Title	Effects of calcium salts of long-chain fatty acids and rumen-protected methionine on plasma concentrations of ghrelin, glucagon-like peptide-1 (7 to 36) amide and pancreatic hormones in lactating cows
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Relation	

Title: Effects of calcium salts of long-chain fatty acids and rumen-protected methionine on plasma concentrations of ghrelin, glucagon-like peptide-1 (7-36) amide and pancreatic hormones in lactating cows

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Abstract

Our objective was to determine the effects of calcium salts of long-chain fatty acids (CLFA) and rumen-protected methionine (RPM) on plasma concentrations of ghrelin, glucagon-like peptide-1 (7-36) amide (GLP-1) and pancreatic hormones in lactating cows. Four midlactation Holstein cows were used in a 4 x 4 Latin square experiment in each 2-wk periods. Cows were fed corn silage-based diets with supplements of CLFA (1.5% added on dry matter basis), RPM (20 g/d), CLFA plus RPM and without supplement. Jugular blood samples were taken from 1 h before to 2 h after morning feeding at 10 min intervals on day 12 of each period. CLFA decreased DMI, but RPM did not affect DMI. Both supplements of CLFA and RPM did not affect metabolizable energy intake and milk yield and composition. Plasma NEFA, triglyceride (TG) and total cholesterol (T-Cho) concentrations were increased with CLFA alone, but increases of plasma TG and T-Cho concentrations were moderated by CLFA plus RPM. CLFA increased plasma ghrelin concentration and the ghrelin concentration with CLFA plus RPM was the highest among the treatments. Plasma GLP-1, glucagon and insulin concentrations were decreased with CLFA, whereas adding RPM moderated the decrease of plasma glucagon concentration by CLFA. These results suggest that the addition of Met to cows given CLFA increases plasma

ghrelin and glucagon concentrations associated with the decrease in plasma TG and T-Cho concentrations.

Key words: Dairy cow; Fatty acid; Ghrelin; Glucagon-like peptide-1 (7-36) amide; Methionine

1. Introduction

Fat supplementation in diets for milk production is valid to increase dietary energy density [1]. However, increasing the dietary fat content often depresses DMI of lactating cows [2]. The mechanism of hypophagia induced by fat supplementation is incompletely understood and assumed to be affected by various factors involving the absorption process and metabolism of fatty acids. Methionine (Met) improves lipid metabolism [3] partially by altering the activities of hormone-sensitive lipase and lipoprotein lipase [4]. In dairy cows, Met is the first limiting amino acid for milk production [5]. Supplementation of Met hydroxyl analog increases milk fat synthesis with hypertriglycemia [6]. Conversely, an insufficiency of Met during the periparturient period results in the development of hepatic lipidosis [7]. These reports

suggest that Met may affect lipid metabolism in dairy cows. In lactating ewes, supplementation of rumen-protected Met (RPM) combined with fat increased DMI, milk yield and milk fat secretion compared with the supplementation of fat only [8], but the effects of Met on plasma metabolite and hormone concentrations were unknown. Recently Relling and Reynolds [9, 10] reported changes in plasma concentrations of some gut hormones such as glucagon-like peptide-1 (7-36) amide (GLP-1) and ghrelin caused by several nutrients in lactating cows, partly because of their role in the regulation of feed intake. Additionally, these gut hormones were reported to influence insulin and glucagon secretion [11, 12]. Therefore, in this study we evaluated the effects of adding calcium salts of long-chain fatty acids (CLFA) and RPM on plasma metabolite and hormone concentrations in lactating cows.

2. Materials and Methods

The procedures used in the present study were carried out in accordance with the principles and guidelines for animal use issued by the National Institute of Livestock

and Grassland Science Animal Care Committee, and which were formulated to comply with Japanese regulations.

Table 1

Animals and Management

One primiparous and three multiparous Holstein cows (143.5 ± 3.9 d in milk, calving number: 1.75 ± 0.25, initial body weight: 547.3 ± 14.7 kg) were fed four diets formulated to meet the nutrient requirements according to the Japanese Feeding Standard for Dairy Cattle [13]. The ingredients and composition of the diets are presented in Table 1. The cows were managed in individual tie stalls, allowed free access to water, and provided experimental diets twice daily at 0900 and 1800 h. They were milked twice daily before each feeding (0840 and 1740 h), and weighed every week.

Treatments and Experimental Design

The cows were used in a 4 × 4 Latin square design in each 2-wk period. Four treatments consist of basal diet only (without supplement), supplemented with CLFA made from palm and soybean oil [Megalac R (Church & Dwight Co., Inc., Princeton, NJ, USA)], with RPM [Lactet SP (Nippon Soda Co., Ltd., Tokyo, Japan)], and with

CLFA plus RPM. The CLFA containing 85% fatty acids was added to 1.5% of the diet DM, following a previous study [14]. The RPM containing 67% of D-, L-Met was supplemented at 20 g/d for RPM or CLFA plus RPM treatment. Cows were offered each diet ad libitum, allowing for 15% refusal for the first 10 d for each treatment. For the last 4 d of each period, the cows were fed 95% of ad libitum intake for estimating metabolizable energy intake (MEI). Refusals were weighed daily before each morning feeding (0800 h).

Sampling

Samples of the diets and the refusals were collected and pooled for the last 4 d of each period. Milk samples were collected for the last 4 d of each period, added with sodium azide as preservative, and stored at 4℃ until analysis. Blood samples were taken at 12 d from the jugular vein catheter (Argyle 14 G CV catheter kit; Nippon Sherwood Medical Industries Ltd., Tokyo, Japan) inserted on 10 d of each period. Blood samples (8 mL) were taken at 10 min intervals from 0800 to 1100 h. Cows were milked during bleeding period (-20 and -10 min sampling time), and they were fed just after 0 min sampling. Blood samples were collected into heparinized tubes with aprotinin [500 kilo inhibitor unit (KIU) /mL of blood; Trasylol, Bayer Leverkusen,

Germany], and centrifuged at 1,500 × *g* for 20 min at 4℃. Harvested plasma samples were stored at -80℃ until assay.

Sample Analysis

The diet samples and refusals were analysed for DM, CP, NDF, crude fat and crude ash contents according to the procedures of AOAC (1990) [15]. Metabolizable energy contents in the treatment diets were calculated based on NRC (2001) [1].

Milk samples were measured for fat, protein, lactose, TS and SNF by infrared analysis (Milko-Scan 1344 A/BN.; Foss Electric Company, Inc., Hillerod, Denmark).

Plasma ghrelin, insulin and GLP-1 concentrations were measured every 10 min by time-resolved fluoro-immunoassay (TR-FIA).

Assay for bioactive ghrelin was conducted as described previously [16]. Ghrelin concentration was measured by competitive solid-phase immunoassay using europium (Eu)-labeled synthetic bovine ghrelin and polystyrene microtiter strips (Nalge Nunc Int., Tokyo, Japan) coated with anti-rabbit γ-globulin. Intra- and inter-assay coefficients of variation were 1.3 and 1.5%, respectively. Least detectable dose and 50% inhibitory concentration in this assay system were 0.025 and 0.831 ng/mL, respectively.

Insulin assay was conducted as described previously [17]. Insulin concentration

was measured by competitive solid-phase immunoassay using Eu-labeled synthetic bovine insulin and polystyrene microtiter strips coated with anti-guinea pig γ-globulin. Intra- and inter-assay coefficients of variation were 2.2 and 1.8%, respectively. Least detectable dose and 50% inhibitory concentration in this assay system were 0.016 and 1.073 ng/mL, respectively.

GLP-1 concentration was measured by competitive solid-phase immunoassay based on the method described by Sugino et al. [16] using rat GLP-1 (Peptide Institute, Inc., Osaka, Japan), Eu-labeled rat GLP-1, polystyrene microtiter strips coated with anti-rabbit γ-globulin and anti-human GLP-1 rabbit serum (1:20,000; Yanaihara Institute Inc., Shizuoka, Japan). Intra- and inter-assay of coefficients of variation were 1.7 and 4.8%, respectively. Least detectable dose and 50% inhibitory concentration in this assay system were 0.024 and 0.172 ng/mL, respectively.

Plasma glucagon levels were measured every 20 min using a commercially available RIA kit (glucagon assay kit, Daiichi Radioisotope Co. Ltd., Tokyo, Japan). Glucagon concentrations were measured in the same assay, and the intra-assay coefficient of variation was 3.4%. Least detectable dose and 50% inhibitory concentration in this assay system were 15.6 and 275 pg/mL, respectively.

Plasma glucose concentrations were determined every 10 min using a glucose

analyzer (GA-1151; Arkray Co, Ltd., Kyoto, Japan). Plasma β-hydroxy butyrate (BHBA), NEFA, triglyceride (TG), total-cholesterol (T-Cho) and urea nitrogen (UN) concentrations were determined every 20 min using an automated biochemistry analyzer (Beckman Coulter, Inc., Tokyo, Japan).

Statistics

Data for feed intake, milk yield, milk composition and plasma amino acid concentrations were analyzed using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC, USA). The mixed model included treatment as a fixed effect, and cow and period as random effects. For the statistical analysis of plasma hormone and metabolite concentration, sampling time and sampling time × treatment were added to the model. Factorial contrasts were used to test the main effects of CLFA supplementation (CLFA vs. non-CLFA), RPM supplementation (RPM vs. non-RPM), and their interaction. Results are reported as least squares means and SEM. Significant differences were set at $P < 0.05$.

3. Results

Feed Intake, Milk Yield, and Milk Composition

DMI, milk yield and milk composition are presented in Table 2. DMI in cows fed CLFA diets compared with cows fed non-CLFA diets was lower (P = 0.025), whereas MEI was not affected. There was no significant effect of RPM on DMI and MEI. Milk yield and composition, except for total solid contents, were unaffected by CLFA and RPM. Milk total solid contents tended to be lower in cows fed CLFA diets compared with cows fed non-CLFA diets (P = 0.107).

Table 2

Plasma Concentration of Hormones and Metabolites

The changes in plasma concentration of hormones and metabolites are presented in Figures 1 and 2, respectively. Plasma ghrelin, insulin, glucose, BHBA, NEFA, TG and UN concentrations varied largely through time (P < 0.002, Figures 1 and 2), and no significance of the interaction time by treatment was observed (P > 0.192). The time effect for plasma ghrelin, glucose and NEFA concentrations was due to a postprandial decrease. Plasma insulin, BHBA, TG and UN concentrations increased after feeding.

The means of plasma ghrelin concentration were higher (P < 0.001, Table 3) in cows fed CLFA compared with cows not fed CLFA. Compared with cows fed

non-CLFA, plasma glucagon and insulin concentrations in cows fed CLFA were lower (P = 0.002 and P = 0.012, respectively), and plasma GLP-1 concentration tended to be lower (P = 0.061). Plasma insulin concentration tended to be higher (P = 0.092) in cows supplemented RPM than those without RPM. There were interactions between CLFA and RPM for ghrelin and glucagon (P = 0.002 and P = 0.041, respectively): CLFA plus RPM increased plasma ghrelin, glucagon, but RPM alone did not show such effects.

Compared to those without CLFA, plasma NEFA, TG, T-Cho and UN concentrations in cows fed CLFA were higher (P < 0.001, P = 0.029, P < 0.001, and P < 0.001, respectively), but with lower plasma BHBA concentration (P = 0.006, Table 3). In cows fed RPM diets, plasma glucose concentration was higher (P = 0.001), plasma T-Cho concentration was lower (P = 0.009), and plasma NEFA and UN concentration tended to be lower (P = 0.15 and P = 0.121, respectively). Interactions between CLFA and RPM were observed for TG and T-Cho (P = 0.049 and P = 0.003, respectively): RPM decreased plasma TG and T-Cho concentrations in cows fed CLFA, but did not affect the cows not fed with CLFA.

Table 3
Figure 1
Figure 2

4. Discussion

High fat inclusion in diets reduces fiber digestion, increases fatty acid absorption, inhibits abomasal motility [18] and increases gut hormone secretion [19, 20]. Consequently, DMI tends to be depressed. CLFA could prevent such negative effects on ruminal fermentation and fiber digestibility in lactating cows [21, 22]. However, DMI was decreased by CLFA in this study consistent with other reports [23, 24]. RPM did not improve such DMI depression by CLFA contrary to results found by Goulas et al. [8] who used lactating ewes. Additionally, Chillard and Doreau [25] observed no improvement effect of RPM on DMI of cows fed a fish oil supplemented diet during midlactation, although such non-protected fat including high polyunsaturated fatty acids could largely inhibit dietary fiber digestion and depress DMI.

The increase in plasma glucose concentration of cows fed RPM was consistent with Berthiaume et al. [26], while inconsistent with Bertics and Grummer [27]. Such discrepancies might be explained by the differences in the physiological state of cows (mid vs. early lactation) and energy balance. Plasma NEFA, T-Cho and TG concentrations increased by CLFA in the current study has been previously observed in cows [19, 28]. On the other hand, RPM in cows fed CLFA decreased plasma T-Cho and TG concentrations and tended to decrease plasma NEFA concentration. Met is a

methyl group donor for phosphatidylcholine in dairy cows [29] to enhance plasma lipoprotein. In laboratory animals, casein (a Met-rich protein) or L-Met induced hypercholesterolemia in rabbits and rats [30, 31]. In calves, however, high fat diet supplemented with L-Met did not increase plasma VLDL concentration [32], and Met hydroxyl analog did not affect hepatic TG accumulation in cows [27]. Whether Met enhanced VLDL that was not measured in this study is unknown. On the other hand, because Met is converted to taurine in dairy cows, plasma taurine concentration increased linearly with an increase of postruminal Met infusion [33]. Taurine conjugates with bile acids to become taurocholate in the liver, and promotes lipid absorption and cholesterol consumption [34]. Yagasaki et al. [3] showed that dietary Met and Gly reduced serum T-Cho and increased fecal sterol excretion in rats. Furthermore, Met, as a sulfur amino acid, suppressed serum NEFA and TG concentration through the control of hormone-sensitive lipase activity and the restoration of lipoprotein lipase activity in peripheral tissues of hepatoma-bearing rats [4]. In this study, therefore, suppression of plasma T-Cho, NEFA and TG with CLFA plus Met might be induced by enhanced uptake and use of lipids by various tissues.

The increase in plasma ghrelin concentration of cows fed CLFA is consistent with that of rats that ingested some fatty acids [35]. In addition, RPM decreased plasma

ghrelin concentration in cows not fed CLFA, whereas CLFA plus RPM compared with CLFA alone tended to increase plasma ghrelin concentration. Thus, only a simultaneous inclusion of CLFA and RPM may enhance ghrelin secretion via the changes in lipid metabolism. Ghrelin enhances food intake in both nonruminants [36] and ruminants [37, 38]. As a result, we could not find the relationship between plasma ghrelin concentration and feed intake in this study. Since reports on ghrelin responses to lipid metabolism in ruminants are limited, further research is desirable.

Many researchers have reported that high fat supplementation increased GLP-1 concentration [20, 39, 40]. Litherland et al. [40] demonstrated that abomasal infusion of fat increased plasma GLP-1 concentration dependent on dosage. Compared with others, the decrease of plasma GLP-1 concentration with CLFA in this study could be attributed to a lessor fat ingestion level of 280 g/d. GLP-1 plays a role in food intake [41] and Relling and Reynolds [20] have reported that dietary fats increased GLP-1 in dairy cows. Additionally, Relling et al. [42] observed that intrajugular infusion of GLP-1 tended to decrease DMI in growing wethers. In this study however, plasma GLP-1 concentration was decreased by CLFA, suggesting that GLP-1 did not mediate DMI.

Plasma insulin concentration decreased in cows fed CLFA with decreasing DMI

consistent with previous reports [23, 43]. We consider that insulin depression might be due to lessor volatile fatty acids (VFA) production caused by decreased DMI. Although we did not determine plasma VFA concentrations, CLFA decreased plasma BHBA concentration, derived from butyrate by rumen fermentation. And, we also observed that plasma GLP-1 concentration tended to decrease at the same time, suggesting decreased GLP-1, which stimulates insulin secretion, may be related to insulin depression in cows fed CLFA.

CLFA also decreased plasma glucagon concentration. Our result is consistent with that of Cummins and Sartin [43], but inconsistent with that of Khorasani and Kennelly [44]. In several species, elevated NEFA decreases plasma glucagon concentration [45, 46]. However, the present results showed that the decrease of plasma glucagon with CLFA was attenuated by RPM. In our previous study, glucagon secretion was more strongly enhanced when amino acids and ghrelin were simultaneously administered compared with the administration of ghrelin alone in lactating cows [11]. In addition, RPM tended to depress plasma NEFA concentration of cows fed CLFA. Therefore, we consider that increased Met absorption, higher plasma ghrelin concentration and a decreased plasma NEFA concentration might modulate plasma glucagon depression in CLFA-fed cows.

The present study did not show any favorable effects of RPM on feed intake and milk production in cows fed with CLFA although plasma ghrelin concentration increased. Further studies are desirable to elucidate the effects of lactating stage and Met supplemental level with CLFA on DMI and milk production.

In conclusion, responses to CLFA are associated with increases in plasma concentration of ghrelin and decreases of insulin, glucagon and GLP-1. RPM plus CLFA modulated plasma lipid concentrations and concomitantly elevated plasma ghrelin concentration. These metabolic and endocrine changes were induced by absorbed long-chain fatty acid and methionine and not by energy intake because MEI was not different among treatments.

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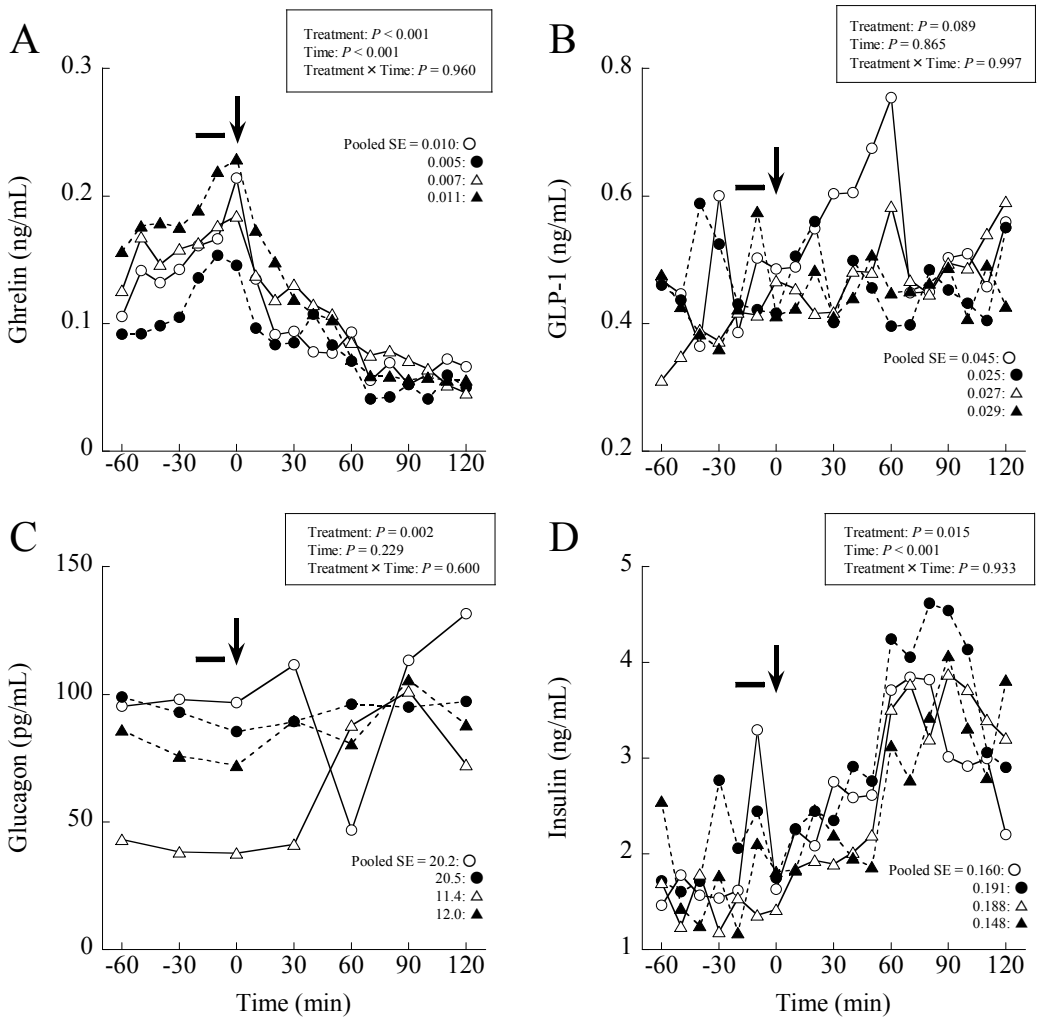
Figure captions:

Figure 1. Plasma hormone (ghrelin: A, GLP-1: B, glucagon: C and insulin: D) concentrations in cows fed each treatment diet: non-CLFA + non-RPM (○ with a solid line), non-CLFA + RPM (● with a dotted line), CLFA + non-RPM (△ with a solid line), and CLFA + RPM (▲ with a dotted line). Values are expressed as least square of means (n=4). *P*-value for effects of treatment, time and the interaction between treatment and time. The horizontal bar and arrow show the milking period and feeding

time, respectively.

Figure 2. Plasma metabolite (glucose: A, BHBA: B, NEFA: C, TG: D, T-Cho: E and UN: F) concentrations in cows fed each treatment diet: non-CLFA + non-RPM (○ with a solid line), non-CLFA + RPM (● with a dotted line), CLFA + non-RPM (△ with a solid line), and CLFA + RPM (▲ with a dotted line). Values are expressed as least square of means (n=4). *P*-value for effects of treatment, time and the interaction between treatment and time. The horizontal bar and arrow show the milking period and feeding time, respectively.


A
Treatment: P < 0.001
Time: P < 0.001
Treatment × Time: P = 0.960
Pooled SE = 0.010:
0.005:
0.007:
0.011:
Ghrelin (ng/mL)
0.3
0.2
0.1
0
-60 -30 0 30 60 90 120
B
Treatment: P = 0.089
Time: P = 0.865
Treatment × Time: P = 0.997
Pooled SE = 0.045:
0.025:
0.027:
0.029:
GLP-1 (ng/mL)
0.8
0.6
0.4
0.2
-60 -30 0 30 60 90 120
C
Treatment: P = 0.002
Time: P = 0.229
Treatment × Time: P = 0.600
Pooled SE = 20.2:
20.5:
11.4:
12.0:
Glucagon (pg/mL)
150
100
50
0
-60 -30 0 30 60 90 120
Time (min)
D
Treatment: P = 0.015
Time: P < 0.001
Treatment × Time: P = 0.933
Pooled SE = 0.160:
0.191:
0.188:
0.148:
Insulin (ng/mL)
5
4
3
2
1
-60 -30 0 30 60 90 120
Time (min)

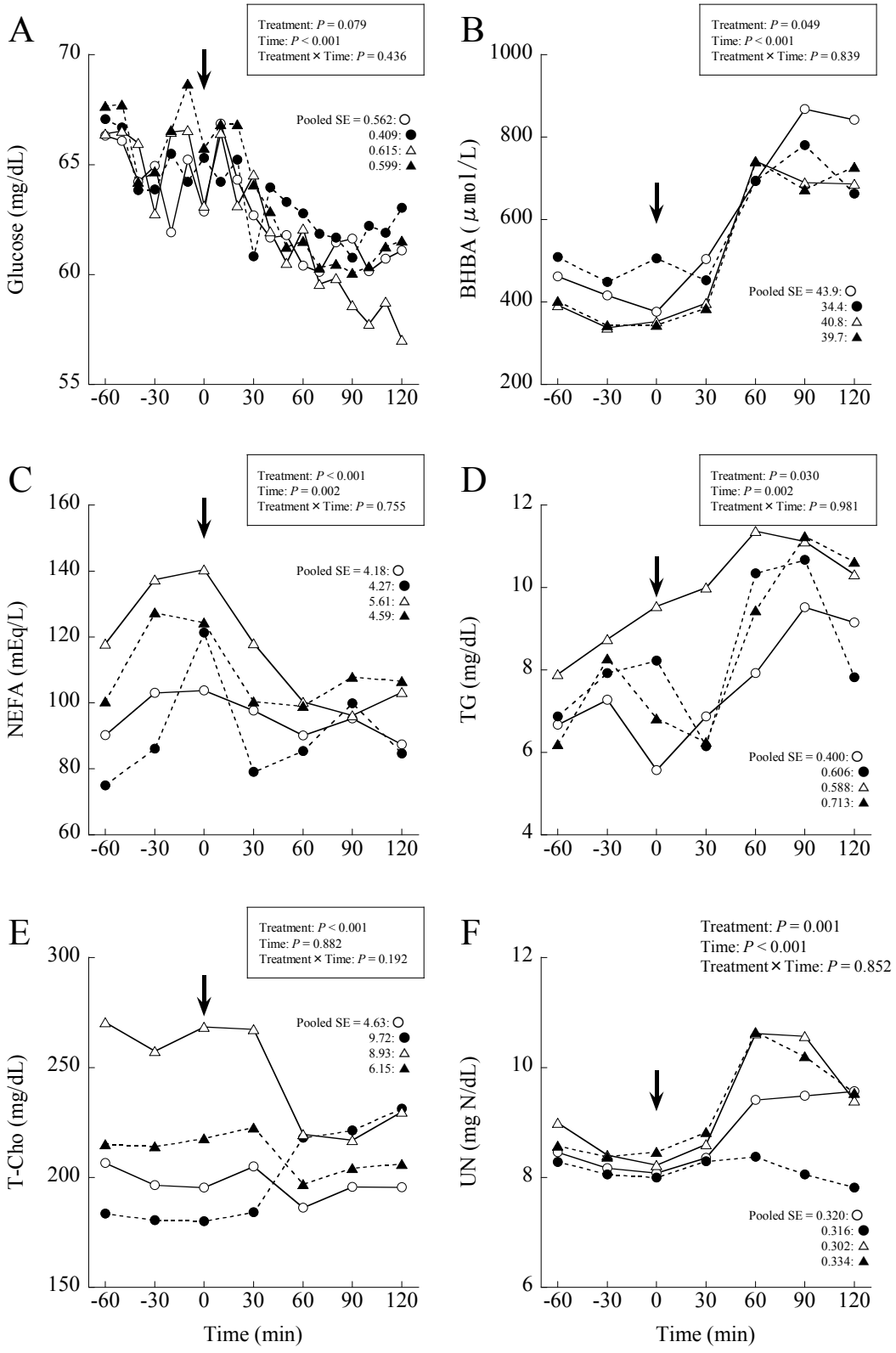

A
Treatment: P = 0.079
Time: P < 0.001
Treatment × Time: P = 0.436
Pooled SE = 0.562:
0.409:
0.615:
0.599:
Glucose (mg/dL)
70
65
60
55
-60 -30 0 30 60 90 120
B
Treatment: P = 0.049
Time: P < 0.001
Treatment × Time: P = 0.839
Pooled SE = 43.9:
34.4:
40.8:
39.7:
BHBA (μmol/L)
1000
800
600
400
200
-60 -30 0 30 60 90 120
C
Treatment: P < 0.001
Time: P = 0.002
Treatment × Time: P = 0.755
Pooled SE = 4.18:
4.27:
5.61:
4.59:
NEFA (mEq/L)
160
140
120
100
80
60
-60 -30 0 30 60 90 120
D
Treatment: P = 0.030
Time: P = 0.002
Treatment × Time: P = 0.981
Pooled SE = 0.400:
0.606:
0.588:
0.713:
TG (mg/dL)
12
10
8
6
4
-60 -30 0 30 60 90 120
E
Treatment: P < 0.001
Time: P = 0.882
Treatment × Time: P = 0.192
Pooled SE = 4.63:
9.72:
8.93:
6.15:
T-Cho (mg/dL)
300
250
200
150
-60 -30 0 30 60 90 120
Time (min)
F
Treatment: P = 0.001
Time: P < 0.001
Treatment × Time: P = 0.852
Pooled SE = 0.320:
0.316:
0.302:
0.334:
UN (mg N/dL)
12
10
8
6
-60 -30 0 30 60 90 120
Time (min)

Table 1. Ingredient and chemical composition of the diets

Item	Non-CLFA	CLFA[a]
Ingredient (DM basis)		
Corn silage (%)	42.2	41.6
Alfalfa hay cubes (%)	7.60	7.48
Sudan grass hay (%)	5.68	5.60
Concentrate[b] (%)	43.9	43.2
Calcium salts of fatty acids[c] (%)	0.00	1.50
Calcium Carbonate (%)	0.60	0.60
Salt (%)	0.01	0.01
Vitamin premix (%)	0.01	0.01
Chemical composition (DM basis)		
OM (%)	93.0	92.7
CP (%)	14.1	13.9
NDF (%)	32.2	31.7
Crude fat (%)	3.24	4.45
ME[d] (Mcal/kg)	2.42	2.48

[a]LCFA: Calcium salts of long-chain fatty acids.

[b]Concentrate contained 39% corn grain, 17% corn gluten feed, 16% beet pulp pe
10% soybean meal,6% canola meal, 5% wheat middlings, 5% wheat bran, 2% m

[c]Megalac R: Declared fatty acids contained 26% palmitic acid, 4% stearic acid,
33% oleic acid, 32% linoleic acid, and 5% linolenic acid.

[d]Metabolizable energy: Estimated value from NRC (2001).

Table 2. Feed intake, milk production and composition during experimental period in lactating cows

	Treatment							
	non-CLFA		CLFA[a]			Contrast[c] (*P*-value)		
Item	non-RPM	RPM	non-RPM	RPM[b]	SEM	CLFA	RPM	CLFA × RPM
DMI (kg/d)	22.4	22.2	21.3	21.7	0.675	0.025	0.803	0.322
MEI[d] (Mcal/d)	56.7	56.2	55.4	56.4	1.72	0.463	0.794	0.325
Milk yield (kg/d)	27.2	27.1	27.3	27.5	1.83	0.508	0.982	0.655
4% FCM[e] (kg/d)	29.9	28.8	30.0	30.3	1.63	0.505	0.766	0.574
Milk composition								
Fat (%)	4.46	4.35	4.48	4.42	0.44	0.825	0.685	0.909
Protein (%)	3.51	3.56	3.47	3.45	0.132	0.177	0.729	0.508
Lactose (%)	4.62	4.59	4.61	4.61	0.059	0.736	0.743	0.662
Total solid (%)	13.6	13.5	12.7	12.5	1.01	0.107	0.759	0.966
Solid non-fat (%)	9.13	9.11	9.08	9.11	0.124	0.736	0.973	0.710
Body weight changes (kg)	14.3	18.5	13.5	12.7	4.60	0.359	0.612	0.474

Data are shown by least squares means (LSM) and SEM.

[a]CLFA: Calcium salts of long-chain fatty acids.

[b]RPM: Rumen-protected methionine.

[c]*P*-value for factorial contrasts: CLFA, RPM and the interaction between CLFA and RPM.

[d]MEI: Metabolizable energy intake.

[e]4% FCM: 4% fat corrected milk (kg/d) = 0.4 × Milk yield (kg/d) + 15 × Milk yield (kg/d) × Milk fat (%).

Table 3. Plasma hormone and metabolite concentrations during feeding period in lactating cows

	Treatment							
	non-CLFA		CLFA[a]			Contrast[c] (*P*-value)		
Item	non-RPM	RPM	non-RPM	RPM[b]	SEM	CLFA	RPM	CLFA × RPM
Hormones								
Ghrelin (ng/mL)	0.106	0.087	0.116	0.126	0.027	<0.001	0.301	0.002
GLP-1[d] (ng/mL)	0.519	0.464	0.452	0.448	0.128	0.061	0.185	0.258
Glucagon (pg/mL)	99.1	93.6	60.3	85.5	46.2	0.002	0.187	0.041
Insulin (ng/mL)	2.51	2.86	2.36	2.41	0.128	0.012	0.092	0.202
Metabolites								
Glucose (mg/dL)	62.9	63.4	62.1	63.6	1.91	0.872	0.001	0.344
BHBA[e] (μmol/L)	595	579	514	517	43.4	0.006	0.796	0.729
NEFA (μEq/L)	95.4	90.2	116	110	7.13	<0.001	0.150	0.842
TG[f] (mg/dL)	7.57	8.29	9.86	8.41	0.82	0.029	0.501	0.049
T-Cho[g] (mg/dL)	197	200	247	211	14.9	<0.001	0.009	0.003
UN[h] (mgN/dL)	8.79	8.13	9.26	9.23	0.70	<0.001	0.121	0.152

Data are shown by least squares means (LSM) and SEM.

[a]CLFA: Calcium salts of long-chain fatty acids.

[b]RPM: Rumen-protected methionine.

[c]*P*-value for factorial contrasts: CLFA, RPM and the interaction between CLFA and RPM.

[d]GLP-1: Glucagon-like peptide-1 (7-36) amide.

[e]BHBA: β-hydroxy butyrate.

[f]TG: Triglyceride

[g]T-Cho: Total-cholesterol.

[h]UN = Urea nitrogen.