



J. Dairy Sci. 99:1–14
<http://dx.doi.org/10.3168/jds.2016-11208>
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Metabolic responses, performance, and reticuloruminal pH of early-lactating cows fed concentrates treated with lactic acid, with or without inorganic phosphorus supplementation

A. Khol-Parisini,^{*1} E. Humer,^{*1} H. Harder,^{*} E. Mickdam,^{*†} and Q. Zebeli^{*2}

^{*}Institute of Animal Nutrition and Functional Plant Compounds, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, 1210 Vienna, Austria

[†]Faculty of Veterinary Medicine, South Valley University, 83523 Qena, Egypt

ABSTRACT

Recent data indicate beneficial effects of treating grains with lactic acid (LA) in alleviating the need for inorganic phosphorus supplementation during ruminal fermentation *in vitro*. The aim of this study was to evaluate the effects of feeding concentrates treated with LA with or without inorganic phosphorus supplementation on feed intake, performance, blood variables, and reticuloruminal pH in dairy cows. A total of 16 early-lactating cows (12 Simmental and 4 Brown Swiss) were included in this study from d 1 until d 37 postpartum. Cows were fed 3 total mixed rations differing in supplementation of inorganic phosphorus and treatment of concentrates. The control (CON) and LA (+P) diets included a concentrate mixture containing 0.8% mono-calcium phosphate, and the LA (–P) diet contained no inorganic phosphorus source. The concentrates of the LA (+P) and LA (–P) diets were treated with 5% LA for 24 h before feeding, and the concentrate of the CON diet was not treated. Dry matter intake and milk yield were recorded daily, and milk composition and blood variables were determined on several occasions during the trial. Reticuloruminal pH was measured using indwelling sensors that allowed for continuous measurement during the experimental period. Data showed depressed dry matter intake in cows receiving LA-treated concentrates, but milk yield, body weight, and body weight changes remained similar among treatment groups. Cows receiving the LA-treated diets had lower concentrations of serum nonesterified fatty acids, cholesterol, and insulin, and they tended to have higher serum phosphorus levels. On the other hand, reticuloruminal pH was lower and duration of the pH being <6.0 was longer in cows in the LA-treated groups. Aspartate

aminotransferase, gamma-glutamyltransferase, and concentrations of bilirubin and bile acids were lower in the LA (–P) group. Taken together, the 5% LA-treated diet without inorganic phosphorus supplementation did not exert any negative effects on performance. The observed beneficial effects on blood metabolites related to lipid metabolism, insulin sensitivity, and liver variables, as well as the tendency for greater systemic phosphorus circulation, suggest that diets including concentrates treated with 5% LA may allow for savings of inorganic phosphorus supplementation in dairy cows. Treatment with 5% LA enhanced cows' risk of developing sub-acute rumen acidosis, although this condition showed no adverse effects with respect to liver variables and the inflammatory response.

Key words: concentrate chemical treatment, dairy cow nutrition, phosphorus, blood metabolites

INTRODUCTION

Diets of high-producing dairy cows commonly contain large amounts of concentrates to enhance energy and metabolizable protein intake. Concentrates fed to dairy cows typically consist of cereal grains, oil-seeds, and various industrial by-products. Besides being rich sources of energy and protein, these concentrates also contain large amounts of minerals, especially phosphorus (P). Phosphorus is a key mineral for rumen microbes and the host ruminant animal (Kincaid et al., 2005). In high-producing dairy cows, the proportion of grains, grain by-products, and oil-seeds has increased to a level that dietary phosphorus is provided primarily via concentrates (Humer and Zebeli, 2015). The phosphorus in these concentrates is stored mainly in the form of phytates. Although ruminal microbial phytase is capable of hydrolyzing phytate-bound phosphorus, degradation is not complete, especially during sub-optimal rumen fermentation conditions (Jarrett et al., 2014). Thus, inclusion of high levels of concentrates in the diets of high-producing dairy cows that impair

Received March 23, 2016.

Accepted April 14, 2016.

¹These authors contributed equally to this work.

²Corresponding author: qendrim.zebeli@vetmeduni.ac.at

rumen conditions likely also limit phosphorus availability for rumen fermentation (Harder et al., 2015a) and the host ruminant (Humer and Zebeli, 2015). The phosphorus requirements of cows increase with milk production level. Because of the limited availability of phytate-P, the diets of high-producing cows are typically supplemented with inorganic phosphorus sources, which are highly available and safe for meeting the phosphorus requirements of the milking cow. On the other hand, phosphorus is considered one of the most polluting nutrients from animal production, in that the low availability of phytate-P enhances phosphorus excretion (Kincaid et al., 2005). To minimize the pollution risk associated with high levels of phosphorus excretion, it is of utmost importance to enhance the efficiency of phosphorus use, especially of phytate-P sources (Humer and Zebeli, 2015).

More and more research efforts are aimed at establishing ways to improve the efficiency of phosphorus use in animal production, including feed technological settings and phytase supplementation (Brask-Pedersen et al., 2013; Humer et al., 2015a; Jarrett et al., 2014). Treatment of grains with organic acids, such as citric acid or lactic acid (LA), has been shown to increase phytate-P availability in the rumen *in vitro* (Harder et al., 2015a). The potential of treating grains with LA to enhance degradation of cereal phytate has been suggested in earlier studies (Haraldsson et al., 2004) and was confirmed by more recent data from our team, both *in vitro* (Metzler-Zebeli et al., 2014) and *in vivo* (Khol-Parisini et al., 2015). Interestingly, we have found that phytate degradation increases linearly with the concentration of LA, from 0 to 0.5, 1, and 5% (Metzler-Zebeli et al., 2014). However, so far, no *in vivo* studies have been carried out testing the potential of treating concentrates with LA to avoid inorganic phosphorus supplementation when diets of cows are free from inorganic phosphorus sources.

Earlier studies in sheep and cattle have reported that LA treatment enhances propionate production in the rumen (Morgan and L'Estrange 1976, 1977). Other nutritive effects of LA treatment of grains were enhanced content of slowly degradable starch (Deckardt et al., 2014; Harder et al., 2015b,c) and bypass starch (Khol-Parisini et al., 2015), resulting in increased energy value in the LA-treated grain (Humer et al., 2015b). Modification of the rumen fermentation toward propionate, enhanced content of bypass starch flowing to the duodenum, and increased energy value in the feed suggest that gluconeogenesis and glucose supply are improved, leading to enhanced energy status in the cows, as observed by Iqbal et al. (2010). On the other hand, inclusion of LA in the diet of high-producing cows that

consume more than 10 kg of concentrates daily may increase the proton load in the rumen and jeopardize its acid-base balance and pH regulation (Aschenbach et al., 2011).

The hypothesis of the present study was that steeping concentrates that contain grains and grain by-products in 5% LA enhances the availability of phosphorus, enabling diet formulation without inorganic phosphorus supplementation, and without impairing milk production, ruminal pH, or the metabolic health of early-lactating dairy cows. The main objective was to evaluate the effects of feeding concentrate treated with 5% LA—with or without inorganic phosphorus supplementation—on feed intake, milk production performance, blood metabolites, and reticuloruminal pH dynamics in early-lactating dairy cows.

MATERIALS AND METHODS

Animals, Experimental Design, and Diets

All procedures involving animal handling and treatment were approved by the institutional ethics committee of the University of Veterinary Medicine (Vetmeduni), Vienna, Austria, and the national authority according to §26 of the Law for Animal Experiments, Tierversuchsgesetz 2012-TVG (GZ 68.205/0093-II/3b/2013). The trial was conducted at the dairy research farm “Kremesberg” of the University of Veterinary Medicine. A total of 18 pregnant cows were initially assigned to the experiment, but 2 cows were excluded at the beginning of the trial due to illnesses not related to the experiment, such as acute mastitis. The experiment included 16 cows (4 primiparous and 12 multiparous); 12 were Simmental (initial BW: 798 ± 23.9 kg, mean \pm SD) and 4 were Brown Swiss (initial BW: 642 ± 22.3 kg). Cows were followed from d 1 after parturition and sampled until d 37 postpartum. They were blocked according to calving date, lactation number, and milk yield in the previous lactation, and they were allocated to 1 of 3 different experimental groups: 2 groups of 5 cows and 1 group of 6. As part of routine management practice on the farm, all cows received 500 g of Rindavital (Schaumann, Pinneberg, Germany) dissolved in 20 L water as a rapid energy, electrolyte, and micronutrient supply within 8 h of calving. This supplement contained 72% glucose, 12% sodium chloride, 10% monocalcium phosphate, and 2% sodium hydrogen carbonate; and 4,000 mg vitamin E, 50 mg cobalt, 10 mg selenium, and 19.0×10^9 cfu *Saccharomyces cerevisiae* per kg of DM. After parturition, cows were housed in 1 group in a loose-housing stable with straw bedding and examined by a veterinarian daily. Feeding troughs were equipped

with electronic weighing scales and computer-regulated access gates (Insentec B.V., Marknesse, Netherlands) to control individual feed distribution and the intake of each cow. From d 1 to 9 postpartum, all cows had ad libitum access to the same fiber-rich close-up TMR (Supplementary Table S1; <http://dx.doi.org/10.3168/jds.2016-11208>), and they had limited access to increasing amounts of a TMR for early-lactating cows (Table 1) in separate controlled feeding troughs (Insentec B.V.). The daily allowance of TMR for early-lactating cows was set at 5 kg DM per cow on the first day after parturition and increased daily by 1.5 kg DM per cow

until d 7 and by 1 kg DM per cow from d 7 to 9 DIM. Starting from 10 DIM until the end of the experiment on 37 DIM, cows had ad libitum access to the respective early-lactating TMR only.

The close-up TMR did not differ among treatment groups, but we formulated 3 different TMRs for early-lactating cows that differed with respect to the treatment of the concentrate mixture and inorganic phosphorus supplementation (Table 1). The concentrates of the control (CON) and LA-treated, phosphorus-supplemented [LA (+P)] diets were supplemented with 0.8% monocalcium phosphate and a conventional

Table 1. Ingredients, nutrient composition, and physically effective fiber of the TMR containing untreated concentrates supplemented with inorganic phosphorus (CON), treated with 5% lactic acid (LA), and supplemented with inorganic phosphorus (+P) or treated with 5% LA not supplemented (–P)

Item	CON	LA (+P)	LA (–P)
Ingredient (% of DM)			
Meadow hay	10.0	10.0	10.0
Grass silage	10.0	10.0	10.0
Corn silage	33.0	33.0	33.0
Concentrate with inorganic P, without LA treatment ¹	47.0	—	—
Concentrate with inorganic P, with LA treatment ²	—	47.0	—
Concentrate without inorganic P, with LA treatment ³	—	—	47.0
Nutrient composition (% of DM unless stated)			
DM (% of fresh feed)	43.1	43.1	42.7
Ash	7.11	7.00	6.97
CP	18.5	18.9	19.5
NDF	34.8	33.5	31.6
ADF	22.3	21.7	19.7
Ether extract	2.10	2.16	2.22
NFC ⁴	37.5	38.4	39.8
Calcium (g/kg of DM)	9.63	9.66	9.58
Phosphorus (g/kg of DM)	4.53	4.64	4.13
NE _L (MJ/kg DM)	7.06	7.23	7.17
Particle size distribution (g/kg of DM)			
19 mm	438	406	412
8 mm	116	116	107
1.18 mm	360	391	393
Pan	86	88	88
peNDF _{>8} (g/kg of DM) ⁵	188	174	171
peNDF _{>1.18} (g/kg of DM) ⁵	311	304	301

¹CON concentrate contained 55% rolled barley grain, 25% soybean meal, 9% rapeseed meal, 4% wheat bran, 3% dried beet pulp, 0.45% limestone, 0.25% sodium chloride, 0.8% monocalcium phosphate, 0.5% urea, and 2% mineral vitamin premix (contained 16% calcium, 4% phosphorus, 7% magnesium, 10% sodium, 3,325 mg/kg of manganese, 5,000 mg/kg of zinc, 600 mg/kg of copper, 1,000,000 IU/kg of vitamin A, 100,000 IU/kg of vitamin D₃, 4,000 mg/kg of vitamin E).

²LA (+P) concentrate contained 55% rolled barley grain, 25% soybean meal, 9% rapeseed meal, 4% wheat bran, 3% dried beet pulp, 0.45% limestone, 0.25% sodium chloride, 0.8% monocalcium phosphate, 0.5% urea, and 2% mineral vitamin premix (contained 16% calcium, 4% phosphorus, 7% magnesium, 10% sodium, 3,325 mg/kg of manganese, 5,000 mg/kg of zinc, 600 mg/kg of copper, 1,000,000 IU/kg of vitamin A, 100,000 IU/kg of vitamin D₃, 4,000 mg/kg of vitamin E). This concentrate mixture was soaked in 5% LA (vol/vol) for 24 h before feeding as TMR.

³LA (–P) concentrate contained 56.65% rolled barley grain, 25% soybean meal, 9% rapeseed meal, 4% wheat bran, 2% dried beet pulp, 0.60% limestone, 0.25% sodium chloride, 0.5% urea, and 2% mineral vitamin premix (contained 16% calcium, 4% magnesium, 10% sodium, 4,000 mg/kg of manganese, 6,000 mg/kg of zinc, 1,200 mg/kg of copper, 800,000 IU/kg of vitamin A, 80,000 IU/kg of vitamin D₃, 2,500 mg/kg of vitamin E). This concentrate mixture was soaked in 5% LA (vol/vol) for 24 h before feeding as TMR.

⁴NFC = 100 – (% NDF + % CP + % ether extract + % ash).

⁵peNDF = physically effective neutral detergent fiber inclusive of particles >8 mm (peNDF_{>8}) or >1.18 mm (peNDF_{>1.18}) (Kononoff et al., 2003; Zebeli et al., 2012).

mineral premix that contained inorganic phosphorus sources. The concentrate of the LA-treated, non-supplemented [LA (-P)] diet was not supplemented with any inorganic phosphorus source. The concentrates of the LA (+P) and LA (-P) diets were soaked daily in 700 mL of LA solution per kg of DM, containing 5% LA (656.25 mL of H₂O + 43.75 mL of LA; DL-lactate, 80% wt/wt; Brenntag CEE GmbH, Vienna, Austria) for 24 h before mixing them into the TMR, so that the LA solution was completely absorbed by the concentrate mixture. The soaking procedure was the same as described by Harder et al. (2015a). The TMR were prepared using an automatic system (Trioliet Triomatic T15, Oldenzaal, the Netherlands) and offered twice daily at 0800 and 1630 h, so that 50% of the TMR was fed between 0000 and 1200 h and the remaining 50% was fed between 1200 and 2400 h. Feed residuals, between 5 and 10% of the offered feed, were removed before offering fresh feed in the morning of the next day. Fresh water was available ad libitum for all cows. Diets were formulated according to GfE (2001) guidelines for cows in early lactation.

Feed Sampling, Particle Size Distribution, and Chemical Analyses

We collected samples of the TMR weekly for analysis of nutrient composition. All chemical analyses were done in duplicate according to the *German Handbook of Agricultural Experimental and Analytical Methods* (VDLUFA, 2006). Total DM was determined by oven-drying at 105°C overnight. Samples were analyzed for ash by combustion overnight at 580°C. Both NDF and ADF were analyzed based on Van Soest et al. (1991) (Fibretherm FT12 Fa., C. Gerhardt GmbH & Co. KG, Königswinter, Germany), using heat-stable α -amylase (Termamyl 120 L, 120 KNU-T/g, Novozymes A/S, Bagsvaerd, Denmark) in the NDF procedure and were expressed exclusive of residual ash. Crude protein content was analyzed using the Kjeldahl method (CP = N \times 6.25). Phosphorus content was determined photometrically using the vanado-molybdate method, which measures the color intensity at 436 nm after wet-ashing the lyophilized samples in HNO₃ and H₂O₂ via microwave (MLS-Ethos plus Terminal 320, Leutkirch, Germany). Calcium content was determined by flame atomic absorption spectrophotometry (AA Spectrometer 4100, PerkinElmer, Brunn am Gebirge, Austria). Particle size distribution was determined using a Penn State Particle Separator with 3 sieves (Kononoff et al., 2003). Particles retained on each fraction were oven-dried at 55°C. Physical effectiveness factors (**pef**, or the cumulative proportion of feed DM retained on sieves

of the particle separator) were nominated as $\text{pef}_{>8}$ and $\text{pef}_{>1.18}$, respectively. The physically effective NDF of 2 (**peNDF**_{>8}) and 3 sieves (**peNDF**_{>1.18}) were calculated by multiplying the fraction of $\text{pef}_{>8}$ and $\text{pef}_{>1.18}$ with the NDF content (Kononoff et al., 2003), respectively.

Milk Data

Cows were milked at 0600 and 1530 h in a tandem milking parlor, and the milk yield of each cow was recorded daily. Milk samples were collected at the morning and afternoon milking from d 34 to 37 postpartum and analyzed for fat, protein, lactose, ash, MUN, and SCC using MilkoScan (Foss Electric, Hillerød, Denmark).

Blood Collection and Analyses

Blood samples were taken from each cow from the jugular vein 1 h before the morning feeding on d 11, 18, 25, 36, and 37 postpartum. On d 36 and 37, further samples were collected 2 h (d 36) and 4 and 12 h (d 37) after feeding to determine postprandial blood metabolites. Serum samples were collected using a sterile needle into 10-mL Vacutainer tubes (Vacurette, Greiner Bio-One, Kremsmünster, Austria) and were allowed to clot for 1 h at room temperature. After centrifugation at 3,000 \times g at 18°C for 10 min, serum samples were decanted and stored at -20°C until analysis. Serum insulin levels were measured using a commercially available ELISA kit (Monobind Inc., Lake Forest, CA). Serum concentrations of nonesterified fatty acids (**NEFA**), BHB, glucose, lactate, BUN, total protein, bile acids, bilirubin, cholesterol, and liver enzymes such as aspartate aminotransferase (**AST**), glutamate dehydrogenase (**GLDH**), and gamma-glutamyltransferase (**GGT**), as well as calcium and phosphorus, were measured by a conventional large-scale analyzer for clinical chemistry at the laboratory of the Central Clinical Pathology Unit, University of Veterinary Medicine, Vienna (Humer et al., 2016). We performed standard enzymatic colorimetric analyses with a fully automated autoanalyzer for clinical chemistry (Cobas 6000/c501, Roche Diagnostics GmbH, Vienna, Austria). We determined concentrations of the acute-phase protein serum amyloid A (**SAA**) using a commercially available ELISA bovine kit according to the method described above (Klevenhusen et al., 2013). Serum samples for SAA were initially diluted 1:200, and samples with optical density values above the range of the standard curve were diluted further (1:400 or 1:250) and reanalyzed. All samples were tested in duplicate, and optical density values were read using an iMark microplate absorbance reader (Bio-Rad Laboratories GmbH, Vienna, Austria).

at 450 nm. Intraassay variation was controlled by limiting the coefficient of variation to $\leq 10\%$ for insulin and SAA, and $< 5\%$ for other blood variables.

Reticuloruminal pH and Temperature Monitoring

For continuous monitoring of reticuloruminal pH from d 1 to 37 postpartum, all cows received indwelling wireless ruminal pH sensors (smaXtec Animal Care Sales GmbH, Graz, Austria) that allow measurements every 10 min. These sensors were recently validated in an experiment with rumen-cannulated cows (Klevenhusen et al., 2014), and the procedure of measurement was the same as described earlier (Humer et al., 2015b). The pH data were analyzed as daily mean, maximum, and minimum pH, as well as time (min/d) below specific cutoff points (5.8 and 6.0). Temperature data were analyzed as daily mean temperature and duration during which the temperature was above 39.5°C .

Statistical Analysis

Data were analyzed by ANOVA using the MIXED procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC). For each response variable tested, the model included the fixed effects of diet, time (i.e., day and hour relative to the morning feeding), and the resulting 2-way interaction between time and diet. Breed and lactation number were considered random effects. Data gained from the same cow at different times were considered repeated measures in the model, with first-order autoregressive variance-covariance matrices. Comparisons among treatments were evaluated using Tukey's test. Degrees of freedom were estimated using the Kenward-Roger method. Furthermore, the significance of the overall effect of LA treatment was tested using a linear contrast involving the average of the 2 LA groups versus CON. Moreover, for assessing the effect of inorganic phosphorus supplementation, we performed a linear contrast as well [average of CON and LA (+P) vs. LA (-P)]. Significance was set at $P \leq 0.05$, and a trend was considered for $0.05 < P \leq 0.10$.

RESULTS

DMI, Milk Yield and Composition, BW, and BW Changes

Dry matter intake, expressed as a percentage of the metabolic BW ($\text{BW}^{0.75}$) of the close-up diet from 1 to 9 DIM, did not differ among groups and averaged 2.3% ($P = 0.15$). On the other hand, intake from 1 to 37 DIM of the 3 different TMR for early-lactating

cows was higher in the CON group than in the groups treated with LA ($P < 0.01$; Figure 1).

Overall milk yield did not differ among treatment groups (Table 2). Milk protein and fat content, as well as the ratio of fat to protein, did not differ among treatment groups (Table 2). However, we observed a tendency toward lower milk lactose concentration in group LA (+P) compared with CON ($P = 0.07$). Also, contrast analysis of the overall effects of the LA treatments revealed lower milk lactose concentrations compared with the untreated CON diet ($P = 0.02$). We observed no differences in SCC, MUN, or milk pH among the treatment groups. Both BW and BW changes were similar among treatments (Table 2).

Serum Analyses

Preprandial serum analyses at 4 different measurement days revealed a tendency toward higher glucose concentrations in the LA (-P) diet compared with the LA (+P) diet ($P = 0.10$; Figure 2A). Serum NEFA concentrations tended to be lower in the diets treated with LA ($P = 0.07$, Figure 2B). Cholesterol was also lower in the diets treated with LA ($P < 0.01$, Figure 2C), but the ratio of NEFA to cholesterol remained unchanged (Figure 2F). Serum phosphorus tended to be higher in the LA-treated groups compared with CON ($P = 0.10$, Figure 2D), resulting in a lower calcium-to-phosphorus ratio ($P = 0.05$, Figure 2E). However, we observed no differences in serum BHB, lactate, BUN, total protein, or calcium among all treatment groups (Table 3). We observed a significant day effect for several variables. For instance, glucose concentrations were lower at d 11 than at d 25 and 36. Concentrations of NEFA were higher at d 11 than at d 36, but the opposite was true for total protein. Cholesterol increased linearly with increasing distance to calving, but the opposite was true for the NEFA-to-cholesterol ratio.

Preprandial blood analysis revealed lower values for AST and GGT, and for bilirubin and bile acids in the LA (-P) diet compared with the groups supplemented with phosphorus (Table 3). On the other hand, GLDH and SAA were not affected by dietary treatment. An interaction between dietary treatment and time revealed higher concentrations of AST on d 11 and 18 ($P = 0.05$) in the LA (+P) group compared with the other groups. We observed an overall effect of LA treatment solely for bile acids, showing 40% lower concentrations compared with the CON diet ($P = 0.01$). We observed a significant effect of day for several variables: AST was higher on 11 DIM compared with 18, 25, and 36 DIM; GGT was higher on 36 DIM compared with 11 and 18 DIM; bilirubin was higher on 11 DIM compared

Table 2. Average DMI of the 3 TMR for early-lactating cows, milk yield and composition, and BW in dairy cows fed TMR containing untreated concentrates supplemented with inorganic phosphorus (CON), concentrates treated with 5% lactic acid (LA) and supplemented with inorganic phosphorus (+P), or treated with 5% LA and not supplemented (-P)

Item ¹	Diet			SEM	P-value ²				
	CON	LA (+P)	LA (-P)		Day	Diet	Day × Diet	CON vs. LA	+P vs. -P
DMI (% of BW ^{0.75})	13.8 ^a	12.3 ^b	12.0 ^b	0.77	<0.01	<0.01	0.19	<0.01	0.02
Milk yield (kg/d)	33.4	34.9	32.4	0.59	<0.01	0.17	0.89	0.82	0.13
Milk composition (%)									
Fat	3.88	4.21	3.68	0.169	0.73	0.55	0.13	0.88	0.44
Protein	3.27	3.08	3.14	0.032	0.23	0.31	0.32	0.20	0.85
Lactose	4.79 ^a	4.66 ^b	4.68 ^{ab}	0.016	0.65	0.07	0.58	0.02	0.33
Fat:protein	1.24	1.38	1.18	0.059	0.58	0.57	0.05	0.79	0.48
SCC (cells/mL)	41,769	24,685	33,984	10,417	0.32	0.35	0.26	0.23	0.94
MUN (mg/dL)	38.45	38.43	36.41	0.582	0.03	0.43	0.13	0.45	0.20
Milk pH	6.54	6.52	6.53	0.012	0.88	0.95	0.54	0.75	0.93
BW (kg)	745.3	700.8	701.4	10.61	<0.01	0.35	0.90	0.16	0.41
BW change (kg)	-26.6	-26.4	-23.2	3.79	<0.01	0.98	0.49	0.91	0.83

^{a,b}Different superscripts indicate differences among LSM in the same row at $P < 0.05$ after Tukey correction.

¹DMI, BW, and milk yield were measured daily from d 1 to 37; milk samples for milk composition were measured daily from d 34 to 37; BW change was measured as difference between BW of d 37 and 1 postpartum.

²Effect of measurement day (Day), dietary treatment (Diet), interactive effect of dietary treatment and measurement day (Day × Diet), overall effect of LA (CON vs. LA), overall effect of inorganic phosphorus supplementation (+P vs. -P).

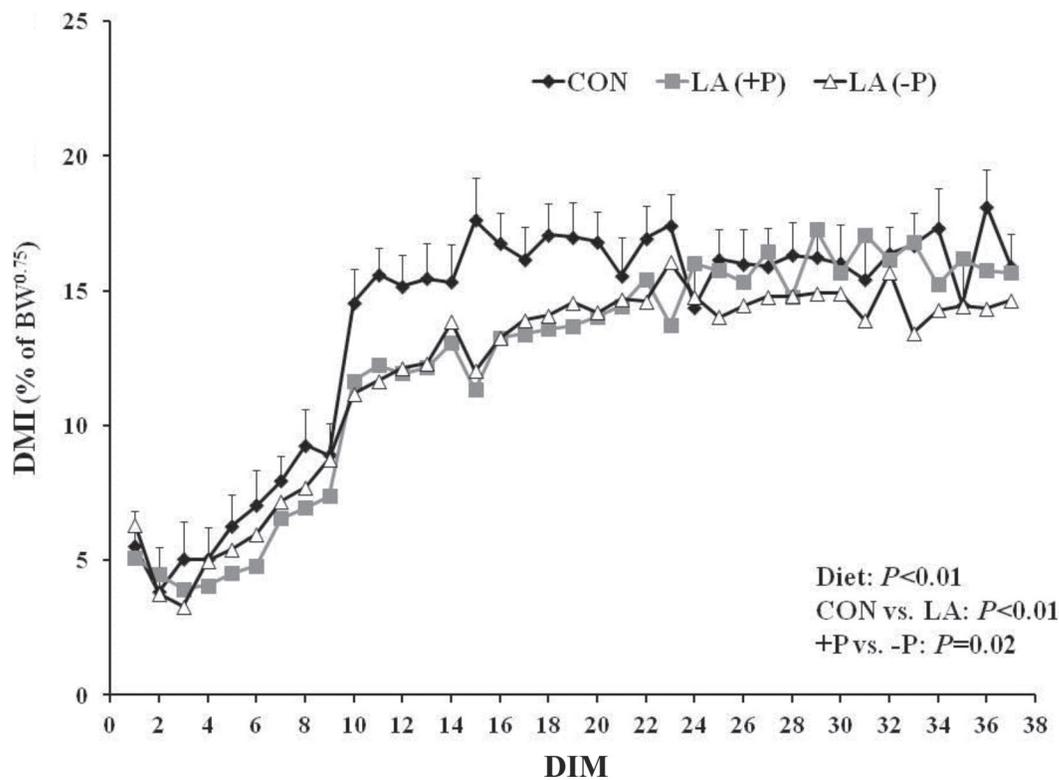


Figure 1. Daily DMI of TMR in relation to metabolic BW in early-lactating cows fed a control TMR containing untreated concentrates supplemented with inorganic phosphorus (CON; $n = 5$), treated with 5% lactic acid (LA) and supplemented with inorganic phosphorus [LA (+P); $n = 5$], or without inorganic phosphorus supplementation [LA (-P); $n = 6$]. Data are presented as LSM \pm SEM.

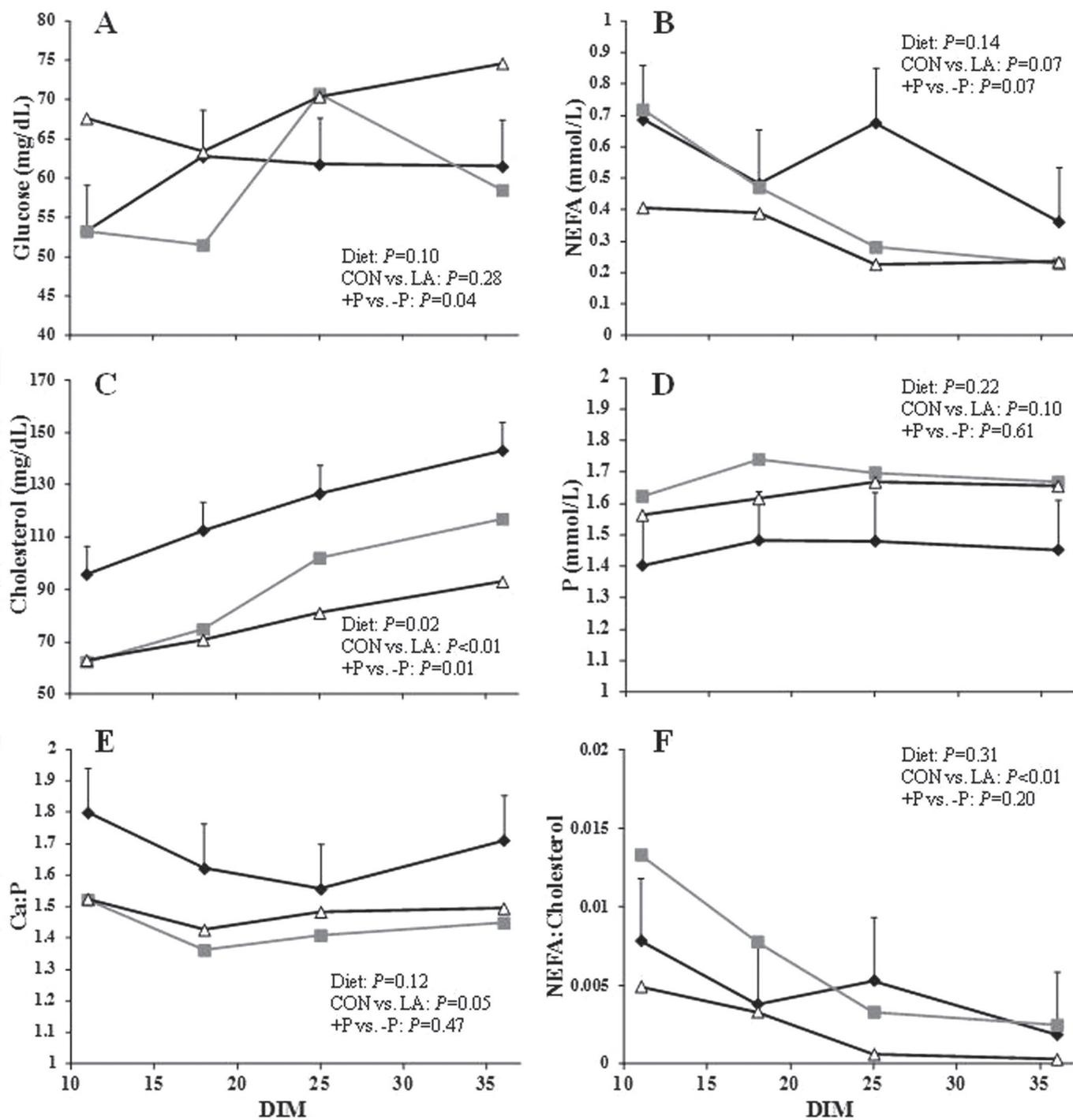


Figure 2. Preprandial serum concentrations of glucose (A), nonesterified fatty acids (NEFA; B), cholesterol (C), phosphorus (D), calcium-to-phosphorus ratio (E), and NEFA to cholesterol ratio (F) at 4 different sampling days in early-lactating cows fed a control TMR containing untreated concentrates supplemented with inorganic phosphorus (CON, diamonds, $n = 5$), treated with 5% lactic acid (LA) and supplemented with inorganic phosphorus [LA (+P), squares, $n = 5$], or without inorganic phosphorus supplementation [LA (-P), triangles, $n = 6$]. Data are presented as LSM \pm SEM.

with 25 and 36 DIM; and SAA decreased linearly with increasing distance to calving.

Analysis of the postprandial kinetics of selected serum variables revealed that serum concentrations of NEFA ($P < 0.01$, Figure 3B) and insulin ($P = 0.03$, Figure 3D) were higher at different time points after feeding in CON cows than in LA-treated cows. Nevertheless, postprandial serum analyses of glucose (Figure 3A), BHB (Figure 3C), lactate, phosphorus, calcium, and calcium-to-phosphorus ratio at different hours relative to the morning feeding were not affected by treatment. We noticed a significant hour effect for BHB, showing lowest concentrations before feeding ($P < 0.01$), but NEFA was highest before the morning feeding compared with 2, 4, and 12 h afterward ($P < 0.01$). Glucose concentrations were higher before the morning feeding than 2 h after the morning feeding ($P = 0.04$).

Reticuloruminal pH and Temperature Dynamics

Mean reticuloruminal pH from 1 to 37 DIM showed an average of 0.3 pH units lower in the LA groups compared with CON [6.12, 5.77, and 5.90 in the CON, LA (+P), and LA (-P) diets, respectively; $P < 0.01$; Figure 4A]. An interaction between dietary treatment and DIM ($P < 0.01$) revealed smaller differences between CON and LA (-P) during the last 7 d of the observation period. Maximum and minimum pH readings were highest in the CON group and lowest in the LA (+P) group ($P < 0.01$, Table 4). Furthermore, the duration of pH < 6.0 was lowest in the CON group [on average

599 min/d compared with 1,149 min/d in LA (+P) and 950 min/d in LA (-P); $P < 0.02$, Figure 4B]. An interaction for dietary treatment and DIM ($P < 0.01$) revealed that the differences between the CON and the LA-treated diets were most pronounced from 19 to 28 DIM. Moreover, from 29 DIM until the end of the observation period, we found no differences between CON and LA (-P). The duration of pH below 5.8 exceeded the SARA threshold of 330 min/d very early after parturition, but we determined no significant differences between the treatment groups ($P = 0.38$, Table 4).

Mean reticuloruminal temperature was interactively affected by dietary treatment and DIM ($P = 0.02$), showing higher temperature readings in the LA (+P) group during the first 2 wk of the measurement period. However, the duration of temperature above 39.5°C did not differ between groups ($P = 0.56$).

DISCUSSION

Feed Intake, Performance, and Blood Variables Related to Energy Status and Lipomobilization

We undertook this study primarily to test the hypothesis that LA treatment of concentrates containing mainly grains and oil-seed by-products would enhance the availability of organically bound phytate-P, enabling diet formulations to exclude inorganic phosphorus supplementation without impairing performance and metabolic health in dairy cows during very early lactation. We based our hypothesis on newly published

Table 3. Data of selected blood variables in dairy cows fed TMR containing untreated concentrates and supplemented with inorganic phosphorus (CON), concentrates treated with 5% lactic acid (LA) and supplemented with inorganic phosphorus (+P), or treated with 5% LA and not supplemented (-P)¹

Item ²	Diet			SEM	<i>P</i> -value ³				
	CON	LA (+P)	LA (-P)		Day	Diet	Day × Diet	CON vs. LA	+P vs. -P
BHB, mmol/L	0.52	0.61	0.51	0.039	0.37	0.70	0.42	0.81	0.63
Lactate, mmol/L	0.69	0.76	0.72	0.026	0.24	0.80	0.24	0.57	0.99
BUN, mg/dL	36.7	34.3	36.9	0.83	<0.01	0.39	0.79	0.56	0.48
Total protein, g/dL	7.27	7.11	7.37	0.071	0.28	0.59	0.98	0.90	0.41
Calcium, mmol/L	2.36	2.38	2.39	0.021	0.22	0.87	0.84	0.61	0.70
AST, U/L	71.38 ^b	90.19 ^a	63.81 ^b	3.364	0.02	<0.01	0.05	0.35	0.01
GLDH, U/L	21.92	14.44	8.41	1.566	0.47	0.29	0.34	0.13	0.17
GGT, U/L	22.26 ^a	23.40 ^a	15.73 ^b	0.567	0.03	<0.01	0.98	0.15	<0.01
Bilirubin, mg/dL	0.109	0.122	0.069	0.011	0.04	0.18	0.60	0.54	0.08
Bile acids, μmol/L	58.64 ^a	42.13 ^{ab}	28.47 ^b	4.221	0.96	0.02	0.56	0.01	0.01
SAA, μg/mL	38.13	28.45	24.84	3.902	0.02	0.69	0.99	0.41	0.52

^{a,b}Different superscripts indicate differences among LSM in the same row at $P < 0.05$ after Tukey correction.

¹Blood samples were taken 1 h before the morning feeding on experimental d 11, 18, 25, 36, and 37.

²AST = aspartate aminotransferase; GLDH = glutamate dehydrogenase; GGT = gamma-glutamyltransferase; SAA = serum amyloid A.

³Effect of measurement day (Day), dietary treatment (Diet), interactive effect of dietary treatment and measurement day (Day × Diet), overall effect of LA (CON vs. LA), overall effect of inorganic phosphorus supplementation (+P vs. -P).

data from our team indicating the potential of 5% LA treatment to fully alleviate the lack of inorganic phosphorus supplementation during ruminal fermentation and microbial activity in vitro (Harder et al., 2015a). The diet without inorganic phosphorus supply in the present study consisted of 4.1 g of phosphorus/kg of DM, meeting the theoretical current phosphorus recommendations for dairy cattle according to the NRC (2001) and GfE (2001), which recommend 3.2 to 3.8 g of phosphorus/kg of DM or 3.4 to 4.2 g of phosphorus/kg of DM, respectively. However, the phosphorus con-

tained in these diets came solely from organic phosphorus sources, thus containing less readily available phosphorus than inorganic phosphorus supplementation.

Feeding of LA-treated concentrates lowered DMI, so it seems that high LA concentrations negatively affected the palatability of the offered TMR. An increase of propionate in the rumen fluid due to LA treatment could also have been the reason for the lowered DMI in our study; propionate has a specific hypophagic effect, affecting satiety and hunger (Martin and Baile, 1972; Oba and Allen, 2003). Research conducted by Morgan

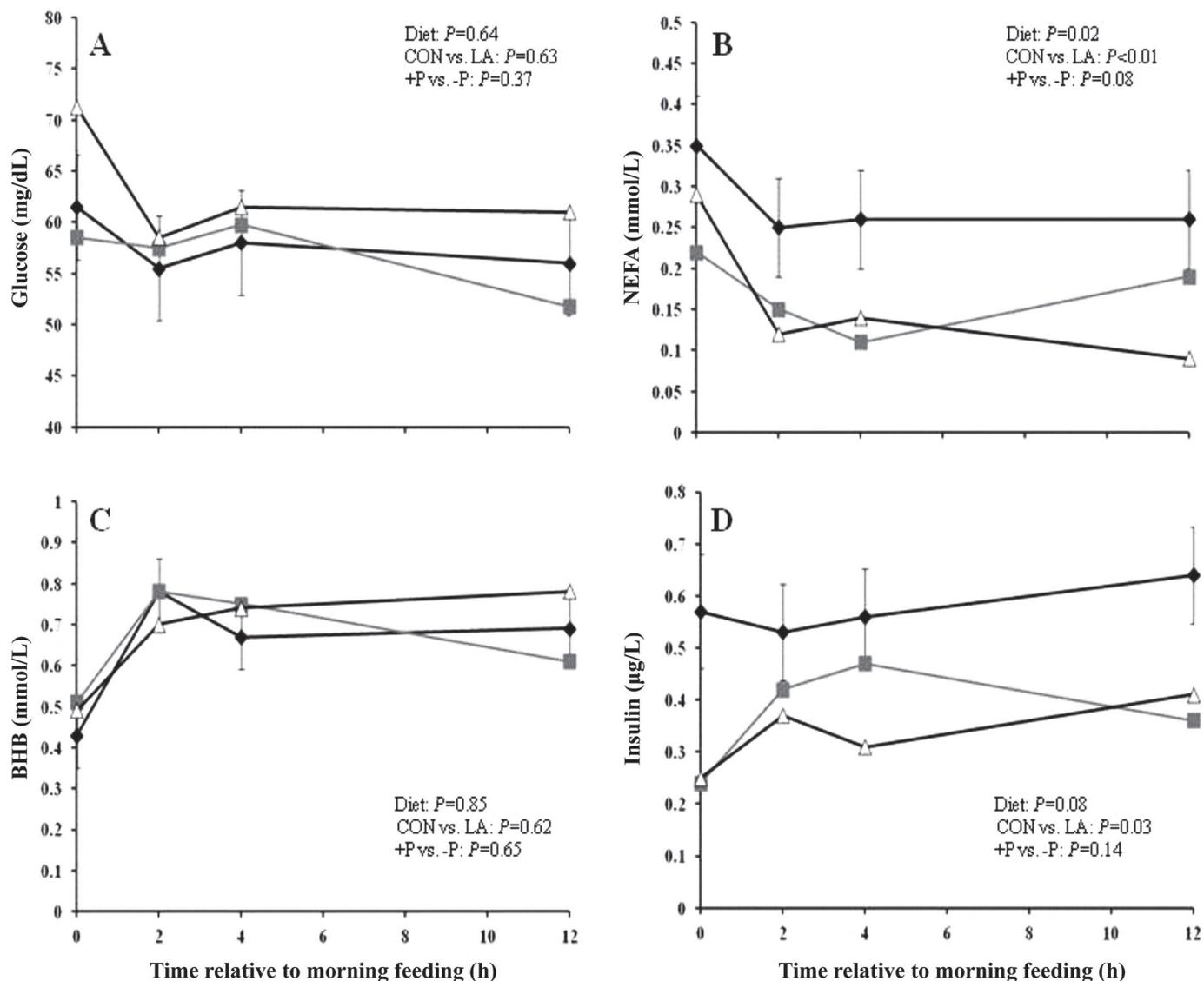


Figure 3. Diurnal variation of serum concentrations of glucose (A), nonesterified fatty acids (NEFA; B), BHB (C), and insulin (D) at 4 different time points relative to the morning feeding in early-lactating cows fed a control TMR containing untreated concentrates supplemented with inorganic phosphorus (CON, diamonds, $n = 5$), treated with 5% lactic acid (LA) and supplemented with inorganic phosphorus [LA (+P), squares, $n = 5$], or without inorganic phosphorus supplementation [LA (-P), triangles, $n = 6$]. Data are presented as LSM \pm SEM.

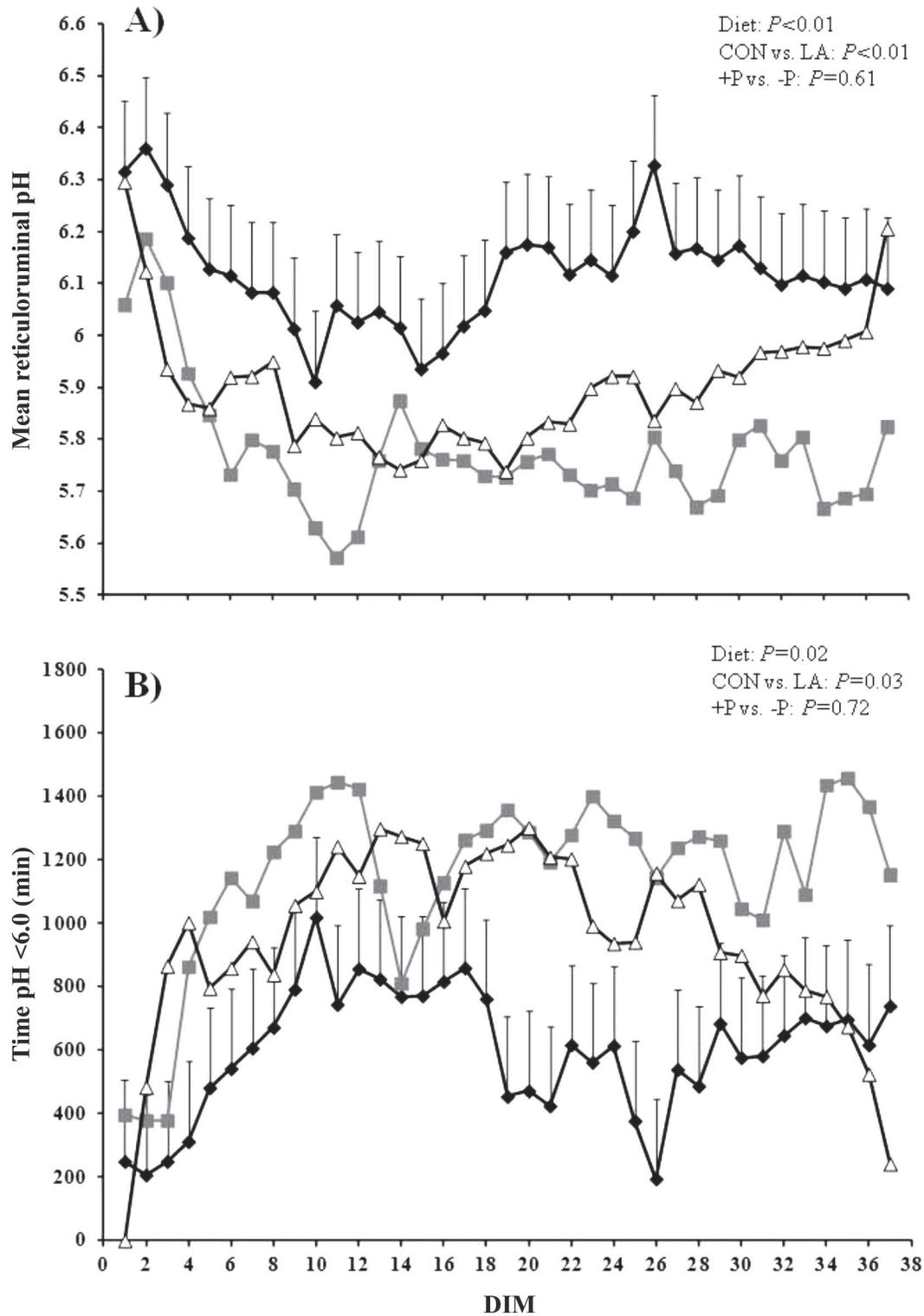


Figure 4. Mean reticularuminal pH (A) and duration of reticularuminal pH < 6.0 (B) in lactating cows fed a control TMR containing untreated concentrates supplemented with inorganic phosphorus (CON, diamonds, $n = 5$), treated with 5% lactic acid (LA) and supplemented with inorganic phosphorus [LA (+P), squares, $n = 5$], or without inorganic phosphorus supplementation [LA (-P), triangles, $n = 6$]. Data are presented as LSM \pm SEM.

and L'Estrange (1976) also observed decreased DMI in cattle that were fed diets treated with 500 and 700 mmol LA per kg of DM, and this effect went along with increased propionate concentration in the rumen. Previous studies in dairy cows using lower concentrations of LA (i.e., 0.5 or 1%), and treating only the barley portion of the concentrate, found no effect on DMI (Iqbal et al., 2009, 2012; Khol-Parisini et al., 2015).

However, despite the differences in DMI, we observed no difference in milk yield, BW, and BW changes among the treatment groups. Studies conducted by Iqbal et al. (2009) and Iqbal et al. (2012) also found no effect of treating barley grain with 0.5 or 1% LA on the milk yield of cows. One explanation for the missing effect of the reduced DMI in the LA groups could be that the addition of 5% LA contributed to greater energy values in the feed. Further important mechanisms that might have compensated for the DMI deficit in milk production and enhanced cows' energy intake were an increase in ruminal propionate production (Harder et al., 2015a; Morgan and L'Estrange, 1976) and an increase in bypass starch (Khol-Parisini et al., 2015) with LA treatment. Propionate serves as the major precursor of glucose via hepatic gluconeogenesis (Reynolds, 2006), and an enhanced proportion of propionate in the rumen fluid is assumed to contribute beneficially to energy supply in high-producing dairy cows during early lactation (Aschenbach et al., 2011). On the other hand, bypass starch escapes degradation in the rumen and is hydrolyzed by host enzymes in the duodenum, increasing the net glucose supply in ruminants (Reynolds, 2006). Increased total NE_L content in barley treated with 1% LA of almost 0.5 MJ/kg of DM has been also confirmed in an in vivo study in wethers by Humer et al. (2015b).

The higher phosphorus and lower calcium-to-phosphorus ratios in the serum of the cows receiving LA-treated concentrates support the hypothesis of improved phosphorus availability in concentrates treated with LA. Previous studies have shown that treatments with LA trigger the hydrolysis of phytate to inorganic phosphorus (Haraldsson et al., 2004) with lower phosphorylated inositolphosphates as intermediates (Metzler-Zebeli et al., 2014; Khol-Parisini et al., 2015), and improve ruminal phosphorus disappearance (Harder et al., 2015a; Khol-Parisini et al., 2015). One explanation might be the pH-lowering effect of LA, providing optimum pH values for endogenous phytase activity (Humer et al., 2015a). However, the exact mechanisms behind the phytate degradation due to LA treatment are largely unknown and require further investigation.

With regard to blood metabolites measured on several occasions starting from d 11, the data showed that cows fed LA-treated diets had lower serum concentrations of NEFA and insulin than cows receiving the CON diet. Because concomitantly elevated blood concentrations of insulin and NEFA during early lactation suggest insulin resistance that aggravates lipolysis (Reaven et al., 1988), these findings suggest improved energy status, improved insulin sensitivity, and lowered lipomobilization in early-lactating cows by feeding concentrates treated with 5% LA.

In the present study, we found no differences in BHB among the dietary treatments despite observed differences in NEFA. This can be explained by the fact that BHB levels originate not only from NEFA oxidation in the liver during high lipid mobilization, but also from alimentary sources of butyrate and its synthesis in the rumen fluid being metabolized in the rumen epithelium and contributing to blood BHB (Penner et al., 2009).

Table 4. Reticuloruminal pH and temperature in lactating cows fed TMR containing untreated concentrates and supplemented with inorganic phosphorus (CON), concentrates treated with 5% lactic acid (LA) and either supplemented with inorganic phosphorus (+P), or treated with 5% LA and not supplemented (−P)¹

Item	Diet				P-value ²				
	CON	LA (+P)	LA (−P)	SEM	Day	Diet	Day × Diet	LA vs. CON	+P vs. −P
Mean pH	6.12 ^a	5.77 ^b	5.90 ^{ab}	0.011	<0.01	<0.01	<0.01	<0.01	0.61
Maximum pH	6.46 ^a	6.16 ^c	6.27 ^b	0.011	<0.01	<0.01	0.14	<0.01	0.49
Minimum pH	5.85 ^a	5.35 ^c	5.55 ^b	0.013	<0.01	<0.01	0.02	<0.01	0.52
Time <5.8, min/d	396	653	504	20.8	0.14	0.38	0.11	0.37	0.93
Mean temperature	39.10	39.27	39.17	0.012	0.08	0.20	0.02	0.26	0.93
Time >39.5°C, min/d	447	566	477	14.30	0.01	0.56	0.09	0.55	0.75

^{a-c}Different superscripts indicate differences among LSM in the same row at $P < 0.05$ after Tukey correction.

¹pH and temperature were measured every 10 min from d 1 until 37 postpartum.

²Effect of DIM (Day), dietary treatment (Diet), interactive effect of DIM and dietary treatment (Day × Diet), overall effect of LA (LA vs. CON), and overall effect of inorganic P supplementation (+P vs. −P).

Indeed, it has been shown previously that LA enhances the proportion of butyric acid (Morgan and L'Estrange, 1976; Iqbal et al., 2012; Metzler-Zebeli et al., 2015) and modulates butyrate-producing bacteria (Harder et al., 2015a; Metzler-Zebeli et al., 2015) in the rumen.

Serum cholesterol concentrations decreased in the LA-treated diets. Because blood cholesterol in ruminants derives mainly from endogenous synthesis, whereby acetate is the main precursor of cholesterol synthesis in the small intestines or liver (Liepa et al., 1978), lowered concentration of acetate in the rumen fluid due to LA treatment (Morgan and L'Estrange, 1976; Harder et al., 2015a; Metzler-Zebeli et al., 2015) might have led to lowered cholesterol synthesis.

Rumen pH, Systemic Inflammation, and Reactants of Liver Health

Diets in the present study contained 47% barley grain-based concentrates, whereby barley is rapidly fermentable in the rumen (Zebeli et al., 2012). Diets also met the general recommendations for dairy cows both in terms of $\text{peNDF}_{>8}$ and $\text{peNDF}_{>1.18}$ (Zebeli et al., 2012). However, the finding of depressed reticuloruminal pH immediately after calving indicates higher peNDF requirements for these cows to maintain average ruminal pH above 6.0, compared with cows that are already in lactation. Stronger depression of reticuloruminal pH in treatment groups indicates that adding 5% LA to each kilogram of concentrate decreases further dietary fiber adequacy and regulation of the acid-base balance of the rumen. Previous studies using lower concentrations of LA (i.e., 0.5 or 1%) and treating only the barley portion of the grain found either no effect on ruminal pH dynamics (Humer et al., 2015b; Khol-Parisini et al., 2015), or a slight improvement (Iqbal et al., 2009, 2012). Thus, it seems that the previously observed positive effects of high amounts of LA on the enhancement of slowly degradable starch (Deckardt et al., 2014; Harder et al., 2015b,c) or bypass starch (Khol-Parisini et al., 2015) were not sufficient to counterbalance the high input of protons in the rumen of a very potent organic acid such as LA at a 5% dosage.

Besides monitoring the effect on reticuloruminal pH dynamics, the present study also focused on liver-associated variables, because it is well known that metabolic stress associated with low ruminal pH during early lactation primarily affects liver function (Bionaz et al., 2007). For instance, dairy cattle affected by diet-induced SARA have exhibited increased concentrations of SAA, an acute phase protein that is rapidly synthesized and released by bovine hepatocytes in response to inflammatory agents released during gastrointestinal dysbiosis in cattle (Plaizier et al., 2008). Our study

showed that the concentrations of SAA were not affected by dietary treatment, but the drop of ruminal pH was already at SARA levels (Zebeli and Metzler-Zebeli, 2012). Thus, although the cows receiving LA-treated diets experienced SARA-like conditions, they did not experience enhanced inflammatory responses. This indicates that SARA-like conditions in LA-treated cows were not strong enough to induce inflammation, an event that has been repeatedly observed in studies with strong diet-induced SARA conditions (Plaizier et al., 2008; Zebeli and Metzler-Zebeli, 2012).

On the other hand, the increased flow of NEFA to the liver affects the metabolizing capacity of the liver postpartum (Bobe et al., 2004), promoting lesions in the liver tissue that are reflected in augmented levels of enzymes such as AST, GGT, and GLDH (Wille et al., 2010; Bossaert et al., 2012). Overall, AST, GGT and GLDH levels in our study corresponded with previous reports (Wille et al., 2010; Bossaert et al., 2012) and were not impaired by LA treatments. Interestingly, the LA (-P) diet showed the lowest concentrations of AST and GGT, likely reflecting less mobilization of fat depots, as supported by lower serum concentrations of NEFA. Furthermore, LA treatment affected the concentrations of bile acids, showing a strong reduction in the respective groups. Because the precursor of bile acids is cholesterol (Staels and Fonseca, 2009), this reduction seems to stem from the lower serum cholesterol concentrations found in the cows receiving LA-treated diets.

CONCLUSIONS

Feeding early-lactating dairy cows concentrates steeped in 5% LA impaired DMI but did not affect milk production or BW. Lowered serum NEFA, insulin, and cholesterol, as well as a tendency for greater serum phosphorus in cows fed the LA-treated diets, indicate that 5% LA treatment of concentrates is advantageous with regard to energy and mineral status in dairy cows fed high levels (i.e., 47% of DM) of dietary concentrate. The results suggest that concentrates treated with 5% LA have the potential to save on inorganic phosphorus supplements without impairing the metabolic status of early-lactating dairy cows. However, the depression of reticuloruminal pH is a concern and needs to be addressed in further studies, although it did not seem to impair liver health and showed no adverse effects in terms of inflammatory response.

ACKNOWLEDGMENTS

We acknowledge the assistance with the trial of T. Kapfenberger, J. Singer, M. Sulzbacher (Stiftsgymna-

sium Admont, Austria), M. Neuper, and M. Roschitz (HBLFA Raumberg-Gumpenstein, Irdning, Austria). We thank A. Dockner, M. Wild, and S. Eisen (Institute of Animal Nutrition and Functional Compounds, University of Veterinary Medicine, Vienna) for their assistance with laboratory analyses, as well as Dr. W. Pohl, E. Draxler, Dr. H. Huber, and the technical staff of the dairy research station “Kremesberg” at Vetmeduni Vienna for their excellent help and care of the cows. We gratefully acknowledge the support for this research from the “Sparkling Science” program (project “Added Value in Nutrition”) of the Austrian Federal Ministry of Science, Research and Economy (BMWFV).

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