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Effects of rumen-protected *Capsicum* oleoresin on productivity and responses to a glucose tolerance test in lactating dairy cows

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ABSTRACT

The objective of this experiment was to investigate the effects of rumen-protected *Capsicum* oleoresin (RPC) supplementation on feed intake, milk yield and composition, nutrient utilization, fecal microbial ecology, and responses to a glucose tolerance test in lactating dairy cows. Nine multiparous Holstein cows were used in a replicated 3 × 3 Latin square design balanced for residual effects with three 28-d periods. Each period consisted of 14 d for adaptation and 14 d for data collection and sampling. Treatments were 0 (control), 100, and 200 mg of RPC/cow per day. They were mixed with a small portion of the total mixed ration and top-dressed. Glucose tolerance test was conducted once during each experimental period by intravenous administration of glucose at a rate of 0.3 g/kg of body weight. Dry matter intake was not affected by RPC. Milk yield tended to increase for RPC treatments compared to the control. Feed efficiency was linearly increased by RPC supplementation. Concentrations of fat, true protein, and lactose in milk were not affected by RPC. Apparent total-tract digestibility of dry matter, organic matter, and crude protein was linearly increased, and fecal nitrogen excretion was linearly decreased by RPC supplementation. Rumen-protected *Capsicum* oleoresin did not affect the composition of fecal bacteria. Glucose concentration in serum was not affected by RPC supplementation post glucose challenge. However, compared to the control, RPC decreased serum insulin concentration at 5, 10, and 40 min post glucose challenge. The area under the insulin concentration curve was also decreased 25% by RPC. Concentration of nonesterified fatty acids and β-hydroxybutyrate in serum were not affected by RPC following glucose administration. In this study, RPC tended to increase milk production and increased feed efficiency in dairy cows. In addition, RPC decreased serum insulin concentration during the glucose tolerance test, but glucose concentration was not affected by treatment.
Key words: *Capsicum* oleoresin, insulin, milk production, dairy cow

INTRODUCTION

Capsicum oleoresin, an acetone or hexane extract from *Capsicum* fruits, has been studied as a modifier of ruminal fermentation in cattle (Calsamiglia et al., 2007). Capsaicin, the main active compound in *Capsicum* oleoresin, has a phenolic structure and has been shown to exhibit antimicrobial effects in the rumen and to modify rumen fermentation (Calsamiglia et al., 2007). In beef cattle studies, *Capsicum* oleoresin applied as a feed additive decreased acetate proportion and increased ammonia concentration (Fandiño et al., 2008; Rodríguez-Prado et al., 2012). *Capsicum* oleoresin, however, had no effect on rumen fermentation in dairy cows (Tager and Krause, 2011; Oh et al., 2015).

Recent studies with dairy cows suggested that *Capsicum* may exhibit physiological effects directly on the host animal. For example, abomasal infusion of *Capsicum* oleoresin increased a subtype of T lymphocytes related to adaptive immunity (Oh et al., 2013). In addition, dietary supplementation of *Capsicum* oleoresin increased serum BHB concentration and neutrophil counts with no effect on rumen fermentation (Oh et al., 2015). In studies with nonruminants, *Capsicum* or capsaicin has been investigated with regard to its physiological effects (Lee et al., 2013a; Liu et al., 2014; Srinivasan, 2016). The regulatory effects of capsaicin include changes in feed intake, digestive enzyme secretion, fat mobilization, and hormone regulation. Capsaicin treatment stimulated gastric emptying and decreased leptin levels, which resulted in higher food intake in humans and rats (McCann et al., 1988; Debreceni et al., 1999; Hsu and Yen, 2007). Capsaicin increased the activity of digestive enzymes such as lipase and trypsin in pancreatic homogenate of rats (Platel and Srinivasan, 2000). Dietary inclusion or topical administration of capsaicin reduced adipose tissue and increased blood free fatty

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acids (**FA**) in rats (Yoshioka et al., 2000; Lee et al., 2013b). In addition, capsaicin reportedly increased or decreased pancreatic hormones in rats and human subjects (Dömötör et al., 2006; Chaiyasit et al., 2009). In particular, insulin concentration in blood was decreased by capsaicin after an intravenous glucose challenge in rats (van de Wall et al., 2005, 2006). Because insulin has a pivotal role in glucose homeostasis and homeorhesis in dairy cows (Bell and Bauman, 1997), the effect of capsaicin on insulin secretion may increase glucose availability to the mammary gland and thus milk production in dairy cows.

Based on existing literature with nonruminants and our previous experiments, we hypothesized that *Capsicum* acts in the digestive tract postruminally and may positively affect feed intake, nutrient utilization, gut microbial ecology, fat mobilization, and hormone regulation in dairy cows. Thus, the objective of the experiment was to investigate the effects of rumen-protected *Capsicum* oleoresin (**RPC**) supplementation on feed intake, milk production and composition, total-tract digestibility, nitrogen excretion, fecal bacterial population, and responses during a glucose tolerance test (**GTT**) in lactating dairy cows.

MATERIALS AND METHODS

Animals and Treatments

The Pennsylvania State University Animal Care and Use Committee approved all procedures used in this experiment. The experiment was a replicated 3 × 3 Latin square design, balanced for residual effects, and it was conducted at the tie-stall barn of The Pennsylvania State University's Dairy Teaching and Research Center. The experiment involved 9 Holstein cows (milk yield, 47 ± 5.7 kg/d; DIM, 100 ± 9.1 d; and BW, 665 ± 83.3 kg, at the beginning of the experiment). Cows were grouped in squares based on parity and milk yield. Each period consisted of 28 d: 14 d for adaptation and 14 d for data collection including sampling. An immune challenge was conducted on d 24 in each experimental period, and data generated after the challenge, including intake, milk production, and acute phase immune responses are presented in our companion paper (Oh et al., 2017). Cows were fed once daily ad libitum targeting 5 to 10% refusals and had free access to fresh water. During feeding, RPC was mixed with a small amount of the TMR and top-dressed on the feed. Treatments in this experiment were 3 levels of RPC: 0 mg/d (control), 100 mg/d (**C100**), and 200 mg/d (**C200**). The RPC product used in the experiment was Nexulin (X50-7035; 15.5% *Capsicum* oleoresin; 0.93% capsaicinoids; Pancosma, S. A., Geneva, Switzerland). The dose amounts were determined based on previous work in our laboratory (Oh et al., 2015). In a separate in situ test, ruminal DM disappearance rate of RPC ranged from 3.30 to 27.8% in 24-h incubation (data not shown). All cows were fed the same basal TMR (Table 1). Intake, refusal weights, and milk production were recorded daily. Recombinant bST (500 mg, i.m., Posilac; Elanco Co., Greenfield, IN) was administered at the beginning and middle of each experimental period.

Sampling and Analyses

Weekly composite samples of the TMR and refusals were prepared from subsamples collected twice weekly. Forages and concentrate feeds were sampled weekly, and composite samples were made for each experimental period. Composite samples of the TMR, forages, and concentrates were stored frozen, oven-dried to constant weight (65°C), and ground through a 1-mm sieve before being analyzed for CP (AOAC International, 2000), NDF (Van Soest et al., 1991), ADF (AOAC International, 2000), ether extract (AOAC International, 2006), Ca (AOAC International, 2000), P (AOAC International, 2000), and estimated NFC (NRC, 2001) and NE_L (NRC, 2001) by Cumberland Valley Analytical Services (Maugansville, MD). Fecal and TMR samples were incinerated for 4 h at 600°C for analysis of ash and OM. Fecal, urine, blood, and milk samples were collected during the last week of each experimental period (Figure 1). Fecal and urine samples were collected by stimulating defecation from the rectum and by massaging the vulva, respectively, at 1000, 1600, and 2200 h on d 20; 0400, 1300, and 1900 h on d 21; and 0100 and 0700 h on d 22. Fecal samples (approximately 300 g) were oven-dried at 65°C in a forced-air oven for 48 h. After drying, samples were ground through a 1-mm sieve (Wiley mill), composited on an equal weight basis per cow and period, and analyzed for OM (as indicated previously for feed samples) and NDF and ADF (Ankom A200 fiber analyzer; Ankom Technology, Macedon, NY; Van Soest et al., 1991). Heat-stable amylase (Ankom Technology) and sodium sulfite (Fisher Scientific, Waltham, MA) were used in the NDF procedure. Dried fecal samples were pulverized at 30 Hz/s for 2 min in a Mixer Mill MM 200 (Retsch GmbH, Haan, Germany) and analyzed for CP (N × 6.25) on a Costech ECS 4010 C/N/S elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA). Fecal and TMR samples were analyzed for indigestible NDF as an intrinsic digestibility marker to estimate apparent total-tract digestibility of nutrients according to Huhtanen et al. (1994), with the exception

Table 1. Ingredient and chemical composition of the basal diet fed during the experiment

Item	% of diet DM
Ingredients	
Corn silage[1]	43.5
Haylage[2]	12.0
Cottonseed, hulls	3.2
Corn grain, ground	6.0
Candy by-product meal[3]	6.5
Soybean seeds, whole heated[4]	8.65
Canola meal[5]	8.65
Molasses[6]	3.5
Vitamin and mineral premix[7]	3.0
SoyPLUS[8]	5.0
Composition,[9] % of DM (or as indicated)	
CP[9]	16.1
RDP[10]	9.8
RUP[10]	6.9
NDF[9]	30.9
ADF[9]	23.2
NE_L, Mcal/kg[9]	1.69
Ca[9]	1.02
P[9]	0.39
NFC[10]	45.2
Average NE_L balance,[11] Mcal/d	(4.5, 3.6, 3.2)
Average MP balance,[11] g/d	(359, 330, 304)

[1]Corn silage was 46.5% DM and contained (DM basis) 6.7% CP, 32.4% NDF, and 42.7% starch.

[2]Haylage was 58.0% DM and contained (DM basis) 21.0% CP and 43.7% NDF.

[3]Candy by-product meal (Graybill Processing, Elizabethtown, PA) contained (DM basis) 16.9% CP and 26.7% NDF.

[4]Soybean seeds contained (DM basis) 40.0% CP.

[5]Canola meal contained (DM basis) 40.9% CP.

[6]Molasses (Westway Feed Products, Tomball, TX) contained (DM basis) 3.9% CP and 66% total sugar.

[7]The premix (Cargill Animal Nutrition, Cargill Inc., Roaring Spring, PA) contained (%, as-is basis) trace mineral mix, 0.86; MgO (56% Mg), 8.0; NaCl, 6.4; vitamin ADE premix (Cargill Animal Nutrition, Cargill Inc.), 0.48; limestone, 37.2; selenium premix (Cargill Animal Nutrition, Cargill Inc.), 0.07; and dry corn distillers grains with solubles, 46.7. Ca, 14.1%; P, 0.39%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 362 mg/kg; Zn, 1,085 mg/kg; Fe, 186 mg/kg; vitamin A, 276,717 IU/kg; vitamin D, 75,000 IU/kg; and vitamin E, 1,983 IU/kg.

[8]SoyPLUS (West Central Cooperative, Ralston, IA) contained (DM basis) 47.2% CP.

[9]Values calculated using the chemical analysis (Cumberland Valley Analytical Services Inc., Maugansville, MD) of the ingredients of the diet.

[10]Estimated by NRC (2001).

[11]Estimated based on NRC (2001) using actual DMI, milk yield, milk composition, and BW of the cows throughout the experiment. Results are given as (control, C100, and C200), respectively. Treatments were 0, 100, or 200 mg/d of rumen-protected *Capsicum* oleoresin for control, C100, and C200, respectively.

of 25-μm pore size filter bags (Ankom Technology) being used. Urine samples were acidified (pH < 3) using 2 M H_2SO_4 (pH was verified using litmus paper), diluted 1:10 with distilled water, composited on an equal volume basis per cow and period, and stored frozen at −20°C. Urine samples were analyzed for N on a Costech ECS 4010 C/N/S elemental analyzer (Costech Analytical Technologies Inc.), urea N (Stanbio Urea Nitrogen Kit 580; Stanbio Laboratory Inc., San Antonio, TX), allantoin (Chen et al., 1992), uric acid (Stanbio Uric Acid kit 1045; Stanbio Laboratory Inc.), and creatinine (Stanbio Creatinine Kit 0400; Stanbio Laboratory Inc.). Urinary creatinine concentration was used for estimation of daily volume of excreted urine. Creatinine was assumed to be excreted at a rate of 29 mg/kg of BW (Hristov et al., 2011). Excretion of total N, urinary urea N, and purine derivatives (**PD**; allantoin and uric acid) was calculated using estimated urine output. Fresh fecal samples were composited on an equal wet-weight basis by cow and experimental period and frozen immediately at −20°C. These samples were analyzed for bacterial population diversity by MR DNA (Molecular Research, Shallowater, TX) as described in Oh et al. (2015). In period 1 of the study, fecal and urine samples were collected following LPS challenge (see Oh et al., 2017). In periods 2 and 3, samples were collected before the LPS challenge. As DMI decreased significantly following LPS challenge in period 1, digestibility and urine data from periods 2 and 3 only are presented here.

Milk samples for composition analyses (fat, protein, lactose, SCC, MUN) were collected on d 20 during each experimental period (afternoon/evening and morning milkings) and submitted to Dairy One laboratory for analysis (Pennsylvania DHIA, University Park, PA). Milk composition was analyzed using infrared spectroscopy (MilkoScan 4000; Foss Electric, Hillerød, Denmark), and data were weighted for the corresponding afternoon/evening and morning milk yield.

Catheters (14-gauge × 13 cm, MILA International Inc., Erlanger, KY) were inserted with an extension set into the jugular vein of all cows on the day before GTT. Catheters were flushed with 8 mL of heparinized saline (10 IU/mL) every 6 h and during blood sampling. Feeding was suspended for 12 h before the glucose challenge, but cows had access to water. Glucose (50% D-glucose, Nova-Tech Inc., Grand Island, NE) was administered intravenously at 0.3 g/kg of BW within 3 min with a 60-mL syringe. The dose of glucose was determined based on previous studies with dairy cows (Pires et al., 2007; Zachut et al., 2013). The GTT was performed once during the last week of each experimental period (Figure 1). Blood samples were collected from the jugular catheters at 0 (before glucose infusion), 5, 10, 15, 20, 30, 40, 50, 65, 80, and 110 min relative to glucose administration. Whole blood samples were collected into vacuumed tubes containing silica clot activator (SST Tube; BD Biosciences, Franklin Lakes, NJ), placed at room temperature for 1 h to allow clotting, and centrifuged at 3,000 × g at

room temperature for 15 min to separate blood serum. Serum samples were kept frozen in a −80°C freezer until analyses for glucose, insulin, nonesterified fatty acids (**NEFA**), BHB, and leptin. Samples from all time points were used for all the analyses except BHB, which was analyzed only at 0, 30, and 65 min post glucose challenge. Serum glucose concentration was analyzed by a chemistry analyzer (VetTest Chemistry Analyzer, Idexx Laboratories, Inc., Westbrook, ME). A radioimmunoassay kit (PI-12K, EMD Millipore, Billerica, MA) was used for insulin analysis. The specificity for bovine insulin in the assay kit was 90% as suggested in the manufacturer's manual. Recovery tests were conducted to correct the underestimation of insulin concentration. The minimum detection level of insulin in the kit was 1.611 μU/mL. An enzymatic colorimetric method [NEFA-HR(2), Wako Diagnostics, Mountain View, CA] was used for NEFA, and inter- and intra-assay precision of this method was 0.61 to 0.75% CV and 0.75 to 4.91% CV, respectively. The minimum detectable level of the method was 0.0014 μmol/L (oleic acid equivalent). A biochemistry analyzer (Cobas 6000; Roche, Germany) was used for BHB analysis. Leptin concentration in serum was analyzed using radioimmunoassay (XL-85K, EMD Millipore). Inter- and intra-assay precision of the leptin kit was 6.5 to 8.7% CV and 2.8 to 3.6% CV, respectively. The minimum detection level of leptin in the kit was 0.801 ng/mL.

Calculations and Statistical Analysis

Microsoft EXCEL (Microsoft Corp., Seattle, WA) was used to fit exponential curves for glucose and insulin data using the following equation (Pires et al., 2007):

$$\mathrm{F}(t) = A \times \mathrm{e}^{(-\mathrm{k} \times t)},$$

where $\mathrm{F}(t)$ is the concentration at time t; A is the maximum concentration; t is the time after the glucose challenge; and k is the regression coefficient. Clearance rate (**CR**), time to half-maximal concentration ($\mathrm{T}_{1/2}$), and time to reach basal concentration ($\mathrm{T}_{\mathrm{basal}}$) were calculated by the following equations:

$$\mathrm{CR}\ (\%/\mathrm{min}) = \{[\ln\ (\mathrm{ta}) - \ln\ (\mathrm{tb})] \div (\mathrm{tb} - \mathrm{ta})\} \times 100,$$

$$T_{1/2} = \{[\ln\ (2)] \div \mathrm{CR}\} \times 100,$$

$$T_{\mathrm{basal}} = \{[\ln\ (\mathrm{ta}) - \ln\ (\mathrm{tb})] \div \mathrm{CR}\} \times 100,$$

where ta is the concentration at time a, and tb is the concentration at time b.

The trapezoidal method was used for calculating the area under the glucose and insulin concentration curve (**AUC**) post glucose challenge using the positive incremental area method (Cardoso et al., 2011). Concentrations at 0 h (before the glucose challenge) were used as baseline concentrations.

All data were analyzed using the MIXED procedure of SAS 9.3 (2003; SAS Institute Inc., Cary, NC). Milk yield, DMI, and estimated feed efficiency data for 9 d after adaptation period of each experimental period were used in the statistical analysis. The averaged milk

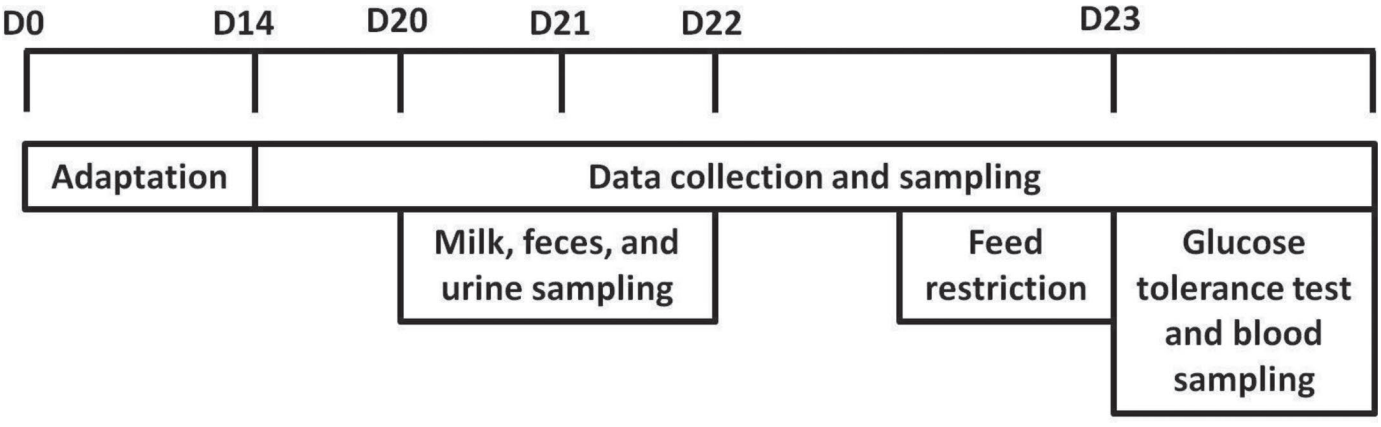

Figure 1. Schematic diagram of the experiment. Milk samples were collected on afternoon/evening and morning milkings on D (day of experimental period) 20. Fecal and urine samples were collected 8 times: D20 at 1000, 1600, and 2200 h; D21 at 0400, 1300, and 1900 h; D22 at 0100 and 0700 h. Catheters for glucose tolerance test were inserted on D22, and cows were restricted on feed from 2100 h on D22. Glucose challenge occurred at 0900 h on D23 and was followed by blood sampling.

yield and milk composition data were used to calculate yields of milk fat, true protein, lactose, and ECM. Data were tested for normality using the UNIVARIATE procedure of SAS. Log-transformed data were analyzed when the W statistic of the Shapiro-Wilk test was < 0.05.

Nutrient intake, digestibility, urinary and fecal N excretions, fecal bacterial population, milk composition, and GTT data (glucose, insulin, NEFA, BHB, and leptin) for each sampling time post glucose administration including AUC, peak and basal concentration, CR, $T_{1/2}$, and T_{basal} were analyzed by ANOVA Latin square. The model used was as follows:

$$Y_{ijkl} = \mu + S_i + C(S)_{ij} + P_k + T_l + PT_{kl} + e_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is the overall mean, S_i is the square, $C(S)_{ij}$ is the cow within square, P_k is the kth period, T_l is the lth treatment, and PT_{kl} is the period × treatment effect with the error term e_{ijkl}. Square and cow within square were random effects, and all others were fixed.

Dry matter intake, milk yield, feed efficiency, and GTT data (glucose, insulin, NEFA, BHB, and leptin) for all the sampling times were analyzed as repeated measures assuming an AR(1) covariance structure. The model used was as follows:

$$Y_{ijklm} = \mu + S_i + C(S)_{ij} + P_k + T_l + D_m + PT_{kl} + TD_{lm} + e_{ijklm},$$

where Y_{ijklm} is the dependent variable, μ is the overall mean, S_i is the square, $C(S)_{ij}$ is the cow within square, P_k is the kth period, T_l is the lth treatment, D_m is the time effect, PT_{kl} is the period × treatment interaction, and TD_{lm} is the treatment × time interaction with the error term e_{ijklm}. Square and cow within square were random effects, and all others were fixed.

Orthogonal contrasts were used to evaluate RPC treatments versus control and linear effect of RPC supplementation. Statistical differences were considered significant at $P < 0.05$ and a trend at $0.05 \leq P < 0.10$. Data are presented as least squares means.

RESULTS

The basal diet used in this experiment was formulated to meet NE_L and MP requirements of cows milking 45 kg/d (Table 1). The experimental diet supplied NE_L and MP in excess of cow requirements (NRC, 2001). Supplementation of RPC on the basal diet did not affect DMI (Table 2). Milk yield tended to be increased ($P = 0.06$) by RPC. A linear increase ($P < 0.01$) in feed efficiency was observed with RPC. Milk fat, true

Table 2. Effect of rumen-protected *Capsicum* oleoresin on milk yield and composition in dairy cows

	Treatment[1]				*P*-value[3]	
Item	Control	C100	C200	SEM[2]	Con vs. RPC	Linear
DMI,[4] kg/d	29.4	30.0	29.2	0.74	0.72	0.55
Milk yield, kg/d	42.8	44.7	43.9	1.27	0.06	0.22
Feed efficiency,[5] kg/kg	1.48	1.52	1.57	0.056	0.03	<0.01
Milk fat, %	3.89	3.92	3.97	0.220	0.73	0.65
Yield, kg/d	1.64	1.72	1.73	0.102	0.34	0.38
ECM,[6] kg/d	44.7	46.8	46.5	1.62	0.19	0.26
ECM feed efficiency,[7] kg/kg	1.33	1.41	1.39	0.052	0.15	0.25
Milk true protein, %	3.12	3.11	3.10	0.088	0.69	0.68
Yield, kg/d	1.32	1.38	1.36	0.041	0.21	0.35
Milk lactose, %	4.72	4.75	4.71	0.052	0.88	0.77
Yield, kg/d	2.03	2.14	2.07	0.046	0.22	0.53
TS, %	12.7	12.6	12.7	0.39	0.89	0.99
Yield, kg/d	5.38	5.60	5.55	0.169	0.22	0.33
MUN, mg/100mL	10.4	9.24	10.4	0.542	0.30	0.98
SCC, × 10^3 cells/mL	36.5	33.8	73.2	24.41	0.50	0.21

[1]Control = 0 mg/d rumen-protected *Capsicum* oleoresin (RPC); C100 = 100 mg/d RPC; C200 = 200 mg/d RPC.

[2]Highest SEM shown; n = 215 for DMI, n = 202 for milk yield and feed efficiency, n = 24 for all other variables (n represents number of observations used in the statistical analysis).

[3]Con vs. RPC = control vs. RPC treatment; Linear = linear effect of RPC.

[4]Control vs. C100, $P = 0.23$; Control vs. C200, $P = 0.54$.

[5]Milk yield ÷ DMI.

[6]Energy-corrected milk (kg/d) = milk production (kg/d) × (383 × % fat + 242 × % true protein + 165.4 × % lactose + 20.7) ÷ 3,140 (Sjaunja et al., 1990).

[7]ECM ÷ DMI.

Table 3. Effect of rumen-protected *Capsicum* oleoresin on total-tract digestibility of nutrients in dairy cows[1]

	Treatment[2]				*P*-value[4]	
Item	Control	C100	C200	SEM[3]	Con vs. RPC	Linear
Intake, kg/d						
DM	29.6	29.7	29.0	1.13	0.73	0.46
OM	27.6	27.7	27.0	1.09	0.72	0.44
CP	4.71	4.74	4.61	0.186	0.70	0.42
NDF	9.45	9.49	9.28	0.375	0.74	0.50
ADF	7.25	7.30	7.11	0.287	0.72	0.44
Apparent digestibility, %						
DM	63.6	63.8	66.2	0.89	0.06	0.02
OM	64.8	64.9	67.4	0.85	0.06	0.02
CP	59.1	60.0	64.9	1.90	0.09	0.03
NDF	35.5	35.5	39.6	1.47	0.18	0.06
ADF	31.3	33.6	38.5	1.95	0.16	0.10

[1]Digestibility data were collected in experimental periods 2 and 3 only.
[2]Control = 0 mg/d rumen-protected *Capsicum* oleoresin (RPC); C100 = 100 mg/d RPC; C200 = 200 mg/d RPC.
[3]Highest SEM shown; n = 18 for all variables (n represents number of observations used in the statistical analysis).
[4]Con vs. RPC = control vs. RPC treatment; Linear = linear effect of RPC.

protein, and lactose concentrations and TS, ECM yield, ECM feed efficiency, MUN, and SCC were not affected by treatment.

Total-tract digestibility of DM, OM, and CP was linearly increased ($P \leq 0.03$), whereas that of NDF and ADF tended to be linearly increased ($P \leq 0.10$) with RPC compared to the control (Table 3). Although excretion of urine N and urinary urea N was similar among treatments, fecal N excretion and total excreta N tended to be linearly decreased ($P \leq 0.07$) by RPC (Table 4). The proportion of fecal N excreted to N intake was linearly decreased ($P = 0.03$) by RPC. Uric acid, allantoin, and total urinary PD excretions were not affected by treatment.

The predominant genera (>10% of total sequences) of fecal bacteria in this experiment were *Ruminococcus*, *Bifidobacterium*, *Barnesiella*, and *Fecalibacterium* (Table 5). The relative abundance of fecal bacteria was not affected by RPC.

Serum glucose concentration peaked at 5 min and returned to the basal level at 80 min after glucose challenge (Figure 2). Glucose concentration was not affected by RPC at all sampling times, except trends for decrease ($P \leq 0.07$) at 40 and 110 min post glucose

Table 4. Effect of rumen-protected *Capsicum* oleoresin on nitrogen utilization in dairy cows[1]

	Treatment[2]				*P*-value[4]	
Item	Control	C100	C200	SEM[3]	Con vs. RPC	Linear
N intake, g/d	754	759	738	29.1	0.70	0.41
N secretion and excretion, g/d						
Urine N	154	145	154	15.7	0.69	1.00
Urine urea N	99.6	89.7	106	5.63	0.72	0.36
Fecal N	303	298	260	20.9	0.13	0.06
Total excreta N	466	443	414	15.8	0.09	0.07
As proportion of N intake, %						
Urine N	21.7	19.7	20.6	2.89	0.51	0.68
Fecal N	40.9	40.0	35.1	1.89	0.09	0.03
Total excreta N	61.8	59.9	56.0	1.65	0.16	0.09
Creatinine, m*M*	7.25	8.33	8.63	0.626	0.07	0.09
Urinary PD[5] excretion, mmol/d						
Allantoin	452	431	472	35.2	1.00	0.69
Uric acid	68.8	52.3	64.5	6.43	0.12	0.52
Total PD	511	494	528	38.8	0.99	0.77

[1]Urine data were collected in experimental periods 2 and 3 only.
[2]Control = 0 mg/d rumen-protected *Capsicum* oleoresin (RPC); C100 = 100 mg/d RPC; C200 = 200 mg/d RPC.
[3]Highest SEM shown; n = 18 for all variables (n represents number of observations used in the statistical analysis).
[4]Con vs. RPC = control vs. RPC treatment; Linear = linear effect of RPC.
[5]PD = purine derivatives.

challenge. Glucose concentration was also not different among treatments when analyzed as repeated measure across all sampling times (Table 6). Basal and peak levels, CR, $T_{1/2}$, T_{basal}, and AUC of glucose also did not differ among treatments. Insulin concentration peaked at 10 min for the control and at 5 min for C100 and C200, and returned to the basal level at 65 min after glucose administration (Figure 3). Serum insulin concentration was decreased ($P \leq 0.04$) at 5, 10, and 40 min and tended to be decreased ($P \leq 0.08$) at 30 and 50 min by RPC compared to the control. Basal insulin concentration, CR, $T_{1/2}$, and T_{basal} were not affected by the treatment (Table 6). However, peak concentration of insulin tended to decrease ($P = 0.07$) and the insulin AUC decreased ($P = 0.04$) with RPC by an average of 25% compared to the control. Serum NEFA concentration decreased following glucose challenge, reached the lowest levels at 30 or 40 min, and returned to the basal level at 120 min (Figure 4). Overall, NEFA was not affected by RPC during GTT, except linear increases ($P = 0.03$) at 65 and 80 min. We also observed no effect of RPC on NEFA concentration during GTT when data were analyzed as repeated measure (Table 7). Serum concentration of BHB was not affected by RPC, although numerical increases ($P \geq 0.15$) were observed for RPC before and after glucose challenge (Table 7). Serum BHB concentration in this experiment was below the level for subclinical ketosis (1,200 to 2,900 μmol/L; Oetzel, 2004). Leptin concentration in serum started to increase at 10 or 15 min after glucose challenge (Figure 5). The control had a peak level of leptin at 30 min, whereas C100 and C200 peaked at 50 and 80 min, respectively. Leptin concentration returned to the basal level at 110 min after glucose infusion for all the groups. Serum leptin concentration post glucose challenge tended to be linearly decreased ($P = 0.08$) by RPC when analyzed as repeated measure (Table 7). However, a treatment × period interaction ($P < 0.01$) was observed for the leptin data. The interaction was caused by a variable response to treatment during the experimental periods. Thus, leptin data should be interpreted with caution.

DISCUSSION

The lack of effect of RPC on DMI in the current experiment is in accordance with previous studies with dairy cows (Tager and Krause, 2011; Oh et al., 2013, 2015). Supplementation of *Capsicum* through the feed or directly into the abomasum in dairy cows did not affect DMI, although *Capsicum* supplementation increased DMI and water consumption in studies with beef cattle (Cardozo et al., 2006; Rodríguez-Prado et al., 2012). This discrepancy between beef and dairy studies is probably a result of the amounts of *Capsicum* used in dairy cows being relatively smaller than those used in beef cattle as previously discussed by Oh et al. (2015).

Table 5. Effect of rumen-protected *Capsicum* oleoresin on relative abundance (as percentage[1] of total sequences) of major fecal bacterial genera in dairy cows[2]

	Treatment[3]				*P*-value[5]	
Item	Control	C100	C200	SEM[4]	Con vs. RPC	Linear
Ruminococcus	20.5	23.2	22.0	1.60	0.34	0.49
Bifidobacterium	14.1	13.3	7.89	6.668	0.75	0.75
Barnesiella	12.3	10.3	11.2	3.63	0.65	0.77
Fecalibacterium	10.1	9.43	10.8	0.810	0.98	0.56
Clostridium	8.23	6.58	8.30	0.965	0.46	0.95
Bacteroides	4.37	4.36	5.09	0.734	0.68	0.48
Coprococcus	3.55	4.79	4.39	0.771	0.26	0.41
Blautia	2.77	3.09	3.13	0.465	0.43	0.49
Dermatophilus	2.54	2.76	3.25	0.313	0.26	0.16
Pseudobutyrivibrio	1.76	1.93	1.98	0.163	0.57	0.91
Azospira	1.41	1.59	1.45	0.384	0.69	0.77
Butyrivibrio	1.02	1.44	1.65	0.290	0.16	0.13
Papillibacter	1.11	1.34	1.36	0.155	0.33	0.37
Ruminiclostridium	1.03	1.06	1.14	0.248	0.82	0.77
Streptococcus	0.54	0.59	0.75	0.099	0.35	0.23
Prevotella	0.59	0.52	0.71	0.112	0.84	0.48

[1]The percentage represents the percentage of the total sequences analyzed within the sample.

[2]Data were collected in experimental periods 2 and 3 only.

[3]Control = 0 mg/d rumen-protected *Capsicum* oleoresin (RPC); C100 = 100 mg/d RPC; C200 = 200 mg/d RPC.

[4]Highest SEM shown; n = 18 for all variables (n represents number of observations used in the statistical analysis).

[5]Con vs. RPC = control vs. RPC treatment; Linear = linear effect of RPC.

Table 6. Effect of rumen-protected *Capsicum* oleoresin on serum glucose and insulin concentration response to glucose tolerance test in dairy cows

	Treatment[1]				*P*-value[3]	
Item	Control	C100	C200	SEM[2]	Con vs. RPC	Linear
Glucose,[4] mg/dL	133	126	127	7.5	0.17	0.24
Basal, mg/dL	72.0	69.1	67.8	3.39	0.36	0.32
Peak, mg/dL	366	330	335	56.4	0.59	0.64
CR,[5] %/min	3.97	4.10	3.78	1.288	0.98	0.89
$T_{1/2}$,[6] min	18.8	19.7	21.5	3.95	0.68	0.58
T_{basal}, min	181	202	202	29.9	0.65	0.68
AUC,[7] mg/dL × min	4,342	4,078	4,194	531.5	0.76	0.84
Insulin,[4] μIU/mL	32.9	21.4	24.5	5.08	0.16	0.26
Basal, μIU/mL	8.75	5.95	5.81	1.81	0.52	0.50
Peak, μIU/mL	77.6	56.7	59.6	14.63	0.07	0.11
CR, %/min	10.2	18.3	14.1	3.32	0.25	0.52
$T_{1/2}$, min	8.36	5.43	7.43	1.581	0.38	0.70
T_{basal}, min	71.9	45.5	59.1	17.65	0.11	0.21
AUC, μIU/mL × min	1,762	1,249	1,389	252.6	0.04	0.07

[1]Control = 0 mg/d rumen-protected *Capsicum* oleoresin (RPC); C100 = 100 mg/d RPC; C200 = 200 mg/d RPC.

[2]Highest SEM shown; n = 249 for glucose and insulin, n = 23 for all other variables (n represents number of observations used in the statistical analysis).

[3]Con vs. RPC = control vs. RPC treatment; Linear = linear effect of RPC.

[4]Data were analyzed as repeated measures.

[5]CR = clearance rate.

[6]$T_{1/2}$ = half-life.

[7]AUC = area under the curve.

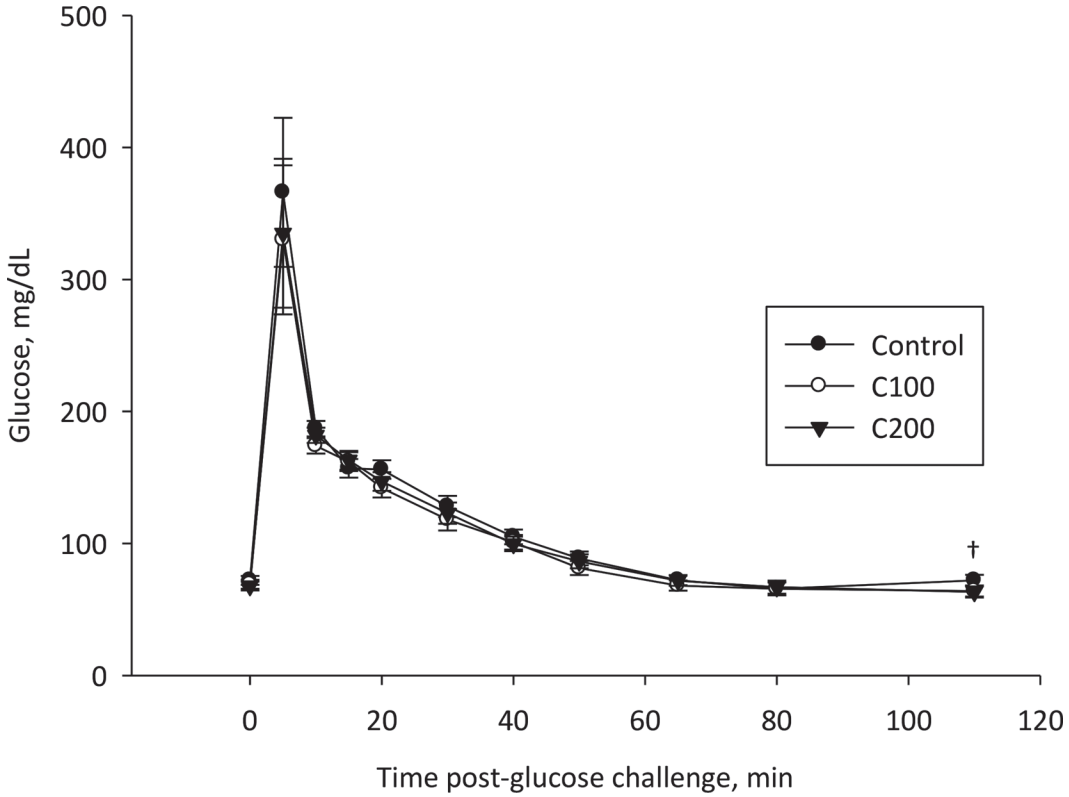

Figure 2. Effect of rumen-protected *Capsicum* oleoresin (RPC) on serum glucose concentration (mean ± SE) following intravenous administration of glucose in dairy cows. Control = 0 mg/d RPC; C100 = 100 mg/d RPC; C200 = 200 mg/d RPC. Orthogonal contrast between control and treatments (n = 23; n represents number of observations used in the statistical analysis), †$P = 0.07$ control vs. RPC.

Total-tract digestibility in ruminants is affected by degradation and passage rates in the rumen and enzymatic digestion in the lower gut. We did not measure rumen fermentation parameters or the passage rate in the current experiment, but Tager and Krause (2011) and Oh et al. (2015) reported no effect of *Capsicum* on ruminal VFA and ammonia concentration, and no difference was observed in total PD excretion as an indicator of ruminal microbial synthesis among treatments in the current experiment. Although the major site for digestion of feed in ruminants is the rumen, contribution of the lower gut is not negligible and has been reported to be 35.3, 21.2, and 19.5% to total-tract digestibility for OM, cell wall, and starch, respectively, in a comprehensive review by Archimède et al. (1997). The increase in total-tract digestibility of dietary nutrients by RPC in the current experiment may be due to stimulatory effects of capsaicin on digestive enzymes in the lower gut. Dietary supplementation of capsaicin has been reported to increase secretion of enzymes such as lipase, amylase, trypsin, and chymotrypsin in the pancreas of rats (Platel and Srinivasan, 1996, 2000). Ramakrishna Rao et al. (2003) also found an increase of amylase activity in pancreas homogenate with capsaicin administration. The linear decreases in fecal N excretion by RPC were in accordance with the total-tract digestibility data in the current experiment. Previous experiments with dairy cows, however, reported no effect of *Capsicum* oleoresin on total-tract digestibility of DM, OM, CP, NDF, and ADF and on fecal N excretion in dairy cows (Tager and Krause, 2011; Oh et al., 2015). In addition, supportive data such as secretion or activity of secreted intestinal digestive enzymes were not collected. It should also be pointed out that the digestibility data are from 6 cows only and should therefore be interpreted with caution.

Capsicum has been known to exhibit inhibitory effects on bacteria in the intestine as well as in the rumen (Cichewicz and Thorpe, 1996; Calsamiglia et al., 2007). The phenolic group of capsaicin may suppress the growth of bacteria by decreasing membrane stability because of its hydrophobicity (Burt, 2004). However, in vivo experiments with dairy cows showed no effect of *Capsicum* on intestinal bacteria. In a short-term (5-d) abomasal pulse-dose study, we reported that *Capsicum* treatment did not affect fecal bacterial populations in dairy cows (Oh et al., 2013). In the current experiment, dietary supplementation of RPC for a longer period (20

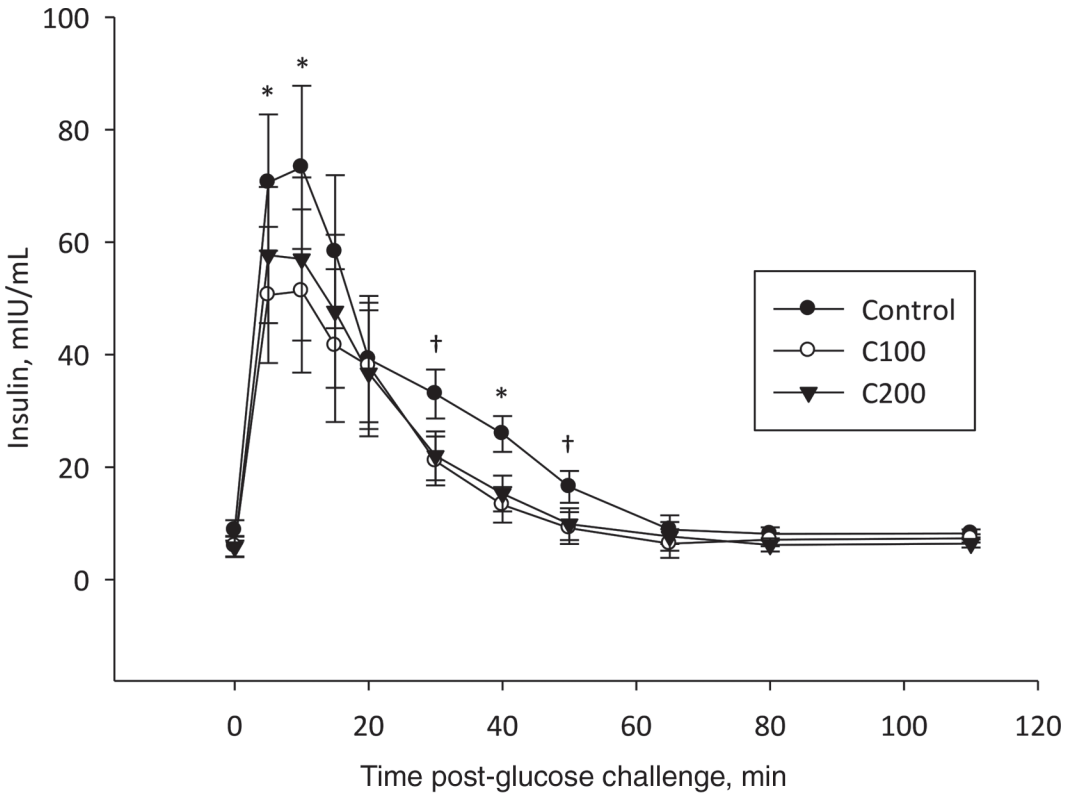

Figure 3. Effect of rumen-protected *Capsicum* oleoresin (RPC) on serum insulin concentration (mean ± SE) following intravenous administration of glucose in dairy cows. Control = 0 mg/d RPC; C100 = 100 mg/d RPC; C200 = 200 mg/d RPC. Orthogonal contrast between control and treatments (n = 23; n represents number of observations used in the statistical analysis), $^*P \leq 0.05$ and $\dagger 0.05 < P \leq 0.10$ control vs. RPC.

Table 7. Effect of rumen-protected *Capsicum* oleoresin on concentration of nonesterified fatty acids (NEFA), BHB, and leptin in serum following glucose tolerance test in dairy cows

	Treatment[1]				*P*-value[3]	
Item	Control	C100	C200	SEM[2]	Con vs. RPC	Linear
NEFA,[4] μmol/L	365	358	435	64.8	0.56	0.23
Basal, μmol/L	614	522	628	101.1	0.71	0.90
AUC,[5] μmol/L × min	37,750	39,035	46,781	5,592.0	0.33	0.14
BHB,[6] μmol/L	262	309	313	26.6	0.17	0.21
Basal, μmol/L	310	403	391	41.9	0.15	0.21
Leptin,[7] ng/mL	5.31	5.53	4.81	0.675	0.56	0.08
Basal, ng/mL	4.27	4.93	4.33	0.903	0.65	0.94
AUC, ng/mL × min	159	126	148	26.7	0.57	0.81

[1]Control = 0 mg/d rumen-protected *Capsicum* oleoresin (RPC); C100 = 100 mg/d RPC; C200 = 200 mg/d RPC.

[2]Highest SEM shown; n = 255 for NEFA, n = 69 for BHB, n = 216 for leptin, and n = 23 for all other variables (n represents number of observations used in the statistical analysis).

[3]Con vs. RPC = control vs. RPC treatment; Linear = linear effect of RPC.

[4]Oleic acid equivalent.

[5]AUC = area under the curve.

[6]BHB was measured only at 0, 30, and 65 min post-glucose challenge.

[7]Period × treatment, $P < 0.01$.

d) also did not affect fecal bacterial populations of the cows.

Serum insulin concentration was decreased by RPC, but glucose concentration remained similar among treatments during GTT in the current experiment. Similar results were reported in rats with capsaicin (van de Wall et al., 2005, 2006). These authors observed lower insulin levels in rats neonatally treated with cap-

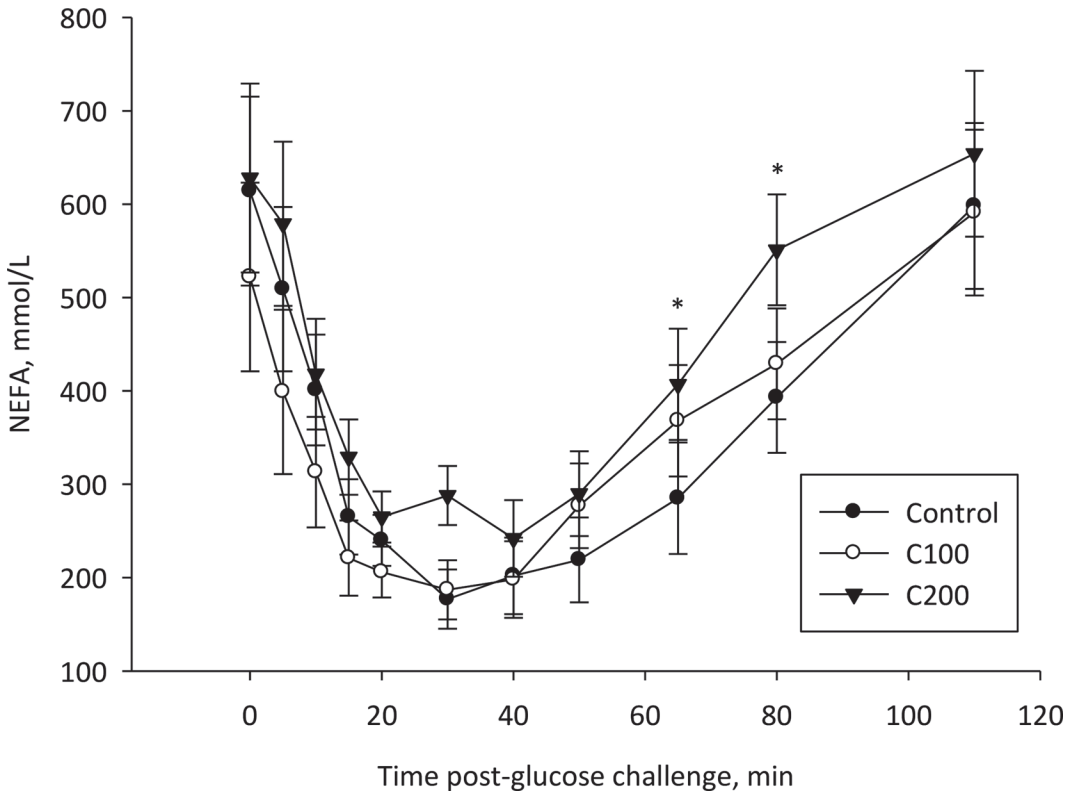

Figure 4. Effect of rumen-protected *Capsicum* oleoresin (RPC) on serum nonesterified fatty acid (NEFA) concentration (mean ± SE) following intravenous administration of glucose in dairy cows. Control = 0 mg/d RPC; C100 = 100 mg/d RPC; C200 = 200 mg/d RPC. Orthogonal contrast between control and treatments (n = 23; n represents number of observations used in the statistical analysis), $^*P \leq 0.05$ linear effect.

saicin during intravenous GTT, whereas glucose was not affected by treatment. In human subjects, regular consumption of cayenne pepper containing 2,000 ppm capsaicin lowered hyperinsulinemia after meals, with no effect on glucose concentration in blood (Ahuja et al., 2006). The inhibitory effect of capsaicin on insulin might be exhibited via calcitonin gene-related peptide (**CGRP**), a neuropeptide produced in neurons, which is known to decrease insulin secretion from the pancreas (Pettersson and Ahrén, 1990; Tanaka et al., 2013). Demirbilek et al. (2004), for example, observed that subcutaneously administered capsaicin increased plasma CGRP concentration in rats. Oral administration of a red chili homogenate in human subjects also increased CGRP concentration in a dose-dependent manner (van Oosterhout et al., 2015). In the current experiment, RPC may have stimulated CGRP production and decreased insulin secretion. Another explanation of the insulin and glucose results in the current experiment could be that insulin sensitivity of insulin-dependent tissues was increased by RPC and glucose concentration was controlled with less insulin. However, insulin sensitivity was not determined in the current study.

Decreased insulin concentration in cows fed RPC may lower glucose uptake in insulin-dependent tissues such as skeletal muscle and adipose tissue (De Koster and Opsomer, 2013). Spared glucose could be used by an insulin-independent tissue, for example, by the mammary gland for lactose synthesis in cows receiving RPC. This hypothesis is supported by the similar glucose concentration among treatments during GTT and higher milk yield and feed efficiency in RPC cows, although milk lactose yield was only numerically higher for RPC.

Nevertheless, data from studies on the effects of capsaicin on glucose and insulin levels have been conflicting. A single dose of 5 g of *Capsicum frutescens* decreased plasma glucose concentration at 30 and 45 min post oral glucose challenge and increased plasma insulin concentration at 60, 75, 105, and 120 min in a human study (Chaiyasit et al., 2009). Akiba et al. (2004) demonstrated that capsaicin treatment increased insulin released from pancreatic islet β-cells by

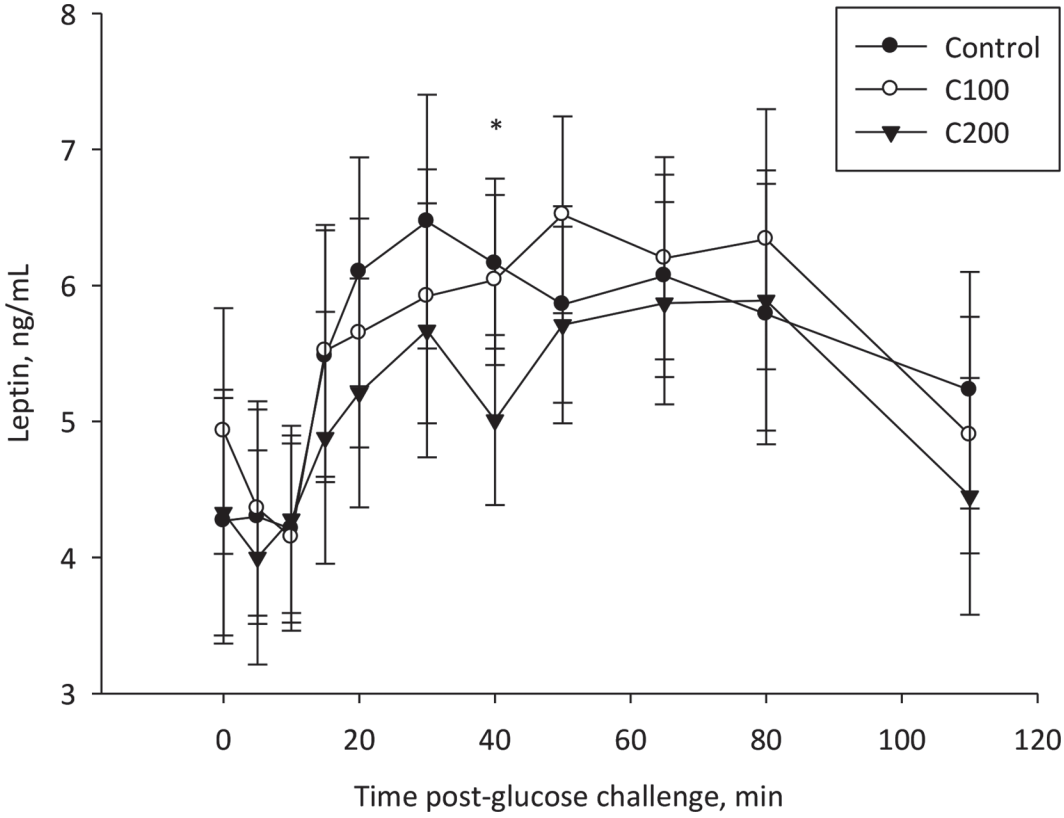

Figure 5. Effect of rumen-protected *Capsicum* oleoresin (RPC) on serum leptin concentration (mean ± SE) following intravenous administration of glucose in dairy cows. Control = 0 mg/d RPC; C100 = 100 mg/d RPC; C200 = 200 mg/d RPC. Orthogonal contrast between control and treatments (n = 23; n represents number of observations used in the statistical analysis), $^*P = 0.03$ linear effect.

increasing Ca^{2+} influx through the capsaicin receptor expressed on β cells. Dömötör et al. (2006) observed increases in serum glucose and glucagon concentration after oral glucose challenges when 400 μg of capsaicin was administrated to human subjects, whereas serum insulin was not affected by capsaicin. In dairy cows, we previously observed no effect of *Capsicum* on insulin concentration in plasma (Oh et al., 2015). However, blood samples were collected only 2 times in 1 d of each experimental period.

Fat mobilization depends on energy supply in the lactating dairy cow (Drackley et al., 2005). Decreases in NEFA concentration for all treatments after glucose administration were accompanied with increased glucose concentration in the current experiment. Limited effect of RPC on circulating NEFA is also consistent with the glucose results in the current experiment, although we observed subtle increases at 65 and 80 min post glucose challenge in cows fed RPC. Studies reported that dietary supplementation of capsaicin increased fat mobilization and blood free FA in rats (Kawada et al., 1986; Yoshioka et al., 2000). According to Lee et al. (2013b), capsaicin increased gene expression of lipoprotein lipase and adiponectin in mesenteric adipose tissues of capsaicin-treated rats, both of which are related to fat accumulation reduction and FA combustion (Yamauchi et al., 2001; Koike et al., 2004). Limited studies exist on the effect of *Capsicum* on NEFA and BHB in dairy cows. In our previous study, plasma BHB concentration was increased by *Capsicum* supplementation, but NEFA was not affected. However, we found only numerical increases in BHB concentration with RPC in the current experiment.

In the current study, increases in serum leptin concentration for all treatments post glucose challenge were likely due to increased insulin concentration. Insulin is known to stimulate leptin synthesis in adipose tissue (Amstalden et al., 2000; Block et al., 2003). In addition, the response in serum leptin to glucose challenge in the current experiment was in the direction opposite that in NEFA concentration. Because the adipose tissue is mainly responsible for leptin concentration in ruminants, fat mobilization could negatively affect leptin concentration (Chilliard et al., 2001). Studies with dairy cows and beef cattle have shown positive correlation between leptin and body fatness (Kokkonen et al., 2005; Foote et al., 2016), which is in agreement with the leptin and NEFA data in the current experiment. We found a time × treatment interaction in serum leptin when the data were analyzed as repeated measure in the current study. This interaction was caused by highly variable leptin concentration during the 3 experimental periods (data not shown). Thus, although a trend existed for a linear decrease in leptin concentration with RPC, the leptin data have to be interpreted with caution.

CONCLUSIONS

Dietary supplementation of RPC tended to increase milk production and increased feed efficiency in dairy cows. In addition, RPC decreased serum insulin concentration, but glucose concentration did not change during GTT. Our data suggest that, by decreasing insulin secretion, RPC fed at 100 to 200 mg/d may have redirected glucose for milk production in lactating dairy cows. Production data from this experiment should be interpreted with caution because of the low number of experimental units, short experimental periods, and the crossover experimental design.

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