



Research article

***In vitro* characterization of ruminal fermentation and lipid metabolism in dairy diets associated with high versus low concentrations of conjugated linoleic acid in milk**

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Abstract: Conjugated linoleic acid (CLA) is an important bioactive fatty acid in ruminant milk due to its potential anticarcinogenic, anti-inflammatory, and antiatherogenic properties. However, milk CLA concentration varies considerably among dairy production systems, and the ruminal mechanisms underlying this variation remain poorly understood. This study aimed to evaluate rumen fermentation characteristics, nutrient digestibility, partial volatile fatty acid (VFA) profiles, methane production, and lipid biohydrogenation characteristics of diets associated with extremely high and low milk CLA concentrations. In total, 300 milk samples were collected from dairy farms in Pangalengan, Kebon Pedes, and Kunak. Fifteen diets associated with the highest milk CLA concentrations (>2.4% of milk fat) and fifteen diets associated with the lowest CLA concentrations (<0.92% of milk fat) were selected and reformulated according to field feeding practices. The diets were evaluated using *in vitro*

fermentation and digestibility techniques. Measured parameters included ruminal pH, ammonia, total and partial VFAs, gas and methane production, microbial protein synthesis, nutrient digestibility, and unsaturated fatty acid biohydrogenation indices. Substantial variation was observed among diets in terms of fermentation, digestibility, and lipid metabolism. Total VFA concentration ranged from 93.37 to 144.37 mM, while methane production ranged from 5.03 to 23.31 mL/g dry matter (DM). Dry matter digestibility and organic matter digestibility ranged across 52.68–80.19% and 51.18–80.64%, respectively. Several high-CLA-associated diets exhibited relatively lower acetate-to-propionate ratios (1.80–1.96) and higher incomplete biohydrogenation (IBH) ratios, although substantial overlap occurred between high- and low-CLA-associated diets. This overlap indicated that general fermentation intensity alone could not fully explain CLA variation. Milk CLA variation showed relatively stronger associations with ruminal lipid biohydrogenation characteristics, particularly IBH and the persistence of unsaturated fatty acid intermediates, than with overall fermentation characteristics. However, substantial overlap among diets prevented the identification of a single VFA profile or biohydrogenation index as a consistent characteristic associated with milk CLA concentration.

Keywords: conjugated linoleic acid; rumen biohydrogenation; volatile fatty acids; *in vitro* fermentation; dairy cow nutrition

1. Introduction

Conjugated linoleic acid (CLA), particularly the *cis*-9, *trans*-11 isomer, is one of the major bioactive fatty acids naturally present in ruminant milk [1]. Dietary polyunsaturated fatty acids (PUFAs), particularly linoleic acid and α -linolenic acid, provide substrates for ruminal lipid transformation. In the rumen, these fatty acids undergo lipolysis, isomerization, and sequential biohydrogenation, producing several *trans*-C18:1 and conjugated fatty acid intermediates, including vaccenic acid (C18:1 *trans*-11) [2,3]. A substantial proportion of milk *cis*-9, *trans*-11 CLA is subsequently synthesized in the mammary gland through Δ 9-desaturation of vaccenic acid [3]. Therefore, the final milk CLA concentration may reflect not only the dietary PUFA supply but also the extent and direction of ruminal biohydrogenation and subsequent mammary fatty acid metabolism. Owing to its well-documented health-promoting properties and its contribution to the nutritional value of dairy products, increasing milk CLA concentration remains an important objective in dairy nutrition research [4,5]. However, substantial variation in milk CLA concentration is commonly observed among dairy farms under practical production conditions, suggesting that the biological mechanisms underlying variation in milk CLA synthesis remain incompletely understood.

Although numerous studies have attempted to increase milk CLA through dietary lipid supplementation, the responses are often inconsistent under practical feeding conditions [6]. This inconsistency may arise because total dietary lipid concentration does not necessarily reflect the amount and composition of polyunsaturated fatty acids (PUFAs) available as substrates for ruminal biohydrogenation. Dietary manipulation can substantially modify the fatty acid profile of ruminant products, as the type and composition of dietary fatty acids influence their subsequent transformation and deposition in animal tissues [7]. Supplementation with ensiled pomegranate by-products increased vaccenic acid, rumenic acid, α -linolenic acid, and total PUFAs in lamb meat without affecting nutrient

digestibility or growth performance [8]. These responses indicate that changes in the fatty acid composition of ruminant products may occur independently of major changes in conventional measures of nutrient utilization. Previous field observations showed that dairy farms with generally comparable feeding practices exhibited substantial variation in milk CLA concentrations [9,10], indicating that factors beyond total dietary lipid supply, including dietary fatty acid composition and ruminal biohydrogenation, may contribute to the regulation of ruminal CLA formation. However, the extent to which dietary composition and nutrient supply account for the observed variation in milk CLA under heterogeneous field conditions remains unclear. These findings suggest that variation in milk CLA may be associated with specific ruminal lipid metabolic processes, particularly biohydrogenation, rather than with overall rumen fermentation intensity alone [11]. Therefore, a more comprehensive evaluation of ruminal metabolic characteristics associated with naturally occurring variation in milk CLA production is warranted.

Rumen biohydrogenation is a complex microbial process involving lipolysis, isomerization, hydrogenation, and the transformation of unsaturated fatty acids into various intermediates and end products. This process is strongly influenced by the ruminal environment, including pH, fermentation characteristics, microbial population, hydrogen balance, and nutrient availability [12,13]. Changes in ruminal conditions may alter the extent and direction of biohydrogenation and, consequently, the relative formation and persistence of trans-C18:1 and other unsaturated fatty acid intermediates. These intermediates may subsequently contribute to the pool of fatty acids available for mammary metabolism and CLA formation. Thus, general fermentation parameters may describe the ruminal environment but may not fully capture the specific lipid transformations associated with variation in milk CLA. Previous studies have shown that low rumen pH and high-concentrate feeding may promote more complete biohydrogenation toward stearic acid formation [9]. However, this interpretation may not fully represent the complexity of ruminal biohydrogenation. Kazemi [14] reported that high-concentrate diets associated with lower ruminal pH can alter ruminal lipid metabolism and increase the formation of trans- and conjugated fatty acid intermediates. Thus, changes in ruminal pH may alter the extent and direction of biohydrogenation and the formation of trans-C18:1 intermediates, while the subsequent availability of specific intermediates such as vaccenic acid may contribute to mammary cis-9, trans-11 CLA synthesis [11].

Despite extensive studies on dietary lipid supplementation, most previous research has focused primarily on general rumen fermentation parameters such as total volatile fatty acids (VFAs), ammonia, and digestibility [15]. Information regarding the relationships among partial VFA profiles, methane production, biohydrogenation indices, and CLA production under practical feeding systems remains limited. In particular, little is known about how rumen lipid metabolism differs between dairy production systems naturally producing extremely high and low milk CLA concentrations under field conditions. Ruminal fatty acid transformation has also been shown to be influenced by alternative feed sources. The inclusion of whole pomegranate by-products was associated with increased total PUFAs, vaccenic acid, and rumenic acid, together with a lower C18:1 trans-10/trans-11 ratio, indicating that ruminal biohydrogenation may be modulated by both fatty acid substrates and bioactive compounds [14]. Comparing feeding systems from the extreme ends of the milk-CLA spectrum allows for a more comprehensive characterization of ruminal metabolic characteristics associated with CLA variation and lipid biohydrogenation.

Therefore, this study aimed to evaluate the rumen fermentation characteristics, partial VFA profiles, methane production, nutrient digestibility, and biohydrogenation indices of dairy rations

associated with extremely high and low milk CLA concentrations. It was hypothesized that diets associated with higher milk CLA concentrations would be associated with ruminal metabolic characteristics consistent with the relatively greater persistence of unsaturated fatty acid intermediates and IBH than diets associated with lower milk CLA concentrations.

2. Materials and methods

2.1. Ethical approval for the research

The experimental protocol used in this study was approved by the Animal Ethics Committee of IPB University (approval number 395/KEH/SKE/XI/2025).

2.2. Dietary treatments

Ration selection was based on the CLA concentrations in 300 milk samples collected from dairy farms in Pangalengan, Kebon Pedes, and Kunak. Fifteen rations associated with the highest milk CLA concentrations and fifteen associated with the lowest CLA concentrations were selected. Milk production, CLA concentration and yield, and PUFA concentration of the selected milk samples are presented in Table 1.

The rations used by the selected farms were identified and subsequently reformulated using locally available feed ingredients according to field proportions. These rations were further evaluated for rumen fermentability, digestibility, and lipid biohydrogenation using *in vitro* methods to characterize the ruminal responses associated with the field-observed variation in milk CLA concentrations. Because the experimental rations were reconstructed from field feeding practices, they were not formulated to differ in a single nutritional factor, such as dietary fat concentration. Therefore, the observed milk CLA concentrations in Table 1 represent the characteristics of the original field-associated production systems, while the feed ingredient composition presented in Table 2 and the corresponding nutrient composition presented in Table 3 describe the reconstructed rations evaluated *in vitro*. The comparison between Tables 1 and 3 was therefore used to explore associations between field-associated milk CLA production and dietary characteristics rather than to establish a direct causal relationship between individual dietary nutrients and milk CLA concentration.

The 30 experimental rations consisted of varying proportions of forage and concentrate ingredients (as detailed in Table 2). Fresh grasses and forage sources comprised approximately 10.38–82.28% of the rations' dry matter (DM), while concentrate ingredients, including commercial concentrate, rice bran, cassava by-products, corn by-products, tofu waste, and other agro-industrial residues, ranged from 0.83% to 82.64%. Rice bran and energy-rich by-products were included at approximately 4.42–61.54%, whereas wet agro-industrial by-products such as tofu waste and cassava waste ranged from 2.44% to 80.00%. Protein-rich ingredients and concentrate mixtures were incorporated at approximately 2.56–30.97%, while mineral and vitamin supplements were included at low levels (<5%). The wide variation in ingredient composition reflected the diversity of tropical smallholder dairy feeding systems and was maintained to evaluate the relationships among rations' characteristics, rumen fermentation, nutrient digestibility, lipid biohydrogenation, and milk CLA production. The experimental diets were not formulated to be compositionally or nutritionally matched because they were reconstructed to represent the feeding practices observed on the selected farms.

Consequently, the high- and low-CLA classifications should be regarded as field-associated groupings rather than controlled dietary treatments differing only in their capacity to produce milk CLA. The nutrient composition of the experimental rations is presented in Table 3.

Table 1. Milk production and fatty acid profiles of tested rations associated with the highest and lowest milk CLA concentrations from smallholder farmers.

Treat ment	Milk yield (kg/d)	Milk fat (%)	CLA (% of milk fat)	CLA (% of milk)	CLA Yield (g/d)	UFA (% of milk fat)	UFA (% of milk)
P1	15	1.05	4.00	0.04	6.31	56.03	0.59
P2	19	4.45	3.7	0.16	31.24	62.31	2.77
P3	18	4.18	3.38	0.14	25.47	58.2	2.43
P4	20	1.54	3.08	0.05	9.46	52.07	0.8
P5	19	1.44	2.94	0.04	8.01	53.41	0.77
P6	14	5.04	2.94	0.15	20.73	62.6	3.15
P7	12	6.63	2.94	0.19	23.37	68.74	4.56
P8	13	4.39	2.78	0.12	15.85	62.22	2.73
P9	16	4.63	2.77	0.13	20.48	64.78	3
P10	13	2.21	2.69	0.06	7.70	52.75	1.16
P11	16	1.52	2.61	0.04	6.33	60.44	0.92
P12	14	3.62	2.55	0.09	12.93	64.07	2.32
P13	16	4.52	2.51	0.11	18.18	62.01	2.8
P14	16	3.64	2.41	0.09	14.05	60.78	2.21
P15	14	5.11	2.4	0.12	17.17	66.99	3.42
P16	20	2.53	0.43	0.01	2.15	37.45	0.95
P17	11	6.48	0.54	0.04	3.85	55.86	3.62
P18	16	3.37	0.59	0.02	3.20	56.56	1.91
P19	12	3.18	0.62	0.02	2.36	47.29	1.5
P20	11	4.8	0.63	0.03	3.35	51.01	2.45
P21	15	2.77	0.68	0.02	2.81	59.16	1.64
P22	16	1.59	0.72	0.01	1.83	47.72	0.76
P23	21	3.37	0.73	0.02	5.13	52.45	1.76
P24	11	2.96	0.74	0.02	2.40	52.47	1.55
P25	13	3.72	0.76	0.03	3.67	50.99	1.89
P26	13	3.19	0.78	0.02	3.22	65.15	2.08
P27	16	3.82	0.85	0.03	5.18	60.43	2.31
P28	10	3.95	0.86	0.03	3.38	67.91	2.68
P29	13	3.22	0.87	0.03	3.66	27.07	0.87
P30	11	3.98	0.92	0.04	4.05	62.2	2.48

Note: P1–15 = diets associated with high CLA; P16–30 = diets associated with low CLA.

Table 2. Feed compositions of tested rations associated with the highest and lowest milk CLA concentrations from smallholder farmers.

Treatment	Feed ingredients (% DM)															
	RC	PC	NG	CSL	RS	TOW	RB	MF	TEW	EG	VW	CW	CH	CSW	CL	MV
P1	17.59		5.25													
P2	20.63			8.67	4.64	4.22	7.95									
P3						2.81		4.82	2.50	11.93						
P4			2.69			4.62		1.97			8.85					
P5	16.92									14.68		0.46				
P6		6.66				11.08				5.87						
P7			14.19			4.87		4.17								
P8	8.92	8.78	5.58							10.14						
P9						8.78		1.88		5.82	2.74					
P10		12.37				5.14				10.91						
P11						1.87		3.27	1.66				19.50			
P12		0.72				2.98				15.78						
P13		4.03				4.60				13.32						
P14		6.17				10.29				6.82						
P15		7.07			3.57	7.35				7.79						
P16	10.49	10.32	4.17							7.59				2.48		
P17		20.78	5.32							9.67						
P18	8.91		17.28			1.37										
P19		13.67			9.82					5.36					9.54	
P20			3.53			9.07		2.59					7.64			0.04
P21	10.35	5.09	3.29							5.99					12.00	
P22	6.42		6.18			0.80				5.62		3.53				
P23	14.67		5.83							10.61						
P24	7.33		11.67											1.25	9.45	

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Treatment	Feed ingredients (% DM)															
	RC	PC	NG	CSL	RS	TOW	RB	MF	TEW	EG	VW	CW	CH	CSW	CL	MV
P25	25.13		5.00							9.09						
P26						9.23		1.97		3.92			6.67			
P27		5.82				5.33				12.15						
P28		8.66			5.25	4.50				9.55						
P29		17.32								15.27						
P30		9.62				8.00				8.48						

Note: P1–15 = diets associated with high CLA; P16–30 = diets associated with low CLA; RC = regular concentrate; PC = premium concentrate; NG = natural grass; CSL = corn silage; RS = rice straw; TOW = tofu waste; RB = rice bran; MF = Mulyo feed; TEW = tempeh waste; EG = elephant grass; VW = vegetable waste; CW = cabbage waste; CH = corn husk; CSW = cassava waste; CL = corn leaves; MV = multivitamins.

Table 3. Nutrient composition of rations associated with high- and low-milk CLA production systems under smallholder farming conditions.

Treatment	Ash*	Crude protein*	Ether extract*	Crude fiber*	NFE ^a	TDN ^b
	% DM					
P1	13.84	11.62	1.93	23.49	49.12	62.18
P2	9.32	8.94	2.95	20.77	58.02	65.39
P3	8.41	6.21	3.66	27.46	54.26	59.97
P4	13.31	17.74	5.54	13.29	50.12	74.81
P5	10.90	6.32	1.96	27.29	53.53	58.35
P6	9.57	10.42	5.32	21.15	53.54	67.47
P7	11.23	10.91	3.27	23.55	51.04	63.47
P8	11.36	7.93	1.97	25.88	52.86	59.79
P9	9.23	10.36	4.54	20.78	55.09	67.09
P10	7.92	8.61	3.64	24.50	55.33	62.92
P11	15.97	5.93	1.90	26.38	49.82	58.54
P12	9.01	5.40	2.55	29.78	53.26	56.79
P13	8.26	7.50	3.34	26.62	54.28	60.62
P14	5.71	10.55	4.79	21.44	57.51	67.11
P15	8.14	8.92	4.02	23.81	55.11	63.89
P16	9.48	7.96	1.83	22.75	57.98	62.50
P17	10.95	9.21	1.90	24.53	53.41	61.12
P18	13.68	11.49	2.07	24.84	47.92	61.15
P19	12.99	9.21	1.14	23.04	53.62	61.51
P20	5.81	10.45	4.36	21.78	57.60	66.40
P21	10.81	8.75	1.91	26.15	52.38	59.69
P22	14.04	10.03	2.15	24.18	49.60	61.51
P23	11.43	8.13	1.99	26.56	51.89	59.25
P24	12.16	10.00	1.63	25.30	50.91	60.24
P25	11.61	9.24	2.14	24.20	52.81	61.57
P26	5.20	9.42	4.41	22.70	58.27	65.54
P27	8.38	8.01	3.57	25.55	54.49	61.82
P28	10.14	7.91	2.94	24.69	54.32	61.80
P29	10.20	6.84	1.97	26.84	54.15	58.89
P30	6.97	9.84	4.34	22.62	56.23	65.46

Note: P1–15 = diets associated with high CLA; P16–30 = diets associated with low CLA. *Nutrient composition was estimated using Near-Infrared Reflectance Spectroscopy (NIRS) with the local calibration database developed by Despal et al. [16].

^aNitrogen-free extract (NFE), calculated using the following formula: $NFE = 100 - (\%Ash + \%CP + \%EE + \%CF)$, where CP is crude protein, EE is ether extract, and CF is crude fiber. ^bTotal digestible nutrient (TDN), according to Wardeh [17].

2.3. Preparation of rumen inoculum

Rumen fluid was collected before the morning feeding from three rumen-fistulated Holstein-Friesian bulls maintained under the supervision of a licensed veterinarian. Rumen fluid was collected on three separate occasions at weekly intervals. At each collection occasion, rumen fluid obtained from

the three bulls was pooled and thoroughly mixed to obtain a homogeneous rumen inoculum. The pooled rumen inoculum was then used for all 30 dietary treatments within the corresponding experimental run. The individual donor bulls were therefore not considered to be independent experimental units. Immediately after collection, the rumen fluid was filtered through four layers of cheesecloth under continuous CO₂ flushing to maintain anaerobic conditions. The filtered rumen fluid was transferred into prewarmed (39–40 °C) thermos flasks and transported immediately to the laboratory for *in vitro* incubation.

2.4. *In vitro* procedure

In vitro analyses were conducted using two complementary techniques, namely the methods of Tilley and Terry [18] and Theodorou et al. [19]. Tilley and Terry's method was used to determine rumen fermentation characteristics, microbial protein synthesis (MPS), nutrient digestibility, and biohydrogenation indices, whereas Theodorou et al.'s method was used to evaluate gas and methane production.

For Tilley and Terry's method [18], 0.5 g of each feed sample was weighed into a 100-mL fermentation tube, followed by the addition of 40 mL of McDougall's buffer and 10 mL of rumen fluid. The mixture was homogenized, flushed with CO₂ for 30 s to maintain anaerobic conditions, sealed with a vented rubber stopper, and incubated in a shaking water bath at 39 °C. Fermentation was conducted for 4 h for determining the rumen fermentation characteristics and microbial parameters. After incubation, 1 mL of the fermentation fluid was collected for protozoal enumeration, 9 mL for partial VFA analysis, and 20 mL for MPS analysis, whereas pH was measured immediately. The remaining incubation fluid was centrifuged at 3000 rpm for 15 min, and the supernatant was preserved with two drops of a saturated HgCl₂ solution for the NH₃ and total VFA analyses.

For biohydrogenation analysis, whole incubation contents (solid and liquid fractions) were collected separately from designated fermentation tubes at 0 and 48 h of incubation. For determining *in vitro* digestibility, samples were incubated for 48 h under the same fermentation conditions. Fermentation was terminated by adding two drops of a saturated HgCl₂ solution, followed by centrifugation at 3000 rpm for 10 min. The supernatant was discarded, and the residue was resuspended in 50 mL of a 0.2% pepsin–HCl solution. Enzymatic digestion was continued for an additional 48 h under aerobic conditions.

Meanwhile, in Theodorou et al.'s [19] method, 0.75 g of each feed sample was weighed into 100-mL serum bottles, followed by the addition of 50 mL of McDougall's buffer and 25 mL of rumen fluid. The bottles were flushed with CO₂ for 1 min to maintain anaerobic conditions, sealed with rubber stoppers and aluminum crimp seals, and incubated in a water bath at 39 °C for 48 h.

2.5. *Fermentation characteristics*

Rumen pH was measured after 4 h of incubation using a calibrated digital pH meter (standard buffers at pH 4.0 and 7.0). Measurements were performed on incubation fluid prior to the addition of a saturated HgCl₂ solution. Ammonia (NH₃) concentration was determined using Conway's microdiffusion method. Briefly, 1 mL of a saturated Na₂CO₃ solution and 1 mL of supernatant from the 4-h incubation were placed in separate compartments of a Conway dish, while the center well contained 1 mL of a boric acid indicator solution (bromocresol green:methyl red, BCG:MR). The dish was sealed and incubated at room temperature for 24 h. Subsequently, the trapped ammonia was

quantified by titration with 0.005 N H₂SO₄.

Total VFA concentration was determined using the steam distillation method. Briefly, 5 mL of supernatant from the 4-h incubation was mixed with 1 mL of 15% H₂SO₄ and subjected to steam distillation. The distillate was collected into 5 mL of 0.5 N NaOH until a final volume of 250 mL was obtained and subsequently titrated with 0.5 N HCl. A blank sample containing 15% H₂SO₄ without incubation fluid was analyzed simultaneously.

Partial VFA concentrations were determined by gas chromatography–mass spectrometry (GC–MS) following the method described by Sarwono et al. [20]. Following 4 h of incubation, samples were centrifuged at 3000 rpm for 15 min to obtain the supernatant. A 1.25-mL aliquot of the supernatant was transferred into a 1.5-mL microcentrifuge tube containing 30 mg of 5-sulfosalicylic acid dihydrate and centrifuged at 12,000 rpm for 15 min at 4 °C. The clarified supernatant (1 µL) was injected into an Agilent 8890 GC–MS system equipped with an AOC-20i+s autosampler and an HP-INNOWAX MS capillary column (length, 30 m; inner diameter (i.d.), 0.25 mm; film thickness, 0.25 µm). Ultrahigh-purity helium (99.99%) was used as the carrier gas at a flow rate of 3 mL/min. Samples were injected with a split ratio of 50:1, and the solvent delay was set at 3 min. The injector, transfer line, and ion source temperatures were maintained at 250 °C. The oven temperature was initially set at 100 °C and held for 9 min, followed by an increase to 200 °C at a rate of 10 °C/min and held for an additional 10 min. The total run time was approximately 29 min. Individual VFA fractions were identified and quantified using a volatile free acid mix standard (Supelco, CRM46975).

2.6. Microbial measurements

Protozoal population was determined using the staining method of Ogimoto and Imai [21]. Briefly, 1 mL of rumen fluid collected after 4 h of incubation was mixed with 1 mL of a trypan blue formal saline (TBFS) solution, loaded onto a Fuchs–Rosenthal counting chamber, covered with a cover glass, and examined under a light microscope at 40× magnification. Protozoal population was expressed as cells/mL of rumen fluid.

MPS was determined according to the method of Makkar et al. [22] using the Lowry protein assay [23]. Briefly, 20 mL of anaerobic incubation fluid collected after 4 h of incubation was centrifuged at 400 rpm for 45 s, followed by centrifugation at 408 × g for 5 min. Subsequently, 10 mL of the resulting supernatant was mixed with 2.5 mL of 64.5% trichloroacetic acid (TCA) and centrifuged at 15,000 rpm for 20 min. The supernatant was discarded, and the pellet was washed with distilled water and centrifuged again under the same conditions. The pellet was then resuspended in 30 mL of 0.25 N NaOH, heated in a water bath for 10 min, and cooled to room temperature.

Microbial protein concentration was measured using the Lowry assay. The reagents consisted of 2% sodium carbonate in 0.1 N NaOH, 0.5% copper sulfate in 1% sodium potassium tartrate, freshly prepared alkaline copper reagent (50:1, v/v), and Folin–Ciocalteu reagent diluted 1:1 with distilled water. Aliquots of the sample solution and bovine serum albumin (BSA) standards were adjusted to a final volume of 4 mL with distilled water. Subsequently, 5.5 mL of alkaline copper reagent was added and incubated for 10–15 min, followed by the addition of 0.5 mL diluted Folin–Ciocalteu reagent. After approximately 30 min of reaction, absorbance was measured at 650 nm using a spectrophotometer, and the microbial protein concentration was calculated from the bovine serum albumin (BSA) standard curve.

2.7. Gas production and methane concentration

Total gas production during *in vitro* fermentation was measured at 2, 4, 6, 8, 10, 12, 24, and 48 h of incubation using a 50-mL gas syringe. Gas volume was determined by inserting the syringe needle through the rubber stopper of the fermentation bottle and recorded directly from the syringe scale. At the same incubation times, gas samples for methane analysis were collected into preconditioned vacuum vials and analyzed by gas chromatography (GC) at the Greenhouse Gas Laboratory, Indonesian Agricultural Environment Research Institute.

2.8. Digestibility measurements

Dry matter digestibility (DMD) and organic matter digestibility (OMD) were determined according to the second stage of the *in vitro* Tilley and Terry [18] procedure described previously. Following 48 h of ruminal incubation and subsequent pepsin–HCl digestion, digestion residues were filtered through Whatman No. 41 filter paper using vacuum filtration. The residues were dried at 105 °C for 24 h to determine the DM residue and subsequently ashed at 650 °C for 4 h to determine the organic matter residue.

Fiber digestibility was analyzed using the Van Soest method with an ANKOM 200 fiber analyzer. Residues obtained from *in vitro* digestion were transferred into ANKOM N57 filter bags and sequentially extracted using neutral detergent solution (NDS) at 100 °C for 75 min followed by an acid detergent solution (ADS) at 100 °C for 60 min. Neutral detergent fiber digestibility (NDFD) and acid detergent fiber digestibility (ADFD) were calculated on the basis of the remaining fiber residues.

2.9. Biohydrogenation measurements

In vitro biohydrogenation analysis was conducted using the Tilley and Terry incubation system [18], following the procedure described by Anzhany et al. [24]. Feed samples incubated with rumen fluid and McDougall's buffer were collected at 0 and 48 h of incubation for fatty acid analysis. Immediately after sampling, fermentation was terminated by immersing the samples in ice water, and samples were stored at –20 °C until further analysis.

Sample preparation, lipid extraction, and methylation were performed according to Vargas et al. [25]. Briefly, 50 mg of the lyophilized sample was transferred into a screw-cap tube containing 2148 µL of methanol, 990 µL of toluene, 66 µL of sulfuric acid, 1000 µL of dimethyl sulfoxide (DMSO), and 2000 µL of hexane. The sealed tubes were incubated in a water bath at 80 °C for 2 h and subsequently cooled to room temperature (approximately 25 °C). The hexane layer was collected into a 2-mL microcentrifuge tube and evaporated under a nitrogen stream. The remaining residue was reconstituted in 500 µL of dichloromethane and vortexed for 1 min. Subsequently, 250 µL of the solution was transferred into chromatographic vials and stored at –20 °C until analysis.

Fatty acid composition was determined by injecting 1 µL of the sample into a gas chromatograph (Agilent 7820A GC; Agilent Technologies) equipped with a CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d., 0.20 µm film thickness). The injector and detector temperatures were maintained at 260 °C with a split ratio of 50:1. Chromatographic separation was conducted under constant pressure mode (32 psi) using hydrogen as the carrier gas (40 mL/min), helium as the makeup gas (25 mL/min), and air as the oxidant gas (400 mL/min). The oven temperature program was set at 100 °C for 5 min, increased to 180 °C at 8 °C/min and held for 9 min, followed by an increase to 230 °C

at 1°C/min and a final hold for 15 min. Fatty acid peaks were identified by comparing the retention times with an authentic standard mixture (Supelco 37 Component FAME Mix, CRM47885; Sigma-Aldrich).

Biohydrogenation parameters and related indices were calculated from the changes in fatty acid composition between 0 and 48 h of incubation according to the approach described by Despal et al. [26], using the following equations:

$$\text{C18:2 cis (\%)} = [(C18:2 \text{ cis}_{0h} - C18:2 \text{ cis}_{48h}) / C18:2 \text{ cis}_{0h}] \times 100 \quad (1)$$

$$\text{C18:3 n-3 (\%)} = [(C18:3 \text{ n-3}_{0h} - C18:3 \text{ n-3}_{48h}) / C18:3 \text{ n-3}_{0h}] \times 100 \quad (2)$$

$$\text{C18:3 n-6 (\%)} = [(C18:3 \text{ n-6}_{0h} - C18:3 \text{ n-6}_{48h}) / C18:3 \text{ n-6}_{0h}] \times 100 \quad (3)$$

$$\text{Total PUFA (\%)} = [(Total \text{ PUFA}_{0h} - Total \text{ PUFA}_{48h}) / Total \text{ PUFA}_{0h}] \times 100 \quad (4)$$

$$\text{Saturation index} = (C18:0_{48h} / Total \text{ PUFA}_{48h}) \quad (5)$$

$$\text{IBH ratio} = [(C18:1 \text{ trans}_{48h} + Total \text{ CLA}_{48h}) / C18:0_{48h}] \quad (6)$$

$$\text{Apparent net CLA change (\%)} = [(Total \text{ CLA}_{48h} - Total \text{ CLA}_{0h}) / C18:2 \text{ cis}_{0h}] \times 100 \quad (7)$$

$$\text{C18:1 isomerization index} = [C18:1 \text{ trans}_{48h} / (C18:1 \text{ cis}_{48h} + C18:1 \text{ trans}_{48h})] \quad (8)$$

$$\text{C18:2 isomerization index} = [C18:2 \text{ trans}_{48h} / (C18:2 \text{ cis}_{48h} + C18:2 \text{ trans}_{48h})] \quad (9)$$

$$\text{Total C18 recovery index} = [\sum \text{Final C18 FA} / \sum \text{Initial C18 FA}] \quad (10)$$

$$\text{Unsaturated index}_{0h} = (Total \text{ PUFA}_{0h} / C18:0_{0h}) \quad (11)$$

$$\text{Unsaturated index}_{48h} = (Total \text{ PUFA}_{48h} / C18:0_{48h}) \quad (12)$$

These indices were used as complementary descriptors of the relative extent and pattern of C18 fatty acids' transformation during incubation. They were not considered direct indicators of specific stages or pathways of ruminal biohydrogenation, because individual trans-C18:1 and CLA isomers were not quantified separately [11–13]. Because fatty acid values were expressed as the relative chromatographic composition rather than absolute amounts of fatty acid, the calculated disappearance values represent apparent rather than absolute disappearance. Negative disappearance values indicate that the relative abundance of the respective fatty acid at 48 h exceeded that measured at 0 h and should not be interpreted as net fatty acid synthesis. Because the calculation is expressed relative to the initial abundance at 0 h, a large proportional increase in relative fatty acid abundance at 48 h can result in an extremely negative apparent disappearance value, without representing an equivalent absolute increase in fatty acid mass. The apparent net CLA change (ANCC) represents the change in the relative CLA pool between 0 and 48 h, expressed relative to the initial C18:2-cis concentration, rather than an absolute CLA production yield. Because ANCC is normalized to the initial relative abundance of C18:2-cis, a relatively small denominator can amplify the magnitude of the calculated percentage change. Positive values indicate an apparent net increase in the measured CLA pool, whereas negative values indicate an apparent net decrease during incubation. Accordingly, these indices reflect changes in the relative fatty acid profile during incubation rather than an absolute fatty acid mass balance.

2.10. Design experiment and data analysis

This study combined field-based exploratory research and laboratory experiments. The field study

used a survey approach with cluster sampling to identify dairy farms producing extremely high and extremely low milk CLA concentrations. Subsequently, the laboratory experiment employed an *in vitro* technique using a randomized complete block design with 30 dietary treatments, comprising 15 rations associated with high CLA and 15 associated with low CLA. The three rumen-fluid collection occasions conducted at weekly intervals constituted the three experimental blocks. At each collection occasion, rumen fluid from the three rumen-fistulated Holstein-Friesian bulls was pooled and used as a common rumen inoculum for all 30 dietary treatments. Thus, each dietary treatment was evaluated once within each block, resulting in three experimental replicates per treatment ($n = 3$) and 90 treatment $\times$ block observations in total. The three donor bulls were not considered to be independent experimental units because their rumen fluids were pooled at each collection occasion. For each dietary treatment within each collection occasion, multiple fermentation vessels were used for different analytical endpoints, including fermentation, digestibility, gas and methane production, and biohydrogenation. These vessels were not considered to be independent replicates. The experimental unit for each analysis was one dietary treatment within one rumen fluid collection block; thus, each treatment had three independent experimental replicates ($n = 3$).

Data were analyzed using analysis of variance (ANOVA) based on the general linear model (GLM) procedure in IBM SPSS Statistics version 27, with dietary treatment as the fixed effect and rumen fluid collection occasion as the blocking factor. The 30 reconstructed diets were treated as individual dietary treatments to characterize the variation among field-derived feeding systems. When a significant treatment effect was detected ($P < 0.05$), subsequent contrast-based comparisons were performed to identify differences among individual dietary treatments, and the results are presented as superscript letters in Tables 4–6. The classifications associated with high and low CLA (P1–P15 and P16–P30, respectively) were used as field-associated groupings and were not interpreted as controlled dietary treatments. Statistical significance was declared at $P < 0.05$. Principal component analysis (PCA) was conducted after variable standardization using the *prcomp* function in R version 4.4.2 (RStudio, Posit Software, PBC, Boston, MA, USA). 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 in R, with only statistically significant correlations (95% confidence level) presented.

3. Results

3.1. *In vitro* rumen fermentation characteristics

The *in vitro* rumen fermentation characteristics of the experimental diets are presented in Table 4. Significant differences ($P < 0.05$) were observed among diets for total VFA concentration, ammonia (NH_3), gas production, and MPS, whereas ruminal pH and protozoal population did not differ significantly among diets. Ruminal pH ranged from 6.74 to 6.93, while total VFA concentration ranged from 93.37 to 144.37 mM. Ammonia concentration varied from 5.18 to 16.70 mM, and MPS ranged from 5.27 to 14.99 mg/10 mL.

Gas production differed substantially among diets, ranging from 107.89 to 218.56 mL/g DM. The highest gas production was observed in P20 (218.56 mL/g DM), followed by P14 (218.53 mL/g DM) and P6 (215.56 mL/g DM), whereas P1 had the lowest value (107.89 mL/g DM). Protozoal population remained relatively stable among treatments, ranging from 6.19 to 6.34 log cells/mL.

Several diets associated with high milk CLA concentrations, including P6, P9, P14, and P15, exhibited relatively high total VFA concentrations, whereas similarly high values were observed in diets associated with low CLA, such as P20, P25, P26, and P30. High gas production was also observed in both groups. Thus, the variation in individual fermentation parameters occurred across diets associated with both high and low CLA.

Table 4. *In vitro* rumen fermentation characteristics of diets associated with high and low CLA.

Treatment	pH	Total VFAs (mM)	Ammonia (NH ₃) (mM)	Gas production (mL/g DM)	Protozoa (log cell/mL)	MPS (mg/10 mL)
P1	6.80 ± 0.09	96.61 ± 3.58 ^a	10.34 ± 0.61 ^g	107.89 ± 7.40 ^a	6.31 ± 0.26	11.12 ± 2.78 ^d
P2	6.79 ± 0.06	109.14 ± 3.95 ^c	6.35 ± 0.70 ^b	166.73 ± 4.44 ^e	6.29 ± 0.24	12.36 ± 2.18 ^e
P3	6.86 ± 0.04	108.98 ± 3.61 ^c	5.18 ± 0.96 ^a	177.21 ± 8.16 ^f	6.20 ± 0.09	11.99 ± 5.95 ^e
P4	6.79 ± 0.09	121.77 ± 3.75 ^e	16.70 ± 2.46 ⁱ	164.19 ± 8.98 ^d	6.22 ± 0.24	14.40 ± 3.96 ^f
P5	6.93 ± 0.05	97.06 ± 7.31 ^a	7.75 ± 1.01 ^d	148.12 ± 1.58 ^c	6.31 ± 0.16	10.36 ± 3.93 ^d
P6	6.83 ± 0.07	132.30 ± 5.76 ^g	8.19 ± 0.92 ^e	215.56 ± 15.06 ^h	6.26 ± 0.14	12.76 ± 3.44 ^f
P7	6.80 ± 0.09	116.58 ± 6.59 ^d	9.80 ± 1.77 ^f	135.24 ± 0.07 ^b	6.30 ± 0.14	7.67 ± 0.59 ^b
P8	6.83 ± 0.08	104.36 ± 14.33 ^b	7.88 ± 0.40 ^d	139.58 ± 9.52 ^b	6.27 ± 0.13	7.67 ± 1.82 ^b
P9	6.82 ± 0.03	130.38 ± 4.81 ^g	8.31 ± 0.08 ^e	197.88 ± 10.60 ^g	6.26 ± 0.17	10.38 ± 0.48 ^d
P10	6.83 ± 0.10	116.70 ± 13.73 ^d	6.37 ± 0.08 ^b	194.26 ± 9.64 ^g	6.23 ± 0.17	5.27 ± 0.10 ^a
P11	6.90 ± 0.10	114.35 ± 6.59 ^d	6.29 ± 0.07 ^b	134.08 ± 16.08 ^b	6.25 ± 0.19	7.78 ± 0.40 ^b
P12	6.83 ± 0.09	125.83 ± 3.70 ^f	5.44 ± 0.29 ^a	150.79 ± 2.87 ^c	6.29 ± 0.11	7.64 ± 1.23 ^b
P13	6.81 ± 0.05	129.69 ± 2.03 ^f	6.70 ± 1.22 ^b	173.99 ± 8.67 ^e	6.23 ± 0.19	7.52 ± 0.64 ^b
P14	6.83 ± 0.08	132.93 ± 4.22 ^g	7.81 ± 0.63 ^d	218.53 ± 7.38 ^h	6.20 ± 0.06	7.81 ± 0.08 ^b
P15	6.89 ± 0.09	121.33 ± 14.33 ^e	6.24 ± 0.13 ^b	186.01 ± 1.35 ^f	6.23 ± 0.11	8.58 ± 1.74 ^c
P16	6.78 ± 0.06	109.50 ± 7.35 ^c	5.81 ± 0.06 ^a	173.97 ± 3.54 ^e	6.19 ± 0.13	8.97 ± 1.59 ^c
P17	6.80 ± 0.12	107.88 ± 6.46 ^c	7.61 ± 0.23 ^c	156.48 ± 9.87 ^d	6.27 ± 0.12	9.62 ± 0.77 ^c
P18	6.74 ± 0.12	110.05 ± 7.56 ^c	7.09 ± 0.13 ^c	113.66 ± 1.28 ^a	6.20 ± 0.13	9.15 ± 1.72 ^c
P19	6.86 ± 0.10	121.24 ± 0.22 ^e	8.59 ± 0.83 ^e	139.69 ± 4.63 ^b	6.27 ± 0.24	8.20 ± 2.15 ^b
P20	6.75 ± 0.06	133.59 ± 7.68 ^g	9.73 ± 0.06 ^f	218.56 ± 0.97 ^h	6.20 ± 0.14	9.39 ± 1.91 ^c
P21	6.79 ± 0.06	114.17 ± 8.82 ^d	9.46 ± 0.30 ^f	158.82 ± 8.32 ^d	6.30 ± 0.19	9.16 ± 2.29 ^c
P22	6.78 ± 0.06	105.37 ± 11.34 ^b	13.16 ± 1.07 ^h	140.91 ± 11.40 ^b	6.19 ± 0.18	9.18 ± 3.05 ^c
P23	6.75 ± 0.08	93.37 ± 7.17 ^a	8.03 ± 1.33 ^d	143.35 ± 8.15 ^c	6.28 ± 0.14	9.55 ± 3.49 ^c
P24	6.84 ± 0.10	114.67 ± 10.80 ^d	9.83 ± 0.41 ^f	143.94 ± 12.33 ^c	6.34 ± 0.12	9.03 ± 3.49 ^c
P25	6.79 ± 0.06	136.08 ± 4.02 ^h	8.32 ± 0.71 ^e	155.81 ± 4.77 ^d	6.20 ± 0.26	10.70 ± 2.51 ^d
P26	6.76 ± 0.04	144.37 ± 0.27 ^h	9.05 ± 0.71 ^f	170.22 ± 35.56 ^e	6.22 ± 0.21	14.32 ± 0.80 ^f
P27	6.76 ± 0.05	127.41 ± 10.68 ^f	7.66 ± 0.47 ^d	182.41 ± 8.77 ^f	6.24 ± 0.14	11.87 ± 0.25 ^e
P28	6.77 ± 0.08	105.40 ± 6.06 ^b	7.30 ± 0.43 ^c	170.09 ± 1.03 ^e	6.29 ± 0.16	12.10 ± 0.60 ^e
P29	6.84 ± 0.11	96.05 ± 10.41 ^a	6.89 ± 0.51 ^c	160.44 ± 9.58 ^d	6.24 ± 0.13	13.45 ± 0.83 ^f
P30	6.85 ± 0.14	136.01 ± 6.30 ^h	7.64 ± 0.06 ^d	202.95 ± 5.96 ^g	6.24 ± 0.06	14.99 ± 0.76 ^f

Note: P1–15 = diets associated with high CLA; P16–30 = diets associated with low CLA. VFA = volatile fatty acid. Different superscript letters within the same column indicate significantly different dietary treatments based on the subsequent contrast-based comparison ($P < 0.05$).

3.2. Molar proportions of partial VFAs and methane production

The molar proportions of individual VFAs and methane production are presented in Table 5. Significant differences ($P < 0.05$) were observed among diets for acetate (C2), propionate (C3), butyrate (C4), iso-butyrate (iC4), valerate (C5), acetate-to-propionate ratio (C2/C3), and methane production, whereas iso-valerate (iC5) did not differ significantly among diets ($P > 0.05$).

Acetate concentration ranged from 46.43% to 57.03%, while propionate ranged from 21.28% to 27.04%, resulting in acetate-to-propionate ratios ranging from 1.80 to 2.68. Butyrate concentration ranged from 11.30% to 18.57%, whereas iso-butyrate and valerate ranged from 1.04% to 2.89% and 2.57% to 4.19%, respectively. Iso-valerate ranged from 3.23% to 7.86% and did not differ significantly among diets. Methane production ranged from 5.03 to 23.31 mL/g DM.

Several diets associated with high CLA, including P2, P6, P10, P12, P14, and P15, exhibited relatively high propionate proportions and lower C2/C3 ratios. Similar patterns were observed in diets associated with low CLA, particularly P20, P26, P27, and P30. Methane production also varied substantially among diets, with both relatively high and low values occurring within the groups associated with high and low CLA.

3.3. Nutrient digestibility

The nutrient digestibility of the experimental diets is presented in Table 6. Significant differences ($P < 0.05$) were observed among diets for DMD, OMD, NDFD, and ADFD. DMD ranged from 52.68% to 80.19%, whereas OMD ranged from 51.18% to 80.64%. NDFD ranged from 26.61% to 55.64%, while ADFD ranged from 24.04% to 40.21%.

Several diets associated with high CLA, including P3, P6, P9, and P15, exhibited relatively high DMD and OMD values. However, similarly high digestibility was observed in diets associated with low CLA, including P19, P20, P25, P26, and P30. NDFD and ADFD also varied considerably within both dietary groups. Overall, substantial overlap in nutrient digestibility was observed between diets associated with high and low milk CLA concentrations.

3.4. Unsaturated fatty acid profiles during ruminal biohydrogenation

The changes in unsaturated fatty acid profiles during *in vitro* ruminal biohydrogenation are presented in Table 7. Significant differences ($P < 0.05$) were observed among diets in terms of the apparent disappearance of cis-C18:2, C18:3 n-3, C18:3 n-6, and total PUFAs. The apparent disappearance of cis-C18:2 ranged from -245.18% to 18.98%, while that of C18:3 n-3 ranged from -21.95% to 7.82%. The apparent disappearance of C18:3 n-6 ranged from -48.62% to 39.16%, whereas that of total PUFAs ranged from -12.96% to 24.70%. Markedly negative apparent disappearance values for cis-C18:2 were observed in several diets, including P9, P16, P20, P26, and P27. Similar variability was observed for C18:3 n-3, C18:3 n-6, and total PUFAs in diets associated with both high and low CLA. Overall, substantial overlap was observed between the two dietary groups. Because the fatty acid values were expressed as the relative chromatographic composition, negative apparent disappearance values represent an increase in relative fatty acid abundance at 48 h compared with 0 h. The extreme value observed for P27 (-245.18% $\pm$ 432.00%) corresponds to a relative abundance at 48 h approximately 3.45 times the initial value at 0 h and therefore does not represent net fatty acid synthesis or an equivalent absolute increase in C18:2-cis.

Table 5. Molar proportions of partial VFA and methane production of diets associated with high and low CLA.

Treatment	C2 (%)	C3 (%)	C4 (%)	iC4 (%)	C5 (%)	iC5 (%)	C2/C3	Methane production (mL/g DM)
P1	54.68 ± 2.39 ^h	21.63 ± 0.59 ^a	13.02 ± 2.18 ^b	2.59 ± 0.29 ^d	3.08 ± 0.16 ^e	4.99 ± 0.60	2.53 ± 0.13 ⁱ	5.03 ± 3.64 ^a
P2	46.43 ± 0.31 ^a	25.76 ± 1.09 ^h	18.45 ± 1.72 ^d	2.19 ± 0.20 ^c	3.02 ± 0.11 ^d	4.15 ± 0.41	1.80 ± 0.07 ^a	12.59 ± 3.90 ^c
P3	49.19 ± 0.70 ^d	25.24 ± 1.07 ^g	16.63 ± 1.96 ^c	2.17 ± 0.24 ^c	2.75 ± 0.14 ^b	4.03 ± 0.52	1.95 ± 0.07 ^c	10.39 ± 7.08 ^c
P4	54.27 ± 0.84 ^g	23.45 ± 0.92 ^d	11.30 ± 1.16 ^a	2.89 ± 0.21 ^d	4.19 ± 0.78 ^g	3.92 ± 0.36	2.32 ± 0.10 ^h	9.86 ± 2.44 ^b
P5	49.53 ± 0.57 ^d	23.56 ± 1.01 ^d	17.82 ± 1.56 ^d	2.21 ± 0.20 ^c	2.94 ± 0.13 ^d	3.94 ± 0.39	2.10 ± 0.07 ^d	11.00 ± 1.95 ^c
P6	48.88 ± 0.68 ^c	26.63 ± 0.90 ⁱ	16.19 ± 1.95 ^c	2.01 ± 0.19 ^b	2.84 ± 0.13 ^c	3.45 ± 0.38	1.84 ± 0.05 ^a	23.31 ± 0.00 ^e
P7	55.96 ± 0.73 ⁱ	22.32 ± 0.75 ^b	11.92 ± 1.27 ^a	2.44 ± 0.26 ^d	2.82 ± 0.12 ^c	4.55 ± 0.55	2.51 ± 0.08 ⁱ	10.16 ± 0.04 ^b
P8	51.54 ± 0.70 ^f	23.00 ± 0.94 ^c	17.22 ± 1.24 ^c	1.15 ± 1.34 ^a	2.96 ± 0.11 ^d	4.14 ± 0.51	2.24 ± 0.10 ^g	9.08 ± 0.90 ^b
P9	53.83 ± 0.49 ^g	24.64 ± 0.86 ^e	13.39 ± 1.48 ^b	1.14 ± 1.33 ^a	3.31 ± 0.07 ^f	3.70 ± 0.50	2.19 ± 0.06 ^f	18.22 ± 2.95 ^c
P10	49.30 ± 0.88 ^d	25.69 ± 0.97 ^h	17.47 ± 1.90 ^d	1.04 ± 1.22 ^a	2.87 ± 0.14 ^c	3.62 ± 0.53	1.92 ± 0.06 ^b	12.46 ± 2.14 ^c
P11	48.92 ± 0.55 ^c	22.99 ± 0.94 ^c	17.83 ± 1.68 ^d	2.53 ± 0.30 ^d	2.74 ± 0.18 ^b	5.00 ± 0.68	2.13 ± 0.07 ^e	11.26 ± 2.54 ^c
P12	50.12 ± 0.92 ^e	25.57 ± 1.22 ^h	16.74 ± 1.84 ^c	1.11 ± 1.31 ^a	2.72 ± 0.18 ^b	3.75 ± 0.71	1.96 ± 0.09 ^c	15.26 ± 1.42 ^d
P13	49.30 ± 0.69 ^d	25.18 ± 0.93 ^g	16.85 ± 1.91 ^c	2.13 ± 0.24 ^c	2.79 ± 0.17 ^b	3.76 ± 0.55	1.96 ± 0.05 ^c	12.96 ± 3.14 ^d
P14	49.02 ± 0.61 ^c	26.58 ± 0.76 ⁱ	16.45 ± 1.85 ^c	1.53 ± 1.04 ^b	2.85 ± 0.15 ^c	3.57 ± 0.43	1.84 ± 0.04 ^a	19.28 ± 2.45 ^c
P15	48.78 ± 0.65 ^c	25.46 ± 1.00 ^g	17.10 ± 1.83 ^c	2.12 ± 0.19 ^c	2.82 ± 0.14 ^c	3.73 ± 0.41	1.92 ± 0.05 ^b	15.50 ± 3.03 ^d
P16	49.23 ± 0.60 ^d	24.96 ± 0.84 ^f	18.23 ± 2.17 ^d	1.46 ± 0.99 ^a	2.73 ± 0.13 ^b	3.39 ± 0.47	1.97 ± 0.05 ^c	12.49 ± 2.21 ^c
P17	50.01 ± 0.55 ^e	23.35 ± 1.10 ^d	13.46 ± 8.34 ^b	2.20 ± 0.22 ^c	3.11 ± 0.29 ^c	7.86 ± 7.51	2.15 ± 0.08 ^c	11.09 ± 3.10 ^c
P18	57.03 ± 0.41 ^j	21.28 ± 0.74 ^a	11.41 ± 1.02 ^a	2.55 ± 0.27 ^d	2.91 ± 0.15 ^c	4.83 ± 0.63	2.68 ± 0.08 ^k	6.42 ± 1.68 ^a
P19	49.00 ± 0.65 ^c	22.20 ± 0.81 ^b	18.25 ± 1.62 ^d	2.53 ± 0.24 ^d	3.08 ± 0.14 ^c	4.93 ± 0.49	2.21 ± 0.06 ^g	12.43 ± 0.20 ^c
P20	50.06 ± 0.60 ^e	26.19 ± 0.65 ⁱ	15.96 ± 1.57 ^c	1.94 ± 0.17 ^b	2.57 ± 0.08 ^a	3.28 ± 0.32	1.91 ± 0.03 ^b	20.03 ± 1.93 ^c
P21	50.60 ± 0.35 ^e	22.72 ± 1.28 ^c	16.89 ± 1.42 ^c	2.41 ± 0.32 ^d	2.91 ± 0.19 ^c	4.47 ± 0.79	2.23 ± 0.11 ^g	10.58 ± 4.14 ^c
P22	56.35 ± 0.59 ⁱ	21.67 ± 0.81 ^a	11.99 ± 1.19 ^a	2.44 ± 0.29 ^d	3.25 ± 0.14 ^f	4.30 ± 0.62	2.60 ± 0.08 ^j	10.82 ± 0.63 ^c
P23	50.81 ± 0.48 ^f	22.75 ± 1.03 ^c	17.16 ± 1.62 ^c	2.27 ± 0.27 ^c	2.89 ± 0.17 ^c	4.13 ± 0.64	2.24 ± 0.09 ^g	7.06 ± 0.49 ^a
P24	54.97 ± 0.56 ^h	21.72 ± 0.88 ^a	13.37 ± 0.92 ^b	2.47 ± 0.25 ^d	2.76 ± 0.17 ^b	4.71 ± 0.56	2.53 ± 0.09 ⁱ	8.19 ± 0.16 ^b

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Treatment	C2 (%)	C3 (%)	C4 (%)	iC4 (%)	C5 (%)	iC5 (%)	C2/C3	Methane production (mL/g DM)
P25	49.59 ± 0.27 ^d	23.30 ± 1.03 ^d	17.84 ± 1.25 ^d	2.24 ± 0.24 ^c	3.00 ± 0.17 ^d	4.02 ± 0.57	2.13 ± 0.09 ^e	11.70 ± 1.74 ^e
P26	49.11 ± 0.58 ^c	27.04 ± 0.87 ⁱ	16.16 ± 1.75 ^c	1.89 ± 0.19 ^b	2.57 ± 0.17 ^a	3.23 ± 0.43	1.82 ± 0.04 ^a	16.17 ± 3.39 ^d
P27	49.33 ± 0.72 ^d	25.19 ± 0.96 ^g	16.91 ± 1.92 ^c	2.08 ± 0.26 ^c	2.79 ± 0.16 ^b	3.69 ± 0.57	1.96 ± 0.05 ^c	10.79 ± 1.66 ^c
P28	48.91 ± 0.49 ^c	24.87 ± 1.01 ^f	17.41 ± 1.73 ^d	2.14 ± 0.27 ^c	2.82 ± 0.19 ^c	3.84 ± 0.56	1.97 ± 0.07 ^c	13.06 ± 1.42 ^d
P29	48.24 ± 0.32 ^b	24.28 ± 1.12 ^e	18.57 ± 1.38 ^d	2.15 ± 0.24 ^c	2.89 ± 0.19 ^c	3.87 ± 0.58	1.99 ± 0.09 ^d	12.58 ± 2.18 ^c
P30	49.21 ± 0.77 ^d	25.70 ± 0.80 ^h	16.66 ± 1.95 ^c	2.04 ± 0.19 ^b	2.83 ± 0.17 ^c	3.56 ± 0.47	1.92 ± 0.04 ^b	20.86 ± 0.43 ^e

Note: P1–15 = diets associated with high CLA; P16–30 = diets associated with low CLA. C2 = acetate; C3 = propionate; C4 = butyrate; iC4 = iso-butyrate; C5 = valerate; iC5 = iso-valerate; C2/C3 = acetate/propionate ratio. Different superscript letters within the same column indicate significantly different dietary treatments based on the subsequent contrast-based comparison ($P < 0.05$).

Table 6. *In vitro* nutrient digestibility of diets associated with high and low CLA.

Treatments	DMD (%)	OMD (%)	NDFD (%)	ADFD (%)
P1	52.68 ± 1.77 ^a	51.18 ± 1.29 ^a	49.83 ± 7.67 ^f	37.27 ± 5.68 ^g
P2	66.01 ± 2.10 ⁱ	67.19 ± 2.77 ⁱ	49.02 ± 4.53 ^f	35.17 ± 6.23 ^f
P3	65.09 ± 1.62 ⁱ	64.85 ± 2.27 ^h	47.95 ± 3.57 ^e	35.27 ± 5.42 ^f
P4	74.67 ± 2.11 ^m	72.51 ± 2.96 ^l	26.61 ± 6.28 ^a	25.77 ± 7.98 ^b
P5	59.30 ± 2.84 ^f	56.03 ± 0.94 ^c	52.85 ± 6.09 ^g	38.52 ± 6.94 ^g
P6	78.51 ± 1.50 ⁿ	78.63 ± 2.09 ^m	28.54 ± 6.64 ^a	24.33 ± 8.64 ^a
P7	59.22 ± 2.31 ^f	58.33 ± 2.87 ^d	48.15 ± 4.89 ^e	36.59 ± 5.97 ^f
P8	58.51 ± 1.46 ^f	56.73 ± 2.40 ^c	52.83 ± 5.07 ^g	37.85 ± 5.88 ^g
P9	73.83 ± 1.06 ^m	73.70 ± 1.17 ^l	29.93 ± 5.36 ^b	26.04 ± 7.15 ^b
P10	67.54 ± 1.12 ^j	67.06 ± 1.56 ⁱ	44.77 ± 6.48 ^e	34.48 ± 7.45 ^e
P11	55.65 ± 2.18 ^c	59.35 ± 1.15 ^e	43.33 ± 9.64 ^d	31.12 ± 7.52 ^d
P12	58.48 ± 2.22 ^f	57.53 ± 2.85 ^d	42.77 ± 4.56 ^d	31.08 ± 3.92 ^c
P13	62.63 ± 0.54 ^g	61.90 ± 1.08 ^f	48.59 ± 4.95 ^f	35.40 ± 4.38 ^f
P14	73.83 ± 2.41 ^m	72.65 ± 1.43 ^l	30.46 ± 5.11 ^b	25.09 ± 3.86 ^b
P15	70.31 ± 1.33 ^k	70.95 ± 1.98 ^k	40.43 ± 4.39 ^c	30.85 ± 3.35 ^c
P16	63.76 ± 1.46 ^h	63.24 ± 1.23 ^g	46.08 ± 10.80 ^e	34.18 ± 6.19 ^c
P17	58.83 ± 1.14 ^f	57.31 ± 1.82 ^d	49.12 ± 7.03 ^f	35.06 ± 6.40 ^e
P18	54.36 ± 1.09 ^b	52.80 ± 1.28 ^b	44.32 ± 2.39 ^e	33.06 ± 2.87 ^e
P19	55.99 ± 1.25 ^d	57.28 ± 1.64 ^d	48.40 ± 6.47 ^f	33.89 ± 6.35 ^e
P20	72.13 ± 2.58 ^l	71.70 ± 2.39 ^k	32.58 ± 7.90 ^b	24.04 ± 6.10 ^a
P21	61.01 ± 1.97 ^g	59.95 ± 2.88 ^e	52.27 ± 5.73 ^g	36.33 ± 2.60 ^f
P22	57.71 ± 1.48 ^e	55.07 ± 2.40 ^c	52.47 ± 5.69 ^g	38.27 ± 1.07 ^g
P23	55.17 ± 0.54 ^c	53.17 ± 0.91 ^b	55.64 ± 5.36 ^g	40.21 ± 1.10 ^g
P24	56.62 ± 1.58 ^d	55.26 ± 2.41 ^c	51.37 ± 4.37 ^g	37.22 ± 2.30 ^g
P25	57.68 ± 1.69 ^e	55.73 ± 2.26 ^c	53.15 ± 3.30 ^g	38.74 ± 1.75 ^g
P26	80.19 ± 1.05 ^o	80.64 ± 1.37 ⁿ	43.26 ± 3.38 ^d	32.70 ± 5.10 ^d
P27	66.49 ± 2.70 ^j	64.43 ± 1.74 ^g	49.25 ± 5.78 ^f	36.06 ± 3.09 ^f
P28	63.87 ± 2.07 ^h	64.11 ± 2.86 ^g	50.75 ± 3.76 ^f	37.09 ± 1.66 ^f
P29	60.02 ± 2.34 ^g	58.90 ± 2.86 ^c	54.51 ± 5.74 ^g	39.45 ± 3.02 ^g
P30	72.25 ± 2.82 ^l	70.09 ± 1.10 ^j	39.61 ± 5.11 ^c	31.40 ± 2.70 ^d

Note: P1–15 = diets associated with high CLA; P16–30 = diets associated with low CLA. DMD = dry matter digestibility; OMD = organic matter digestibility; NDFD = neutral detergent fiber digestibility; ADFD = acid detergent fiber digestibility. Different superscript letters within the same column indicate significantly different dietary treatments based on the subsequent contrast-based comparison ($P < 0.05$).

Table 7. Unsaturated fatty acid profiles during *in vitro* ruminal biohydrogenation of diets associated with high and low CLA.

Treatment	C18:2-cis (%)	C18:3 n3 (%)	C18:3 n6 (%)	Total PUFAs (%)
P1	-6.47 ± 14.46	-3.63 ± 14.17	37.47 ± 23.14	-4.83 ± 22.94
P2	11.05 ± 15.86	4.17 ± 14.13	30.30 ± 40.76	-1.88 ± 19.14
P3	-11.40 ± 48.17	6.16 ± 17.41	-13.81 ± 61.77	-0.95 ± 22.76
P4	15.08 ± 47.08	-2.58 ± 17.73	5.74 ± 16.64	2.75 ± 17.77
P5	-23.73 ± 52.23	-1.68 ± 7.56	8.09 ± 38.24	11.68 ± 9.95
P6	-17.59 ± 50.08	0.29 ± 8.99	-2.86 ± 43.10	-3.68 ± 20.90
P7	-3.53 ± 20.73	1.56 ± 7.14	1.27 ± 19.70	-5.82 ± 27.87
P8	2.93 ± 27.41	2.32 ± 17.36	12.06 ± 18.88	6.24 ± 18.13
P9	-145.81 ± 109.89	-9.05 ± 25.91	-21.67 ± 33.31	12.51 ± 5.00
P10	-1.99 ± 25.17	-2.94 ± 24.49	19.65 ± 39.27	-3.88 ± 13.44
P11	8.02 ± 25.80	-1.47 ± 12.73	3.43 ± 27.99	-2.43 ± 24.92
P12	18.98 ± 19.30	-10.38 ± 18.79	-19.30 ± 25.82	7.97 ± 13.46
P13	-28.72 ± 88.36	4.65 ± 8.38	14.43 ± 11.45	10.36 ± 12.74
P14	10.01 ± 34.75	7.82 ± 10.82	10.64 ± 18.81	1.27 ± 26.52
P15	-20.05 ± 65.35	0.70 ± 15.76	14.17 ± 34.36	0.91 ± 15.91
P16	-120.36 ± 263.11	-7.85 ± 25.47	39.16 ± 22.27	9.50 ± 23.73
P17	-3.32 ± 34.86	-3.72 ± 4.00	25.90 ± 18.63	4.48 ± 25.92
P18	0.98 ± 27.64	-6.32 ± 13.99	24.15 ± 25.51	0.07 ± 40.36
P19	-3.24 ± 79.53	3.02 ± 14.62	23.23 ± 32.89	12.87 ± 9.71
P20	-109.32 ± 232.81	3.02 ± 11.23	-17.07 ± 46.94	11.70 ± 15.22
P21	3.38 ± 10.90	3.25 ± 8.41	30.83 ± 50.26	13.32 ± 13.65
P22	-11.58 ± 99.39	-8.47 ± 9.04	17.87 ± 19.38	-12.96 ± 30.04
P23	-12.16 ± 37.19	-0.79 ± 14.03	33.04 ± 29.02	24.70 ± 10.15
P24	-32.01 ± 46.72	-13.08 ± 16.22	-1.85 ± 28.24	-5.30 ± 15.60
P25	-13.63 ± 51.96	-1.89 ± 9.23	13.56 ± 31.90	13.89 ± 17.69
P26	-70.88 ± 106.61	-7.73 ± 14.84	14.95 ± 23.78	-1.08 ± 14.36
P27	-245.18 ± 432.00	-21.95 ± 16.74	-2.70 ± 37.83	-11.46 ± 13.30
P28	-21.41 ± 44.61	-5.69 ± 17.06	7.01 ± 60.60	-5.75 ± 6.79
P29	-51.20 ± 96.73	-13.04 ± 13.10	1.17 ± 40.68	14.42 ± 11.63
P30	-22.24 ± 65.85	-0.81 ± 15.42	-48.62 ± 75.86	16.09 ± 20.60

Note: P1–15 = diets associated with high CLA; P16–30 = diets associated with low CLA. Negative values represent an increase in relative fatty acid abundance at 48 h compared with 0 h and do not indicate net fatty acid synthesis or absolute fatty acid production.

3.5. Biohydrogenation indices and CLA formation

The biohydrogenation indices and CLA formation characteristics of the experimental diets are presented in Table 8. Significant differences ($P < 0.05$) were observed among diets for saturation index (SI), IBH ratio, ANCC, C18:1 isomerization index (C18:1-II), C18:2 isomerization index (C18:2-II), total C18 unsaturated recovery index (TC18URI), and unsaturated index before and after incubation (UI_{0h} and UI_{48h}). The SI ranged from 1.45 to 2.66, whereas IBH ranged from 0.20 to 0.32. ANCC showed the greatest variation, ranging from -71.45% to 125.29%. The C18:1 and C18:2 isomerization indices ranged from 0.33 to 0.50 and from 0.10 to 0.23, respectively. The TC18URI ranged from 0.87 to 1.24, while UI_{0h} and UI_{48h} ranged from 0.48 to 0.89 and from 0.39 to 0.72, respectively. Considerable variation was observed among diets for all biohydrogenation-related indices, with substantial overlap between diets associated with high CLA and low CLA.

Table 8. Biohydrogenation indices and CLA formation of diets associated with high and lowCLA.

Treatment	SI	IBH ratio	ANCC (%)	C18:1-II	C18:2-II	TC18URI	UI _{0h}	UI _{48h}
P1	1.87 ± 0.83	0.24 ± 0.07	-18.24 ± 19.57	0.34 ± 0.04	0.11 ± 0.07	0.90±0.20	0.48±0.07	0.62 ± 0.27
P2	1.69 ± 0.46	0.25 ± 0.11	-27.34 ± 69.94	0.35 ± 0.05	0.15 ± 0.07	0.90±0.08	0.57±0.09	0.63 ± 0.19
P3	1.85 ± 0.48	0.23 ± 0.04	10.12 ± 40.21	0.40 ± 0.10	0.21 ± 0.13	0.98±0.16	0.55±0.11	0.57 ± 0.16
P4	2.02 ± 0.61	0.30 ± 0.06	7.61 ± 20.44	0.48 ± 0.11	0.22 ± 0.11	0.98±0.09	0.57±0.10	0.54 ± 0.18
P5	2.16 ± 0.48	0.24 ± 0.03	16.03 ± 19.45	0.44 ± 0.08	0.15 ± 0.05	1.06±0.15	0.58±0.16	0.48 ± 0.10
P6	1.74 ± 0.68	0.32 ± 0.19	54.45 ± 79.26	0.41 ± 0.05	0.21 ± 0.10	0.97±0.20	0.54±0.06	0.69 ± 0.41
P7	1.87 ± 0.46	0.26 ± 0.05	-9.00 ± 45.08	0.38 ± 0.06	0.14 ± 0.05	0.90±0.06	0.51±0.18	0.56 ± 0.15
P8	1.92 ± 0.76	0.29 ± 0.05	12.17 ± 33.87	0.41 ± 0.09	0.14 ± 0.06	0.96±0.17	0.61±0.22	0.59 ± 0.24
P9	2.29 ± 0.59	0.24 ± 0.02	25.11 ± 84.29	0.36 ± 0.03	0.15 ± 0.02	1.08±0.20	0.50±0.17	0.46 ± 0.10
P10	1.97 ± 0.56	0.23 ± 0.08	-71.45 ± 45.44	0.44 ± 0.09	0.17 ± 0.08	0.93±0.08	0.54±0.12	0.55 ± 0.19
P11	1.92 ± 0.73	0.28 ± 0.12	40.63 ± 62.95	0.44 ± 0.10	0.18 ± 0.05	0.98±0.01	0.56±0.13	0.59 ± 0.26
P12	2.03 ± 0.46	0.24 ± 0.03	-3.47 ± 22.90	0.44 ± 0.08	0.20 ± 0.10	1.00±0.07	0.57±0.03	0.51 ± 0.13
P13	1.99 ± 0.95	0.23 ± 0.07	11.02 ± 162.44	0.41 ± 0.07	0.16 ± 0.09	0.87±0.19	0.52±0.07	0.57 ± 0.21
P14	2.16 ± 0.46	0.25 ± 0.06	54.64 ± 47.23	0.45 ± 0.09	0.19 ± 0.06	1.11±0.15	0.59±0.14	0.48 ± 0.11
P15	2.09 ± 0.94	0.26 ± 0.12	34.48 ± 80.60	0.35 ± 0.05	0.14 ± 0.07	0.98±0.20	0.58±0.26	0.57 ± 0.26

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Treatment	SI	IBH ratio	ANCC (%)	C18:1-II	C18:2-II	TC18URI	UI _{0h}	UI _{48h}
P16	1.98 ± 0.71	0.25 ± 0.08	90.44 ± 302.46	0.36 ± 0.08	0.16 ± 0.04	1.04±0.19	0.62±0.13	0.56 ± 0.22
P17	2.44 ± 1.00	0.27 ± 0.14	7.07 ± 36.98	0.41 ± 0.05	0.18 ± 0.09	1.13±0.23	0.53±0.11	0.49 ± 0.29
P18	2.52 ± 0.80	0.22 ± 0.03	−56.43 ± 37.59	0.44 ± 0.11	0.16 ± 0.03	1.02±0.33	0.53±0.35	0.42 ± 0.11
P19	2.29 ± 0.55	0.21 ± 0.04	18.40 ± 42.31	0.47 ± 0.08	0.20 ± 0.12	1.05±0.13	0.63±0.21	0.46 ± 0.12
P20	2.06 ± 1.36	0.29 ± 0.07	79.75 ± 174.91	0.41 ± 0.07	0.16 ± 0.03	1.07±0.18	0.72±0.34	0.62 ± 0.29
P21	2.44 ± 0.08	0.20 ± 0.03	−19.71 ± 39.91	0.38 ± 0.09	0.18 ± 0.05	1.23±0.20	0.71±0.38	0.41 ± 0.01
P22	1.80 ± 0.64	0.27 ± 0.14	11.70 ± 37.73	0.50 ± 0.14	0.23 ± 0.08	0.91±0.17	0.51±0.07	0.63 ± 0.30
P23	2.66 ± 0.62	0.23 ± 0.04	−46.25 ± 77.43	0.40 ± 0.09	0.14 ± 0.07	1.20±0.28	0.70±0.35	0.39 ± 0.10
P24	1.62 ± 0.38	0.28 ± 0.05	35.32 ± 60.10	0.40 ± 0.05	0.13 ± 0.06	0.98±0.08	0.58±0.22	0.65 ± 0.16
P25	1.98 ± 0.57	0.24 ± 0.07	24.16 ± 46.50	0.37 ± 0.05	0.16 ± 0.11	1.15±0.24	0.73±0.19	0.54 ± 0.17
P26	1.63 ± 0.32	0.32 ± 0.09	−4.51 ± 41.24	0.40 ± 0.06	0.13 ± 0.06	0.95±0.08	0.61±0.21	0.64 ± 0.14
P27	1.45 ± 0.30	0.30 ± 0.07	125.29 ± 324.00	0.41 ± 0.06	0.10 ± 0.02	0.90±0.09	0.51±0.14	0.72 ± 0.18
P28	1.69 ± 0.39	0.24 ± 0.05	−21.19 ± 46.95	0.33 ± 0.04	0.13 ± 0.05	0.98±0.16	0.66±0.45	0.62 ± 0.16
P29	1.85 ± 0.40	0.25 ± 0.04	−23.85 ± 61.85	0.43 ± 0.08	0.14 ± 0.07	0.99±0.17	0.64±0.20	0.56 ± 0.12
P30	1.99 ± 0.21	0.25 ± 0.06	−14.49 ± 55.50	0.38 ± 0.05	0.15 ± 0.07	1.24±0.43	0.89±0.61	0.51 ± 0.05

Note: P1–15 = diets associated with high CLA; P16–30 = diets associated with low CLA. SI = saturation index; IBH ratio = incomplete biohydrogenation ratio; ANCC = apparent net CLA change; C18:1-II = C18:1 isomerization index; C18:2-II = C18:2 isomerization index; TC18URI = total C18 unsaturated recovery index; UI_{0h} = unsaturated index at 0 hours of incubation; UI_{48h} = unsaturated index at 48 hours of incubation.

3.6. Principal component analysis of rumen fermentation, digestibility, and biohydrogenation characteristics

The principal component analysis (PCA) biplot presented in Figure 1 illustrates the multivariate relationships among rumen fermentation characteristics, nutrient digestibility, VFA profiles, methane production, and biohydrogenation parameters. The first two principal components (PC1 and PC2) explained 32.4% of the total variation. Variables related to nutrient digestibility (DMD and OMD), gas production, total VFAs, and methane production were primarily associated with the negative direction of PC1, whereas NDFD, ADFD, iC4, iC5, and C2/C3 were distributed toward the positive direction of PC1. Along PC2, IBH and UI_{48h} were separated from SI, TC18URI, and total PUFAs. Substantial overlap was observed between diets associated with high and low CLA in the PCA space, indicating that the first two principal components did not clearly discriminate between the two field-associated groups. Nevertheless, some individual diets appeared spatially closer to biohydrogenation-related variables, including IBH and UI_{48h}, than to variables representing general fermentation characteristics. These spatial relationships should be interpreted cautiously as exploratory associations rather than evidence of group separation or a specific biohydrogenation pattern.

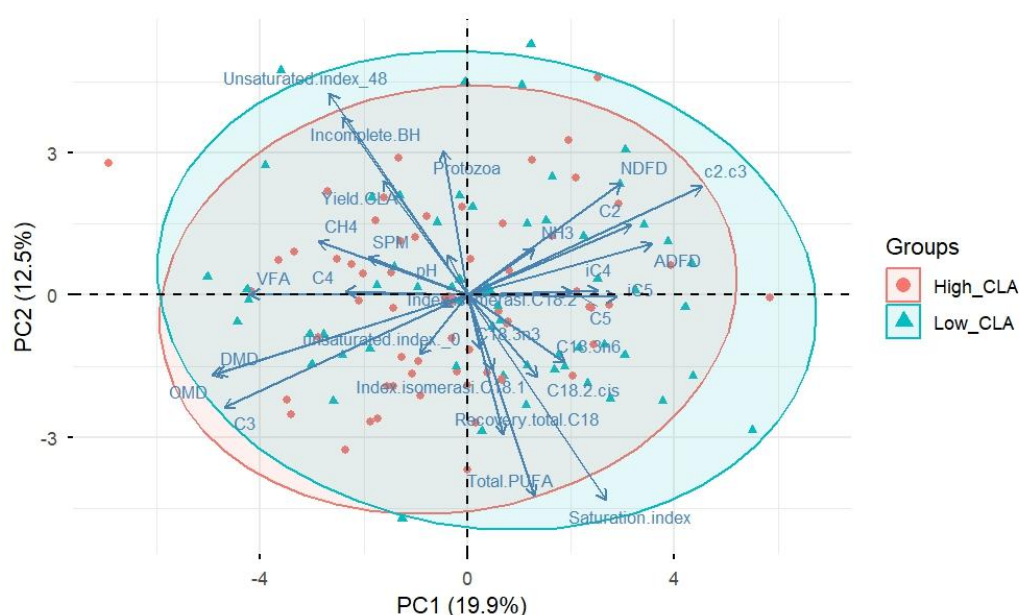


Figure 1. Principal component analysis (PCA) biplot illustrating the relationships among rumen fermentation characteristics, nutrient digestibility, fatty acid biohydrogenation parameters, and the distribution of dairy rations associated with high- and low-CLA milk production systems.

3.7. Correlation analysis among rumen fermentation, digestibility, and biohydrogenation variables

The correlation matrix illustrating the relationships among milk CLA concentration, rumen fermentation characteristics, nutrient digestibility, partial VFA profiles, methane production, and biohydrogenation indices is presented in Figure 2. Milk CLA concentration showed weak correlations with most conventional rumen fermentation variables, whereas stronger associations were observed

among nutrient digestibility, VFA profiles, gas production, methane production, and several biohydrogenation-related variables.

DMD and OMD were positively correlated with total VFA concentration and gas production, and were also strongly correlated with each other. Acetate proportion (C2) and C2/C3 showed positive correlations with methane production, whereas propionate (C3) showed negative correlations with methane production. Several biohydrogenation indices, including IBH, CLA-related indices, and unsaturated fatty acid recovery, were positively associated with one another but generally showed weaker relationships with conventional fermentation variables. Overall, milk CLA concentration showed a limited association with general rumen fermentation characteristics, whereas variables related to ruminal lipid transformation exhibited stronger interrelationships.

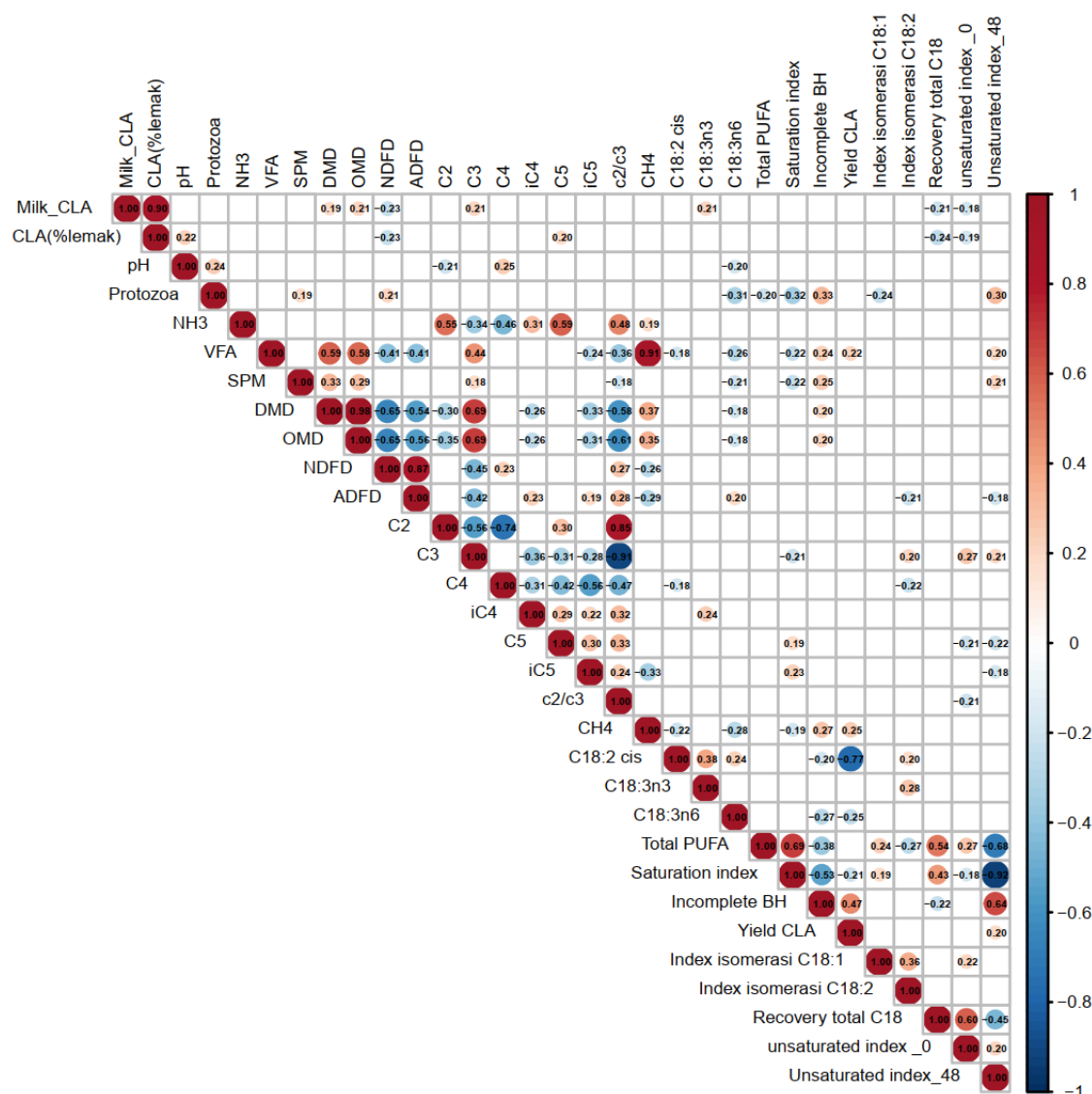


Figure 2. Correlation matrix heatmap showing the relationships among milk CLA concentration, rumen fermentation characteristics, nutrient digestibility, volatile fatty acid profiles, and lipid biohydrogenation parameters in dairy rations associated with the high- and low-CLA milk production systems.

4. Discussion

4.1. *In vitro rumen fermentation characteristics*

Ruminal pH values ranged from 6.74 to 6.93 across all treatments (Table 4), with most values falling within or close to the reported physiological range of 6.65–6.90 for optimal microbial fermentation activity [27]. No distinct separation between diets associated with high CLA and those associated with low CLA was observed for pH, although several high-CLA diets tended to show slightly higher pH values than some low-CLA diets. In general, a pH of 6 is adequate for the rumen to support CLA production [28]. Several milk fatty acids, particularly *de novo* and preformed fatty acids, have been reported to be significantly correlated (positively and negatively, respectively) with ruminal pH, where low ruminal pH conditions decrease *de novo* fatty acid synthesis [29]. This finding is consistent with the slightly higher pH observed in several diets associated with high-CLA. The low ruminal pH can alter lipid biohydrogenation pathways that affect the synthesis of milk fatty acids in the mammary gland [30].

Total VFA concentration varied considerably among treatments, ranging from 93.37 to 144.37 mM (Table 4), which is within the reported normal range of 70–150 mM [31]. Relatively high VFA concentrations were observed in both diets associated with high CLA (P6, P9, and P14) and low CLA (P20, P25, P26, and P30). These patterns were associated with diets containing relatively high ether extract (EE) and total digestible nutrient (TDN), suggesting that total VFA production primarily reflected differences in overall nutrient supply and fermentability rather than CLA classification [32]. Therefore, total VFA concentration did not clearly distinguish diets associated with high and low milk CLA concentrations. Ammonia concentration ranged from 5.18 to 16.70 mM (Table 4), reflecting substantial variation in ruminal nitrogen degradation and utilization among diets. The highest ammonia concentration was observed in P4, whereas the lowest was found in P3, P12, and P15. Previous studies showed a negative correlation between NH_3 and MPS [33]. However, in this study, different statistical patterns were observed for ammonia concentration and MPS. The MPS ranged from 5.27 to 14.99 mg/10 mL (Table 4). The highest MPS values were observed in P4, P6, P26, P29, and P30, representing diets from groups associated with both high and low CLA. This suggests differences among diets in nitrogen utilization efficiency and in the synchronization between nitrogen and energy supply for MPS [33,34].

Gas production differed significantly among diets, ranging from 107.89 to 218.56 mL/g DM. The highest values were observed in P20, P14, and P6, whereas P1 and P18 exhibited relatively low gas production. Greater gas production generally reflects greater fermentation of fermentable organic substrates, particularly readily degradable carbohydrates and other fermentable nutrients [35,36]. However, high gas production occurred in diets associated with high and low CLA. Therefore, although gas production provides an indicator of differences in fermentability among the reconstructed diets, it did not consistently distinguish feeding systems associated with high and low milk CLA concentrations.

Likewise, protozoal populations remained relatively stable across treatments, ranging within the normal range ($10^6/\text{mL}$; [31]) from 6.19 to 6.34 log cell/mL (Table 4). This result indicates that total gas production and protozoal abundance was not markedly altered between diets associated with high and low CLA. The fermentation parameters observed in this study demonstrate that general rumen fermentation characteristics, including pH, total VFA and ammonia concentration, total gas production, protozoa population, and MPS, were highly variable among diets but did not clearly differentiate

between high- and low-CLA production systems. These findings suggest that variation among the reconstructed diets was more closely reflected in specific ruminal lipid metabolism and biohydrogenation characteristics than in general fermentation intensity alone.

4.2. Molar proportions of partial VFA and methane production

The partial VFA profiles showed substantial variation among the reconstructed diets (Table 5). Acetate ranged from 46.43% to 57.03%, whereas propionate ranged from 21.28% to 27.04%, resulting in C2/C3 ratios of 1.80–2.68. Several diets associated with high CLA, including P2, P6, P10, P12, P14, and P15, exhibited relatively high propionate proportions and lower C2/C3 ratios. However, similar profiles occurred in those associated with low CLA diets, particularly P20, P26, P27, and P30. Thus, the shift toward propionate-dominant fermentation was not exclusive to diets associated with high milk CLA.

Higher propionate production may reflect greater fermentation of readily fermentable carbohydrates and increased incorporation of metabolic hydrogen into propionate rather than methane [37,38]. Conversely, diets with relatively high acetate proportions, such as P7, P18, P22, and P24, tended to have higher C2/C3 ratios. These diets also contained relatively high proportions of natural grass (Table 2), consistent with the tendency of fiber-rich diets to favor acetate production through fibrolytic fermentation [32,39]. Acetate itself is a major precursor of *de novo* milk fatty acid synthesis in the mammary glands [38]. Natural grass was reported to contain higher DM than Napier grass, while its crude protein, EE, and crude fiber were comparable. However, its overall digestibility was lower than that of Napier grass [40].

In addition, methane production ranged from 5.03 to 23.31 mL/g DM and differed significantly among the experimental diets ($P < 0.05$). Some diets with relatively low methane production were characterized by higher propionate proportions and lower C2/C3 ratios, whereas several diets with relatively high methane production showed lower propionate proportions and higher C2/C3 ratios. However, this relationship was not consistently observed across all diets, indicating that methane production was not solely determined by the acetate-to-propionate balance. Methanogenesis is a biochemical process that can occur through several pathways and is influenced by multiple factors [41]. The tendency for higher methane production to occur with higher C2/C3 ratios is consistent with the classical concept of methanogenesis as a major hydrogen sink that contributes to maintaining ruminal thermodynamic equilibrium [42]. However, the variation among diets suggests that other factors may also contribute to hydrogen disposal and methanogenesis under the present conditions.

The butyrate (C4) proportion ranged from 11.30% to 18.57%, while isobutyrate (iC4) ranged from 1.04% to 2.89% and valerate (C5) from 2.57% to 4.19%, with significant variation among treatments ($P < 0.05$). Isovalerate (iC5) showed relatively little variation among treatments and had no difference among them ($P > 0.05$). Isovalerate, isobutyrate, and 2-methylbutyrate are branched-chain VFAs generally associated with amino acid deamination and microbial protein metabolism of leucine, valine, and isoleucine, respectively [43]. The relatively stable iC5 concentration despite significant variation in iC4 suggests that the dietary treatments may have affected specific pathways of branched-chain amino acid metabolism rather than overall branched-chain VFA production. The lowest C4 concentrations were observed in P4, P7, P18, and P22, which also tended to have higher acetate proportions and C2/C3 ratios. However, acetate and butyrate are both important precursors for milk fat synthesis [38].

Although several diets associated with high CLA exhibited relatively high propionate proportions

and lower C2/C3 ratios, similar fermentation patterns were also observed in several diets associated with low CLA. For example, the propionate proportion in P20 (26.19%), a diet associated with low CLA, was similar to or higher than that observed in several high-CLA diets. Therefore, propionate concentration and the C2/C3 ratio did not consistently distinguish diets associated with high and low milk CLA concentrations. This substantial overlap indicates that partial VFA profiles alone could not fully explain the differences in milk CLA concentrations observed among the heterogeneous feeding systems. Instead, the observed variation suggests that differences among the reconstructed diets may be more closely associated with specific lipid biohydrogenation characteristics than with broad shifts in carbohydrate fermentation patterns alone.

The increase in total VFA concentration in this study did not always align with the concentration of partial VFA profiles. This suggests that the nutrient composition of both diets groups could produce high fermentative end-products but had different fermentation characteristics when viewed according to the proportions of their partial VFA profiles. Nevertheless, the tendency for some high-CLA diets to exhibit slightly lower C2/C3 ratios may indicate a rumen environment with altered hydrogen utilization dynamics. Changes in hydrogen balance may be related to the extent and direction of ruminal biohydrogenation and the formation or persistence of trans-C18:1 intermediates that may contribute to subsequent mammary CLA synthesis. However, the specific accumulation of vaccenic acid cannot be inferred from the present data because vaccenic acid was not quantified separately. Because similar VFA patterns were also observed in low-CLA diets, these fermentation characteristics alone appear insufficient to explain CLA variations under practical feeding conditions.

4.3. Nutrient digestibility

DMD ranged from 52.68% to 80.19%, while OMD ranged from 51.18% to 80.64% (Table 6). Considerable variation was observed among treatments, indicating substantial differences in substrate degradability and fiber utilization among the tested feeding systems. Variation in dietary carbohydrate and nitrogen sources can substantially influence nutrient utilization and ruminal nitrogen metabolism in cattle, supporting the importance of considering dietary composition when interpreting differences in fermentation and digestibility responses [44]. Relatively higher DMD and OMD were observed among both high-CLA diets and low-CLA diets. Higher digestibility was observed in several high-CLA diets, including P4, P6, P9, P14, and P15, as well as in several low-CLA diets, including P20, P26, and P30. The highest DMD and OMD were observed in P26 (80.19% and 80.64%, respectively). Similar to the higher digestibility, the lower DMD and OMD were also observed in both high- and low-CLA diets, such as P1 and P18. Despite being found in both CLA groups' diets, high-CLA diets tended to contain several treatments with consistently elevated digestibility compared with low-CLA diets.

NDFD varied across 26.61–55.64%, whereas ADFD ranged from 24.04% to 40.21% (Table 6). In contrast to DMD and OMD, fiber digestibility showed an opposite tendency. Diets with high DMD and OMD, such as P4, P6, P9, P14, P15, P20, P26 and P30, generally exhibited lower NDFD and ADFD values. Meanwhile, diets with relatively lower overall digestibility often showed higher fiber digestibility values. This pattern likely reflects differences in dietary composition and fermentable substrate availability. Although the eight treatments consisted of different feeding patterns, they exhibited similarly high EE and TDN content compared with treatments with lower DMD and OMD. High dietary EE and increased TDN content generally improve dietary energy density and may enhance overall DMD and OMD. However, elevated dietary lipid levels may selectively impair fiber

digestion by altering the ruminal fermentation conditions and microbial activity, primarily through reduced access of fibrolytic microorganisms to fiber substrates [45].

Interestingly, the combination of high-CLA diets with relatively high DMD and OMD but lower fiber digestibility indicates that fermentative activity may have shifted toward more rapidly degradable substrates rather than toward extensive fiber degradation. Diets rich in rapidly fermentable nonfiber carbohydrate (NFC) may increase overall digestibility while simultaneously reducing fiber degradation due to competitive effects on fibrolytic microbial activity [46]. Treatments high in DMD and OMD, like P6, P9, P14, P20, P26 and P30, were also supported with high total VFAs (Table 4). Such conditions may alter ruminal hydrogen dynamics and microbial metabolism [47]. Reduced fiber degradation is often associated with lower ruminal acetate production and altered microbial populations [46,47], which may indirectly affect the extent of unsaturated fatty acid hydrogenation.

Nevertheless, the overlapping digestibility values between high- and low-CLA diets on milk CLA production appeared more complex than expected. The results suggest that variation in milk CLA may be associated with interactions among digestibility, ruminal fermentation pathways, and lipid metabolism rather than with individual digestibility parameters alone. A similar dissociation between nutrient utilization and fatty acid responses was reported by Kazemi and Valizadeh [8], where ensiled pomegranate by-products increased vaccenic acid, rumenic acid, and total PUFAs without altering nutrient digestibility. This supports the present finding that digestibility alone may not predict CLA-related lipid responses

4.4. Unsaturated fatty acid profiles during ruminal biohydrogenation

The unsaturated fatty acid profiles during *in vitro* ruminal biohydrogenation are presented in Table 7. Considerable variation was observed among treatments in the apparent disappearance of cis-C18:2, C18:3 n-3, C18:3 n-6, and total PUFAs. The apparent disappearance of cis-C18:2 ranged from -245.18% to 18.98%, while that of C18:3 n-3 ranged from -21.95% to 7.82%. Similarly, that of C18:3 n-6 ranged from -48.62% to 39.16%, and that of total PUFAs ranged from -12.96% to 24.70%.

Several treatments, particularly P9, P16, P20, P26, and P27, exhibited markedly negative apparent disappearance values for cis-C18:2. A negative value occurred when the relative abundance of the fatty acid measured after 48 h exceeded its initial value. Because the calculation is expressed relative to the initial abundance at 0 h, a large proportional increase in relative abundance can result in an extremely negative apparent disappearance value. The -245.18% value observed for P27, for example, corresponds to a relative abundance at 48 h approximately 3.45 times the initial value at 0 h. Therefore, these values do not indicate negative biohydrogenation, net synthesis of cis-C18:2, or accumulation of a specific biohydrogenation intermediate. From a biological perspective, microbial lipolysis and progressive degradation of the feed matrix during incubation may have increased the release or extractability of fatty acids that were less accessible at 0 h [9,10]. In addition, preferential transformation of other fatty acids may have increased the relative proportion of cis-C18:2 in the recovered fatty acid pool [11,44].

From an analytical perspective, the calculations were based on the relative fatty acid composition rather than absolute fatty acid amounts. Consequently, they were sensitive to changes in the proportional denominator. Low initial concentrations, subsampling heterogeneity, variation in lipid extraction and methylation recovery, and chromatographic peak integration may have further magnified the calculated percentages. These potential sources of analytical variation are particularly

relevant because fatty acid determination involved subsampling, lipid extraction, methylation, and chromatographic analysis [21,22]. The extremely large standard deviations accompanying several negative values indicate substantial uncertainty and support a cautious interpretation. Accordingly, these values should be regarded as apparent compositional changes rather than evidence of increased PUFA synthesis, accumulation of specific biohydrogenation intermediates, or activation of a particular biohydrogenation pathway.

Considerable variation was observed among diets, but similar negative apparent disappearance values occurred in both the high-CLA and low-CLA groups. Negative values were observed in the high-CLA diets P5, P9, P13, and P15 and in the low-CLA diets P16, P20, P26, and P27. This overlap indicates that the apparent disappearance of *cis*-C18:2 alone could not distinguish diets associated with high- and low-CLA milk. Instead, the results indicate substantial variation in fatty acid release, recovery, and transformation among the heterogeneous field-derived diets.

The high variability in *cis*-C18:2 and total PUFA responses may have resulted from multiple interacting factors, including substrate composition, lipid accessibility, fermentability, hydrogen availability, microbial activity, and analytical recovery. Diets containing higher proportions of rapidly fermentable substrates may be associated with differences in ruminal redox balance and hydrogen utilization [36,45], thereby influencing fatty acid transformation. However, because individual biohydrogenation intermediates were not quantified and the calculations were based on relative fatty acid composition, the present results cannot determine the predominant biohydrogenation pathway [11,44].

Previous studies have shown that a substantial proportion of *cis*-9, *trans*-11 CLA in milk is synthesized endogenously from vaccenic acid through the activity of mammary Δ^9 -desaturase [43]. However, vaccenic acid was not quantified separately in the present study. Therefore, the observed apparent disappearance patterns cannot be directly linked to vaccenic acid accumulation, vaccenic acid's availability, or mammary CLA synthesis. References to vaccenic acid in the present discussion should therefore be regarded as biological context rather than as evidence of a measured intermediate. The occurrence of similarly variable responses in both dietary groups further indicates that apparent fatty acid disappearance alone was insufficient to explain the variation in milk CLA concentration.

The results demonstrate substantial variation in the apparent transformation of unsaturated fatty acids among diets. Nevertheless, because of the analytical limitations and considerable overlap between the high- and low-CLA groups, these results should be interpreted as exploratory evidence of variation in ruminal fatty acid metabolism rather than as confirmation of IBH or a specific pathway associated with milk CLA production. Milk CLA production is influenced by complex interactions among substrate availability, intermediate formation, and final hydrogenation toward saturated fatty acids [43,44]. Accordingly, the amount of total dietary lipid should be distinguished from the availability of specific PUFA substrates, because diets with similar or higher EE concentrations may provide different quantities and profiles of fatty acids available for ruminal biohydrogenation.

4.5. Biohydrogenation indices and CLA formation

The biohydrogenation indices and CLA formation characteristics of the experimental diets are presented in Table 8. Considerable variation was observed among treatments for SI, IBH, ANCC, isomerization indices, TC18URI, and unsaturated index values before and after incubation. These indices provide complementary descriptors of the relative extent and pattern of C18 fatty acid transformation during incubation rather than direct measurements of specific biohydrogenation

pathways. The relationship between dietary EE and milk CLA concentration was not consistent across the experimental feeding systems. For example, P1 had the lowest dietary EE content (1.93% DM) but was associated with the highest milk CLA concentration (4.00% of milk fat), whereas P4 had the highest dietary EE content (5.54% DM) but a lower milk CLA concentration (3.08% of milk fat) (Tables 1 and 3). This apparent discrepancy indicates that total dietary lipid concentration alone was insufficient to explain the variation in milk CLA under the present field-derived conditions. Importantly, EE represents the total extractable lipid fraction and does not directly indicate the quantity or composition of dietary PUFAs available as substrates for ruminal biohydrogenation. The biological response therefore depends not only on the amount of dietary lipid supplied but also on its fatty acid composition, ruminal transformation, the formation and persistence of biohydrogenation intermediates, and subsequent mammary metabolism. Consistent with this interpretation, P4 showed a higher initial unsaturated index (UI_{0h}) than P1 (0.57 vs. 0.48), despite its lower milk CLA concentration (Table 8). Thus, the variation in milk CLA observed among the field-associated feeding systems cannot be interpreted solely from total dietary EE or overall unsaturated fatty acid abundance. Feed composition, particularly dietary PUFA supply, has been recognized as an important determinant of the fatty acid profile of animal-derived products [7]. This supports the present observation that the type and composition of dietary lipids may be more informative than total EE alone.

The saturation index ranged from 1.45 to 2.66, while the IBH values varied from 0.20 to 0.32. Several treatments, including P4, P6, P26, and P27, exhibited relatively high IBH values, indicating the greater persistence of unsaturated intermediates during ruminal biohydrogenation. However, the relatively high IBH in P4 did not correspond to higher milk CLA, as P4 had a milk CLA concentration of only 3.08%. This suggests that IBH alone may not directly predict milk CLA, as the specific intermediates formed and their subsequent utilization for mammary CLA synthesis may also be important. In contrast, lower IBH values were observed in P8, P11, P19, P20, P21 and P24, indicating more complete hydrogenation toward saturated end products in both dietary groups. Interestingly, except for P8, these diets contained relatively high proportions of corn husk or corn leaves, suggesting that these forage sources might have been associated with more complete ruminal biohydrogenation. A similar tendency has been reported for corn silage diets, which tend to have a higher proportion of saturated fatty acids (SFAs) and short-chain fatty acid (SCFAs) in milk than grass diets, consistent with the greater extent of ruminal biohydrogenation [9,48]. However, the use of corn oil or high-oil corn shifted the biohydrogenation pathway by increasing C18:1-trans10 and C18:2-trans10-cis12 [49]. Therefore, differences in dietary lipid sources and forage types may be associated with variations in ruminal biohydrogenation, although potentially through different biohydrogenation responses [49,50]. Although diets with higher digestibility tended to exhibit greater incomplete biohydrogenation, diets rich in corn husk or corn leaves showed the opposite tendency, suggesting that the extent of biohydrogenation was influenced not only by overall fermentability but also by forage characteristics. However, both high- and low-CLA diets showed overlapping IBH ranges, indicating that IBH alone did not fully explain the variation in milk CLA concentration. A similar dietary effect was summarized by Kazemi [14], where whole pomegranate by-products increased total PUFAs, vaccenic acid, and rumenic acid while lowering the C18:1 trans-10/trans-11 ratio. This suggests that specific fatty acid substrates and bioactive feed components may modify the direction of ruminal biohydrogenation.

An additional limitation is that individual positional isomers of trans-C18:1 were not quantified separately. In the present study, total trans-C18:1 was used to calculate the IBH and C18:1 isomerization indices. Consequently, these aggregate indices could not determine the contribution of

vaccenic acid (trans-11 C18:1) relative to trans-10 C18:1 or other minor trans-C18:1 isomers. The observed responses therefore cannot determine the relative contribution of the conventional trans-11 pathway versus trans-10 or other minor biohydrogenation pathways. Accordingly, the measured indices should be interpreted as indicators of overall unsaturated fatty acid transformation and IBH rather than as evidence of a specific pathway. Isomer-specific quantification of trans-11 C18:1, trans-10 C18:1, and individual CLA isomers would be required to confirm the predominant biohydrogenation pathway. Biologically, the SI reflects the relative abundance of C18:0 compared with residual PUFAs at 48 h, while the IBH ratio describes the relative persistence of trans-C18:1 and CLA in relation to C18:0. The C18:1-II and C18:2-II indices describe the relative distribution of the trans and cis forms within the respective fatty acid pools, whereas TC18URI reflects the relative recovery of the total C18 fatty acid pool after incubation. UI_{0h} and UI_{48h} describe the relative degree of unsaturation before and after incubation.

The apparent net CLA change showed extremely large variation among treatments, ranging from -71.45% to 125.29%. Because ANCC was calculated from relative chromatographic abundances rather than absolute CLA amounts, it should be interpreted as an apparent change in the measured CLA pool rather than as an absolute CLA production yield. Relatively higher positive apparent net CLA changes were observed in several high-CLA diets, including P6, P11, P14, and P15, indicating greater relative accumulation of measured CLA during incubation. Interestingly, several low-CLA diets, particularly P16, P20, and P27, also showed high positive apparent net CLA changes and substantial variability. This apparent CLA change was not directly associated with the IBH ratio. Conversely, strongly negative apparent net CLA changes were observed in treatments such as P10, P18, and P23, indicating a reduction in the relative abundance of measured CLA during incubation. These results indicate substantial variation in the apparent changes in CLA abundance among the heterogeneous feeding systems.

The C18:1 and C18:2 isomerization indices showed moderate variation among treatments. The C18:1 isomerization index ranged from 0.33 to 0.50, while the C18:2 isomerization index ranged from 0.10 to 0.23. The total C18 unsaturated recovery index ranged from 0.87 to 1.24, indicating variation in the extent of unsaturated fatty acid recovery after incubation.

The unsaturated index at 0 h of incubation ranged from 0.48 to 0.89, while the index at 48 h ranged from 0.39 to 0.72. In several treatments, the unsaturated index decreased after incubation, reflecting the progressive hydrogenation of unsaturated fatty acids during ruminal fermentation. However, some treatments maintained relatively high unsaturated indices after incubation, suggesting less complete biohydrogenation and greater persistence of unsaturated intermediates. The changes in the unsaturation index at both time points do not align with the EE content of the diets, suggesting that the effects of dietary lipid metabolism warrant further investigation.

Some diets associated with higher milk CLA concentrations exhibited characteristics consistent with differences in the extent of ruminal C18 fatty acid transformation, including relatively high IBH values, higher ANCC, and greater retention of unsaturated fatty acids in the relative fatty acid profile after incubation. These responses may be consistent with greater persistence of unsaturated fatty acids during incubation; however, the specific contribution of individual trans-C18:1 positional isomers could not be determined. The IBH ratio provides a relative descriptor of the persistence of trans-C18:1 and CLA in relation to C18:0, but it should not be interpreted as a direct indicator of a specific biohydrogenation pathway. Nevertheless, substantial overlap between high-CLA and low-CLA diets was observed for most biohydrogenation indices, indicating that these indices did not consistently

discriminate between the two field-associated dietary groups. Several low-CLA diets also exhibited relatively high IBH and ANCC values, indicating that ruminal biohydrogenation characteristics alone could not fully explain variation in milk CLA under field conditions. This suggests that substrate availability, post-ruminal metabolism, mammary desaturation activity, and animals' physiological status may also contribute to the final CLA concentration in milk.

Overall, diets associated with higher milk CLA concentrations showed some characteristics consistent with differences in ruminal C18 fatty acid transformation, including relatively high IBH values, apparent net CLA change, or retention of unsaturated fatty acids in selected treatments. However, these characteristics were not consistently observed across all high-CLA diets, and several low-CLA diets also exhibited relatively high IBH and ANCC values. Therefore, ruminal biohydrogenation characteristics alone could not fully explain the variation in milk CLA under the present field conditions. Instead, these findings indicate that variation in milk CLA among the field-derived feeding systems may involve interactions among dietary PUFA substrates' availability, ruminal fatty acid transformation, and subsequent post-ruminal and mammary metabolism. The lack of a consistent relationship between dietary EE and milk CLA further supports the interpretation that total dietary lipid concentration is not a sufficient predictor of milk CLA concentration in heterogeneous smallholder feeding systems.

Importantly, the present findings should be interpreted as associations rather than causal effects on milk CLA synthesis. Milk CLA concentrations were obtained under field conditions, whereas the subsequent fermentation, digestibility, methane production, and biohydrogenation measurements were conducted *in vitro* using reconstructed diets. Thus, the observed association between selected biohydrogenation characteristics and milk CLA concentration does not demonstrate that these ruminal characteristics directly caused the differences in milk CLA among feeding systems. Milk CLA concentration may also reflect differences in dietary fatty acid intake and substrate availability, intestinal absorption, post-ruminal lipid metabolism, mammary $\Delta 9$ -desaturation, energy balance, and animal-level variation, none of which were directly represented in the present *in vitro* system. Accordingly, the biohydrogenation indices should be considered as indicators of ruminal lipid transformation associated with field-derived differences in milk CLA rather than direct predictors or causal determinants of milk CLA synthesis.

4.6. PCA of rumen fermentation, digestibility, and biohydrogenation characteristics

The first two principal components explained 32.4% of the total variation. Therefore, the PCA should be interpreted as an exploratory representation of the major relationships among variables rather than as a complete explanation of the biological variation among diets. The opposite orientation between fiber digestibility variables (NDFD, ADFD) and nutrient digestibility variables (DMD, OMD) indicates that the two variable clusters did not always increase simultaneously. Diets with high total digestibility often tended to cluster separately from those with high fiber digestibility, suggesting differences in the relative contribution of rapidly fermentable versus structural carbohydrates among feeding systems. Biologically, PC1 appeared to represent a contrast between overall substrate fermentability and fiber-oriented fermentation. The association of DMD, OMD, total VFAs, and gas production suggests greater degradation of readily fermentable organic matter, whereas the opposite orientation of NDFD, ADFD, and C2/C3 reflects a relatively greater contribution of structural carbohydrate fermentation. Significantly higher DMD and lower NDFD have been reported in high-

starch diets compared with low-starch diets [51]. In addition, the higher EE content in high DMD-OMD diets might alter the microbiome composition, leading to lower fibrolitic activity [45,52]. Nutrient digestibility and gas production were also associated with total VFAs, suggesting that diets with greater gas production generally exhibited higher carbohydrate fermentability and higher fermentable substrate [35]. This is supported by previous findings showing a strong correlation between gas production and VFAs [42]. In contrast, MPS showed only a limited association with digestibility-related variables. Synchronization of nitrogen and energy supply for MPS did not affect the DMD, OMD, and fiber digestibility [33,34].

Most variables related to lipid metabolism showed relatively weak spatial associations with nutrient digestibility, fiber degradation, and fermentation variables, suggesting that these variables varied along dimensions that were partly distinct from general fermentation processes. The distribution of these variables along PC2 appeared to reflect variation in fatty acid transformation. The association of IBH and the unsaturated index after incubation on one side of PC2, and their separation from the saturation index, total C18 recovery, and total PUFAs on the other side, suggests a contrast between the relative persistence of unsaturated fatty acids and measured biohydrogenation intermediates and greater progression toward saturated end products. However, this pattern cannot identify a specific biohydrogenation pathway because individual trans-C18:1 and CLA isomers were not quantified. This exploratory pattern suggests that lipid biohydrogenation characteristics varied along dimensions that were partly distinct from broad ruminal fermentation characteristics. Meanwhile, methane production appeared to be positively associated with acetate proportion (C2) and the acetate-to-propionate ratio (C2/C3), whereas propionate (C3) exhibited a contrasting tendency. This pattern reflects the classical ruminal fermentation relationship in which methanogenesis acts as major hydrogen sink, thereby maintaining thermodynamic equilibrium within the rumen [41,42]. Alternative hydrogen sinks, particularly propionate formation, compete with methanogenesis for metabolic hydrogen in the rumen, thereby lowering methane production and the relative proportion of acetate [41].

The overlap between high- and low-CLA diets in the PCA space indicates that the reconstructed diets exhibited substantial multivariate heterogeneity and were not clearly separated on the basis of the variables included in the analysis. Several high-CLA diets were associated with greater digestibility and altered fermentation characteristics, whereas others clustered near low-CLA diets. This suggests that multiple interacting factors, including ruminal hydrogen balance, substrate composition, fatty acid metabolism, and biohydrogenation-related characteristics, may be associated with the observed variation among the reconstructed diets. Interestingly, variables associated with IBH and unsaturated fatty acid retention tended to show weaker associations with methane production and acetate proportion (C2). This may indicate that conditions favoring less complete biohydrogenation were not necessarily linked to major shifts in the fermentation pathways overall. Instead, relatively subtle differences in ruminal substrate utilization and hydrogen availability may be relevant to the variation in biohydrogenation-related responses observed among the reconstructed diets.

Overall, the PCA suggests that variation among the reconstructed diets was not characterized by a single dominant ruminal parameter but rather by multiple dimensions involving digestibility, fermentation pathways, and lipid biohydrogenation characteristics. Variables associated with general ruminal fermentation and digestibility tended to cluster on the negative side of PC1, whereas fiber degradation-related variables were positioned on the positive side of PC1. In contrast, lipid metabolism-related variables, including biohydrogenation indices and fatty acid saturation indices, were distributed mainly along PC2, suggesting that these responses varied along dimensions that were

partly distinct from the major fermentation characteristics. Although the extensive overlap between the 95% confidence ellipses indicates that the PCA did not clearly discriminate between diets associated with high- and low-CLA milk, the relative distribution of the variables provides an exploratory view of the major multivariate dimensions associated with the observed variation. The multivariate distribution observed in Figure 1 suggests that variation among the reconstructed diets was associated with multiple ruminal characteristics, including lipid biohydrogenation, rather than with general fermentation intensity alone. However, the PCA does not establish that biohydrogenation was a direct determinant of the milk CLA concentrations observed under field conditions.

4.7. Correlation analysis among rumen fermentation, digestibility, and biohydrogenation variables

The correlation matrix presented in Figure 2 illustrates the relationships among milk CLA concentration, rumen fermentation characteristics, nutrient digestibility, VFA profiles, methane production, and biohydrogenation indices. For interpretation, the correlations can be divided into two broad categories: Relationships among directly measured biological variables and relationships involving calculated or derived indices. The analysis revealed that milk CLA concentration was not strongly correlated with most directly measured general rumen fermentation parameters, suggesting that the observed variation in milk CLA was not closely associated with overall fermentation intensity alone. Nevertheless, milk CLA percentage showed a weak positive correlation with ruminal pH ($r = 0.22$), suggesting that variation in ruminal pH may be associated with differences in ruminal biohydrogenation characteristics. The weak positive association observed in the present study suggests that ruminal pH may be related to biohydrogenation characteristics, although it was unlikely to be the primary factor associated with milk CLA concentration. Variation in milk CLA may be related to interactions among dietary PUFAs' availability, ruminal biohydrogenation processes, and subsequent post-ruminal and mammary metabolism [53]. However, these relationships cannot be directly established from the present *in vitro* experiment.

Digestibility variables, particularly DMD and OMD, showed strong positive correlations with total VFA production, total gas production, and several biohydrogenation-related variables. The relationships among DMD, OMD, and the directly measured fermentation variables primarily reflect associations among measured biological responses, indicating that diets with greater fermentability generally supported higher substrate degradation and more active ruminal metabolism. DMD and OMD also exhibited strong positive correlations with each other, confirming the close relationship between total substrate degradation and organic matter utilization. However, their weak negative associations with milk CLA indicate that overall substrate degradation had weaker associations with milk CLA concentration than specific ruminal biohydrogenation characteristics. This pattern is consistent with the regulatory role of ruminal pH in microbial fermentation, whereby a favorable pH supports nutrient degradation and VFA production but does not necessarily promote greater milk CLA synthesis [54].

Acetate proportion (C2) and the acetate-to-propionate ratio (C2/C3) showed positive correlations with methane production, whereas propionate (C3) exhibited negative correlations with methane. These relationships are consistent with established ruminal fermentation pathways in which acetate formation generates hydrogen that can be utilized for methanogenesis, while propionate formation competes for hydrogen and reduces methane production [55,56]. However, ruminal acetate alone was not associated with methane production, indicating methanogenesis is also regulated by hydrogen's availability and methanogen activity [57]. The strong inverse relationship between acetate and

propionate further illustrates the balance between alternative ruminal hydrogen sinks. Nevertheless, these relationships should be interpreted cautiously because methane production was directly measured by gas chromatography, while VFA proportions were measured independently. Therefore, the observed correlations represent associations between independently measured methane production and VFA profiles rather than mathematical relationships derived from the same variables.

Milk CLA concentration tended to show weak or inconsistent correlations with total VFAs, ammonia concentration, protozoal population, and methane production. This suggests that overall ruminal fermentation characteristics had limited explanatory power for the observed variation in milk CLA among feeding systems. Instead, CLA showed relatively stronger associations with variables related to IBH and unsaturated fatty acid metabolism. However, these variables include calculated biohydrogenation indices and should therefore be interpreted differently from correlations involving directly measured biological variables. Milk CLA variation showed closer associations with incomplete ruminal biohydrogenation and unsaturated fatty acid transformation, which may affect the availability of intermediates that are relevant to mammary CLA synthesis [58]. Several biohydrogenation indices, including IBH, unsaturated fatty acid recovery, and apparent net CLA change, exhibited positive relationships with each other. Because these indices were calculated from overlapping fatty acid variables, their correlations may partly reflect mathematical interdependence rather than independent biological relationships. Therefore, these associations should not be interpreted as direct evidence of coordinated biological regulation, although they may also reflect related biological transformations. Dietary lipid sources rich in unsaturated fatty acids may be associated with higher milk CLA through changes in ruminal lipid metabolism and biohydrogenation pathways rather than through direct increases in CLA synthesis [59,60].

Fiber digestibility variables showed different correlation patterns compared with total digestibility variables. NDFD and ADFD tended to correlate positively with acetate production but negatively with some variables related to rapidly fermentable substrates and total digestibility. This reflects the distinct fermentation pathways associated with structural carbohydrate degradation compared with NFC fermentation. Elevated NDF intake stimulated cellulolytic fermentation by stimulating fibrolytic microorganisms, particularly *Ruminococcus* and anaerobic fungi, thereby shifting ruminal fermentation toward greater acetate and butyrate production while increasing hydrogen's availability for methanogenesis. In contrast, higher NFC favored amylolytic and succinate-producing bacteria, resulting in greater propionate productions and reflecting a shift from cellulolytic to rapidly fermentable carbohydrate metabolism [61,62]. The correlation matrix also demonstrated that several biohydrogenation-related variables were weakly associated with major fermentation parameters. The weak associations of NDFD and ADFD with milk CLA suggest that fiber digestibility was not strongly associated with the variation in milk CLA concentration [40]. Likewise, the limited associations of IBH and unsaturated fatty acid retention with total VFAs and ammonia suggest that these lipid-related responses may vary with specific aspects of microbial ecology, hydrogen balance, and substrate availability rather than with overall fermentation output. The correlations observed in this study represent associations rather than causal relationships. This distinction is particularly important because milk CLA concentration was measured in cows under field conditions, whereas the fermentation and biohydrogenation variables were subsequently evaluated *in vitro* using reconstructed diets. Milk CLA may also have been influenced by factors not represented in the *in vitro* system, including fatty acid intake, intestinal absorption, mammary desaturation, energy balance, and individual animal variation. These differences may partly explain the weak correlations between milk

CLA and the measured *in vitro* variables.

Figure 2 provides an exploratory view of the associations among milk CLA concentration, directly measured rumen fermentation and digestibility variables, and derived biohydrogenation indices. The relatively weak correlations between milk CLA and conventional fermentation variables suggest that variation in milk CLA was not closely associated with overall fermentation intensity, whereas some lipid transformation variables showed relatively stronger associations. However, these correlations do not establish specific metabolic regulation or determine the relative contribution of individual biohydrogenation pathways to milk CLA concentration.

Nevertheless, these findings should be interpreted in light of an important limitation of the experimental design. The experimental diets showed substantial heterogeneity in their ingredient and nutrient composition. The high- and low-CLA groups were defined according to milk CLA concentrations observed on the farms from which the diets were selected, rather than through a controlled experiment in which diets differed only in a specific CLA-related factor. Therefore, the group classification was inherently confounded with differences in forage sources, concentrates' ingredients, agro-industrial by-products, dietary fatty acid supply, and nutrient composition. These compositional differences may have independently or interactively affected fermentation, digestibility, hydrogen utilization, and lipid biohydrogenation. Accordingly, the observed *in vitro* responses cannot be attributed solely to whether a diet was classified as being associated with high- or low-CLA milk. The substantial variability within each group and the overlap between groups further indicate that the present results should be interpreted as exploratory associations among heterogeneous field-derived feeding systems. Controlled experiments using compositionally matched diets are required to determine the effects of individual dietary factors on ruminal biohydrogenation and milk CLA production.

5. Conclusions

The present study showed that variation in milk CLA concentration among practical dairy feeding systems was not clearly explained by general rumen fermentation characteristics alone but was associated with ruminal lipid transformation and biohydrogenation characteristics. Diets associated with higher and lower milk CLA concentrations showed substantial overlap in fermentation, digestibility, and biohydrogenation responses, indicating that no single ruminal parameter consistently explained CLA variation. These findings should be interpreted as exploratory associations derived from heterogeneous field-associated feeding systems rather than as evidence of causal dietary effects. The present study did not directly measure ruminal microbial populations, individual biohydrogenation intermediates such as vaccenic acid, or mammary $\Delta 9$ -desaturase activity. Therefore, the specific contributions of these factors to variation in milk CLA cannot be directly established from the present results. Controlled experiments using compositionally matched diets are required to determine the effects of individual dietary factors on ruminal biohydrogenation and milk CLA production.

Author contributions

Despal, Wulansih Dwi Astuti, and Irma Isnafia Arief contributed to the conceptualization of the study, supervised the research, and secured research funding. Muhammad Naufal Farras, Dwitami Anzhany, and Fandini Meilia Anjani conducted sample collection, laboratory analyses, and data

acquisition. Muhammad Naufal Farras, Rika Zahera, and Fandini Meilia Anjani performed data analysis and visualization. Despal, Muhammad Naufal Farras, and Dwitami Anzhany prepared the original manuscript draft. Rika Zahera and Fandini Meilia Anjani critically reviewed and edited the manuscript. All authors contributed to manuscript revision, validated the results, and approved the final version of the manuscript.

Use of AI tools declaration

The authors declare that generative AI and AI-assisted technologies were used solely for language improvement and grammar checking. AI tools were not used for data generation, statistical analysis, interpretation of experimental results, or development of the scientific conclusions. 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.

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Conflict of interest

All authors declare there is no conflict of interest.

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