0.75 mg KOH/g. The acid value was used as an indicator of calcium soap formation, with values below 0.75 mg KOH/g considered indicative of successful saponification.

The dairy ration was prepared using a 40:60 forage-to-concentrate balance, as recommended by Anzhany *et al.* (2022) to enhance CLA-enriched milk production. The formulated diet consisted of 65.53% DM, 11.60% ash, 3.70% ether extract, 14.30% crude protein, 21.41% crude fiber, and 65.24% total digestible nutrients. Rumen fluid was collected from three rumen-fistulated Friesian Holstein cows housed in a closed dairy nutrition facility at the Faculty of Animal Science, IPB University, prior to morning feeding.

In Vitro Fermentability and Digestibility

In vitro fermentability and digestibility were evaluated using a modified two-stage Tilley and Terry (1963)

Table 1. Fatty acid composition of coconut and palm fatty acid distillate

Fatty acid composition (%)	Fatty acid distillate	
	Coconut	Palm
C4:0	0.007	ND
C6:0	ND	ND
C8:0	0.004	ND
C10:0	5.938	ND
C11:0	0.019	ND
C12:0	50.424	ND
C13:0	0.015	ND
C14:0	19.818	0.579
C14:1	0.002	ND
C15:0	0.019	ND
C15:1	0.004	ND
C16:0	9.292	40.430
C16:1	0.034	0.179
C17:0	0.011	0.062
C17:1	0.524	0.021
C18:0	1.881	3.196
C18:1 trans	0.042	0.022
C18:1 cis	5.630	33.767
C18:2 trans	0.015	0.119
C18:2 cis	1.547	19.296
C20:0	0.026	0.222
C18:3n6	0.004	0.020
C20:1	0.026	0.105
C18:3n3	0.015	0.939
C21:0	0.006	0.003
C20:2	0.009	0.009
C22:0	3.449	0.031
C20:3n6	ND	0.014
C22:1n9	0.007	0.004
C20:3n3	0.013	0.010
C20:4n6	0.004	0.949
C23:0	0.006	0.005
C22:2n6	ND	0.008
C24:0	0.006	ND
C20:5n3 (EPA)	0.020	ND
C24:1	1.161	ND
C22:6 (DHA)	0.020	0.010

Note: ND (Not Detected)

procedure, comprising rumen fermentation followed by pepsin–HCl digestion. Each 0.5 g sample was incubated anaerobically at 39 °C with 10 mL of rumen fluid and 40 mL of McDougall's buffer, using five serial tubes per experimental unit. One tube was fermented for 4 h to analyze total VFA, partial VFA, ammonia, protozoa and bacterial counts, and microbial protein synthesis, as this incubation time represents the peak of microbial fermentation activity for most feedstuffs. Two tubes (0 h and 48 h) were designated for biohydrogenation assessment, while the remaining tubes were incubated for 48 h and then digested for 48 h in pepsin–HCl to obtain residues for determining dry matter digestibility (DMD), organic matter digestibility (OMD), neutral detergent fiber digestibility (NDFD), and acid detergent fiber digestibility (ADFD). Rumen pH was measured with a digital pH meter (Hanna HI98191), ammonia concentration was quantified using Conway microdiffusion, and total VFA concentration was determined via steam distillation following a similar method used by Anzhany *et al.* (2024). Total protozoa and bacterial populations were measured using the method of Ogimoto and Imai (1981). Microbial protein synthesis (MPS) was determined using a modified Lowry method as described by Rosmalia *et al.* (2022).

Partial VFA profiles were determined using gas chromatography (Bruker Scion 436-GC equipped with a Bruker-1ms capillary column). Samples were prepared according to standard VFA analytical procedures, and individual VFA peaks were identified and quantified using a certified reference standard (Supelco Volatile Free Acid Mix). Methane production was estimated from the molar proportions of acetate (C2), propionate (C3), and butyrate (C4) using the equation of Moss *et al.* (2000): CH_4 (mM) = 0.45C2 – 0.275 C3 + 0.40 C4.

Rumen C18 fatty acid profiles were analyzed using gas chromatography (GC-7820A, Agilent Technologies) following the procedure of Anzhany *et al.* (2024). Biohydrogenation indices were calculated from changes in fatty acid concentrations between 0 and 48 h. Apparent biohydrogenation of individual and total C18 PUFA was based on proportional loss during incubation, following Vlaeminck *et al.* (2008) with minor modifications. Indices describing incomplete biohydrogenation and CLA yield (intermediate accumulation and CLA formed relative to C18:2 cis disappearance) were adapted from Kalscheur *et al.* (1997) and Cruz-Hernandez *et al.* (2007). Saturation (SFA : total FA ratio) and unsaturation indices (weighted average number of double bonds per FA) were calculated as in Jalc *et al.* (2007). Trans/cis isomerase indices for C18:1 and C18:2 followed the trans/cis ratios used by Kramer *et al.* (2004) and Toral *et al.* (2024). Total C18 recovery was calculated as the ratio of total C18 FA at 48 h to 0 h, similar to Harvatine and Allen (2006).

Hydrogen production, consumption, balance, and recovery were calculated from VFA stoichiometry following the approach of Moss *et al.* (2000). Net H_2 from VFA, H_2 consumed in methane, and H_2 balance were derived using standard hydrogen balance equations, and H_2 recovery was expressed as the proportion of metabolizable hydrogen accounted for by methane formation.

Research Design and Data Analysis

The study employed a 2 × 5 factorial randomized block design with 12 replications. Rumen fluid was collected from three fistulated cows, pooled, and used as the inoculum. Different collection times across incubation runs were treated as blocks. Factor A was the type of protected fatty acid distillate (coconut or palm), and Factor B was the supplementation level (0%, 1%, 2%, 3%, and 4%) in the dairy cattle diet. The measured parameters included rumen fermentability (pH, ammonia, and total and individual VFAs), nutrient digestibility (dry matter, organic matter, NDF, and ADF digestibility), estimated methane production, microbial populations (protozoa and bacteria), and biohydrogenation indices. Data were analyzed using ANOVA, and significant treatment effects were further assessed using Tukey's test. Polynomial contrasts were applied to describe response curves and identify the optimum supplementation levels. All statistical analyses were performed using SPSS version 27.

RESULTS

Fermentation Parameters

Table 2 summarizes the effects of CFAD and PFAD supplementation on rumen fermentation parameters, including pH, ammonia concentration, VFA profiles, and methane production. Rumen pH remained within the normal physiological range across all treatments and was not significantly affected by fat source or supplementation level. A significant FAD × level interaction was observed for NH_3 concentration. In the CFAD treatments, NH_3 decreased from 0% to 2% supplementation before rising sharply at 3%, whereas PFAD showed a more gradual decline up to 3%, followed by a slight increase at 4%. The contrast between fat sources was most pronounced at 2% and 3%, where CFAD resulted in significantly lower (2%) and higher (3%) NH_3 concentrations than PFAD.

Total VFA increased markedly with supplementation ($p<0.05$), with the highest values recorded at 2%–3% inclusion for both fat sources; CFAD consistently produced greater total VFA at these levels. Acetate (C2) was not significantly affected by treatment, although a numerical increase was apparent at 1%–3% inclusion; however, the high variation among replicates prevented this trend from reaching statistical significance. Propionate (C3) decreased at 1% and partially recovered at higher levels, resulting in a significantly elevated acetate-to-propionate ratio (C2:C3) at 1% and 4% ($p<0.05$).

The branched-chain VFA iC4 exhibited a significant interaction, with concentrations declining after 1% CFAD inclusion but remaining relatively high across PFAD levels. No treatment effects were detected for iC5. Straight-chain C4 and C5 were minimally affected, although PFAD yielded a higher mean butyrate (C4) than CFAD ($p<0.05$). Methane production increased from 0% to 1% supplementation ($p<0.05$), remained higher than the control at 3%, and rose again at 4%, with similar patterns for both fat sources.

Table 2. Rumen fermentation variables of dairy cattle rations supplemented with coconut fatty acid distillate (CFAD) and palm fatty acid distillate (PFAD) at different inclusion levels

Variables	Fatty acid distillate	Supplementation level (% DM)					Average
		0	1	2	3	4	
pH	Coconut	6.62±0.08	6.81±0.06	6.77±0.06	6.85±0.08	6.86±0.05	6.78±0.07
	Palm	6.62±0.08	7.00±0.07	6.82±0.05	6.89±0.06	6.87±0.06	6.84±0.07
	Average	6.62±0.08	6.90±0.07	6.80±0.06	6.87±0.07	6.87±0.06	
NH_3 (mM)	Coconut	8.07±0.19^{bc}	6.98±0.32^{abc}	5.63±0.39^{a}	8.81±0.50^{c}	8.04±0.28^{bc}	7.51±0.47
	Palm	8.07±0.19^{bc}	6.85±0.75^{abc}	7.06±0.60^{abc}	6.52±0.72^{ab}	7.69±0.48^{abc}	7.24±0.59
	Average	8.07±0.19	6.92±0.57	6.34±0.54	7.67±0.69	7.86±0.39	
Total VFA (mM)	Coconut	69.14±2.43	104.70±9.28	149.22±4.80	133.56±10.39	118.18±10.43	114.96±11.19
	Palm	69.14±2.43	92.43±12.03	119.37±11.53	112.17±10.12	129.91±6.49	104.60±10.92
	Average	69.14±2.38^{a}	98.56±10.66^{b}	134.30±9.70^{c}	122.86±10.52^{c}	124.05±8.67^{c}	
C2, mol%	Coconut	47.24±0.76	53.92±4.39	53.90±1.96	52.77±3.29	50.08±2.52	51.57±2.83
	Palm	47.24±0.76	51.44±2.46	50.60±3.91	49.15±3.22	55.76±3.67	50.75±3.04
	Average	47.24±0.75	52.81±3.58	52.25±3.06	50.96±3.22	53.06±3.21	
C3, mol%	Coconut	37.77±3.17	30.89±2.06	31.92±2.44	32.22±2.32	29.02±2.86	32.51±2.65
	Palm	37.77±3.17	28.01±1.70	31.57±2.37	30.35±1.93	28.51±2.19	31.49±2.50
	Average	37.77±3.10^{b}	29.59±1.91^{a}	31.74±2.35^{ab}	31.28±2.10^{ab}	28.75±2.47^{a}	
iC4, mol%	Coconut	1.71±0.10^{a}	2.08±0.20^{ab}	1.51±0.17^{a}	1.68±0.16^{a}	1.79±0.17^{ab}	1.75±0.17
	Palm	1.71±0.10^{a}	2.08±0.15^{ab}	2.60±0.14^{b}	2.22±0.19^{ab}	2.21±0.17^{ab}	2.17±0.17
	Average	1.71±0.10	2.08±0.17	2.06±0.22	1.95±0.19	2.01±0.17	
C4, mol%	Coconut	8.71±0.44	8.10±0.28	8.88±0.32	8.63±0.35	8.85±0.42	8.63±0.36^{a}
	Palm	8.71±0.44	9.00±0.29	8.62±0.43	9.47±0.46	9.58±0.53	9.06±0.44^{b}
	Average	8.71±0.43	8.50±0.31	8.75±0.37	9.05±0.42	9.23±0.48	
iC5, mol%	Coconut	5.46±0.14	5.92±0.34	5.73±0.14	5.13±0.16	5.32±0.21	5.52±0.22
	Palm	5.46±0.14	5.96±0.13	5.41±0.15	5.08±0.19	5.37±0.25	5.44±0.19
	Average	5.46±0.13^{ab}	5.93±0.26^{b}	5.57±0.15^{ab}	5.10±0.17^{a}	5.35±0.23^{a}	
C5, mol%	Coconut	1.32±0.05	1.76±0.25	1.37±0.06	1.29±0.07	1.29±0.06	1.41±0.13
	Palm	1.32±0.05	1.40±0.07	1.51±0.12	1.35±0.08	1.42±0.08	1.40±0.08
	Average	1.32±0.05	1.60±0.20	1.44±0.09	1.32±0.07	1.36±0.07	
C2/C3	Coconut	1.34±0.10	1.99±0.36	1.83±0.20	1.78±0.21	1.93±0.21	1.76±0.23
	Palm	1.34±0.10	1.98±0.23	1.67±0.14	1.65±0.08	2.10±0.24	1.73±0.18
	Average	1.34±0.10^{a}	1.99±0.30^{b}	1.75±0.17^{ab}	1.72±0.15^{ab}	2.02±0.22^{b}	
CH_4, mol/100 mol hexose fermented	Coconut	15.04±0.61	19.84±2.50	19.63±1.09	19.01±1.90	18.81±1.01	18.42±1.60
	Palm	15.04±0.61	19.88±1.38	18.58±1.60	18.45±1.23	21.97±1.80	18.67±1.49
	Average	15.04±0.60^{a}	19.86±2.02^{b}	19.10±1.35^{ab}	18.73±1.56^{ab}	20.47±1.52^{b}	

Note: Means ± SEM. Different superscripts denote significant differences ($p < 0.05$). Main effects were tested using marginal means: fat source compares only the overall means of CFAD vs. PFAD; supplementation level compares only the five overall means for 0–4%. Interaction effects: superscripts within treatment cells (2 × 5) indicate significant fat source × level interactions. CH_4 is expressed as mol/100 mol of hexose fermented. Abbreviations: CFAD = Coconut Fatty Acid Distillate; PFAD = Palm Fatty Acid Distillate; NH_3 = ammonia; VFA = volatile fatty acids; C2 = acetate; C3 = propionate; iC4 = isobutyrate; C4 = butyrate; iC5 = isovalerate; C5 = valerate; CH_4 = methane.

Microbial Populations and Microbial Protein Synthesis

Table 3 presents the responses of total bacterial and protozoal populations, along with microbial protein synthesis (MPS), to different levels of CFAD and PFAD supplementation. Total bacterial and protozoal counts decreased progressively with increasing supplementation but remained comparable to the control up to 2%, with both fat sources showing similar patterns. Neither microbial population was significantly affected by the type of fat. In contrast, a significant FAD × supplementation level interaction was observed for MPS. Under CFAD, MPS declined from 0% to 2% inclusion and then increased at higher levels, reaching its maximum at 4%. For PFAD, the decline in MPS was more gradual, reaching the lowest value at 3% before increasing again at 4%.

Digestibility

Table 4 shows the effects of CFAD and PFAD supplementation on nutrient digestibility, including dry matter, organic matter, NDF, and ADF digestibility. Dry matter digestibility (DMD) and organic matter digestibility (OMD) responded differently to CFAD and PFAD supplementation. With CFAD, both DMD and OMD began to decline at 2%–3% inclusion, whereas PFAD produced no significant changes across levels, except for a slight reduction at 1% relative to the control. NDF digestibility was not significantly affected by either fat source or supplementation level, while ADF digestibility showed a modest but consistent decrease as supplementation increased.

Hydrogen Metabolism

Table 5 presents the hydrogen metabolism indices derived from VFA stoichiometry and methane

Table 3. *In vitro* rumen microbial populations and microbial protein synthesis of dairy cattle rations supplemented with coconut fatty acid distillate (CFAD) and palm fatty acid distillate (PFAD) at different inclusion levels

Variables	Fatty acid distillate	Supplementation level (% DM)					Average
		0	1	2	3	4	
Bacteria (log CFU/mL)	Coconut	9.83±0.13	9.84±0.08	9.70±0.09	9.76±0.03	9.55±0.18	9.74±0.11
	Palm	9.83±0.13	9.84±0.03	9.75±0.04	9.55±0.08	9.29±0.06	9.65±0.10
	Average	9.83±0.13^{c}	9.84±0.05^{c}	9.72±0.07^{bc}	9.65±0.07^{ab}	9.42±0.13^{a}	
Protozoa (log cell/mL)	Coconut	6.70±0.00	6.59±0.04	6.56±0.02	6.53±0.03	6.53±0.04	6.58±0.03^{a}
	Palm	6.70±0.00	6.64±0.02	6.64±0.04	6.62±0.03	6.54±0.03	6.63±0.03^{b}
	Average	6.70±0.00^{c}	6.61±0.04^{bc}	6.60±0.04^{bc}	6.58±0.03^{ab}	6.54±0.03^{a}	
MPS (mg/10 mL)	Coconut	25.27±0.89^{b}	19.02±2.55^{ab}	18.15±2.05^{ab}	21.16±1.62^{ab}	36.71±0.93^{c}	24.06±2.59
	Palm	25.27±0.89^{b}	21.10±1.51^{ab}	21.80±2.43^{ab}	14.90±1.62a	19.26±1.36^{ab}	20.47±1.87
	Average	25.27±0.89	20.06±2.07	19.98±2.26	18.03±1.83	27.98±2.81	

Note: Means ± SEM. Different superscripts denote significant differences ($p<0.05$). Main effects were tested using marginal means: fat source compares only the overall means of CFAD vs. PFAD; supplementation level compares only the five overall means for 0–4%. Interaction effects: superscripts within treatment cells (2 × 5) indicate significant fat source × level interactions. Abbreviations: CFU= coliform unit; MPS = Microbial protein synthesis.

Table 4. Dry matter, organic matter, and fiber digestibility in rumen fluid supplemented with coconut fatty acid distillate (CFAD) and palm fatty acid distillate (PFAD)

Variables	Fatty acid distillate	Supplementation level (% DM)					Average
		0	1	2	3	4	
DMD (%)	Coconut	65.96±0.71^{b}	65.73±0.96^{b}	65.21±1.31^{b}	63.34±1.34^{ab}	61.69±1.88^{a}	64.39±1.34
	Palm	65.96±0.71^{b}	63.63±1.24^{ab}	66.48±0.97^{b}	66.27±0.99^{b}	66.85±1.34^{b}	65.84±1.09
	Average	65.96±0.69	64.68±1.13	65.85±1.14	64.81±1.23	64.27±1.77	
OMD (%)	Coconut	67.51±0.69^{c}	66.76±1.60^{c}	64.39±1.38^{abc}	62.84±1.25^{ab}	62.83±1.15^{ab}	64.87±1.33
	Palm	67.51±0.69^{c}	62.72±1.32^{a}	65.30±0.93^{c}	64.89±0.89^{bc}	66.00±1.53^{c}	65.28±1.17
	Average	67.51±0.68	64.74±1.55	64.85±1.16	63.87±1.11	64.41±1.40	
NDFD (%)	Coconut	58.26±0.99	55.88±0.63	55.50±0.84	53.18±1.51	52.57±1.25	55.08±1.21
	Palm	58.26±0.99	54.81±1.20	56.58±0.80	57.00±0.86	57.10±1.12	56.75±1.02
	Average	58.26±0.96	55.35±0.95	56.04±0.82	55.09±1.33	54.84±1.34	
ADFD (%)	Coconut	46.18±0.79	43.91±0.47	42.99±0.41	42.42±0.51	40.61±0.81	43.22±0.80
	Palm	46.18±0.79	42.37±0.71	43.62±0.74	40.84±0.47	43.83±1.07	43.37±0.91
	Average	46.18±0.77^{b}	43.14±0.63^{a}	43.31±0.59^{a}	41.63±0.53^{a}	42.22±1.04^{a}	

Note: Means ± SEM. Different superscripts denote significant differences ($p<0.05$). Main effects were tested using marginal means of supplementation level, comparing only the five overall means for 0%–4%. Interaction effects: superscripts within treatment cells (2 × 5) indicate significant fat source × level interactions. Abbreviations: DMD= dry matter digestibility; OMD= organic matter digestibility; NDFD = neutral detergent fiber digestibility; ADFD = acid detergent fiber digestibility

production, including H_2 produced, H_2 consumed, net H_2 from fermentation, H_2 used in methane formation, H_2 balance, and H_2 recovery. H_2 produced was not significantly affected by fat source or supplementation level. In contrast, H_2 consumed, net H_2 from VFA, H_2 consumed in methane, and H_2 balance were all influenced by supplementation level ($p<0.05$), with the highest values generally occurring at 1% and 4% inclusion.

A significant fat source × level interaction was detected for H_2 recovery (%). Both fat sources increased H_2 recovery relative to the control, but PFAD showed a more pronounced rise, particularly at 2%–4% inclusion, whereas CFAD displayed a more modest and variable increase across levels. Overall, supplementation altered multiple hydrogen-related indices, with PFAD producing a steeper enhancement in H_2 recovery than CFAD.

Fatty Acid Biohydrogenation and Transformation

Changes in fatty acid transformation, isomerization, and biohydrogenation of dairy cattle rations supplemented with CFAD and PFAD at various levels are presented in Table 6. The biohydrogenation of C18:2 cis and C18:3 n-6, the saturation index, and the C18:1 isomerase index were not significantly affected by either the type of FAD or the supplementation level. Incomplete biohydrogenation, CLA yield, and total C18 recovery were influenced only by FAD source, whereas total PUFA biohydrogenation, the C18:2 isomerase index, and the 48-h unsaturation index were affected solely by supplementation level. Interaction effects between FAD type and supplementation level were observed for the biohydrogenation of C18:3 n-3 and the 0-h unsaturation index.

Correlation Between Hydrogen Indices and Biohydrogenation Traits

Correlation analysis revealed several significant associations between hydrogen metabolism variables and C18 fatty acid transformation indices (Table 7). Biohydrogenation of C18:2 cis showed positive correlations with H_2 produced ($r = 0.192$, $p<0.05$) and

Table 5. Hydrogen metabolism indices (H_2 production, consumption, balance, and recovery) derived from VFA stoichiometry and methane output following supplementation of coconut (CFAD) and palm (PFAD) fatty acid distillates

Variables	Fatty acid distillate	Supplementation level					Average
		0	1	2	3	4	
H_2 produced, mol/100 mol hexose fermented	Coconut	206.38±3.03	231.89±17.47	233.35±7.80	228.35±13.38	218.02±10.02	223.56±11.30
	Palm	206.38±3.03	223.77±10.16	219.65±15.23	215.53±12.86	242.21±14.72	221.12±12.14
	Average	206.38±2.96	228.24±14.34	226.50±12.00	221.94±12.95	230.69±12.90	
H_2 consumed, mol/100 mol hexose fermented	Coconut	75.55±6.34	61.77±4.12	63.83±4.88	64.43±4.64	58.05±5.72	65.02±5.30
	Palm	75.55±6.34	56.02±3.41	63.15±4.73	60.71±3.87	57.02±4.37	62.97±5.01
	Average	75.55±6.20^{b}	59.19±3.82^{a}	63.49±4.70^{ab}	62.57±4.20^{ab}	57.51±4.93^{a}	
Net H_2 from VFA, mol/100 mol hexose fermented	Coconut	130.83±4.73	170.12±21.06	169.52±8.90	163.91±15.80	159.98±15.80	158.54±13.33
	Palm	130.83±4.73	167.74±11.50	156.51±14.31	154.82±10.83	185.20±15.68	158.14±12.68
	Average	130.83±4.63^{a}	169.05±17.01^{b}	163.01±11.82^{ab}	159.37±13.29^{ab}	173.19±13.09^{b}	
H_2 consumed in CH_4, mol/100 mol hexose fermented	Coconut	60.15±2.46	79.38±10.00	78.53±4.37	76.04±7.59	75.24±4.04	73.68±6.39
	Palm	60.15±2.46	79.51±5.50	74.31±6.42	73.79±4.93	87.88±7.21	74.68±5.95
	Average	60.15±2.40^{a}	79.44±8.09^{b}	76.42±5.40^{ab}	74.91±6.25^{ab}	81.86±6.07^{b}	
H_2 balance, mol/100 mol hexose fermented	Coconut	70.69±2.29	90.74±11.07	90.99±4.59	87.87±8.23	84.74±4.79	84.86±6.98
	Palm	70.69±2.29	88.23±6.00	82.20±7.93	81.03±5.94	97.32±8.50	83.46±6.77
	Average	70.69±2.24^{a}	89.61±8.93^{ab}	86.59±6.46^{ab}	84.45±7.07^{ab}	91.33±7.06^{b}	
H_2 recovery (%)	Coconut	45.89±0.28^{a}	46.57±0.16^{abcd}	46.28±0.29^{abc}	46.14±0.33^{ab}	47.07±0.34^{abcd}	46.37±0.30
	Palm	45.89±0.28^{a}	47.38±0.17^{bcd}	47.74±0.40^{d}	47.74±0.32^{d}	47.59±0.33^{cd}	47.25±0.37
	Average	45.89±0.27	46.93±0.20	47.01±0.40	46.94±0.39	47.34±0.33	

Note: Means ± SEM. Different superscripts denote significant differences ($p<0.05$). Main effects were tested using marginal means of supplementation level, comparing only the five overall means for 0%–4%. Interaction effects: superscripts within treatment cells (2 × 5) indicate significant fat source × level interactions. Abbreviations: H_2 = hydrogen; VFA = volatile fatty acid; CH_4 = methane.

H_2 consumed via propionate ($r = 0.220$, $p<0.05$), and a negative correlation with H_2 recovery ($r = –0.191$, $p<0.05$). Biohydrogenation of C18:3 n-6 was negatively correlated with net H_2 from VFA ($r = –0.189$, $p<0.05$), H_2 consumed in methane ($r = –0.191$, $p<0.05$), and H_2 balance ($r = –0.186$, $p<0.05$), while positively associated with H_2 consumed via propionate ($r = 0.187$, $p<0.05$).

The saturation index displayed a positive correlation with H_2 recovery ($r = 0.315$, $p<0.01$). CLA yield was also positively correlated with H_2 recovery ($r = 0.170$, $p<0.05$). The C18:1 isomerization index showed a negative correlation with H_2 consumed via propionate ($r = –0.386$, $p<0.01$) and a positive correlation with H_2 recovery ($r = 0.307$, $p<0.01$). Meanwhile, the C18:2 isomerization index exhibited a weak but significant positive correlation with H_2 recovery ($r = 0.174$, $p<0.05$).

Polynomial Regression and Optimization

To determine the optimal supplementation level of CFAD and PFAD, polynomial response curves were evaluated for all parameters showing significant effects ($p<0.05$), as presented in Table 8. The table shows that protozoa population, BH-total PUFA, CFAD-DMD, CFAD-OMD, and PFAD-CH_4 followed linear response patterns, whereas the C18:2 isomerase index, CFAD-MPS, and PFAD-iC4 exhibited quadratic responses. Cubic trends were observed for iC5, the acetate-to-propionate ratio (C2/C3), and CH_4. Meanwhile, total VFA, ADFD, CFAD-NH_3, PFAD-MPS, and the 0-h unsaturation index displayed quartic response curves. The R^2 values were relatively low (<0.50), likely due to the large data pool (n = 120) combined across all blocks in a randomized block design, which inherently increases variability among replicates. Nevertheless, all regression models were statistically significant ($p<0.05$), indicating that the fitted curves reliably captured the overall response patterns.

DISCUSSION

Fermentation and Nitrogen Metabolism

The significant interaction between fatty acid source and supplementation level for NH_3 concentration indicates that rumen nitrogen metabolism responded differently to CFAD and PFAD. Although CFAD and PFAD were supplied as calcium soaps, rumen protection is not absolute. Calcium soaps may partially dissociate under ruminal conditions, allowing a fraction of fatty acids to remain available for interaction with rumen microorganisms and ruminal lipid metabolism. Fat supplementation modified rumen fermentation in a source- and level-dependent manner. The increase in total VFA at moderate inclusion suggests enhanced fermentative efficiency, potentially linked to reduced protozoal grazing pressure.

The greater reduction in NH_3 observed with CFAD than with PFAD may be related to differences in fatty acid composition between the two supplements. CFAD was dominated by medium-chain fatty acids (MCFA), whereas PFAD contained predominantly long-chain fatty acids (LCFA). Accordingly, the observed responses are more likely associated with differences in fatty acid chain length and profile than with saturation alone. Medium-chain fatty acids (MCFA), which dominate CFAD, are widely recognized for their strong antiprotozoal activity. Numerous *in vivo* and *in vitro* studies have demonstrated that MCFA markedly suppress *Entodinium*-dominated protozoal communities

Table 6. Changes in rumen fatty acid transformation, isomerization, and saturation indices following coconut (CFAD) and palm (PFAD) fatty acid distillates supplementation

Variables	Fatty acid distillate	Supplementation level (%)					Average
		0	1	2	3	4	
Biohydrogenation (%)							
BH-C18:2 cis	Coconut	-17.32±0.88	-13.75±12.26	-22.17±16.06	-9.27±17.49	5.24±8.84	-11.03±12.89
	Palm	-17.32±0.88	-41.69±26.46	-23.90±16.16	-2.58±9.09	-4.17±6.64	-17.98±15.37
	Average	-17.32±0.85	-27.72±20.58	-23.03±15.76	-5.93±13.66	0.54±7.77	
BH-C18:3n3	Coconut	16.03±1.09^{b}	4.90±3.83^{ab}	10.52±3.65^{ab}	14.18±2.96^{ab}	8.06±10.07^{ab}	10.29±5.42
	Palm	16.03±1.09^{b}	-5.79±6.67^{a}	1.08±5.03^{ab}	-1.74±6.25^{a}	0.01±2.37^{ab}	0.92±5.22
	Average	16.03±1.05	-0.45±5.55	6.47±4.40	5.87±5.39	4.21±7.39	
BH-C18:3n6	Coconut	-14.14±15.28	9.52±15.31	-57.74±39.95	31.73±14.01	-75.66±45.39	-21.76±31.30
	Palm	-14.14±15.28	-14.13±13.01	-28.17±14.28	-73.90±72.31	1.49±14.16	-25.92±34.79
	Average	-14.14±14.76	-1.79±14.36	-43.60±30.16	-21.08±53.17	-35.41±34.15	
BH- total PUFA	Coconut	-1.20±3.58	3.06±5.83	7.36±7.73	-7.69±12.22	-11.00±8.61	-1.95±8.32
	Palm	-1.20±3.58	-7.50±5.51	-1.97±5.34	-6.86±3.47	-19.17±6.04	-7.78±5.16
	Average	-1.20±3.46^{ab}	1.95±5.76^{b}	2.69±6.64^{b}	-7.27±8.78^{ab}	-15.08±7.37^{a}	
Incomplete-BH (%)	Coconut	13.49±0.67	14.87±0.40	14.46±0.81	14.37±0.53	13.88±0.60	14.22±0.61^{a}
	Palm	13.49±0.67	13.44±0.83	15.80±0.63	15.18±0.64	16.75±1.00	14.94±0.83^{b}
	Average	13.49±0.66	14.16±0.67	15.13±0.74	14.78±0.59	15.32±0.91	
Isomeration indices							
C18:1	Coconut	0.50±0.02	0.56±0.04	0.57±0.06	0.61±0.03	0.58±0.06	0.57±0.05
	Palm	0.50±0.02	0.46±0.05	0.61±0.07	0.59±0.05	0.68±0.07	0.57±0.06
	Average	0.50±0.02	0.51±0.05	0.59±0.06	0.60±0.04	0.63±0.07	
C18:2	Coconut	0.08±0.00	0.17±0.02	0.20±0.03	0.21±0.03	0.18±0.04	0.17±0.03
	Palm	0.08±0.00	0.21±0.03	0.21±0.02	0.17±0.03	0.19±0.02	0.17±0.03
	Average	0.08±0.00^{a}	0.19±0.02^{b}	0.21±0.03^{b}	0.19±0.03^{b}	0.18±0.03^{b}	
Fatty acids transformation							
Saturation index	Coconut	0.58±0.01	0.58±0.01	0.59±0.01	0.60±0.01	0.59±0.01	0.59±0.01
	Palm	0.58±0.01	0.57±0.02	0.58±0.01	0.57±0.02	0.58±0.01	0.58±0.01
	Average	0.58±0.01	0.58±0.01	0.58±0.01	0.59±0.01	0.59±0.01	
0-h unsaturation index	Coconut	0.51±0.01^{ab}	0.54±0.03^{ab}	0.46±0.02^{a}	0.55±0.04^{b}	0.47±0.03^{ab}	0.51±0.03
	Palm	0.51±0.01^{ab}	0.47±0.02^{ab}	0.47±0.03^{ab}	0.49±0.03^{ab}	0.52±0.03^{ab}	0.49±0.02
	Average	0.51±0.01	0.50±0.02	0.47±0.02	0.52±0.04	0.50±0.03	
48-h unsaturation index	Coconut	0.54±0.01	0.50±0.01	0.51±0.02	0.49±0.02	0.51±0.01	0.51±0.01
	Palm	0.54±0.01	0.53±0.03	0.51±0.01	0.52±0.02	0.50±0.02	0.52±0.02
	Average	0.54±0.01^{b}	0.52±0.03^{ab}	0.51±0.02^{ab}	0.51±0.02^{ab}	0.50±0.02^{a}	
CLA Yield (%)	Coconut	-27.65±5.70	6.91±9.75	-2.97±13.89	-10.76±12.50	-32.92±19.83	-12.50±13.83^{a}
	Palm	-27.65±5.70	-2.69±23.36	19.49±16.37	3.84±20.35	26.82±17.78	5.98±18.45^{b}
	Average	-27.65±5.51	2.11±17.56	7.77±15.14	-3.15±16.82	-3.05±20.42	
Total C18 recovery (%)	Coconut	103.15±0.94	104.62±6.03	96.71±4.09	100.63±9.77	88.46±6.43	98.71±6.20^{a}
	Palm	103.15±0.94	101.52±7.76	105.46±6.55	97.79±3.07	108.98±8.84	103.38±6.07^{b}
	Average	103.15±0.92	103.07±6.81	101.08±5.50	99.21±7.10	98.72±8.14	

Note: Means ± SEM. Different superscripts indicate significant differences ($p<0.05$). Main effects were tested using marginal means: fat source compares only the overall means of CFAD vs. PFAD; supplementation level compares only the five overall means for 0%–4%. Interaction effects: superscripts within treatment cells (2 × 5) indicate significant fat source × level interactions. Abbreviations: BH = biohydrogenation; C18:2 cis = linoleic acid; C18:3 n-3 = α-linolenic acid; C18:3 n-6 = γ-linolenic acid; C18:1 trans/cis = trans- or cis-octadecenoic acid isomers; CLA = conjugated linoleic acid.

(Shi *et al.,* 2020; Luan *et al.,* 2023). Although protozoal species were not directly quantified in the present experiment, the decline in NH_3 concentrations at 1%–2% CFAD is consistent with reduced protozoal turnover of bacterial protein, a pattern also observed in coconut-oil–based MCFA supplementation studies (Shi *et al.,* 2020; Joch *et al.,* 2023).

The more pronounced reduction in NH_3 under CFAD supplementation suggests a stronger inhibitory effect on proteolytic and deaminating bacteria, which are known to be sensitive to MCFA. Previous studies have demonstrated that key rumen proteolytic taxa, such as *Prevotella ruminicola, Butyrivibrio fibrisolvens*, and *Clostridium aminophilum*, exhibit reduced abundance or metabolic activity when exposed to MCFA (Patra 2013; Shi *et al.,* 2020). Zahera *et al.* (2024) reported a marked decline in *Bacteroides spp*. with increasing levels of unprotected CFAD. This suppression of proteolytic microbial populations likely reduced deamination activity, resulting in lower ammonia release from dietary protein (Shen *et al.,* 2018; Diether & Willing, 2019; Mitchell *et al.,* 2023).

Compared with CFAD, PFAD exerted a milder effect on nitrogen metabolism, consistent with its greater proportion of LCFA and lower concentration of MCFA. Numerous studies have shown that medium-chain fatty acids, particularly lauric acid (C12:0) and myristic acid (C14:0), exert strong antimicrobial and

Table 7. Pearson correlations between hydrogen metabolism indices and fatty acid biohydrogenation traits in *in vitro* dairy cattle rations supplemented with coconut and palm fatty acid distillates

Variables	H_2 Produced	H_2 Consumed (Propionate)	Net H_2 (VFA)	H_2 Consumed in CH_4	H_2 Balance	H_2 Recovery (%)
BH C18:2 cis	0.192*	0.220*	–	–	–	–0.191*
BH C18:3 n-6	–	0.187*	–0.189*	–0.191*	–0.186*	–
Saturation index	–	–	–	–	–	0.315**
CLA yield	–	–	–	–	–	0.170*
C18:1 Isomerization index	–	–0.386**	–	–	–	0.307**
C18:2 Isomerization index	–	–	–	–	–	0.174*

Note: Only significant Pearson correlation coefficients ($p<0.05$) are presented. Positive values indicate that increases in a hydrogen-related variable are associated with increases in the corresponding biohydrogenation or transformation index, whereas negative values indicate an inverse relationship. Abbreviations: BH C18:2 cis = biohydrogenation of linoleic acid; BH C18:3 n-6 = biohydrogenation of γ-linolenic acid; CLA yield = proportion of CLA formed relative to C18:2 cis disappearance; C18:1 isomerization index = ratio of C18:1 trans to C18:1 cis; C18:2 isomerization index = ratio of C18:2 trans to C18:2 cis; H_2 produced/consumed/balance/recovery = hydrogen indices calculated from VFA stoichiometry and estimated methane output

Table 8. Polynomial regression models and optimal supplementation levels of fatty acid distillates on rumen fermentation and biohydrogenation variables

No	Response variables	Regression equation	Optimal inclusion level (%)	P-value	R^2
1	Total VFA	$Y=69.14-19.8645X+81.7953X^2-37.2247X^3+4.7188X^4$	2.21	4.15×10^{-11}	0.375
2	Protozoa	$Y=6.6793-0.0370X$	-	<0.001	0.203
3	ADFD	$Y=6.6952-0.1883X+0.2108X^2-0.0659X^3+0.0032X^4$	4.47	7.05 x 10-06	0.223
4	iC5	$Y=5.4574+1.1678X-0.8157X^2+0.1292X^3$	0.91	0.001	0.145
5	C2/C3	$Y=1.3632+1.0806X-0.6180X^2+0.0975X^3$	1.5 - 3	<0.05	0.045
6	CH4	$Y=15.0928+8.0908X-4.2325X^2+0.6374X^3$	1.51	0.0017	0.131
7	BH-Total PUFA	$Y=2.7354-3.5448X$	-	0.034	0.04
8	C18:2 isomerase index	$Y=0.0848+0.1002X-0.0194X^2$	2.58	0.00175	0.213
9	NH3-CFAD	$Y=-0.5533X^4+4.1183X^3-8.6124X^2+3.9606X+8.0679$	3.51	3.78×10^{-7}	0.469
10	MPS-CFAD	$Y=25.8434-11.0633X+3.3910X^2$	1.63	0.000	0.566
11	MPS-PFAD	$Y=22.33-18.59X+23.03X^2-9.91X^3+1.31X^4$	0	<0.05	0.281
12	CFAD-DMD	$Y=66.5717-1.0921X$	-	<0.001	0.11
13	CFAD- OMD	$Y=67.5263-1.3287X$	-	0.001	0.168
14	PFAD-iC4	$Y= 1.9826+0.1681X+0.0183X^2$	2.54	0.000168	0.410
15	PFAD-CH_4	$Y=16.0544+1.3078X$	-	0.00611	0.133
16	CFAD-0 h Unsaturated index	$Y=0.5043+0.02718(X)-0.02136X^2+0.00440X^3-0.00018X^4$	3	0.013	0.183

Note: Polynomial regression models were generated for all parameters showing significant responses to supplementation ($p<0.05$). Linear, quadratic, cubic, or quartic equations were selected based on the best model fit. The optimal inclusion level was determined only for parameters with significant nonlinear trends. Values of R^2 reflect the proportion of variance explained by each model. Abbreviations: BH = biohydrogenation; C18:2 isomerase index = ratio of C18:2 trans to C18:2 cis; MPS = microbial protein synthesis; DMD = dry matter digestibility; OMD = organic matter digestibility; iC4/iC5 = isobutyrate and isovalerate fractions; CH_4 = methane estimated from VFA stoichiometry; 0-h unsaturation index = weighted average number of double bonds per fatty acid at 0 h incubation

antiprotozoal effects in the rumen, whereas the long-chain fatty acids that predominate in PFAD generally produce milder microbial responses (Shi *et al*., 2020; Luan *et al*., 2023; Joch *et al*., 2023). This is reflected in the more stable NH_3 concentrations observed under PFAD supplementation and is consistent with previous reports showing minimal changes in rumen ammonia dynamics with LCFA feeding (Mavrommatis *et al.,* 2021; Riestanti *et al.,* 2021). Overall, these patterns indicate that CFAD alters fermentation primarily through MCFA-driven microbial suppression, whereas PFAD exerts more modest and stable effects due to its LCFA profile.

Microbial Populations and Digestibility Shifts

The progressive decline in microbial populations with increasing supplementation level indicates that both CFAD and PFAD exerted inhibitory effects on rumen microorganisms, although the magnitude of suppression tended to differ according to fatty acid composition. Total bacterial and protozoal populations declined with increasing supplementation level, but the magnitude of suppression differed between fat sources. CFAD resulted in stronger microbial reductions than PFAD, indicating that the microbial response differed according to fatty acid composition. This finding is consistent with the stronger antimicrobial activity generally reported for MCFA compared with LCFA (Zahera *et al.,* 2024). MCFA disrupt cell membranes through increased permeability and leakage of intracellular components, particularly in gram-positive and anaerobic rumen microbes (Vadroňová *et al.,* 2023; Obukhova & Murzina, 2024). The stronger protozoal suppression under CFAD aligns with earlier findings

showing that MCFA from coconut oil rapidly depresses *Entodinium*-dominated protozoal communities (Burdick *et al.,* 2022; Zahera *et al.,* 2024).

Although individual taxa were not quantified in this study, previous research consistently reports that key fibrolytic bacteria such as *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes* are highly sensitive to free fatty acids due to their strictly anaerobic metabolism and membrane composition (Carreño *et al.,* 2019; Zhang *et al.,* 2020; Lock *et al.*, 2025). This supports the observed decline in ADF digestibility at higher supplementation levels, as fibrolytic pathways depend heavily on these taxa (Riestanti *et al.,* 2021).

However, NDF digestibility and acetate concentrations remained stable across treatments, indicating that the core fibrolytic capacity of the rumen was largely preserved. Lock *et al*. (2025) also reported improved NDF digestibility with fatty acid supplementation, supporting the present findings. Although *B. fibrisolvens* was not quantified in this study, this species is well known to contribute to hemicellulose and pectin degradation (Palevich *et al.,* 2019; Sengupta *et al.,* 2022). While *B. fibrisolvens* is not a major cellulolytic bacterium, its presence has been shown to enhance overall cellulose digestion (Palevich *et al.,* 2019; Dewanckele *et al.,* 2020), and it is generally less sensitive to moderate levels of fat supplementation compared with strictly cellulolytic species. This interpretation is further supported by Zahera *et al.* (2024), who observed no significant changes in *B. fibrisolvens* abundance with CFAD supplementation up to 3%. Taken together, these results suggest that although certain microbial groups may have been inhibited, the functional components responsible for structural carbohydrate degradation were likely maintained, thereby sustaining NDF digestibility. A similar pattern of selective suppression, where ADF digestibility declines but NDF digestibility remains relatively stable, has also been observed with MCFA supplementation. This likely occurs because MCFA inhibit certain cellulolytic microbes, while others are able to persist and compensate through alternative enzymatic pathways (Vadroňová *et al.,* 2023; Obukhova & Murzina, 2024).

The interaction patterns observed in dry matter digestibility (DMD), organic matter digestibility (OMD), and microbial protein synthesis (MPS) indicate that CFAD induced stronger but more variable microbial metabolic shifts (Luan *et al.,* 2023; Lock *et al*., 2025). This variability mirrors known MCFA effects, where microbial inhibition is both concentration-dependent and species-specific (Patra, 2013; Luan *et al.,* 2023). In contrast to CFAD, PFAD produced smoother digestibility and MPS responses, suggesting that the LCFA-rich profile of PFAD imposed less microbial stress than the MCFA-rich profile of CFAD (Riestanti *et al.,* 2021; Sun *et al.,* 2022a). Collectively, these findings demonstrate that CFAD-driven MCFA imposes greater microbial pressure, whereas PFAD leads to moderated changes more compatible with stable rumen function.

VFA Profiles and Methane Dynamics

No significant source × supplementation level interactions were detected for acetate, propionate, C2:C3 ratio, or methane production, indicating that both CFAD and PFAD produced similar response patterns across supplementation levels. The decrease in propionate, accompanied by an increase in the C2:C3 ratio at certain supplementation levels, suggests a shift in rumen fermentation pathways irrespective of fatty acid source. This response is consistent with the sensitivity of amylolytic bacteria, such as *Streptococcus bovis* and *Selenomonas ruminantium*, to dietary fatty acids, as reported in both *in vitro* and *in vivo* studies (Patra, 2013; Vadroňová *et al*., 2023). In addition, the reduction in branched-chain VFA suggests suppression of amino acid-fermenting bacteria, including *Peptostreptococcus* and *Clostridium* species, which contribute to the formation of isobutyrate and isovalerate (Patra, 2013; Mitchell *et al*., 2023; Hackmann, 2024).

Interestingly, methane production increased at inclusion levels of 1% and 4% despite a decline in protozoal populations. This pattern likely reflects the concurrent reduction in propionate production, which diminished one of the primary hydrogen sinks and redirected more reducing equivalents toward methanogenesis (Moss *et al.,* 2000; Ungerfeld, 2020). Under these conditions, surviving hydrogenotrophic methanogens, particularly *Methanobrevibacter*-related groups, may have remained sufficiently active to utilize the increased availability of H_2. These taxa are known for their adaptability and ability to maintain methanogenic activity even when protozoal populations are suppressed (Danielsson *et al.,* 2017; Ungerfeld, 2020; Khairunisa *et al.,* 2023).

Biohydrogenation and Fatty Acid Transformation

The significant interactions detected for C18:3 n-3 biohydrogenation and the unsaturation index indicate differential responses of CFAD and PFAD during ruminal fatty acid transformation. Most terminal biohydrogenation steps, including the disappearance of C18:2 cis and C18:3 n-6, were not affected, indicating that key terminal hydrogenating groups, such as *Butyrivibrio proteoclasticus*, remained functional at the tested fat levels (Jeyanathan *et al.,* 2016; Toral *et al.,* 2024). The interaction observed for biohydrogenation of C18:3 n-3 likely reflects differences in the sensitivity of early hydrogenating species, particularly *B. fibrisolvens*, to the distinct fatty acid profiles of CFAD and PFAD. Although the fatty acid sources were supplied as calcium soaps, rumen protection is not intended to completely prevent biohydrogenation. Rather, calcium soap protection may reduce the immediate availability of unsaturated fatty acids and thereby limit the extent of biohydrogenation, allowing partial transformation and accumulation of intermediates such as CLA and vaccenic acid (Dewanckele *et al.,* 2020; Toral *et al.,* 2024).

Total PUFA biohydrogenation declined at higher supplementation, consistent with established findings that fatty acids becoming available following partial dissociation of calcium soaps impair microbial attachment and inhibit biohydrogenation enzyme systems (Palmquist & Jenkins 2017; Toral *et al.,* 2024). This decline suggests that excessive fat supplementation

may suppress microbial populations involved in biohydrogenation, thereby reducing the formation of CLA-related intermediates at higher inclusion levels. Compared with CFAD, PFAD generated greater incomplete biohydrogenation and higher CLA-related intermediates, suggesting that the two fatty acid sources differed in their effects on ruminal lipid metabolism (Ventto *et al.,* 2017; Toral *et al.,* 2024). In contrast to PFAD, the stronger microbial suppression associated with CFAD may have reduced the activity of early CLA-forming bacteria such as *B. fibrisolvens*, leading to lower CLA yields (Hussain *et al.,* 2016; Dewanckele *et al.,* 2020; Vadroňová *et al.,* 2023)

Hydrogen Partitioning and Mechanistic Interpretation

The significant interaction observed for H_2 recovery indicates that the effects of supplementation level on hydrogen utilization differed between CFAD and PFAD. Hydrogen metabolism indices revealed distinct partitioning patterns. Although total H_2 production remained stable, supplementation markedly shifted the pathways through which H_2 was utilized. The calculated hydrogen partitioning in this study aligns with established stoichiometric estimates in the literature, where methanogenesis typically accounts for 40%–50% of reducing-equivalent use, propionate for 20%–30%, and biohydrogenation for roughly 20%–30% (Moss *et al.,* 2000; Ungerfeld, 2020). The increase in residual H_2 at higher inclusion levels is consistent with the reduced efficiency of hydrogen sinks described by Ungerfeld (2020).

Under CFAD, the strong suppression of protozoa and amylolytic bacteria, which are key contributors to H_2 production and propionate formation (Maia *et al.,* 2010; Choudhury *et al.,* 2022; Vadroňová *et al.,* 2023), reduced both H_2 generation and the propionate sink, leading to H_2 accumulation. At the same time, CFAD inhibited CLA-forming bacteria and disrupted terminal biohydrogenation pathways (Dewanckele *et al.,* 2020; Vadroňová *et al.,* 2023), limiting the use of H_2 for fatty acid hydrogenation and ultimately reducing total H_2 recovery. In contrast, PFAD exerted a weaker antimicrobial effect, allowing the propionate pathway to remain active, promoting incomplete biohydrogenation and the accumulation of CLA precursors (Sun *et al.,* 2022b; Choudhury *et al.,* 2022; Toral *et al.,* 2024), and increasing methane production as more H_2 became available. These combined effects resulted in greater total H_2 recovery under PFAD. Overall, the type of fat directs hydrogen flow either away from (CFAD) or toward (PFAD) hydrogen-utilizing pathways, thereby shaping biohydrogenation outcomes.

Dose–Response Behavior Based on Polynomial Models

Polynomial regressions revealed clear dose-dependent responses for the main variables. CFAD exhibited nonlinear, quadratic-type patterns, consistent with the threshold-based microbial inhibition typically observed for MCFA-rich fats (Patra, 2013; Vadroňová *et al.,* 2023). In contrast, PFAD generated more linear responses, reflecting the milder and more proportional microbial effects of LCFA (Palmquist & Jenkins, 2017; Toral *et al.,* 2024). Most fermentation and fatty-acid traits reached optimal values at 2%–3% CFAD, a range previously associated with maximal VFA production, improved NH_3 utilization, protozoal suppression, and stable unsaturation indices (Shi *et al.,* 2020; Luan *et al.,* 2023). PFAD showed optimal responses at 1.5%–2%, particularly for CLA yield, incomplete biohydrogenation, and fermentation stability (Toral *et al.,* 2024; Lock *et al.,* 2025). Methane increased linearly with PFAD inclusion, in agreement with prior studies demonstrating proportional methane increases with rising saturated-fat supplementation (Patra, 2013; Tan *et al.,* 2024).

Integrated Interpretation and Practical Implications

The combined fermentation, biohydrogenation, and hydrogen-flux responses demonstrate that CFAD and PFAD modulate rumen metabolism through fundamentally different biochemical mechanisms. CFAD, enriched in medium-chain fatty acids, exerted strong suppression of protozoa and several bacterial groups, reducing NH_3 turnover and enhancing fermentation efficiency at moderate inclusion levels (Maia *et al.,* 2010; Vadroňová *et al.,* 2023). However, this same antimicrobial pressure inhibited CLA-forming species, reduced trans-intermediate formation, redirected hydrogen away from biohydrogenation, and decreased overall H_2 recovery (Ventto *et al.,* 2017; Ungerfeld, 2020; Vadroňová *et al.,* 2023). These patterns support supplementing CFAD at 2.5%–3% DM, a range shown to optimize VFA production, ammonia utilization, and fermentation efficiency while avoiding excessive inhibition of fibrolytic pathways (Luan *et al.,* 2023; Vesga *et al.,* 2024).

In contrast, PFAD, which is characterized by a predominance of long-chain fatty acids, exerted a milder inhibitory effect on rumen microbes, allowing fibrolytic activity to be maintained and promoting greater incomplete biohydrogenation with increased accumulation of CLA precursors (Palmquist & Jenkins, 2017; Toral *et al.,* 2024). Hydrogen partitioning under PFAD favored both methanogenesis and biohydrogenation, increasing H_2 recovery but also elevating methane at higher inclusion levels (Patra, 2013; Ungerfeld, 2020; Kjeldsen *et al.,* 2024). These responses indicate that PFAD is optimally supplemented at 1.5%–2% DM, where fermentation remains stable and CLA-related intermediates are enhanced without excessive methane formation.

The dose–response patterns highlight the importance of aligning fat type with the intended nutritional outcomes. CFAD is more suitable for improving nitrogen efficiency and overall fermentation performance, whereas PFAD is better suited for enhancing CLA precursor formation and maintaining lipid transformation pathways. This approach is particularly valuable for precision lipid supplementation strategies in tropical dairy systems.

CONCLUSION

The effects of CFAD and PFAD supplementation on rumen fermentation, nutrient digestibility, and fatty acid biohydrogenation varied with fatty acid composition and inclusion level. CFAD, rich in medium-chain fatty acids, enhanced fermentation efficiency by increasing total VFA and improving NH_3 utilization while suppressing microbial populations at moderate levels, with an optimal inclusion of 2.5%–3% DM. In contrast, PFAD, dominated by long-chain fatty acids, maintained more stable fermentation and fibrolytic activity and promoted greater accumulation of CLA-related intermediates, with an optimal inclusion of 1.5%–2% DM, although methane production increased at higher levels. These results indicate that CFAD is more suitable for improving rumen fermentation and nitrogen utilization, whereas PFAD is more effective for enhancing CLA precursor formation. However, as this study was conducted *in vitro*, further *in vivo* studies are required to validate these findings and provide more comprehensive recommendations for practical application in dairy cattle.

CONFLICT OF INTEREST

Despal serves as an editor of the Tropical Animal Science Journal but has no role in the decision to publish this article. The authors also declare that there is no conflict of interest.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

The authors declare that artificial intelligence (AI) tools were used solely to assist in language editing, grammar improvement, and refinement of sentence clarity during the preparation of this manuscript. The AI tools did not contribute to the generation of scientific content, data analysis, interpretation of results, or formulation of conclusions. All scientific ideas, experimental design, data collection, analysis, and interpretation presented in this manuscript are entirely the original work of the authors. The authors take full responsibility for the accuracy, integrity, and originality of the content.

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