Milk metabolites as noninvasive indicators of nutritional status of mid-lactation Holstein and Montbéliarde cows

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Interpretative Summary, *Billa et al., page XX.* The evaluation of nutritional deficits in dairy cows may be performed by measuring the concentrations of plasma metabolites, which require invasive blood sampling. The use of milk as source of noninvasive indicators would facilitate monitoring of animals. Concentrations of minor milk constituents were modified during partial restriction, suggesting their potential use as biomarkers of nutritional status.

RUNNING HEAD: MILK METABOLITES DURING UNDERNUTRITION

Milk metabolites as noninvasive indicators of nutritional status of midlactation Holstein and Montbéliarde cows

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ABSTRACT

The objective was to investigate the effects of feed restriction on concentrations of selected milk metabolites in midlactation Holstein and Montbéliarde cows, and explore their correlations with energy balance and classic plasma and milk indicators of nutritional status. Eight Holstein and 10 Montbéliarde cows (165 ± 21 DIM) underwent 6 d of feed restriction during which feed allowance was reduced to meet 50% of their net energy for lactation ($\mathbf{NE_L}$) requirements. The experiment was divided in four periods: Control (CONT; d -3 to -1), restriction (REST; d 1 to 6), WEEK1 (d 7 to 13) and WEEK2 (d 14 to 18) after refeeding at ad libitum intake. Intake, milk production, energy balance and plasma metabolites were used to validate the feed restriction model. Concentrations of seven milk metabolites, i.e. BHB, glucose, glucose-6-phosphate, isocitrate, glutamate, uric acid and free amino groups were measured in morning milk samples, and fatty acids in pooled PM and AM samples. Feed restriction induced a negative energy balance (-42.5 ± 4.4 MJ/d), increased plasma non-esterified fatty acids and BHB, and decreased plasma glucose concentrations. Feed restriction increased milk glucose-6-phosphate and isocitrate (+38% and +39%, respectively) and decreased milk BHB, glucose, glutamate, uric acid and free amino group concentrations (-20%, -57%, -65%, -42% and -14%, respectively), compared to pre- restriction. Milk concentrations of medium chain fatty acids (e.g. sum of C10 to C15) decreased and those of long chain (e.g. 18:0, cis-9 18:1) increased during restriction. Breed differences were not detected for the majority of variables. All studied milk metabolites were significantly correlated with energy balance (r_s = 0.48, 0.63, -0.31, -0.45, 0.61 for BHB, glucose, glucose-6-phosphate, isocitrate and glutamate, respectively). Milk glucose and glutamate were the most correlated with plasma metabolites and milk FA associated with lipomobilization. These

results suggest that milk metabolites may be used as noninvasive indicators of NEB and metabolic status of dairy cows.

INTRODUCTION

Milk composition is modulated by a diversity of factors, including genetics, lactation stage, nutrition and health status, therefore, milk is an obvious source of biomarkers for the monitoring of dairy ruminants. Early lactation is characterized by a rapid increase in milk yield, mobilization of body protein and fat reserves, negative energy balance (**NEB**), and modifications in milk protein, fat and fatty acid composition. Complex homeorhetic and homeostatic adaptations are required to the direct limiting nutrients towards the mammary gland and support milk synthesis during early lactation (Bell and Bauman, 1997). The occurrence of metabolic disorders related to energy metabolism is typical during this period, and plasma concentrations of non-esterified fatty acids (**NEFA)** and BHB are classic indicators for herd troubleshooting (Oetzel, 2004). Experimental feed restriction models are used to induce nutrient deficits and NEB at different stages of lactation, assess the production and metabolic responses (Gross et al., 2011a; Bjerre-Harpøth et al., 2012; Friggens et al., 2016), and the effects of NEB on various biological functions of the dairy cow (Moyes et al., 2009; Abdelatty et al., 2017; Pires et al., 2019). Metabolic responses to feed restriction models are of greater amplitude during early lactation compared to later stages (Bjerre-Harpøth et al., 2012), but midlactation period is more convenient to run experimental protocols and less prone to confounding due to the dynamic nature of early lactation (Contreras et al., 2016).

The reliance on body reserve mobilization for milk synthesis during early lactation is largely driven by genetics (Friggens et al., 2013). Breed differences between Holstein (**HOLS**) and Montbéliarde (**MONT**) cows have been described in various production systems (Dillon et al., 2003; Pomiès et al., 2007; Pires et al., 2015). Holstein cows prioritize milk, fat,

protein and lactose secretion compared with MONT (Dillon et al., 2003; Pomiès et al., 2007; Pires et al., 2015), but experience greater BCS loss and metabolic deviations during early lactation (Dillon et al., 2003; Pires et al., 2015). Therefore, we hypothesized that breed effects might be used as a model to induce different production and metabolic responses to a nutritional challenge.

Milk is a source of novel indicators of nutritional status of dairy cows. Milk sampling is easy to perform in dairy operations, and can be automated for inline analyses. Certain molecules of intermediary metabolism are present in milk and may be indicators of physiological and nutritional state of dairy cows (Gross and Bruckmaier, 2019). For instance, milk is classically used to monitor ketosis by cow-side tests (Oetzel, 2004) and by automated inline BHB measurements. Milk glucose, glucose-6 phosphate, and acid uric acid concentrations are modulated by diet digestibility and correlated with DMI in midlactation cows (Larsen et al., 2016). Furthermore, milk glucose and glucose-6 phospate concentrations vary according to DIM (Larsen and Moyes, 2015; Zachut et al., 2016; Ferris et al., 2018). Milk concentrations of these metabolites may reflect modifications of metabolic pathways in mammary epithelial cells, including glucose utilization for glycolysis and lactose synthesis (Chaiyabutr et al., 1981,), glucose-6-phosphate and isocitrate to produce reducing potential (i.e.; NADPH) for de novo fatty acid synthesis (Garnsworthy et al., 2006; Chaiyabutr et al., 1981), and to counterbalance oxidative stress associated with FA oxidation (Zachut et al, 2016). Milk uric acid originates in part from ruminal digestion of purine bases and has been suggested as an indicator of microbial protein synthesis (Larsen and Moyes, 2010). Milk glutamate and free amino acid content may reflect the availability and metabolism of amino acids.

The effects of NEB and breed on concentrations of minor milk metabolites are still insufficiently documented. We hypothesized that concentrations of selected metabolites in

99 milk are modified during periods of NEB, and may constitute novel indicators of energy
100 balance and metabolic status of dairy cows. Thus, the objective was to determine the effects
101 of NEB induced by partial feed restriction on milk concentrations of selected metabolites,
102 assess potential differences between midlactation HOLS and MONT cows, and evaluate their
103 relationships with classic indicators of metabolic status and lipomobilization.
104
105 **MATERIALS AND METHODS**
106
107 ***Experimental Design, Animals, Diets and Housing***
108 All procedures were approved by the ethic committee on animal experimentation
109 (APAFIS # 3737-2015043014541577v2). Twenty multiparous midlactation cows (165 ± 21
110 DIM), 10 Holstein-Friesian and 10 Montbéliarde (1.5 ± 0.29 BCS, 0 to 5 scale) were used to
111 study the effects of 6 d of feed-restriction to meet 50% of NE_L requirements on milk
112 production, classic plasma and putative milk biomarkers of metabolic status. Two HOLS cows
113 were excluded from the study, one due to clinical mastitis and one due to noncompliance with
114 the restriction protocol. Phenotypic measurements were performed from d -3 to +18 relative
115 of initiation of restriction, corresponding to the following periods: control (**CONT**; d -3 to -1),
116 restriction (**REST**; d 1 to 6), week 1 (**WEEK1**; d 7 to 13), and week 2 (**WEEK2**; d 14 to 18;
117 Figure 1). Five cows of each breed were randomly allocated to a group of 10 animals that
118 initiated the experimental protocol one day apart. Mammary and liver biopsies were
119 performed on d 0 and d 6 for complementary studies (Billa et al., 2019). The experiment was
120 conducted at the INRA Herbipôle experimental farm of ‘Marcenat’ (45°18'21’N, 2°50'13’E;
121 1100 m of altitude; https://doi.org/10.15454/1.5572318050509348E12) during April and early
122 May.

During CONT, WEEK1 and WEEK2 periods, all cows were allowed ad libitum intake of a TMR (Table 1). During REST period, feed allowance was reduced to meet 50% of individual NE_L requirements calculated from BW, feed intake and milk production and composition recorded before restriction (INRA, 2007). Cows had free access to water and were housed in a free stall barn equipped with automatic feed bunks that control individual access and weight feed intake (CRFI, Biocontrol, Rakkestad, Norway). Gates were programmed to divide individual daily feed allowance in 4 equal portions in 6-h periods. Cows ate 3 ± 0.9 kg of hay (58.9% NDF, 31.9% ADF, 11.5% CP and 5.4 MJ /kg of DM) during the two d after refeeding to provide extra fiber and decrease the risk of ruminal acidosis due to the transition. The ration was analyzed for DM content to calculate individual DMI. Energy balance was estimated according to the INRA system (INRA, 2007), in which NE_L is expressed as "unité fourragère lait" (UFL; 1 UFL = 7.12 MJ), as follows:

NE intake (UFL) = UFL/kg DM × DMI (kg) - E; with E corresponding to the "digestive interaction", calculated as a function of the percentage concentrate in the diet (% Conc; DM-basis) and UFL intake (i.e., UFL/kg DM × DMI (kg)), using the formula $E= (0.00063 \times \%Conc^2) - (0.017 \times UFLintake) + (0.002 \times UFLintake^2)$.

NE production (UFL) = milk yield (kg) × [0.44 + (0.0055 × (- 40 + fat content; g/kg)) + (0.0033*(- 31 + protein content; g/kg))];

NE maintenance (UFL) = $0.041 \times BW^{0.75} \times 1.1$;

Energy balance (UFL) = NE intake - NE maintenance - NE production.

Sampling, Measurements and Chemical Analyses

Milk Sampling and Analysis. Cows were milked twice daily at approximately 6:30 and 16:00. Milk yield was recorded and milk composition was determined by mid-infrared spectroscopy (LIAL, Aurillac, France) in morning and evening milk samples. Weighted milk component means were computed according to PM/AM production and composition.

149 Morning milk samples were collected to determine metabolite concentrations on d -3,
150 -2, -1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 18 relative to initiation of feed restriction
151 (Figure 1), before distribution of fresh TMR, and conserved at -20°C until analyses.
152 Enzymatic-fluorometric methods were used to quantify milk content of BHB (Larsen and
153 Nielsen, 2005), uric acid (Larsen and Moyes, 2010), isocitrate (Larsen, 2014), glucose and
154 glucose-6-phosphate (Larsen, 2015), glutamate and free amino groups (**NH_2**) (Larsen and
155 Fernández, 2017). Morning and evening milk samples were collected on d -2, -1, 1, 2, 3, 4, 5,
156 6 and 13 to determine milk fatty acid (**FA**) composition by gas chromatography, as previously
157 described (Lerch et al., 2012). Briefly, samples were lyophilized and pooled according PM
158 and AM production, to provide daily composite samples for each cow. Samples were
159 methylated and injected into 7890A GC system CN10271102 Series gas chromatograph
160 equipped with a flame ionization detector (Agilent technologies, Santa Clara, California,
161 USA). Peaks were routinely identified through comparison of retention times with FA methyl
162 ester standards. Peak integration was conducted using Chemstation software (Agilent
163 technologies, Santa Clara, California, USA).
164 ***Blood Sampling and Analyses.*** Jugular blood samples were collected on d -3, -2, 1, 2,
165 3, 4, 5, 6, 7, 8, 9, 11, 13 and 18 relative to initiation of feed restriction, after morning milking
166 and before feed distribution. Blood samples were drawn into EDTA (1.95 mg/mL; Terumo
167 Europe NV, Leuven, Belgium) and Li-heparin (135 USP U; Terumo Europe NV, Leuven,
168 Belgium) tubes. Plasma was separated by centrifugation at 1,400×g for 15 min at 4°C and
169 conserved at -20°C until analysis. Plasma (EDTA) glucose, BHB, urea and NEFA
170 concentrations were quantified spectrophotometrically and insulin measured by RIA (Pires et
171 al., 2019). Plasma (heparin) glutamine, glutamate and free amino groups (**NH_2**)
172 concentrations were quantified by enzymatic-fluorometric methods (Larsen and Fernández,
173 2017).

Statistical Analyses

Statistical analyses were performed using SAS enterprise guide (Version 9.4; SAS Institute INC, Cary, NC). Daily data was analyzed as repeated measures by mixed models that included day, breed and their interaction as fixed effects, cow as random effect, and Kenward-Roger adjustment for calculation of degrees of freedom. The Schwarz's Bayesian criterion was used to compare the fitting of different variance-covariance structures, including spatial power, AR (1), ARH (1) and CS. The means for each variable within animal and period were calculated in order to compare periods (CONT, REST, WEEK1 and WEEK2) and explore breed by period interactions. The models included the fixed effects of period, breed and their interaction, and the random effect of cow. Significant time effects (i.e., day or period) and breed by time interactions were explored by the Fisher's protected least significant difference using the PDIFF and SLICE options of the LSMEANS statement. Residuals were checked for normality and homoscedasticity. Heterogeneous variance was tested whenever suggested by residual plots. Least squares means (LSM) and standard error of the mean (SEM) were estimated from untransformed values, whereas *P*-values may reflect statistical analysis of log-transformed data when transformation was necessary. Relationships among variables were explored by Spearman rank correlations. Linear regressions between energy balance and milk metabolite concentrations were analyzed using PROC REG procedure of SAS. The significance level was predefined as $P \leq 0.05$ and trends toward significance at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Production Responses

A significant period effect was observed for DMI, energy balance, milk and milk component yields (Table 2 and Supplemental Figure 1). Per design, energy balance became negative (-42.5 ± 4.4 MJ/d) and milk, fat and protein yields decreased during REST. Energy balance returned to pre-restriction values during WEEK1, and DMI, fat and protein yields during WEEK2. The effect of restriction on milk, fat and protein yields are in accordance with previous studies in midlactation Holstein cows (Gross et al., 2011a; Bjerre-Harpøth et al., 2012; Pires et al., 2016).

A significant breed effect was observed for DMI, milk, protein and lactose yields, which were greater in HOLS than in MONT cows (Table 2). Energy balance and fat yield tended to be greater in HOLS than in MONT. Nonetheless, milk yield differences between HOLS and MONT during CONT (+17% for HOLS) were less marked than reported in previous studies during mid lactation (+21%; Pomiès et al., 2007; Ferlay et al., 2010). Differences among studies could be explained by feeding systems, milking frequency, and the lactation stage.

Plasma Metabolite and Insulin Concentrations

Plasma metabolite and insulin concentrations are presented in Table 3 and Supplemental Figures 2 and 3. Significant time effects were observed for all plasma variables. Plasma NEFA, BHB and glutamate increased whereas plasma glucose, glutamine, urea, NH_2 and insulin concentrations decreased during the REST. Plasma NEFA, BHB, glucose and NH_2 returned to CONT values on WEEK1. Plasma glutamine, urea and NH_2 returned to CONT values on WEEK2. Plasma glutamate was lower at the end of WEEK2 than before the challenge. Plasma glucose concentrations returned to CONT value during WEEK1 and became greater than CONT concentrations on WEEK2. Plasma insulin was significantly greater than CONT concentrations on WEEK1, but was lower than CONT on WEEK2.

The increase in plasma NEFA concentrations during REST reflects lipomobilisation (Chilliard et al., 2000a) and is in accordance with previous feed-restriction studies involving mid- and late lactation cows (Bjerre-Harpøth et al., 2012; Gross and Bruckmaier, 2011a; Pires et al., 2016). Nonetheless, the increase in plasma NEFA observed during REST in our experiment was smaller than observed in underfed early lactation cows (Bjerre-Harpøth et al., 2012; Pires et al., 2019). Plasma BHB concentrations increased during restriction (Table 3 and Supplemental Figure 2), but remained below the 1.2 mM threshold of subclinical ketosis (LeBlanc et al., 2005), which is in agreement with previous research in underfed midlactation cows (Moyes et al., 2009; Gross et al., 2011a; Bjerre-Harpøth et al., 2012). As plasma BHB originates in part from rumen butyrate (Miettinen and Huhtanen, 1996), its concentrations observed during REST probably reflect concomitant modifications of DMI and ruminal butyrate synthesis, and incomplete beta-oxidation of mobilized NEFA. Two cows had increased concentrations of BHB the morning after refeeding the TMR at ad libitum intake (d 7; Supplemental Figure 3), which may result from increased DMI and a shift in ruminal butyrate production. Accordingly, insulin was greatest the day after refeeding at ad libitum intake (d 7, Supplemental Figure 2).

The decrease in plasma glutamine and NH_2 concentrations observed during REST may reflect reduced DMI and amino acid absorption. Glutamine and glutamate are intermediates in many anabolic and catabolic pathways, including regulation of metabolic acidosis, lymphocyte proliferation, and casein synthesis (Lobley et al., 2001). Plasma glutamine, glutamate and total amino acid concentrations decrease during periods of NEB, such as early lactation (Meijer et al., 1995) and during experimental feed restriction in midlactation cows (Girard et al., 2019). Decreased supply of amino acids for intestine absorption may have contributed more to these changes than amino acid catabolism, because plasma urea concentration also decreased during REST, as previously suggested (Girard et al., 2019). The

249 increase in plasma glutamate concentration observed during REST (Table 3 and Supplemental
250 Figure 3) may be due in part to spontaneous glutamine deamination.
251 Holstein cows had greater plasma glucose, glutamate and NH_2, concentrations than
252 MONT (Supplemental Figures 2 and 3). The greater glucose concentrations observed in
253 HOLS compared to MONT contrast with previous research during the first weeks of lactation
254 in low input systems (Pires et al., 2015). These discrepancies may be explained by different
255 lactation stage (early vs. midlactation), diet, and undernutrition model (early lactation
256 spontaneous NEB vs. midlactation feed restriction). The greater BHB concentrations observed
257 in HOLS during CONT (Table 3) probably reflect their greater DMI, ruminal butyrate and
258 BHB synthesis compared with MONT, because all animals were in positive energy balance.
259
260 ***Milk Metabolite Concentrations***
261 Significant time effects were observed for milk concentrations of all metabolites
262 studied (Table 4, Figures 2 and 3). Milk BHB, glucose, glutamate, uric acid and NH_2
263 decreased, whereas glucose-6-phosphate and isocitrate concentrations increased during REST.
264 Milk glutamate, isocitrate and NH_2 concentrations returned to pre-restriction values on
265 WEEK1. Milk BHB, glucose and uric acid returned to pre-restriction concentrations on
266 WEEK2. Milk glutamate was greater whereas glucose-6-phosphate and isocitrate
267 concentrations were lower on WEEK2 compared to CONT.
268 The decrease of milk uric acid concentrations observed during REST is in agreement
269 with results obtained when cows were offered low energy diets (Larsen et al., 2016). Milk
270 BHB concentrations decreased during REST despite a small but significant increase in plasma
271 BHB, which suggests that BHB was preferentially metabolized in the mammary gland. In
272 contrast, BHB concentrations increase both in plasma and milk during early lactation, and
273 both matrices are used to diagnose subclinical ketosis (Nielsen et al., 2003; Oetzel, 2004).

Increased milk glucose-6-phosphate and isocitrate, and decreased milk glucose concentrations during REST are in agreement with previous observations in starved goats (Chaiyabutr et al., 1981) and in early lactation cows (Larsen and Moyes, 2015; Zachut et al., 2016). Glucose-6-phosphate and isocitrate are precursors for NADPH synthesis via the pentose phosphate and isocitrate dehydrogenase pathways, respectively. Increased milk glucose-6-phosphate and isocitrate content during REST may reflect a shift in these pathways in mammary epithelial cells, due to low plasma insulin, decreased de novo FA synthesis and synthesis of other milk components.

Significant breed effects were observed for milk uric acid and isocitrate concentrations when individual data were analyzed by period (Table 4). Nonetheless, only a trend was detected for milk isocitrate concentration when daily data were analyzed as repeated measures, probably because many time points measured after refeeding (WEEK1 and WEEK2) did not differ between HOLS and MONT (Figures 2 and 3). Breed by time interactions were not observed for any of the milk metabolites analyzed in this study.

Milk Fatty Acid Concentrations

Milk FA concentrations are presented in Table 5 and Supplemental Figure 4. Significant time effects were observed for all FA and FA classes, except for total odd and branched chain fatty acids (∑ **OBCFA**). Milk FA concentrations returned to prechalenge values on d 13, except for OBCFA.

Concentrations of FA with 10 to 15 carbons (**∑ 10:0 to 15:0**), 16:0 and ∑ OBCFA with carbon chain shorter than 16 (∑ **OBCFA< C16**) decreased during REST, whereas concentration of 18:0 and *cis*-9 18:1 and of FA with carbon chain greater than 16 (**∑ > C16**) increased. These modifications in milk FA profile are in agreement with other feed restriction studies (Chilliard et al., 2000b; Gross et al., 2011b; Abdelatty et al., 2017). The decrease of Σ

299 10:0 to 15:0 and 16:0 during REST reflect a diminution of de novo FA synthesis in mammary
300 gland, due to a decreased availability of precursors (e.g., acetate and propionate) absorbed
301 from rumen (Chilliard et al., 2000b; Gross et al., 2011b). The increase of 18:0, and *cis*-9 18:1
302 and ∑ > C16 FA reflect body fat mobilization (Chilliard et al., 2000b; Gross et al., 2011b ;
303 Pires et al., 2013).

304 The Σ 10:0 to 15:0 and 16:0 decreased gradually until d 3 of restriction (Supplemental
305 Figure 5), indicating a gradual downregulation of de novo FA synthesis. This downregulation
306 would reduce NADPH requirements for mammary gland lipogenesis (Bell and Bauman,
307 1997), and may explain the gradual increase in glucose-6-phosphate and isocitrate
308 concentrations during REST, which became significant at 48 h of REST (Figure 2). This
309 pattern may reflect concomitant effects of limiting plasma glucose availability and reduced
310 glucose uptake by mammary gland during REST, downregulation of lactose synthesis
311 (Chaiyabutr et al., 1981), and decreased NADPH requirements for de novo fatty acid
312 synthesis (Chaiyabutr et al., 1981; Garnsworthy et al., 2006). Milk glucose-6-phosphate and
313 isocitrate concentrations decreased on d 5 and 6 of REST, before refeeding at ad libitum
314 intake. Epithelial cell homeostatic mechanisms may have reestablished an equilibrium
315 between cytosolic concentrations of glucose 6-phosphate and isocitrate and the activity of
316 metabolic pathways for which they are precursors (e.g., NADPH and lactose synthesis).
317 Zachut et al. (2016) proposed that FA oxidation in mammary cells during periods of
318 lipomobilization would increase the oxidative stress, requiring the upregulation of the pentose
319 phosphate pathway to generate reducing potential to neutralize reactive oxygen species.
320 Mammary gland gene expression shows a shift towards increased reliance on β-oxidation for
321 energy and sparing of glucose in underfed early lactation cows (Pawłowski et al., 2019).
322 Oxidative stress may have occurred in our study, inducing a gradual depletion of glucose-6-

phosphate and isocitrate on d 5 and 6 of restriction. Indicators of oxidative stress were not measured in the current study.

The decrease in ∑ OBCFA < C16 observed during restriction (Table 5 and Supplemental Figure 4) may be explained by reduced ruminal synthesis and incorporation of absorbed FA into milk fat. The increase in milk ∑ OBCFA C>16 content (data not shown) during REST suggests that these FA were mobilized from adipose tissue. The majority of milk OBCFA originates from digested rumen bacteria. Branched chain FA are synthesized by elongation of carbon chains originating from branched chain amino acids (e.g. valine, leucine and isoleucine), and part of the odd chain FA are synthesized by elongation of propionate and valerate. A smaller proportion is synthesized de novo from elongation of proponiate (Vlaeminck et al., 2005, 2015).

A breed effect was observed for milk 18:0 content, which was greater in HOLS than MONT cows. No breed by time interaction was observed for milk FA concentrations.

Correlation and regression analyses

Correlations are presented in Tables 6 and 7 and regressions between energy balance and milk metabolites are presented in Figure 4 and Supplemental Figure 5. All variables presented were significantly correlated with energy balance, except plasma urea concentration and milk total OBCFA content. Among plasma metabolites, NEFA and glucose had the greatest absolute correlation with energy balance (r_s = -0.72 and r_s = 0.64, respectively; Table 6). Among milk metabolites, milk glucose and glutamate had the greatest absolute correlations with energy balance (r_s > 0.60) and plasma NEFA (r_s = -0.67, Table 6), which is a classic indicator of lipomobilization. Moreover, milk glucose was correlated with plasma glucose (r_s = 0.61) which is also modulated by NEB (Meijer et al., 1995; Bjerre-Harpøth et al., 2012; Girard et al., 2019). Milk isocitrate and glucose-6-phosphate concentrations were

348 negatively correlated with energy balance (r_s = -0.45 and -0.31, respectively; Table 6). Milk
349 glucose and milk glutamate concentrations present the best regressions with energy balance
350 (r^2 = 0.49 and 0.46, respectively; P < 0.001; Figure 4). Whereas milk glucose-6-phosphate and
351 milk isocitrate present a weak regression with energy balance (r^2 = 0.10 and 0.26,
352 respectively; P < 0.001). These results suggest that milk glucose and glutamate concentrations
353 may be good indicators of NEB and metabolic status of dairy cows.
354 Table 7 presents the milk FA correlations with selected variables. Milk FA composition
355 is used as a biomarker of lipomobilization in this study, and was measured in pooled samples
356 of PM/AM milkings, therefore it integrates modifications occurring during 24 h periods, as
357 for the calculations of energy balance. Milk ∑ > C16, 18:0 and *cis*-9 18:1 were negatively
358 correlated with energy balance (r_s = -0.54 to -0.56), whereas milk ∑ 10:0 to 15:0, 16:0 and ∑
359 OBCFA < C16 were positively correlated with energy balance (r_s = 0.39 to 0.52). These
360 results were expected due to the known associations among NEB, lipomobilization and milk
361 FA composition (Gross et al., 2011b; Pires et al., 2013). Accordingly, FA potentially
362 synthesized de novo in mammary gland, such as ∑ 10:0 to 15:0 and OBCFA < C16, were
363 positively correlated with plasma glucose, and negatively correlated with plasma NEFA and
364 BHB. Opposite correlations were observed among these plasma metabolites and ∑ > C16,
365 which are FA potentially mobilized from adipose. Moreover, milk glucose, glutamate and
366 isocitrate concentrations were significantly correlated with all reported milk FA and FA
367 classes (r_s > 0.45 in absolute value), except for total OBCFA, further supporting potential of
368 milk glucose, glutamate and isocitrate as biomarkers of NEB and metabolic status of dairy
369 cows.
370
371 **CONCLUSIONS**

The feed restriction model induced NEB and modified plasma metabolites and milk FA concentrations in midlactation cows as expected. The absence of marked breed differences for most variables may be explained by the short duration of the nutritional challenge (6 d) and lactation stage, as phenotypical differences exist between HOLS and MONT during early lactation. Milk concentrations of FA and selected metabolites were modulated by feed restriction in midlactation cows. Milk glucose and glutamate concentrations presented the strongest correlations with classic indicators of metabolic status and the best regressions with energy balance under this experimental model. Milk glucose and glutamate concentrations may constitute good noninvasive indicators of energy balance, lipomobilization and metabolic status. Further research is warranted in early lactation cows, because feed restriction models during established lactation lead to relatively smaller deviations of classic indicators of NEB and metabolic status compared to early lactation.

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550 **Table 1**: Diet ingredient and nutrient composition

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Ingredient (% DM)	Corn silage	66.3
	Barley straw	8.0
	Corn grain	7.6
	Soybean meal	17.4
	Mineral and vitamin mix[1]	0.7
Nutrient composition (DM basis)	Net Energy (MJ/kg DM)	6.9
	PDI (g/kg DM)	106
	CP (% DM)	13.8
	Fat (% DM)	2.4
	Starch (% DM)	23.3
	NDF (% DM)	34.6
	ADF (% DM)	18.7

552 [1]Mineral and vitamin contained 4.5% P, 23% Ca, 4.5% Mg, 1% S, 400,000 IU/kg of vitamin

553 A, 100,000 IU/kg of vitamin D3, 1,600 IU/kg of vitamin E, 400 IU/kg of vitamin B1, 1 g/kg

554 of Cu, 5 g/kg of Zn, 4 g/kg of Mn, 0.1 g/kg of I, 40 mg/kg of Co and 24 mg/kg of Se;

555 Galaphos Midi Repro granule, CCPA, Aurillac, France.

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Table 2. Effects of feed restriction on energy balance, DMI, milk yield, and milk component yield in midlactation Holstein (HOLS) and Montbéliarde (MONT) cows[1].

		Period		SEM		*P*-value			
		CONT	REST	WEEK 1	WEEK 2		Breed	Period	Breed × Period
Energy balance (MJ/d)	HOLS	37[a]	-42[b]	34[a]	34[a]	5	0.09	0.001	0.09
	MONT	23	-43	23	32	4			
DMI (kg/d)	HOLS	25[a]	9[c]	22[b]	24[a]	0.8	0.01	0.001	0.08
	MONT	21	8	19	23	0.8			
Milk yield (kg/d)	HOLS	29[a]	19[d]	24[c]	27[b]	1.2	0.01	0.001	0.25
	MONT	24	16	21	24	1.1			
Fat yield (g/d)	HOLS	1030[a]	770[b]	836[b]	964[a]	43	0.07	0.001	0.48
	MONT	896	695	739	919	38			
Protein yield (g/d)	HOLS	883[a]	565[c]	722[b]	844[a]	38	0.03	0.001	0.75
	MONT	785	494	628	782	36			
Lactose yield (g/d)	HOLS	1466[a]	966[d]	1197[c]	1316[b]	68	0.01	0.001	0.28
	MONT	1217	797	1007	1180	63			
Body weight (kg)	HOLS	680[a]	628[c]	-	661[b]	21	0.35	0.001	0.64
	MONT	661	605	-	626	19			

[1] Intake was limited to meet 50% of NE_L requirements during restriction period (REST, d 1 to 6). Cows were allowed ad libitum intake during control period (CONT, d -3 to -1) and after restriction (WEEK1, d 7 to 13; WEEK2, d 14 to 18).

[a, b, c, d] Concern main period effects pooled across both breeds. Period LSMEANS not sharing a common superscript differ ($P \leq 0.05$).

[y, z] Breed LSMEANS not sharing a common superscript differ within the period ($P \leq 0.05$), presented when Breed × Period effect was significant.

Table 3. Effects of feed restriction on plasma metabolite and insulin concentrations in midlactation Holstein (HOLS) and Montbéliarde (MONT) cows[1].

		Period		SEM	*P* -value				
		CONT	REST	WEEK 1	WEEK 2		Breed	Period	Breed × Period
NEFA (mM)	HOLS	0.11[b]	0.69[a]	0.09[b]	0.08[b]	0.03	0.93	0.001	0.98
	MONT	0.11	0.68	0.09	0.10	0.03			
BHB (mM)	HOLS	0.61[y,bc]	0.64[a]	0.56[ab]	0.51[c]	0.04	0.53	0.003	0.05
	MONT	0.48[z]	0.62	0.59	0.52	0.03			
Glucose (mM)	HOLS	3.84[b]	3.50[c]	4.04[ab]	3.95[a]	0.07	0.08	0.001	0.77
	MONT	3.77	3.32	3.93	3.87	0.06			
Glutamate (mM)	HOLS	0.10[b]	0.11[a]	0.09[c]	0.09[c]	0.003	0.07	0.001	0.17
	MONT	0.09	0.10	0.09	0.09	0.003			
Glutamine (mM)	HOLS	0.16[a]	0.11[c]	0.16[b]	0.15[ab]	0.01	0.61	0.001	0.13
	MONT	0.16	0.10	0.13	0.15	0.01			
Urea (mM)	HOLS	4.61[a]	3.50[b]	3.71[b]	4.63[a]	0.41	0.07	0.001	0.3
	MONT	5.36	4.61	4.10	4.99	0.38			
NH_2 (mM)[2]	HOLS	1.43[a]	1.21[b]	1.47[a]	1.48[a]	0.04	0.01	0.001	0.19
	MONT	1.4	1.09	1.28	1.38	0.04			
Insulin (µU/ml)	HOLS	20.3[b]	8.5[d]	25.2[a]	16.1[c]	1.8	0.13	0.001	0.6
	MONT	15.7	7.2	24.5	13.7	1.6			

[1] Intake was limited to meet 50% of NE_L requirements during restriction period (REST, d 1 to 6). Cows were allowed ad libitum intake during control period (CONT, d -3 to -1) and after restriction (WEEK1, d 7 to 13; WEEK2, d 14 to 18).

[a, b, c, d] Concern main period effects pooled across both breeds. Period LSMEANS not sharing a common superscript differ ($P \leq 0.05$).

[y, z] Breed LSMEANS not sharing a common superscript differ within the period ($P \leq 0.05$) , presented when Breed × Period effect was significant.

[2] Free amino groups (NH_2): Estimation of free amino acid concentration

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Table 4. Effects of feed restriction on milk metabolite concentrations in midlactation Holstein (HOLS) and Montbéliarde (MONT) cows[1].

		Period		SEM		*P*-value			
		CONT	REST	WEEK 1	WEEK 2		Breed	Period	Breed × Period
BHB (µM)	HOLS	54.7[b]	40.3[c]	64.5[a]	46.7[b]	5.2	0.93	0.001	0.55
	MONT	49.1	41.6	62.0	51.8	4.6			
Glucose (mM)	HOLS	0.54[a]	0.22[c]	0.47[b]	0.51[ab]	0.03	0.15	0.001	0.17
	MONT	0.47	0.21	0.45	0.45	0.02			
Glucose-6-phosphate (µM)	HOLS	20.5[b]	32.2[a]	14.0[c]	27.2[a]	8.9	0.24	0.001	0.12
	MONT	35.5	45.9	18.8	48.7	7.9			
Glutamate (mM)	HOLS	0.39[a]	0.14[c]	0.39[a]	0.33[b]	0.06	0.77	0.001	0.99
	MONT	0.38	0.13	0.38	0.31	0.06			
Isocitrate (mM)	HOLS	0.12[c]	0.17[a]	0.13[c]	0.13[b]	0.01	0.05	0.001	0.29
	MONT	0.14	0.19	0.13	0.16	0.01			
Uric acid (mM)	HOLS	0.15[a]	0.09[c]	0.13[b]	0.16[a]	0.01	0.003	0.001	0.54
	MONT	0.12	0.07	0.09	0.12	0.01			
NH_2 (mM)[1]	HOLS	0.81[a]	0.71[b]	0.84[a]	0.83[a]	0.04	0.31	0.001	0.72
	MONT	0.79	0.66	0.79	0.79	0.03			

[1] Intake was limited to meet 50% of NE_L requirements during restriction period (REST, d 1 to 6). Cows were allowed ad libitum intake during control period (CONT, d -3 to -1) and after restriction (WEEK1, d 7 to 13; WEEK2, d 14 to 18).

[a, b, c, d] Concern main period effects pooled across both breeds. Period LSMEANS not sharing a common superscript differ ($P \leq 0.05$).

[2] Free amino groups (NH_2): Estimation of free amino acid concentration (Larsen and Fernández, 2017).

Table 5. Effects of feed restriction on milk fatty acid concentrations (g/100g of fatty acids) in midlactation Holstein (HOLS) and Montbéliarde (MONT) cows[1]. Milk fatty acid composition was analyzed in pooled PM/AM samples.

		Period		SEM	P-value			
		CONT	REST	WEEK 1		Breed	Period	Breed × Period
Σ 10:0 to 15:0[2]	HOLS	23.3[a]	14.9[b]	22.8[a]	1.3	0.38	0.001	0.49
	MONT	23.9	14.5	25.3	1.1			
16:0	HOLS	35.0[b]	26.6[c]	35.2[a]	1.1	0.89	0.001	0.18
	MONT	33.6	25.8	37.8	0.9			
18:0	HOLS	7.2[b]	11.1[a]	8.1[b]	0.6	0.02	0.001	0.25
	MONT	6.9	10.4	5.7	0.5			
cis-9 18:1	HOLS	18.0[b]	29.6[a]	17.5[b]	1.6	0.97	0.001	0.4
	MONT	18.7	31.2	15.1	1.4			
Σ >C16[3]	HOLS	33.0[b]	52.2[a]	33.8[b]	2.2	0.43	0.001	0.3
	MONT	33.2	53.2	28.3	1.9			
Σ OBCFA	HOLS	3.9	3.7	4.0	0.2	0.37	0.68	0.11
	MONT	3.7	3.8	3.6	0.2			
Σ OBCFA <C16[4]	HOLS	2.2[b]	1.8[c]	2.5[a]	0.2	0.64	0.001	0.81
	MONT	2.1	1.8	2.4	0.1			

[1] Intake was limited to meet 50% of NE_L requirements during restriction period (REST, d 1 to 6). Cows were allowed ad libitum intake during control period (CONT, d -3 to -1) and after restriction (WEEK1, d 7 to 13; WEEK2, d 14 to 18).

[a, b, c, d] Concern main period effects pooled across both breeds. Period LSMEANS not sharing a common superscript differ ($P \leq 0.05$).

[2]Sum of FA with between 10 and 15 carbons.

[3]Sum of FA with more than 16 carbons.

[4]Sum of OBCFA with less than 16 carbons.

Table 6. Spearman rank correlations among milk metabolites and classic indicators of metabolic status in midlactation Holstein and Montbéliarde cows[1]. Metabolite concentrations were measured in morning milk and plasma samples. Number in brackets correspond to the number of observations (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; † $P < 0.1$).

	Energy balance		Plasma NEFA		Plasma BHB		Plasma Glucose		Plasma Glutamate		Plasma NH_2[1]		Milk BHB		Milk Glucose		Milk Glucose-6-phosphate		Milk Glutamate		Milk Isocitrate		Milk Uric acid		Milk NH_2[1]							
Energy balance	1.00		(306)	-0.72	***	(231)	-0.17	**	(231)	0.64	***	(231)	-0.34	***	(230)	0.51	***	(231)	0.48	***	(286)	0.63	***	(285)	-0.31	***	(286)	0.61	***	(285)	-0.45	***	(286)	0.30	***	(286)	0.41	***	(284)
Plasma NEFA	1.00		(306)	0.17	**	(231)	-0.53	***	(231)	0.45	***	(230)	-0.56	***	(231)	0.44	***	(286)	0.67	***	(285)	0.36	***	(286)	0.67	***	(285)	0.48	***	(286)	0.44	***	(286)	0.46	***	(284)		
Plasma BHB	1.00		(306)	0.17	**	(231)	-0.33	***	(231)	0.28	***	(230)	-0.21	***	(231)	0.17	**	(286)	0.27	***	(285)	0.09	ns	(286)	0.11	†	(285)	0.31	***	(286)	0.31	***	(286)	0.06	ns	(284)		
Plasma glucose	1.00		(306)	0.17	**	(231)	-0.53	***	(231)	0.31	***	(230)	-0.46	***	(231)	0.35	***	(286)	0.61	***	(285)	0.25	***	(286)	0.56	***	(285)	0.30	***	(286)	0.31	***	(286)	0.46	***	(284)		
Plasma glutamate	1.00		(306)	0.17	**	(231)	-0.53	***	(231)	0.45	***	(230)	-0.17	**	(231)	0.14	*	(286)	0.36	***	(285)	0.19	**	(286)	0.39	***	(285)	0.33	***	(286)	0.22	***	(286)	0.25	***	(284)		
Plasma NH_2[1]	1.00		(306)	0.17	**	(231)	-0.53	***	(231)	0.45	***	(230)	-0.56	***	(231)	0.26	***	(286)	0.50	***	(285)	0.20	**	(286)	0.59	***	(285)	0.34	***	(286)	0.50	***	(286)	0.44	***	(284)		

	(248)	(247)	(246)		(247)		(246)		(247)		(247)		(245)			
Milk BHB		1.00	0.30	***	-0.18	**	0.45	***	-0.08	ns	-0.03	ns	0.42	***		
		(304)	(303)		(304)		(303)		(304)		(304)		(302)			
Milk glucose			1.00		-0.18	**	0.70	***	-0.50	***	0.51	***	0.51	***		
			(303)		(303)		(303)		(303)		(303)		(302)			
Milk glucose-6-phosphate					1.00		-0.28	***	0.34	***	0.05	ns	-0.29	***		
					(304)		(303)		(304)		(304)		(302)			
Milk glutamate							1.00		-0.36	***	0.38	***	0.72	***		
							(303)		(303)		(303)		(302)			
Milk isocitrate									1.00		-0.17	**	-0.26	***		
									(304)		(304)		(302)			
Milk uric acid											1.00		0.28	***		
											(304)		(302)			
Milk NH_2[2]													1.00			
													(302)			

[1] Intake was limited to meet 50% of NE_L requirements during restriction period (REST, d 1 to 6). Cows were allowed ad libitum intake during control period (CONT, d -3 to -1) and after restriction (WEEK1, d 7 to 13; WEEK2, d 14 to 18).

[2] Free amino groups (NH_2): Estimation of free amino acid concentrations (Larsen and Fernández, 2017).

Table 7. Spearman rank correlations among milk fatty acids and milk metabolites and classic indicators of metabolic status in midlactation Holstein and Montbéliarde cows[1]. Milk fatty acid composition was analyzed in pooled PM/AM milk samples, and metabolite concentrations were measured in morning milk and plasma samples. Number in brackets corresponds to the number of observations. (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; † $P < 0.1$).

	Σ 10:0 to 15:0[2]	16:0	18:0	*Cis*-9 18:1	Σ >C16[3]	Σ OBCFA <C16[4]	Σ OBCFA									
Energy balance					***	***	-0.56 (162)	***	-0.54 (162)	***	-0.55 (162)	***	0.39 (162)	***	-0.09 (162)	ns
Plasma NEFA					***	***	0.61 (143)	***	0.68 (143)	***	0.69 (143)	***	-0.58 (143)	***	-0.09 (143)	ns
Plasma BHB					***	***	0.37 (143)	***	0.43 (143)	***	0.44 (143)	***	-0.48 (143)	***	-0.27 (143)	**
Plasma glucose					***	***	-0.36 (143)	***	-0.45 (143)	***	-0.44 (143)	***	0.28 (143)	***	-0.07 (143)	ns
Plasma glutamate					***	***	0.52 (143)	***	0.52 (143)	***	0.55 (143)	***	-0.55 (143)	***	-0.13 (143)	ns
Plasma NH_2[5]					***	***	-0.41 (143)	***	-0.55 (143)	***	-0.52 (143)	***	0.51 (143)	***	0.18 (143)	*
Milk BHB					***	***	-0.23 (161)	**	-0.38 (161)	***	-0.35 (161)	***	0.15 (161)	ns	-0.23 (161)	**
Milk glucose					***	***	-0.57 (160)	***	-0.66 (160)	***	-0.66 (160)	***	0.45 (160)	***	-0.06 (160)	ns
Milk glucose-6-phosphate					ns	ns	0.25 (161)	**	0.09 (161)	ns	0.15 (161)	†	-0.05 (161)	ns	0.13 (161)	ns
Milk glutamate					***	***	-0.68 (160)	***	-0.70 (160)	***	-0.71 (160)	***	0.57 (160)	***	0.03 (160)	ns
Milk isocitrate					***	***	0.61 (161)	***	0.56 (161)	***	0.61 (161)	***	-0.47 (161)	***	0.04 (161)	ns
Milk uric acid					***	***	-0.28 (161)	***	-0.48 (161)	***	-0.43 (161)	***	0.53 (161)	***	0.40 (161)	***
Milk NH_2[5]					***	***	-0.43 (160)	***	-0.53 (160)	***	-0.52 (160)	***	0.31 (160)	***	-0.16 (160)	*
Σ 10:0 to 15:0[2]						***	-0.85 (162)	***	-0.95 (162)	***	-0.96 (162)	***	0.89 (162)	***	0.28 (162)	***
16:0							-0.78 (162)	***	-0.91 (162)	***	-0.92 (162)	***	0.70 (162)	***	0.04 (162)	ns
18:0							1.00		0.78	***	0.86	***	-0.74	***	-0.10	ns

	(162)		(162)		(162)		(162)		(162)	
***Cis*-9 18:1**			1.00		0.97	***	-0.85	***	-0.23	**
			(162)		(162)		(162)		(162)	
Σ >C16[3]					1.00		-0.83	***	-0.16	*
					(162)		(162)		(162)	
Σ OBCFA <C16[4]							1.00		0.61	***
							(162)		(162)	
Σ OBCFA									1.00	
									(162)	

[1] Intake was limited to meet 50% of NE_L requirements during restriction period (REST, d 1 to 6). Cows were allowed ad libitum intake during control period (CONT, d -3 to -1) and after restriction (WEEK1, d 7 to 13; WEEK2, d 14 to 18).
[2]Sum of FA with between 10 and 15 carbons.
[3]Sum of FA with more than 16 carbons.
[4]Sum of OBCFA with less than 16 carbons.
[5] Free amino groups (NH_2): Estimation of amino acid concentration (Larsen and Fernández, 2017).

Figure 1: Sampling timeline. Control period (CONT; d -3 to -1); restriction period (REST; d 1 to 6, thick line), when feed intake was restricted to meet 50% of NE_L requirements calculated during the CONT period; week 1 after refeeding (WEEK1; d 7 to 13); week 2 after refeeding (WEEK2: d 14 to 18). ◊ Milk yield and composition each milking. ∞ Milk sampling for milk metabolite analyses (d 1 corresponds to samples collected at 24 h of feed restriction). ○ Blood sampling for plasma metabolite and insulin analyses (d 1 corresponds to samples collected at 24 h of feed restriction). Δ Milk sampling for milk fatty acid composition analyses (pooled PM and AM samples).

Figure 2. Effects of feed restriction on milk concentrations of (A) glucose, (B) glucose-6-phosphate, (C) isocitrate and (D) *β*-hydroxybutyrate (BHB) in midlactation Holstein (HOLS; ●, solid lines) and Montbéliarde (MONT; ▲, dashed lines) cows. Intake was limited to meet 50% of NE_L requirements during restriction period (d 1 to 6). Cows were allowed ad libitum intake during control period (d -3 to -1) and after restriction (d 7 to 18). Values are LSM ± SEM.

Figure 3. Effects of feed restriction on milk concentrations of (A) free amino group (NH_2), (B) glutamate and (C) uric acid in midlactation Holstein (HOLS; ●, solid lines) and Montbéliarde (MONT; ▲, dashed lines) cows. Intake was limited to meet 50% of NE_L requirements during restriction period (d 1 to 6). Cows were allowed ad libitum intake during control period (d -3 to -1) and after restriction (d 7 to 18).Values are LSM ± SEM.

Figure 4. Relationship between energy balance and (A) milk glucose ($y = 171.0x – 62.1$, $r^2 = 0.49$), (B) natural log of milk glutamate ($y = 42.7x + 62.7$, $r^2 = 0.46$), (C) natural log of milk glucose-6-phosphate ($y = -9.5x – 37.8$, $r^2 = 0.10$) and (D) milk isocitrate ($y = -458.4 + 70.7$, $r^2 = 0.26$) in midlactation Holstein (HOLS; ●) and Montbéliarde (MONT; ▲) cows ($P < 0.001$). Intake was limited to meet 50% of NE_L requirements during restriction period (d 1 to 6). Cows were allowed ad libitum intake during control period (d -3 to -1) and after restriction (d 7 to 18).

660 Billa et al. Figure 1

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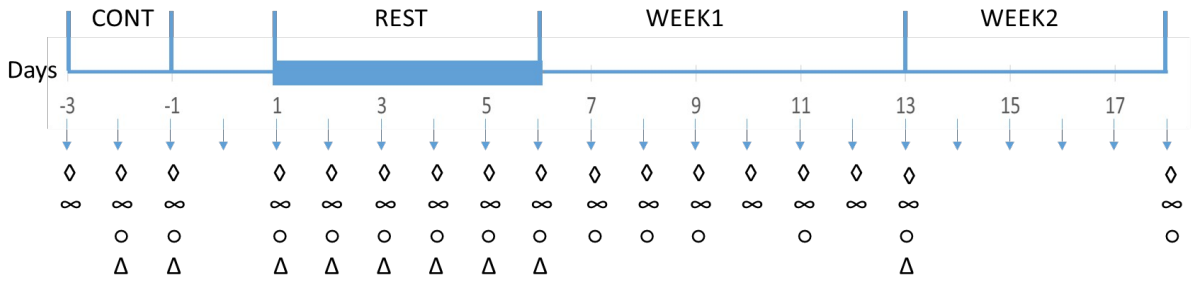

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663 Billa et al. Figure 2

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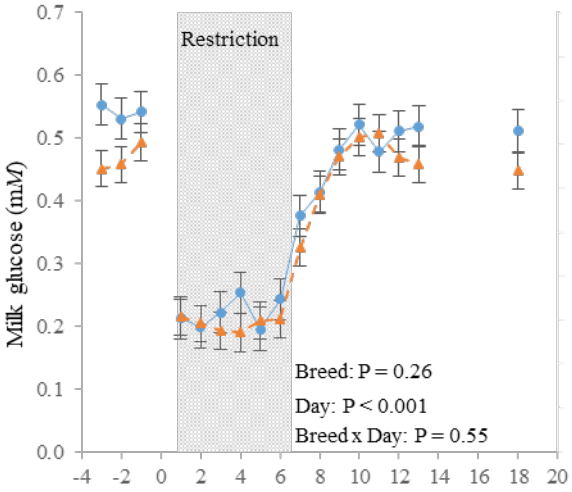
665 **A**



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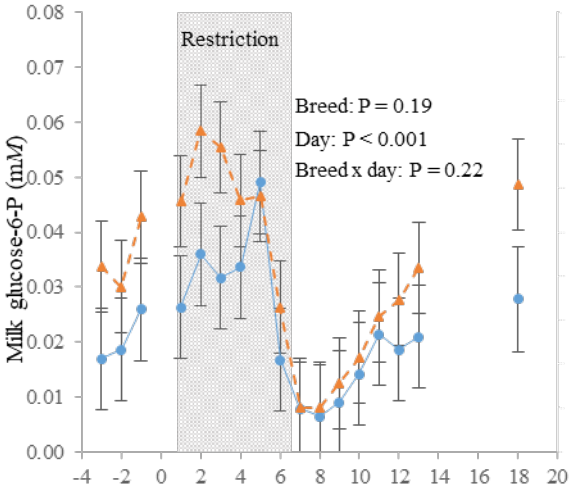

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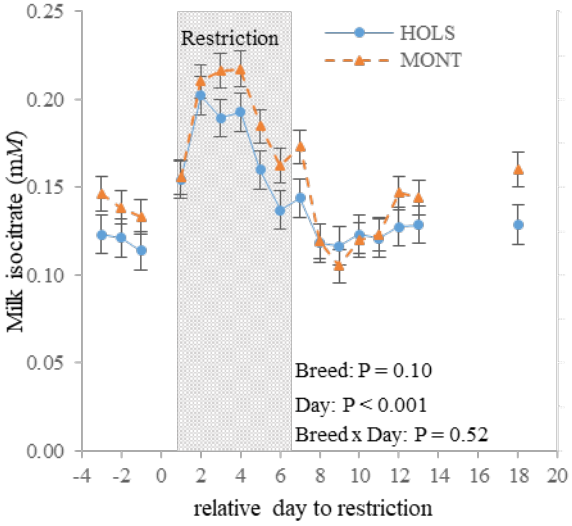
667 **C**



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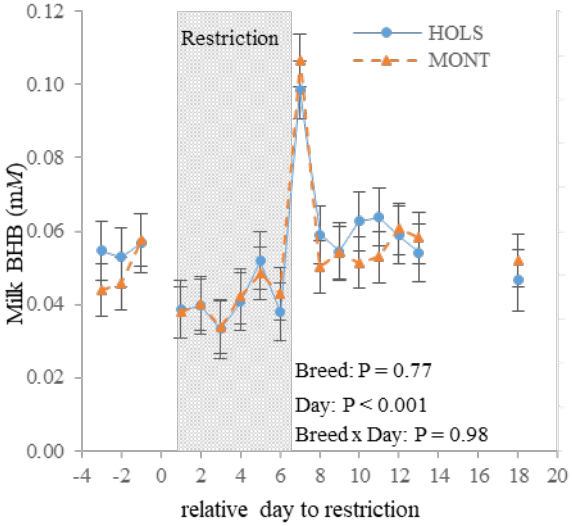

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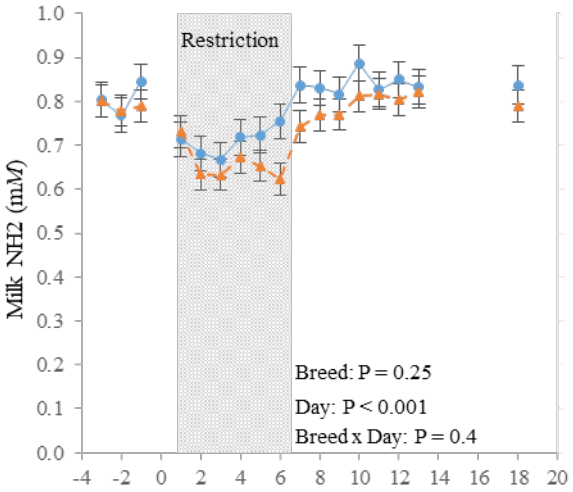
671 Billa et al. Figure 3

672 **A**



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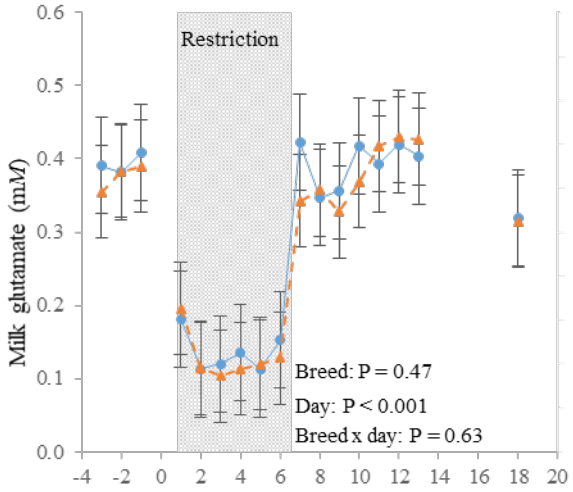
674 **B**



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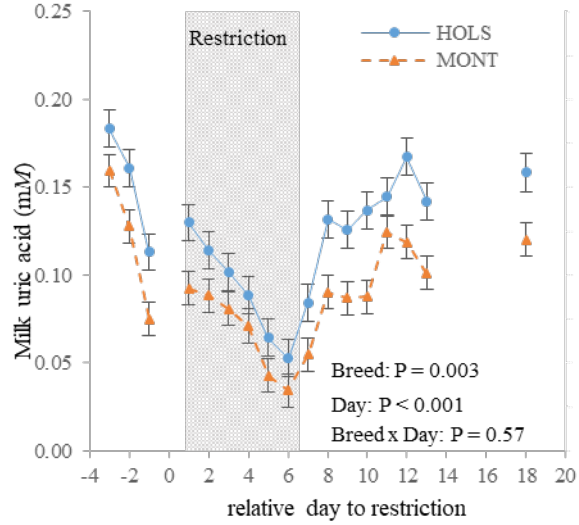
676 **C**



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678 Billa et al. Figure 4
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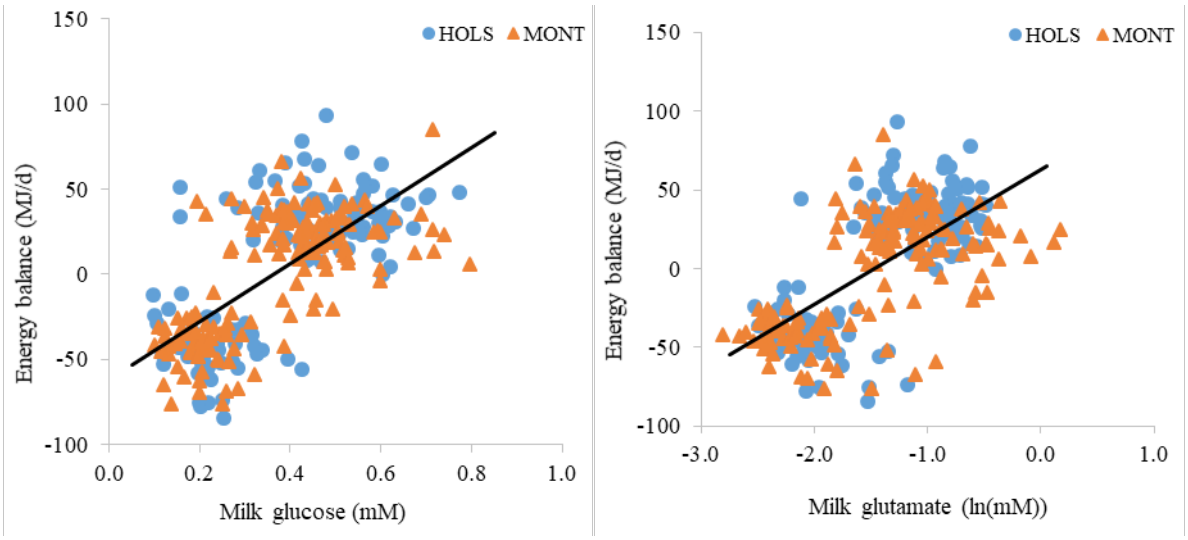
680 A B



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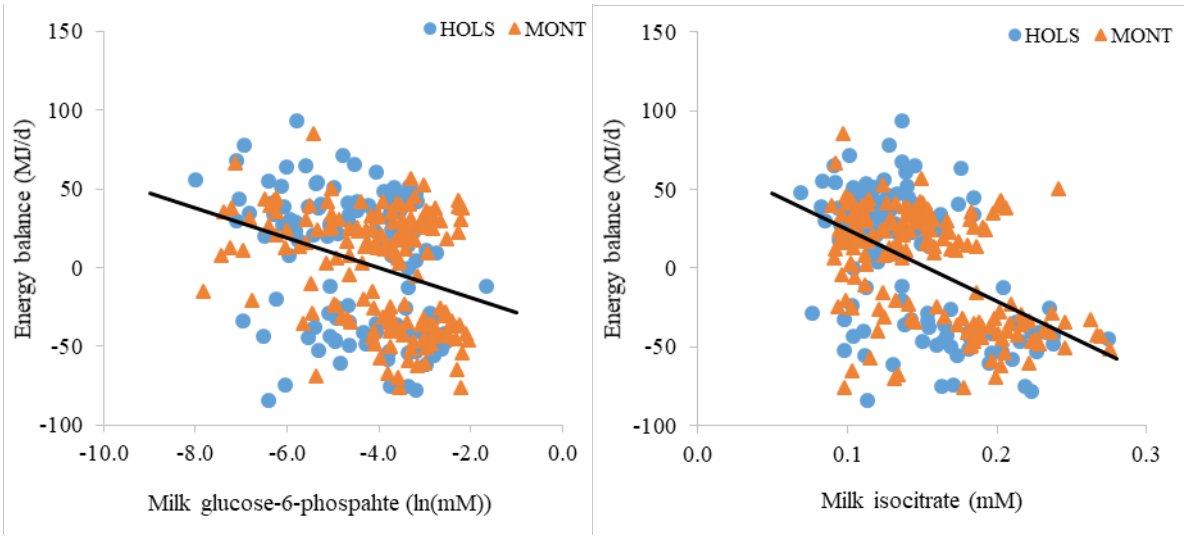
682 C D



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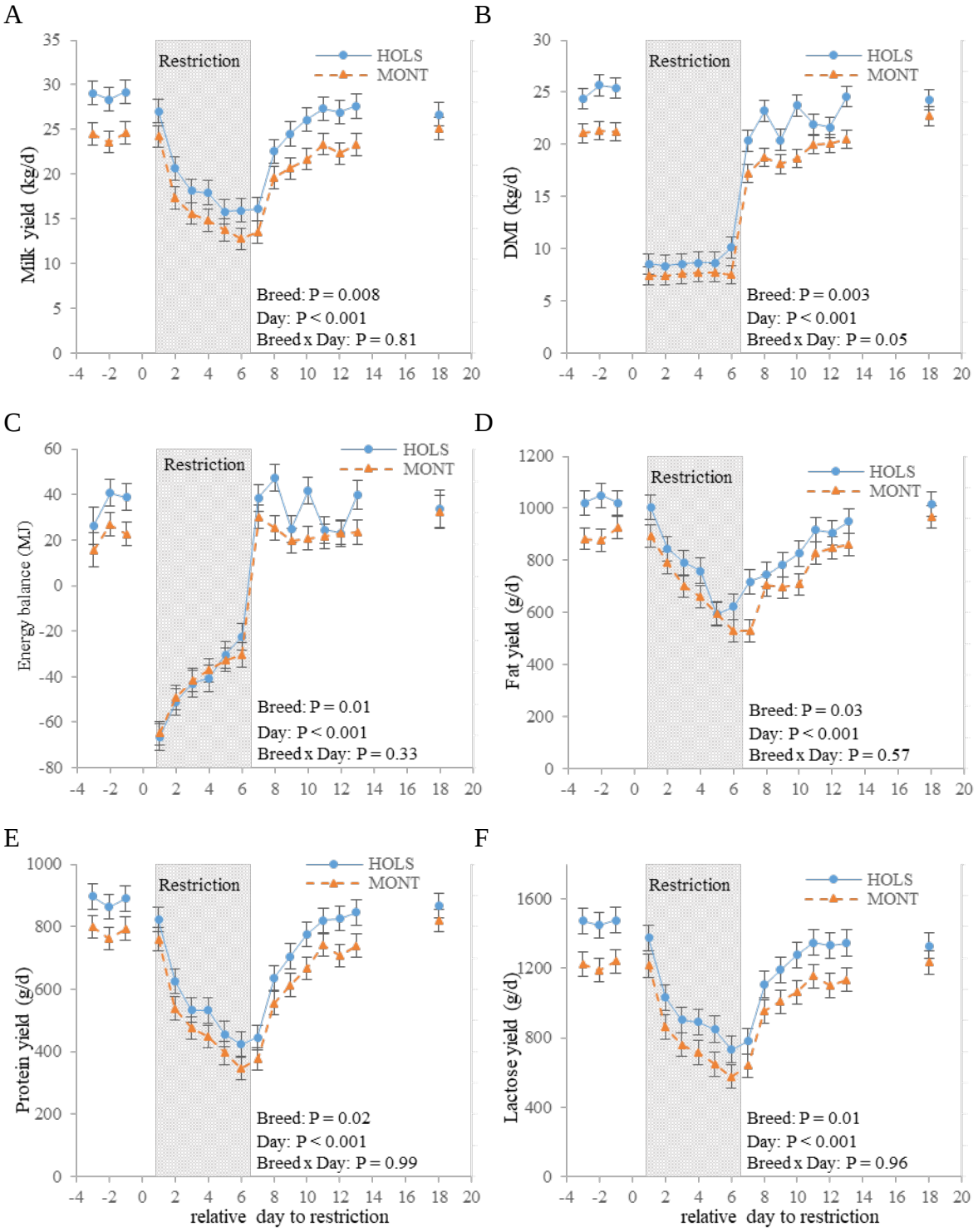
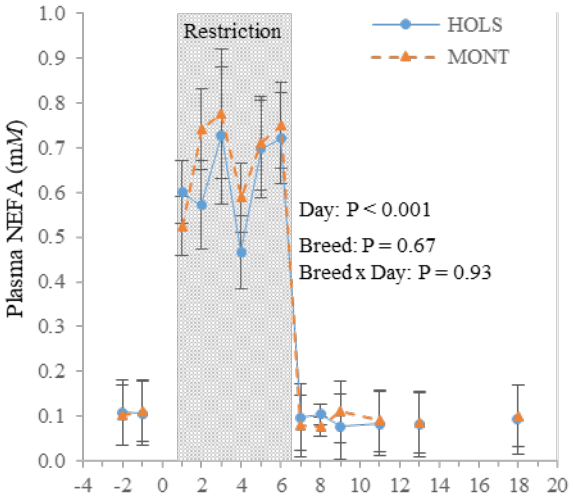
Supplemental Figure 1. Effects of feed restriction on milk yield (A), DMI (B), energy balance (C), fat (D), protein (E) and lactose (F) yield in midlactation Holstein (HOLS; ●, solid lines) and Montbéliarde (MONT; ▲, dashed lines) cows. Intake was limited to meet 50% of NE_L requirements during restriction period (d 1 to 6). Cows were allowed *ad libitum* intake during control period (d -3 to -1) and after restriction (d 7 to 18).Values are LSM ± SEM.



Supplemental Figure 2. Effects of feed restriction on plasma concentrations of non-esterified fatty acid (NEFA) (A), BHB (B), glucose (C) and insulin (D) in midlactation Holstein (HOLS; ●, solid lines) and Montbéliarde (MONT; ▲, dashed lines) cows. Intake was limited to meet 50% of NE_L requirements during restriction period (d 1 to 6). Cows were allowed *ad libitum* intake during control period (d -3 to -1) and after restriction (d 7 to 18).Values are LSM ± SEM.

A



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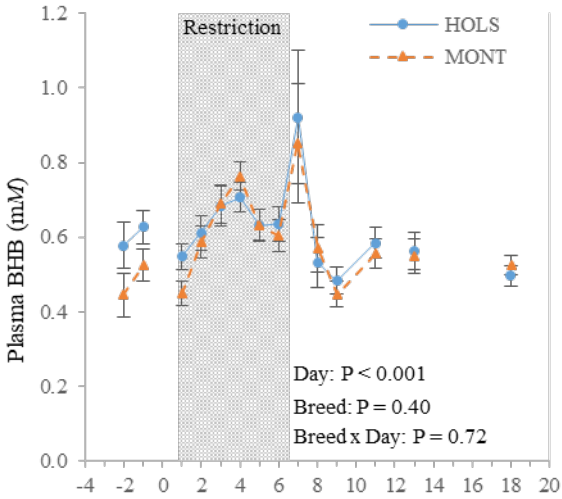
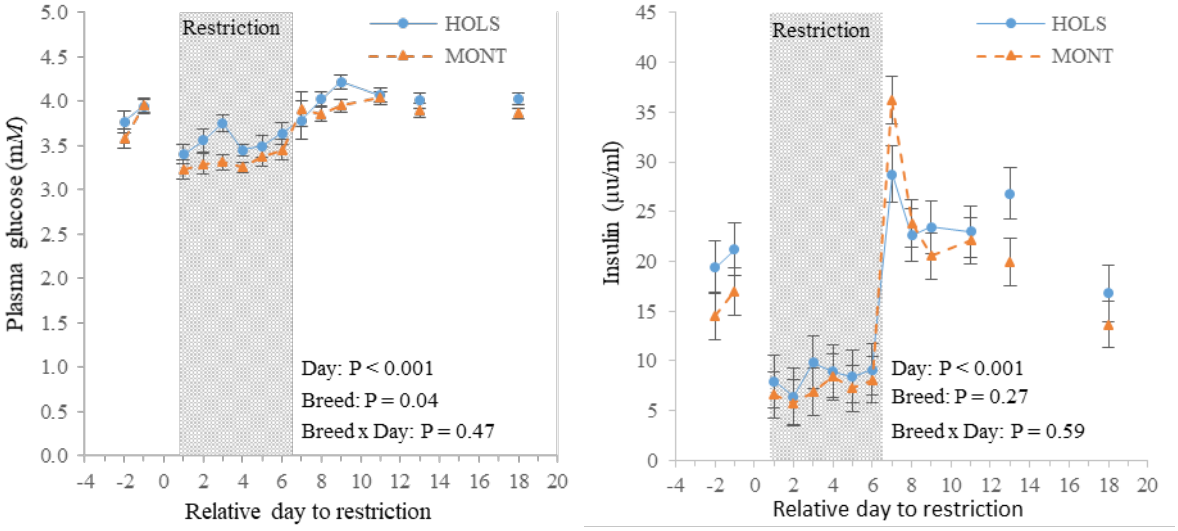


708 C D



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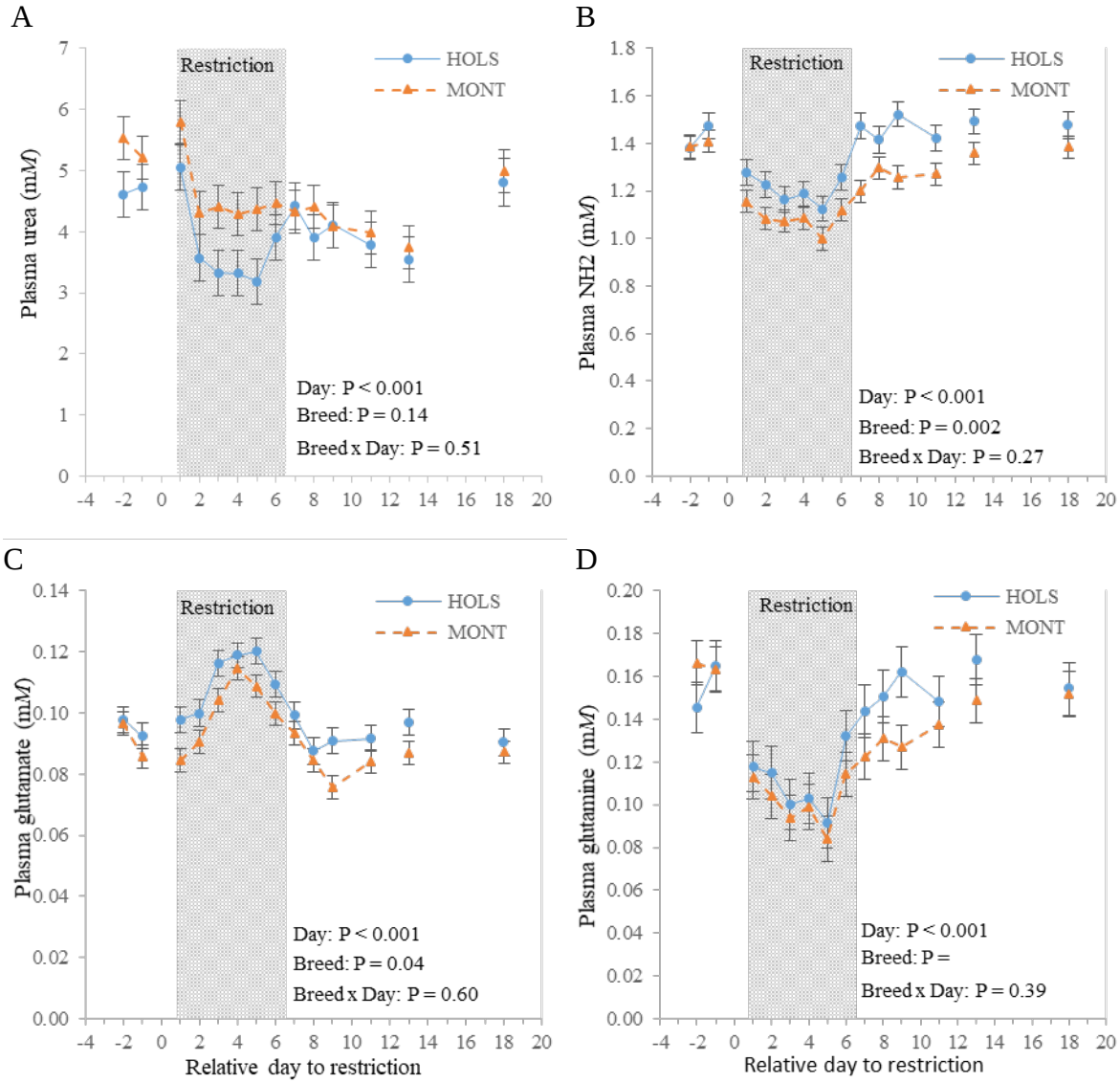
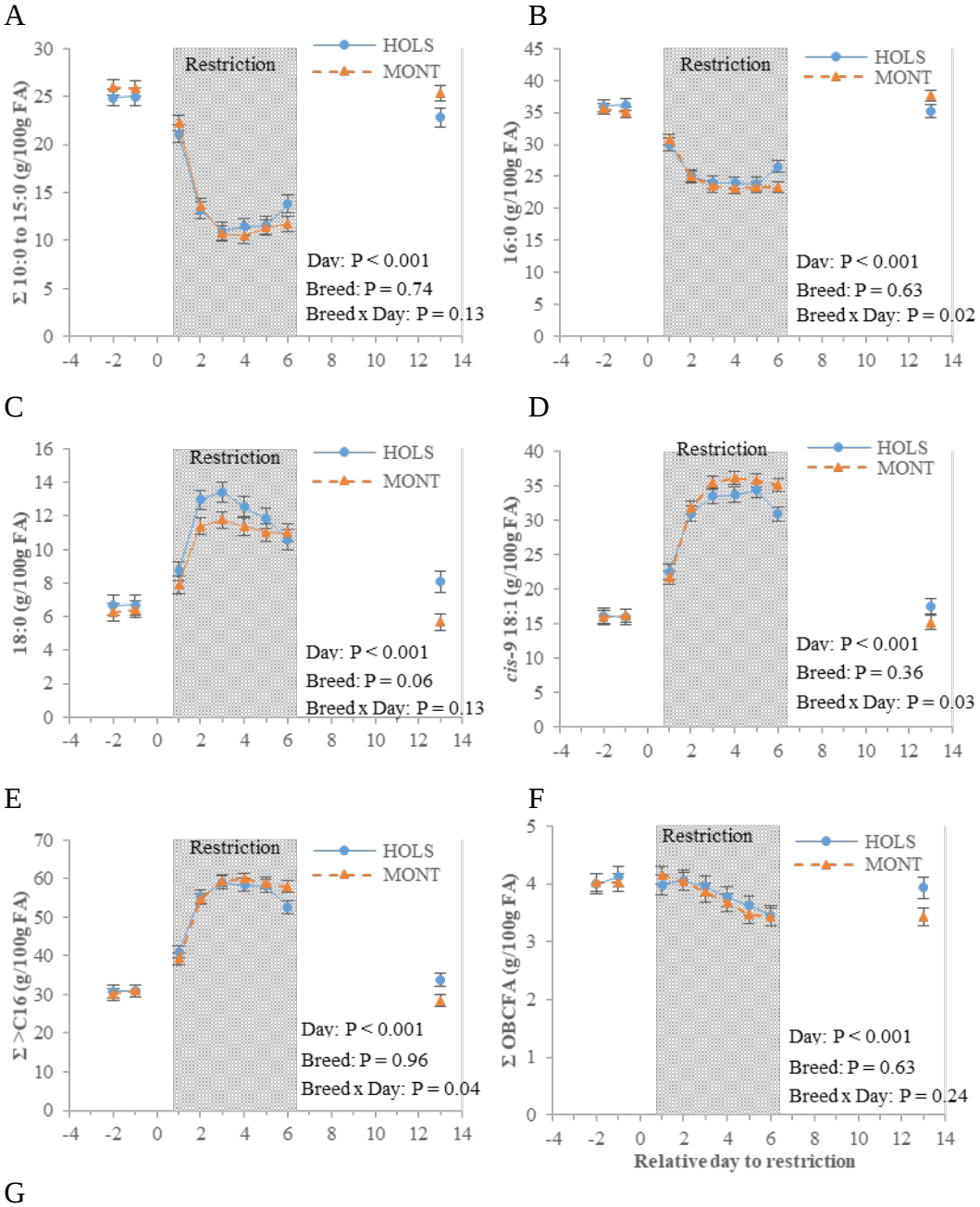
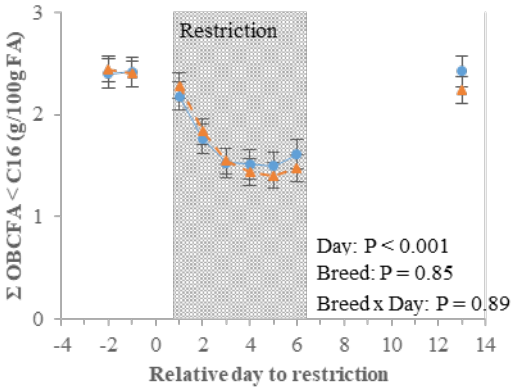
Supplemental Figure 3. Effects of feed restriction on plasma concentrations of urea (A), free amino groups (NH_2) (B), glutamate (C) and glutamine (D) in midlactation Holstein (HOLS; ●, solid lines) and Montbéliarde (MONT; ▲, dashed lines) cows. Intake was limited to meet 50% of NE_L requirements during restriction period (d 1 to 6). Cows were allowed ad libitum intake during control period (d -3 to -1) and after restriction (d 7 to 18).Values are LSM ± SEM.



Supplemental Figure 4. Effects of feed restriction on fatty acid concentrations of sum of FA with 10 to 15 carbons (∑ 10:0 to 15:0) (A), 16:0 (B), 18:0 (C), *cis*-9 18:1 (D), sum greater than C16 carbon (Σ>C16) (E), sum odd and brain chain fatty acid (∑ OBCFA) (D), sum OBCFA with less than 16 carbon (∑ OBCFA<C16) (G) in midlactation Holstein (HOLS; ●, solid lines) and Montbéliarde (MONT; ▲, dashed lines) cows. Fatty acid composition was analyzed in pooled PM/AM milk samples. Intake was limited to meet 50% of NE_L requirements during restriction period (d 1 to 6). Cows were allowed *ad libitum* intake during control period (d -3 to -1) and after restriction (d 7 to 18). Values are LSM ± SEM.





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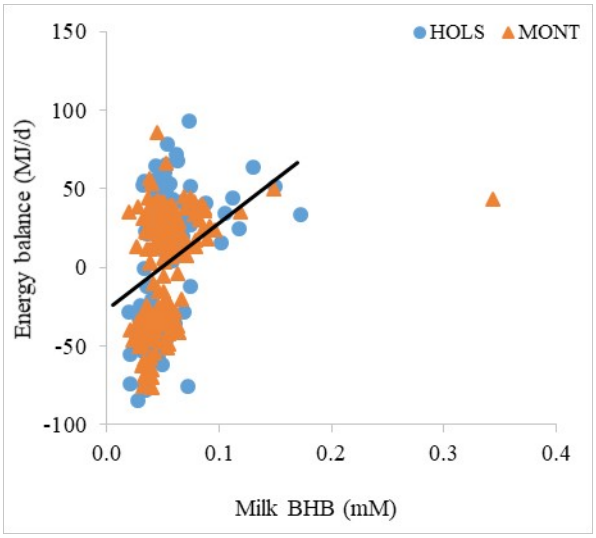
Supplemental Figure 5. Relationship between energy balance and (A) milk BHB ($y = 549.5x - 26.8$, $r^2 = 0.14$), (B) milk uric acid ($y = 288.4x - 27.5$, $r^2 = 0.11$) and (C) milk total free amino groups (NH_2; $y = 129.3x - 96.5$, $r^2 = 0.17$) in midlactation Holstein (HOLS; ●) and Montbéliarde (MONT; ▲) cows ($P < 0.001$). Intake was limited to meet 50% of NE_L requirements during restriction period (d 1 to 6). Cows were allowed ad libitum intake during control period (d -3 to -1) and after restriction (d 7 to 18).

A



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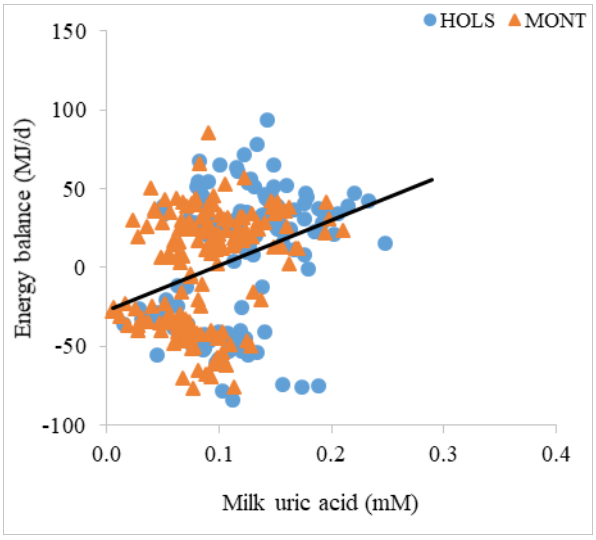

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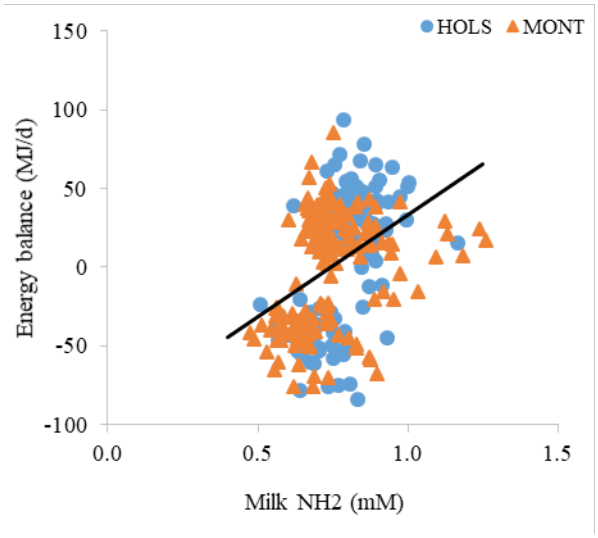

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