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Perturbations of plasma metabolites correlated with the rise of rumen endotoxin in dairy cows fed diets rich in easily degradable carbohydrates

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ABSTRACT

Feeding dairy cows diets high in easily degradable carbohydrates increases the incidence of rumen and systemic metabolic disorders; however, the triggering factor is not well understood. In this study, dairy cows were fed 4 different amounts of barley grain-based concentrate at 15, 30, 45, and 60% (dry matter basis) of a total mixed ration to determine whether alterations in the rumen environment would be associated with perturbations of the plasma profile of selected metabolites. In addition, associations among free rumen endotoxin and several plasma metabolites were determined. The study was a replicated 4 × 4 Latin square design with 8 rumen-cannulated lactating dairy cows (60 ± 15 d in milk). Multiple rumen fluid and blood plasma samples were collected and analyzed for pH and rumen fluid endotoxin and for concentrations of glucose, insulin, cholesterol, β-hydroxybutyrate (BHBA), nonesterified fatty acids (NEFA), and lactate in the plasma. Rumen pH decreased below 6.0, from 8 to 12 h after the morning feeding, with the augmentation of the proportion of concentrate in the diet of ≥30%. Feeding diets with >30% concentrate resulted in a rise of free endotoxin in the rumen fluid (8.87 ± 0.39 μg/mL). Inclusion of 60% concentrate in the total mixed ration was associated with enhanced concentrations of glucose (64.5 ± 1.0 mg/dL) and lactate (540.9 ± 36.5 μmol/L) and lowered cholesterol (265.5 ± 13.7 mg/dL), BHBA (449.1 ± 47.4 μmol/L), and NEFA (138.8 ± 19.1 μEq/L) in the blood plasma. The regression analysis revealed that greater concentrations of plasma lactate and lower concentrations of cholesterol, BHBA, and NEFA were related to the rise of rumen endotoxin. Interestingly, 93% of the increase in the plasma lactate was explained by the rise of rumen endotoxin. Moreover, the analysis revealed inverse relationships of rumen endotoxin with plasma cholesterol ($R^2 = 0.47$), BHBA ($R^2 = 0.37$), and NEFA ($R^2 = 0.50$) and a biphasic response of plasma insulin ($R^2 = 0.58$). Taken together, feeding dairy cows diets rich in rumen-degradable carbohydrates and low in fiber led to lower rumen pH and a large accumulation of rumen endotoxin; the latter was correlated with perturbations of plasma metabolites allied to carbohydrate and lipid metabolism.

Key words: rumen endotoxin, plasma metabolite, barley grain, dairy cattle

INTRODUCTION

A major challenge to the current feeding systems of dairy cattle is how to reconcile feeding of large amounts of cereal grains that support high milk production with the high incidence of metabolic disorders. Although an unbalanced nutrition and, in particular, the feeding of diets high in starch has been proposed as the major causal factor of various metabolic disorders in dairy cows (Ametaj et al., 2005; Goff, 2006), the exact mechanism(s) underlying this association is not yet completely understood.

Research conducted by our team and others demonstrated that feeding dairy cows large amounts of concentrate often results in alterations in the rumen environment, leading to major changes in the composition of rumen microbiota and accumulation of large amounts of potentially harmful compounds such as endotoxin, a bioactive cell-wall component of all gram-negative bacteria (Emmanuel et al., 2008; Khafipour et al., 2009; Ametaj et al., 2010). These studies have shown that the rise in the concentration of rumen endotoxin is associated with activation of an acute phase response (Emmanuel et al., 2008). New evidence suggests that cytokines such as tumor necrosis factor (TNF)-α, IL-1, and IL-6, released by liver macrophages when activated by binding of endotoxin, may alter various physiological functions in the host because most cell types express receptors for those cytokines (Elsasser et al., 2008). As an example, both i.v. (Steiger et al., 1999; Waldron et al., 2003) and intramammary (Waldron et al., 2006) infusions of single doses of endotoxin in cattle have been related to perturbations of different plasma metabolites such as enhanced plasma glucose, devel-

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opment of insulin resistance, greater concentration of lactate in the plasma, and lower circulating BHBA.

Nevertheless, a direct association between the rise of free endotoxin, released into rumen fluid during the feeding of diets high in starch, and the perturbations of plasma variables related to carbohydrate and lipid metabolism in dairy cows has not yet been documented. We hypothesized that alterations in the ruminal environment, including a rise in the concentration of endotoxin, in response to feeding graded amounts of barley grain, might play a role in oscillations of different plasma metabolites. Therefore, the main objectives were to investigate the effects of feeding graded amounts of barley grain on rumen pH patterns, concentration of endotoxin in the rumen fluid, and the profile of selected plasma metabolites in dairy cows. Furthermore, associations among grain-induced rise in the concentration of rumen endotoxin with different plasma metabolites allied to carbohydrate and lipid metabolism in lactating dairy cows were evaluated.

MATERIALS AND METHODS

Animals and Diets

This investigation was part of a larger study designed to investigate the immune and metabolic events associated with the feeding of high-starch diets in lactating dairy cows at Dairy Research and Technology Centre, University of Alberta (Edmonton, AB, Canada). Results related to acute phase response, plasma minerals, milk production, and rumen metabolomics were published previously (Emmanuel et al., 2008; Ametaj et al., 2010). In brief, 8 rumen-fistulated (Ø 100 mm, Bar Diamond, Parma, ID) primiparous Holstein cows were used in a replicated 4 × 4 Latin square design. The experimental period was 21 d in duration with the first 11 d used for diet adaptation. The herd veterinary technician supervised cows daily; all cows remained clinically healthy throughout. At the start, the cows were at 60 ± 15 (mean ± SD) d postpartum. The cows were housed in tie stalls with free access to water and fed once daily at 0800 h. To challenge the cows with different concentrate-to-forage ratios in the diet, different concentrate proportions (i.e., 15, 30, 45, or 60% in DM) were used in the diet. The amount of concentrate was stepped up or down during the adaptation period to avoid potential health implications due to abrupt dietary changes. Daily ration was offered as TMR for ad libitum intake to allow approximately 5% feed refusals. All diets were formulated to meet or exceed the nutrient requirements of a 680-kg lactating cow, following NRC (2001) guidelines. Diet ingredients were analyzed for concentrations of DM, ash, NDF, and ADF. The DM concentration was determined by drying samples at 135°C for 2 h (AOAC, 1990). Ash concentration was determined after 5 h at 500°C in a furnace. Methods of Van Soest et al. (1991) were used in analyses of NDF and ADF using heat-stable amylase and sodium sulfite in the case of NDF. Ingredients and nutrient composition of the TMR are in Table 1. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

Sample Collection

Blood samples were obtained from the tail vein on d 1, 3, 5, 7, and 10 of the measurement period at 0800 h, shortly before the morning feeding. Sodium heparinized 10-mL Vacutainer tubes (Becton Dickinson, Franklin Lake, NJ) were used to collect blood samples. Immediately after collection, blood samples were stored on ice and plasma was separated within 20 min from withdrawal by centrifuging at 3,000 × *g* at 4°C for 20 min (Rotanta 460 R, Hettich Zentrifugan, Tuttlingen, Germany). Plasma samples were stored at −20°C until analysis.

Samples from rumen fluid (100 mL) were obtained on d 1, 3, 5, 7, and 10 of the measurement period shortly before the morning feeding (i.e., 0800 h). All rumen fluid samples were collected through the cannula using a tube fitted with a strainer and a syringe into a 140-mL plastic container. Subsequently, rumen fluid samples were centrifuged at 6,000 × *g* for 15 min, and the supernatant was stored at −20°C until analysis for concentration of endotoxin. Other samples of rumen fluid were obtained at 0, 2, 4, 6, 8, 10, and 12 h after the morning feeding on d 21 to determine the postprandial changes of rumen pH. The pH of rumen fluid was measured immediately after collection by a mobile pH meter (Accumet AP61, Fischer Scientific, Ottawa, Ontario, Canada).

Determination of Rumen Endotoxin and Plasma Metabolites

Concentration of cell-free endotoxin in the rumen fluid supernatant, collected only before the morning feeding, was determined by the Pyrochrome *Limulus* amebocyte lysate assay (Associates of Cape Cod Inc., East Falmouth, MA) as described previously (Emmanuel et al., 2008). In brief, 10 mL of rumen fluid sample was centrifuged at 6,000 × *g* for 15 min, and the supernatant was stored at −20°C. For use in the assay, 1.5 mL of the supernatant was centrifuged again at 10,000 × *g* for 30 min. The supernatant was passed

Table 1. Ingredients and nutrient composition of the 4 experimental TMR, differing in the level of concentrate inclusion

	Concentrate proportion of TMR			
Item	15%	30%	45%	60%
Ingredient (% of DM)				
Alfalfa hay	15.0	15.0	15.0	15.0
Alfalfa silage	12.0	12.0	12.0	12.0
Barley silage	58.0	43.0	28.0	13.0
Rolled barley	—	15.0	30.0	45.0
Gluten meal	6.00	6.00	6.00	6.00
Fish meal	1.03	1.03	1.03	1.03
Canola meal	0.98	0.98	0.98	0.98
Dairy premix[1]	0.58	0.58	0.58	0.58
Megalac[2]	1.79	1.79	1.79	1.79
Limestone	0.58	0.58	0.58	0.58
Biofos[3]	0.40	0.40	0.40	0.40
Magnesium oxide	0.35	0.35	0.35	0.35
Sodium bicarbonate	0.76	0.76	0.76	0.76
Vitamin E (5,000 IU/kg)	0.09	0.09	0.09	0.09
Vitamin D_3 (500,000 IU/kg)	0.17	0.17	0.17	0.17
Molasses	0.35	0.35	0.35	0.35
Hydrogenated tallow	1.87	1.87	1.87	1.87
Nutrient composition (% of DM, unless otherwise stated)				
ME (Mcal/kg of DM)	2.45	2.42	2.40	2.39
CP	16.2	16.4	16.5	16.7
NDF	32.8	30.2	27.6	25.0
ADF	21.8	19.4	17.0	14.6
NFC[4]	35.4	38.8	42.1	45.5
Ca	1.30	1.30	1.20	1.20
P	0.40	0.40	0.50	0.50
DCAD (mEq/kg)	300	274	248	223

[1]Contained calcium, 0.1%; phosphorous, 0.6%; sodium, 11.5%; magnesium, 0.3%; potassium, 0.7%; sulfur, 0.23%; zinc, 5,000 mg/kg; copper, 1,170 mg/kg; manganese, 3,100 mg/kg; iodine, 80 mg/kg; cobalt, 6.2 mg/kg; vitamin A, 1,265,000 IU/kg; vitamin D, 142,000 IU/kg; and vitamin E, 3,800 IU/kg.

[2]Contained 85% fat as fatty acids and 9.6% calcium with a NE_L of 6.52 Mcal/kg (Champion Feed Services Ltd., Barrhead, Alberta, Canada).

[3]Contained monocalcium phosphate and dicalcium phosphate in the ratio 2:1 (Champion Feed Services Ltd.).

[4]Nonfiber carbohydrates = 100 – (% ash + % CP + % NDF + % ether extracts).

through a disposable 0.22-μm sterile, pyrogen-free filter (Fischer Scientific, Fairlawn, NJ) and diluted 1,000-fold using pyrogen-free *Limulus* amebocyte lysate reagent water and pyrogen-free test tubes (Associates of Cape Cod Inc.). Samples were tested in duplicate, and the optical density values were read on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corporation, Sunnyvale, CA) at a wavelength of 405 nm. Intra-assay coefficient of variation was <10% for all the assays.

The concentration of glucose in the plasma was quantified by an enzymatic method using a commercially available kit (Diagnostic Chemicals Ltd., Charlottetown, PE). Briefly, the procedure involves phosphorylation and oxidization of glucose in samples, resulting in the production of NADH, which produces a color proportional to the glucose concentration in the sample. All samples were tested in duplicate and the plasma glucose was determined by reading on a microplate spectrophotometer at an optical density of 340 nm. According to the manufacturer instructions, the lower detection limit of the test was 0.06 mg/dL.

Enzymatic quantization of BHBA by BHBA dehydrogenase was used for quantifying plasma concentration of BHBA using a commercially available kit (Stanbio Laboratory, Boerne, TX). The principle of the test involves conversion of BHBA in the samples to acetoacetate and NADH at pH 8.5 by BHBA dehydrogenase in the presence of NAD. The NADH produced reacts with 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) in the presence of diaphorase to produce a color proportional to the concentration of BHBA in the sample. Plasma BHBA was measured in duplicate by reading a microplate spectrophotometer at an optical density of 505 nm. The lower detection limit of the assay was 0.125 μmol/L.

Quantitative determination of plasma NEFA was by an enzymatic colorimetric method using a commercially available kit (Wako Chemicals, Richmond, VA). The principle of the test involves acylation of coenzyme A (**CoA**) by fatty acids in the sample in presence of acyl-CoA synthetase and production of hydrogen peroxide in presence of acyl-CoA oxidase. Hydrogen peroxide, in presence of peroxidase, permits the oxidative condensa-

tion of 3-methyl-N-ethyl-N-β-hydroxy ethyl-O-aniline with 4-aminoantipyrine to form a purple colored adduct, which is proportional to the concentration of NEFA in the sample. Samples were tested in duplicate and the optical density was measured at 550 nm on a microplate spectrophotometer. The lower detection limit of the assay was 0.50 μEq/L.

Plasma insulin was measured by a commercially available bovine ELISA kit, supplied by Mercodia AB (Mercodia, Uppsala, Sweden). This procedure involves a solid phase 2-site enzyme immunoassay. It is based on the direct sandwich technique in which 2 monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microtitration wells. After simple washing, which removes unbound enzyme-labeled antibody, the bound conjugate is detected by reaction with 3,3′,5,5′-tetramethylbenzidine. The reaction is stopped by the addition of stop solution, giving colorimetric endpoint that is read spectrophotometrically at 450 nm.

Plasma cholesterol was measured using a commercially available kit (Diagnostic Chemicals Ltd., Charlottetown, Prince Edward Island, Canada). The colorimetric method is based on the principle of hydrolyzing the cholesterol esters to free cholesterol and oxidation of free cholesterol to cholest-4ene-3-one with simultaneous production of hydrogen peroxide. The hydrogen peroxide couples with 4-aminoantyrine and *p*-hydroxybenzoate in the presence of peroxidase to yield a chromogen whose intensity is proportional to the concentration of cholesterol in the sample. All samples were tested in duplicate and plasma cholesterol was determined by reading the optical density values on a microplate spectrophotometer at 505 nm.

A commercially available kit from Biomedical Research Service Center (Buffalo, NY) was used to measure concentration of lactate in plasma. The principle of the test involves reduction of tetrazolium salt INT in a NADH-coupled enzymatic reaction to formazan, which exhibits a red color whose intensity is proportional to the concentration of lactate. The lactate standard provided in the kit was diluted to set a detection range of 125 to 1,000 μ*M*. All samples were tested in duplicate and the lactate concentration was determined by reading the optical density values on a microplate spectrophotometer at 492 nm.

Statistical Analyses

All data were analyzed using the MIXED procedure of SAS (SAS Institute, 2006) according to the model

$$Y_{ijklmn} = \mu + p_i + a_j + a(s)_{jk} + d_l + t_m + (dt)_{lm} + e_{ijklmn},$$

where Y_{ijklmn} is the observation for dependent variables, μ represents the population mean, p_i is the fixed effect of period i ($i = 1$ to 4), a_j is the fixed effect of cow j ($j = 1$ to 8), $a(s)_{jk}$ is the random effect of cow j within the square k ($k = 1$ to 2), d_l is the fixed effect of measurement day l ($l = 1$ to 5), t_m represents the fixed effect of diet m ($m = 1$ to 4), $(dt)_{lm}$ is the diet by day interaction, and e_{ijklmn} is the residual error, assumed to be normally distributed. The covariance structure of the repeated measures at different days for each response variable was modeled separately according to the smallest values of the fit statistics based on the Bayesian information criteria and an appropriate structure fitted. To test linear or quadratic effects of treatment on blood variables, the orthogonal contrasts with CONTRAST statement of SAS (SAS Institute, 2006) were used. Significance was declared at $P \leq 0.05$ and a tendency was considered at $0.05 < P \leq 0.10$.

Correlations between independent and response variables were evaluated by the MIXED modeling procedure of SAS (SAS Institute, 2006) and quantified using the option SOLUTION as well as by fitting the data to different straight-line, break-point, nonlinear models with the NLMIXED procedure of SAS. The fitted models were adjusted for experimental errors related to animal-block effect as well as the random effects of period and measurement day, whereby the latter was considered as repeated measures with first-order autoregressive covariance structure. Only significant relationships were considered ($P < 0.05$). Root mean square error (RMSE), *P*-value, and R^2 were computed and used to evaluate the goodness of fit. Graphical depiction of the relationships obtained was conducted with the GPLOT procedure of SAS.

RESULTS

Metabolic Responses to Increasing Amounts of Concentrate

Changes in the postprandial patterns of ruminal pH are in Figure 1, whereas concentrations of selected plasma metabolites are in Table 2. Overall data demonstrated that increasing the proportion of concentrate in the diet lowered ruminal pH in a linear fashion ($P < 0.01$). Starting from a content of 30% concentrate in the diet (i.e., 30, 45, and 60%) rumen pH decreased below 6.0 from 8 to 12 h post-feeding. Furthermore, separate comparison of means at 8, 10, and 12 h post-feeding showed that rumen pH was lower in cows fed 30, 45,

Table 2. Plasma variables in lactating cows fed graded amounts of concentrate[1]

	Concentrate proportion of TMR					*P*-value[2]	
Item	15%	30%	45%	60%	SEM	Linear	Quadratic
Glucose (mg/dL)	60.6[a]	64.7[b]	64.3[b]	64.5[b]	1.00	<0.01	0.02
Insulin (μg/dL)	3.57[a]	4.19[b]	4.51[c]	4.10[b]	0.09	0.01	<0.01
Lactate (μmol/L)	410.0[a]	454.7[ab]	498.4[bc]	540.9[c]	36.5	<0.01	0.27
Cholesterol (mg/dL)	280.8[a]	287.2[a]	272.9[ab]	265.5[b]	13.7	0.09	<0.01
BHBA (μmol/L)	690.5[a]	523.2[b]	511.1[bc]	449.1[cd]	47.4	<0.01	0.05
NEFA (μEq/L)	335.7[a]	212.3[b]	182.9[b]	138.8[c]	19.1	<0.01	0.03

[a–d]Within a row, least squares means with different superscripts differ at $P < 0.05$.

[1]Data across d 12, 14, 16, 18, and 21 of each experimental period (blood samples were collected shortly before the morning feeding).

[2]Linear or quadratic effects of dietary barley grain inclusion: data are presented as least squares means ± standard error of the means; n = 8.

and 60% concentrate compared with those fed the diet with 15% concentrate ($P < 0.05$; Figure 1). Time after the morning feeding affected the ruminal pH, whereby it reached the nadir at 8 to 10 h post-feeding (Figure 1).

The feeding of graded amounts of concentrate increased the concentration of plasma glucose (Table 2; $P < 0.01$). The cows fed 15% concentrate had the lowest, whereas the cows fed 30% concentrate in the diet had the greatest concentration of glucose. Dietary treatment affected the overall concentration of insulin in the plasma ($P < 0.01$). Interestingly, cows fed 15 and 60% concentrate showed the lowest concentration of insulin in the plasma, and this response was reflected by a quadratic effect of the treatment ($P < 0.01$; Table 2).

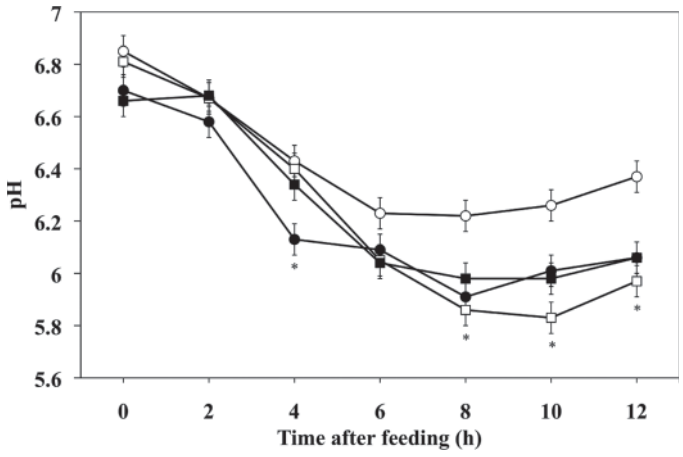

Figure 1. Postprandial patterns of rumen fluid pH measured at different hours after the morning feeding in lactating Holstein cows fed 15 (●), 30 (○), 45 (□), or 60% (■) concentrate in a TMR [(LSM ± SEM; n = 8); treatment effect, $P < 0.01$; time effect, $P < 0.01$; treatment by time effect, $P < 0.01$; within each sampling time, (*) indicates differences at $P < 0.05$ (at 4 h post-feeding, 30% differed from 15, 45, and 60% concentrate in the diet; at 8 h, 10 h, and 12 h post-feeding, 15% differed from 30, 45, and 60% concentrate in the diet)].

Dietary treatment affected the overall concentration of cholesterol in plasma in a curvilinear fashion ($P < 0.01$). The analysis showed that plasma cholesterol concentration was lowest in cows fed 60% concentrate compared with cows fed 15% or 30% grain (Table 2). Plasma cholesterol concentration did not differ between cows fed 45 and 60% concentrate (Table 2). The concentration of lactate in the plasma increased linearly with the increasing of the amount of concentrate in the diet ($P < 0.01$; Table 2). Plasma lactate concentration was lowest for cows fed 15% concentrate and increased with the increase of the amount of grain in the diet.

Dietary treatment affected significantly plasma concentration of BHBA, whereby cows fed 15% concentrate had the greatest plasma concentration of BHBA (Table 2). Feeding increasing proportions of concentrate decreased the concentration of NEFA in the plasma (Table 2). Overall, plasma NEFA concentrations were highest in cows fed 15% concentrate, and lowest in cows fed 60% concentrate in the diet.

Correlations Among Plasma Metabolites and Rumen Endotoxin

Changes in the concentration of plasma metabolites were related to the rise of rumen endotoxin induced by the increasing starch proportion in the diet. The straight-line, threshold model that fit best to the data of plasma cholesterol concentration in response to rumen endotoxin revealed an inverse relationship between the latter predictor and cholesterol concentration, particularly when rumen endotoxin increased up to 7,788 ng/mL (Figure 2). The latter threshold corresponded to an asymptotic plateau of plasma cholesterol concentration of 265.3 mg/dL. Also, a similar relationship between rumen endotoxin and plasma NEFA concentration was detected, whereby the former predictor variable explained 50% of the variation in the response of plasma

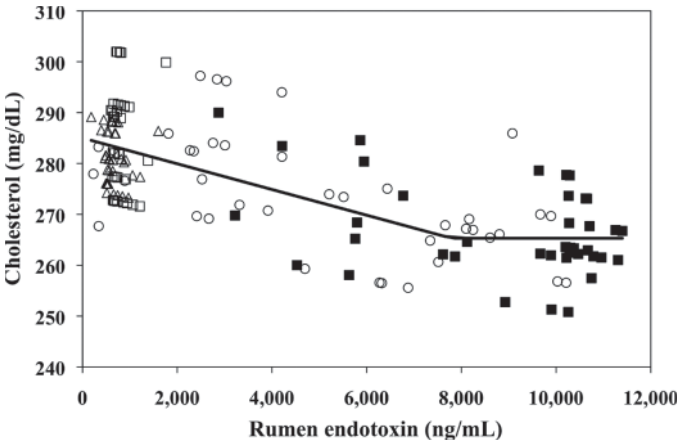

Figure 2. Relationship between concentration of plasma cholesterol (CHOL) and concentration of endotoxin in the rumen fluid of lactating primiparous Holstein cows fed 15% (Δ), 30% (□), 45% (○), or 60% (■) concentrate in the TMR: CHOL = 285.0 – 0.00253 × endotoxin, if endotoxin ≤7,788 ng/mL (asymptotic plateau of CHOL = 265.3 mg/dL, root mean square error = 8.39, $R^2 = 0.47$, $P < 0.001$).

NEFA (Figure 3). The threshold of the asymptotic response of NEFA was reached in response to lower concentrations of rumen endotoxin (i.e., 4,890 ng/mL) compared with the aforementioned response of plasma cholesterol (Figure 2). Further, the analysis indicated that the increase of rumen endotoxin was associated with a linear depletion of plasma BHBA in this study (Figure 4).

Interestingly, when rumen endotoxin increased, the fitted model indicated an abrupt increase in the concentration of plasma lactate, and the predictor variable explained 93% of the variation of the response of plasma lactate (Figure 5). In contrast, augmentation of rumen endotoxin up to 5,973 ng/mL resulted in lower concentrations of plasma insulin (Figure 6). Thus, a biphasic response of plasma insulin to increasing amounts of rumen endotoxin occurred in this study.

DISCUSSION

This study tested the hypothesis that feeding dairy cows diets rich in rumen-degradable starch is associated with alterations in the rumen environment and selected plasma metabolites. Indeed, rumen pH decreased below 6.0 from 8 to 12 h after the morning feeding with the augmentation of the proportion of concentrate in the diet beyond 30%. Feeding of diets with more than 30% concentrate resulted in a rise in the concentration of free endotoxin in the rumen fluid, and major changes in the profile of plasma metabolites associated with carbohydrate and lipid metabolism. The increase in the concentration of endotoxin in the rumen fluid indicates shedding of LPS from the bacterial membrane or lysis of gram-negative bacteria in response to the unfavorable sub-acidotic conditions in the rumen (Emmanuel et al., 2008; Khafipour et al., 2009).

It is well established that blood cholesterol in ruminants derives mainly from endogenous synthesis in the epithelial cells of the gastrointestinal tract, adipose tissue, and liver hepatocytes (Liepa et al., 1978). Acetate and glucose are the major precursors of the de novo synthesis of cholesterol in dairy cows. Because both rumen acetate and plasma glucose were greatest in the 60% concentrate group of this study (Ametaj et al., 2010), it was assumed that those cows should have had

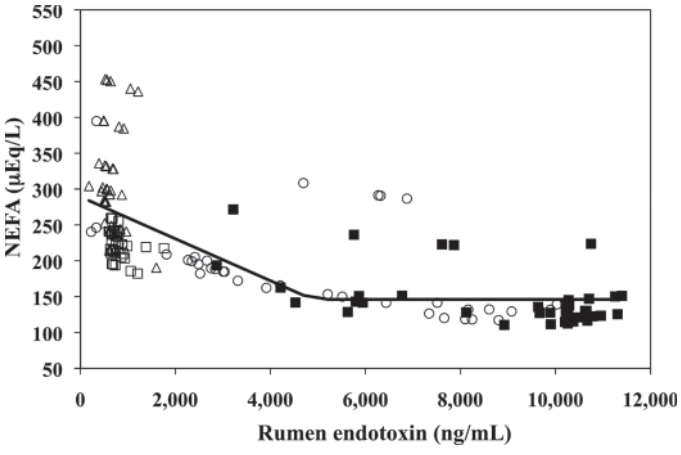

Figure 3. Association between concentration of plasma NEFA and concentration of endotoxin in the rumen fluid of lactating primiparous Holstein cows fed 15% (Δ), 30% (□), 45% (○), or 60% (■) concentrate in the TMR: NEFA = 289.3 – 0.029 × endotoxin, if endotoxin ≤4,890 ng/mL (asymptotic plateau of NEFA = 146.2 μEq/L; root mean square error = 57.5, $R^2 = 0.50$, $P < 0.001$).

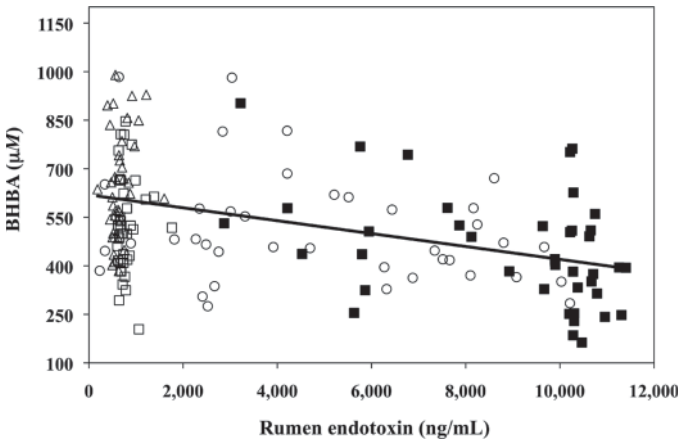

Figure 4. Association between concentration of plasma BHBA and concentration of endotoxin in the rumen fluid of lactating primiparous Holstein cows fed 15% (Δ), 30% (□), 45% (○), or 60% (■) concentrate in the TMR: BHBA = 619.1 – 0.0199 × endotoxin (root mean square error = 103.9, $R^2 = 0.37$, $P < 0.001$).

greater plasma cholesterol concentration; however, that was not the case. This suggested that other mechanisms were involved in the changes of plasma cholesterol concentration.

It is postulated that the decreasing concentration of plasma cholesterol in response to feeding of large amounts of concentrate might be linked to the general inflammatory state triggered by translocation of endotoxin into the systemic circulation. Evidence exists that endotoxin permeates rumen and colon tissues, and that the mucosal barrier functions are affected by sub-acidic pH values (Emmanuel et al., 2007; Khafipour et al., 2009). Indeed, increasing the proportion of concentrate in the diet more than 30% caused a decrease in ruminal pH below 6.0 from 8 to 12 h post-feeding in this study. Rumen endotoxin and low ruminal pH were associated with enhanced concentrations of plasma acute phase proteins like serum amyloid A, LPS-binding protein, and C-reactive protein in the plasma (Emmanuel et al., 2008). Because cholesterol is the main precursor for the synthesis of bile acids, the decrease in plasma cholesterol with augmentation of endotoxin concentration in the rumen fluid may be related to the increased need for secretion of bile acids (Parlesak et al., 2007). Bertok (2004) demonstrated that bile acid-devoid rats developed endotoxemia, suggesting that bile acids are able to split the molecule of endotoxin into nontoxic fragments that are bound and neutralized by gastrointestinal proteins to prevent endotoxin translocation into the host blood circulation.

This investigation showed that circulating NEFA and BHBA decreased as the amount of concentrate in the diet increased. The release of NEFA in the plasma of dairy cows is related predominantly to their mobilization from adipose tissue triacylglycerol stores through the process of lipolysis, whereas plasma BHBA derives mainly either from oxidation of NEFA in the hepatocytes or from the metabolism of butyrate in the rumen epithelium (Drackley, 1999). Increasing the amount of concentrate in the diet improved the energy balance of the cows and led to greater concentrations of rumen propionate and plasma glucose in this study (Ametaj et al., 2010). Availability of propionate and glucose might have contributed to the lowered concentration of NEFA in the plasma due to their inhibitory effect on NEFA release by adipose tissue (van Knegsel et al., 2007).

Furthermore, this study demonstrated an asymptotic inverse relationship between rumen endotoxin and plasma NEFA. Although this finding suggests a role for rumen endotoxin in the perturbation of plasma NEFA, the precise mechanism(s) underlying this relationship is not fully understood. In the published literature, conflicting reports exist on the effects of endotoxin on plasma NEFA. For example, infusion of endotoxin decreased plasma NEFA concentration in sheep (Naylor and Kronfeld, 1986), but not in heifers (Steiger et al., 1999), whereas in dairy cows, the circulating NEFA were initially decreased and then increased (Waldron et al., 2003).

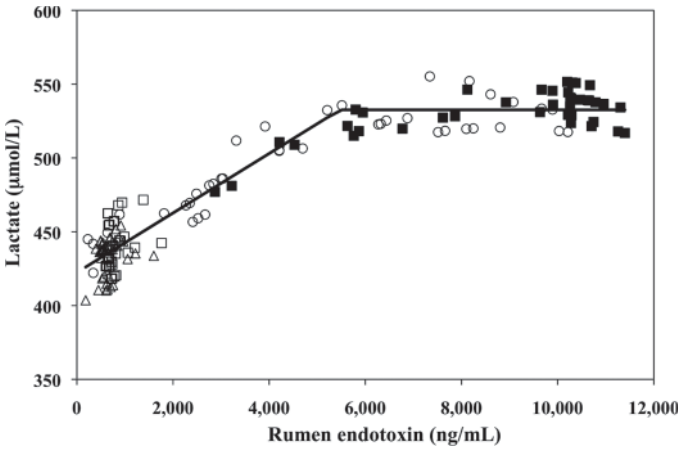

Figure 5. Relationship between concentration of plasma lactate and concentration of endotoxin in the rumen fluid of lactating primiparous Holstein cows fed 15% (Δ), 30% (□), 45% (○), or 60% (■) concentrate in the TMR: lactate = 422.4 + 0.0202 × endotoxin, if endotoxin ≤5,468 ng/mL (asymptotic plateau of lactate = 532.6 µmol/L, root mean square error = 12.4, $R^2 = 0.93$, $P < 0.001$).

A negative relationship was observed between rumen endotoxin and plasma BHBA concentration. Our data are in agreement with previous reports indicating lowering of ketone bodies (including BHBA) in the plasma of experimental animals during *Escherichia coli*-induced sepsis (Lanza-Jacoby et al., 1990). Although several in-

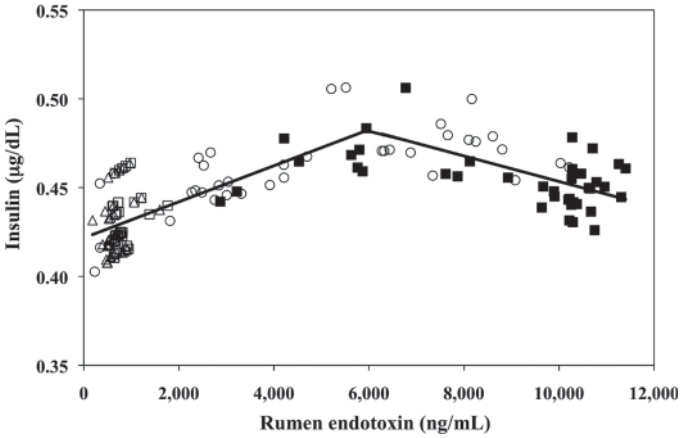

Figure 6. Relationship between concentration of plasma insulin and concentration of endotoxin in the rumen fluid of lactating primiparous Holstein cows fed 15% (Δ), 30% (□), 45% (○), or 60% (■) concentrate in the TMR: insulin = 0.4217 + 0.000010 × endotoxin, if endotoxin ≤5,973 ng/mL; otherwise, insulin = 0.4217 + 0.000010 × 5,973 – 0.00000718 × endotoxin – 5,973 (inflection point of insulin = 0.48 µg/L, root mean square error = 0.014, $R^2 = 0.58$, $P < 0.001$).

vestigators suggested a decreased ketogenesis in the liver as a plausible reason for the lowered plasma BHBA concentration following the administration of LPS (Naylor and Kronfeld, 1986; Steiger et al., 1999; Waldron et al., 2003), this was not the case in the experimental rats as reported by Lanza-Jacoby et al. (1990). The concentration of lactate in the plasma increased with the amount of concentrate in the diet, with cows consuming 60% concentrate having the greatest level of lactate in the plasma. Other investigators reported that switching ruminant animals from low- to high-concentrate diets was associated with greater plasma concentration of lactate (Huntington et al., 1981).

Another finding of this study was the positive, nonlinear correlation between rumen endotoxin and plasma lactate. Because most of the variation (i.e., 93%) in the response of plasma lactate was explained by rumen endotoxin, this suggests a major role for rumen endotoxin and its resulting inflammatory conditions in the increase of plasma lactate in cows fed large amounts of cereal grain. The effect of endotoxin on plasma lactate can be explained by the direct role of proinflammatory cytokines, stimulated by endotoxin, on glycogenolysis and lactate metabolism (Elsasser et al., 2008). This assumption is supported by studies involving experimental endotoxemia. For example, Steiger et al. (1999) demonstrated that heifers challenged with endotoxin had greater concentrations of plasma lactate due to increased glycogenolysis and lowered capacity of extrahepatic tissues to utilize lactate. Furthermore, Caton et al. (2009) reported that endotoxemia contributed to greater plasma lactate concentration through downregulation of mitochondrial phosphoenolpyruvate carboxykinase, a key liver enzyme involved in the process of gluconeogenesis.

Our data regarding greater plasma glucose concentrations in cows fed $\geq$30% concentrate in the diet support other reports demonstrating that feeding diets containing large amounts of fermentable carbohydrates is associated with greater plasma glucose concentrations (van Knegsel et al., 2007). Interestingly, the analysis revealed that, in contrast to the response of plasma glucose, the concentration of insulin in the plasma responded in a typical biphasic manner to the rise of free endotoxin in the rumen fluid. This finding does not indicate a causal relationship between the dramatic rise of rumen endotoxin and insulin response in blood. Further research is warranted to investigate associations among rumen endotoxin, and insulin responses during feeding of high-grain diets.

In conclusion, feeding dairy cows a TMR containing 15, 30, 45, and 60% concentrate, for a period of 21 d, was associated with a large accumulation of endotoxin in the rumen fluid and major alterations in the plasma concentrations of selected metabolites related to carbohydrate and lipid metabolism. Increasing rumen endotoxin concentrations correlated with the decreasing concentrations of cholesterol, BHBA, and NEFA, increasing concentrations of lactate, and the biphasic response of insulin in the plasma. Taken together, our data emphasize the importance of feeding diets balanced in forage-to-concentrate ratio to minimize the risk of accumulation of endotoxin in the rumen and its resulting systemic inflammatory states to prevent perturbations in the metabolic profiles of dairy cows.

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