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

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

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8 **RUNNING HEAD: MILK METABOLITES DURING UNDERNUTRITION**

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

13

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ABSTRACT

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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 (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

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

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INTRODUCTION

53 Milk composition is modulated by a diversity of factors, including genetics, lactation
54 stage, nutrition and health status, therefore, milk is an obvious source of biomarkers for the
55 monitoring of dairy ruminants. Early lactation is characterized by a rapid increase in milk
56 yield, mobilization of body protein and fat reserves, negative energy balance (**NEB**), and
57 modifications in milk protein, fat and fatty acid composition. Complex homeorhetic and
58 homeostatic adaptations are required to the direct limiting nutrients towards the mammary
59 gland and support milk synthesis during early lactation (Bell and Bauman, 1997). The
60 occurrence of metabolic disorders related to energy metabolism is typical during this period,
61 and plasma concentrations of non-esterified fatty acids (**NEFA**) and BHB are classic
62 indicators for herd troubleshooting (Oetzel, 2004). Experimental feed restriction models are
63 used to induce nutrient deficits and NEB at different stages of lactation, assess the production
64 and metabolic responses (Gross et al., 2011a; Bjerre-Harpøth et al., 2012; Friggens et al.,
65 2016), and the effects of NEB on various biological functions of the dairy cow (Moyes et al.,
66 2009; Abdelatty et al., 2017; Pires et al., 2019). Metabolic responses to feed restriction models
67 are of greater amplitude during early lactation compared to later stages (Bjerre-Harpøth et al.,
68 2012), but midlactation period is more convenient to run experimental protocols and less
69 prone to confounding due to the dynamic nature of early lactation (Contreras et al., 2016).

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

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

79 Milk is a source of novel indicators of nutritional status of dairy cows. Milk sampling
80 is easy to perform in dairy operations, and can be automated for inline analyses. Certain
81 molecules of intermediary metabolism are present in milk and may be indicators of
82 physiological and nutritional state of dairy cows (Gross and Bruckmaier, 2019). For instance,
83 milk is classically used to monitor ketosis by cow-side tests (Oetzel, 2004) and by automated
84 inline BHB measurements. Milk glucose, glucose-6 phosphate, and acid uric acid
85 concentrations are modulated by diet digestibility and correlated with DMI in midlactation
86 cows (Larsen et al., 2016). Furthermore, milk glucose and glucose-6 phosphate concentrations
87 vary according to DIM (Larsen and Moyes, 2015; Zachut et al., 2016; Ferris et al., 2018).
88 Milk concentrations of these metabolites may reflect modifications of metabolic pathways in
89 mammary epithelial cells, including glucose utilization for glycolysis and lactose synthesis
90 (Chaiyabutr et al., 1981), glucose-6-phosphate and isocitrate to produce reducing potential
91 (i.e.; NADPH) for de novo fatty acid synthesis (Garnsworthy et al., 2006; Chaiyabutr et al.,
92 1981), and to counterbalance oxidative stress associated with FA oxidation (Zachut et al,
93 2016). Milk uric acid originates in part from ruminal digestion of purine bases and has been
94 suggested as an indicator of microbial protein synthesis (Larsen and Moyes, 2010). Milk
95 glutamate and free amino acid content may reflect the availability and metabolism of amino
96 acids.

97 The effects of NEB and breed on concentrations of minor milk metabolites are still
98 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

Experimental Design, Animals, Diets and Housing

108

109 All procedures were approved by the ethic committee on animal experimentation
110 (APAFIS # 3737-2015043014541577v2). Twenty multiparous midlactation cows (165 ± 21
111 DIM), 10 Holstein-Friesian and 10 Montbéliarde (1.5 ± 0.29 BCS, 0 to 5 scale) were used to
112 study the effects of 6 d of feed-restriction to meet 50% of NE_L requirements on milk
113 production, classic plasma and putative milk biomarkers of metabolic status. Two HOLS cows
114 were excluded from the study, one due to clinical mastitis and one due to noncompliance with
115 the restriction protocol. Phenotypic measurements were performed from d -3 to +18 relative
116 of initiation of restriction, corresponding to the following periods: control (**CONT**; d -3 to -1),
117 restriction (**REST**; d 1 to 6), week 1 (**WEEK1**; d 7 to 13), and week 2 (**WEEK2**; d 14 to 18;
118 Figure 1). Five cows of each breed were randomly allocated to a group of 10 animals that
119 initiated the experimental protocol one day apart. Mammary and liver biopsies were
120 performed on d 0 and d 6 for complementary studies (Billa et al., 2019). The experiment was
121 conducted at the INRA Herbipôle experimental farm of 'Marcenat' ($45^{\circ}18'21''N$, $2^{\circ}50'13''E$;
122 1100 m of altitude; <https://doi.org/10.15454/1.5572318050509348E12>) during April and early
May.

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

135 NE intake (UFL) = UFL/kg DM \times DMI (kg) - E; with E corresponding to the
136 “digestive interaction”, calculated as a function of the percentage concentrate in the diet (%
137 Conc; DM-basis) and UFL intake (i.e., UFL/kg DM \times DMI (kg)), using the formula $E =$
138 $(0.00063 \times \%Conc^2) - (0.017 \times UFLintake) + (0.002 \times UFLintake^2)$.

139 NE production (UFL) = milk yield (kg) \times $[0.44 + (0.0055 \times (-40 + \text{fat content; g/kg}))$
140 $+ (0.0033 \times (-31 + \text{protein content; g/kg}))]$;

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

142 Energy balance (UFL) = NE intake - NE maintenance - NE production.
143

144 **Sampling, Measurements and Chemical Analyses**

145 **Milk Sampling and Analysis.** Cows were milked twice daily at approximately 6:30
146 and 16:00. Milk yield was recorded and milk composition was determined by mid-infrared
147 spectroscopy (LIAL, Aurillac, France) in morning and evening milk samples. Weighted milk
148 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).

174

175 ***Statistical Analyses***

176 Statistical analyses were performed using SAS enterprise guide (Version 9.4; SAS
177 Institute INC, Cary, NC). Daily data was analyzed as repeated measures by mixed models that
178 included day, breed and their interaction as fixed effects, cow as random effect, and Kenward-
179 Roger adjustment for calculation of degrees of freedom. The Schwarz's Bayesian criterion
180 was used to compare the fitting of different variance-covariance structures, including spatial
181 power, AR (1), ARH (1) and CS. The means for each variable within animal and period were
182 calculated in order to compare periods (CONT, REST, WEEK1 and WEEK2) and explore
183 breed by period interactions. The models included the fixed effects of period, breed and their
184 interaction, and the random effect of cow. Significant time effects (i.e., day or period) and
185 breed by time interactions were explored by the Fisher's protected least significant difference
186 using the PDIFF and SLICE options of the LSMEANS statement. Residuals were checked for
187 normality and homoscedasticity. Heterogeneous variance was tested whenever suggested by
188 residual plots. Least squares means (LSM) and standard error of the mean (SEM) were
189 estimated from untransformed values, whereas *P*-values may reflect statistical analysis of log-
190 transformed data when transformation was necessary. Relationships among variables were
191 explored by Spearman rank correlations. Linear regressions between energy balance and milk
192 metabolite concentrations were analyzed using PROC REG procedure of SAS. The
193 significance level was predefined as $P \leq 0.05$ and trends toward significance at $0.05 < P \leq$
194 0.10.

195

196 **RESULTS AND DISCUSSION**

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198 ***Production Responses***

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199 A significant period effect was observed for DMI, energy balance, milk and milk
200 component yields (Table 2 and Supplemental Figure 1). Per design, energy balance became
201 negative (-42.5 ± 4.4 MJ/d) and milk, fat and protein yields decreased during REST. Energy
202 balance returned to pre-restriction values during WEEK1, and DMI, fat and protein yields
203 during WEEK2. The effect of restriction on milk, fat and protein yields are in accordance with
204 previous studies in midlactation Holstein cows (Gross et al., 2011a; Bjerre-Harpøth et al.,
205 2012; Pires et al., 2016).

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

213

214 ***Plasma Metabolite and Insulin Concentrations***

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

224 The increase in plasma NEFA concentrations during REST reflects lipomobilisation
225 (Chilliard et al., 2000a) and is in accordance with previous feed-restriction studies involving
226 mid- and late lactation cows (Bjerre-Harpøth et al., 2012; Gross and Bruckmaier, 2011a; Pires
227 et al., 2016). Nonetheless, the increase in plasma NEFA observed during REST in our
228 experiment was smaller than observed in underfed early lactation cows (Bjerre-Harpøth et al.,
229 2012; Pires et al., 2019). Plasma BHB concentrations increased during restriction (Table 3 and
230 Supplemental Figure 2), but remained below the 1.2 mM threshold of subclinical ketosis
231 (LeBlanc et al., 2005), which is in agreement with previous research in underfed midlactation
232 cows (Moyes et al., 2009; Gross et al., 2011a; Bjerre-Harpøth et al., 2012). As plasma BHB
233 originates in part from rumen butyrate (Miettinen and Huhtanen, 1996), its concentrations
234 observed during REST probably reflect concomitant modifications of DMI and ruminal
235 butyrate synthesis, and incomplete beta-oxidation of mobilized NEFA. Two cows had
236 increased concentrations of BHB the morning after refeeding the TMR at ad libitum intake (d
237 7; Supplemental Figure 3), which may result from increased DMI and a shift in ruminal
238 butyrate production. Accordingly, insulin was greatest the day after refeeding at ad libitum
239 intake (d 7, Supplemental Figure 2).

240 The decrease in plasma glutamine and NH_2 concentrations observed during REST may
241 reflect reduced DMI and amino acid absorption. Glutamine and glutamate are intermediates in
242 many anabolic and catabolic pathways, including regulation of metabolic acidosis,
243 lymphocyte proliferation, and casein synthesis (Lobley et al., 2001). Plasma glutamine,
244 glutamate and total amino acid concentrations decrease during periods of NEB, such as early
245 lactation (Meijer et al., 1995) and during experimental feed restriction in midlactation cows
246 (Girard et al., 2019). Decreased supply of amino acids for intestine absorption may have
247 contributed more to these changes than amino acid catabolism, because plasma urea
248 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).

274 Increased milk glucose-6-phosphate and isocitrate, and decreased milk glucose concentrations
275 during REST are in agreement with previous observations in starved goats (Chaiyabutr et al.,
276 1981) and in early lactation cows (Larsen and Moyes, 2015; Zachut et al., 2016). Glucose-6-
277 phosphate and isocitrate are precursors for NADPH synthesis via the pentose phosphate and
278 isocitrate dehydrogenase pathways, respectively. Increased milk glucose-6-phosphate and
279 isocitrate content during REST may reflect a shift in these pathways in mammary epithelial
280 cells, due to low plasma insulin, decreased de novo FA synthesis and synthesis of other milk
281 components.

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

288

289 ***Milk Fatty Acid Concentrations***

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

294 Concentrations of FA with 10 to 15 carbons (Σ **10:0 to 15:0**), 16:0 and Σ OBCFA with
295 carbon chain shorter than 16 (Σ **OBCFA < C16**) decreased during REST, whereas
296 concentration of 18:0 and *cis*-9 18:1 and of FA with carbon chain greater than 16 (Σ **> C16**)
297 increased. These modifications in milk FA profile are in agreement with other feed restriction
298 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 $\Sigma > C16$ 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-

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

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

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

336

337 ***Correlation and regression analyses***

338 Correlations are presented in Tables 6 and 7 and regressions between energy balance
339 and milk metabolites are presented in Figure 4 and Supplemental Figure 5. All variables
340 presented were significantly correlated with energy balance, except plasma urea concentration
341 and milk total OBCFA content. Among plasma metabolites, NEFA and glucose had the
342 greatest absolute correlation with energy balance ($r_s = -0.72$ and $r_s = 0.64$, respectively; Table
343 6). Among milk metabolites, milk glucose and glutamate had the greatest absolute
344 correlations with energy balance ($r_s > 0.60$) and plasma NEFA ($r_s = -0.67$, Table 6), which is a
345 classic indicator of lipomobilization. Moreover, milk glucose was correlated with plasma
346 glucose ($r_s = 0.61$) which is also modulated by NEB (Meijer et al., 1995; Bjerre-Harpøth et
347 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 $\Sigma > C16$, 18:0 and *cis*-9 18:1 were negatively
358 correlated with energy balance ($r_s = -0.54$ to -0.56), whereas milk $\Sigma 10:0$ to $15:0$, $16:0$ and Σ
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 $\Sigma 10:0$ 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 $\Sigma > C16$,
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

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

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Table 1: Diet ingredient and nutrient composition

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 ¹Mineral 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.

556

557 **Table 2.** Effects of feed restriction on energy balance, DMI, milk yield, and milk component
 558 yield in midlactation Holstein (HOLS) and Montbéliarde (MONT) cows¹.
 559

	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			

560
 561 ¹ Intake was limited to meet 50% of NE_L requirements during restriction period (REST, d 1 to
 562 6). Cows were allowed ad libitum intake during control period (CONT, d -3 to -1) and after
 563 restriction (WEEK1, d 7 to 13; WEEK2, d 14 to 18).

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

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

568

569 **Table 3.** Effects of feed restriction on plasma metabolite and insulin concentrations in
 570 midlactation Holstein (HOLS) and Montbéliarde (MONT) cows¹.
 571

		Period	SEM	P -value					
		CONT	RES T	WEEK 1	WEEK 2	Breed	Period	Breed × Perio d	
NEFA (mM)	HOLS	0.11 ^b	0.69 ^a	0.09 ^b	0.08 ^b	0.03	0.93	0.001	0.98
	MON T	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
	MON T	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
	MON T	3.77	3.32	3.93	3.87	0.06			
Glutamat e (mM)	HOLS	0.10 ^b	0.11 ^a	0.09 ^c	0.09 ^c	0.003	0.07	0.001	0.17
	MON T	0.09	0.10	0.09	0.09	0.003			
Glutamin e (mM)	HOLS	0.16 ^a	0.11 ^c	0.16 ^b	0.15 ^{ab}	0.01	0.61	0.001	0.13
	MON T	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
	MON T	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
	MON T	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
	MON T	15.7	7.2	24.5	13.7	1.6			

572
 573 ¹ Intake was limited to meet 50% of NE_L requirements during restriction period (REST, d 1 to
 574 6). Cows were allowed ad libitum intake during control period (CONT, d -3 to -1) and after
 575 restriction (WEEK1, d 7 to 13; WEEK2, d 14 to 18).

576 ^{a, b, c, d} **Concern main period effects pooled across both breeds.** Period LSMEANS not sharing a
 577 common superscript differ ($P \leq 0.05$).

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

580 ² Free amino groups (NH₂): Estimation of free amino acid concentration

581

583 **Table 4.** Effects of feed restriction on milk metabolite concentrations in midlactation Holstein
 584 (HOLS) and Montbéliarde (MONT) cows¹.

	Period	SEM		P-value					
		CON T	RES T	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			

585
 586 ¹ Intake was limited to meet 50% of NE_L requirements during restriction period (REST, d 1 to 6).
 587 Cows were allowed ad libitum intake during control period (CONT, d -3 to -1) and after
 588 restriction (WEEK1, d 7 to 13; WEEK2, d 14 to 18).

589 ^{a, b, c, d} **Concern main period effects pooled across both breeds.** Period LSMEANS not sharing a
 590 common superscript differ ($P \leq 0.05$).

591 ² Free amino groups (NH₂): Estimation of free amino acid concentration (Larsen and Fernández,
 592 2017).

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 595

596 **Table 5.** Effects of feed restriction on milk fatty acid concentrations (g/100g of fatty acids) in
 597 midlactation Holstein (HOLS) and Montbéliarde (MONT) cows¹. Milk fatty acid composition
 598 was analyzed in pooled PM/AM samples.

	Period	SEM		P-value				
		CON T	RES T	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			
<i>cis</i> -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			

599
 600 ¹ Intake was limited to meet 50% of NE_L requirements during restriction period (REST, d 1 to 6).
 601 Cows were allowed ad libitum intake during control period (CONT, d -3 to -1) and after
 602 restriction (WEEK1, d 7 to 13; WEEK2, d 14 to 18).

603 ^{a, b, c, d} **Concern main period effects pooled across both breeds.** Period LSMEANS not sharing a
 604 common superscript differ ($P \leq 0.05$).

605 ²Sum of FA with between 10 and 15 carbons.

606 ³Sum of FA with more than 16 carbons.

607 ⁴Sum of OBCFA with less than 16 carbons.

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Table 6. Spearman rank correlations among milk metabolites and classic indicators of metabolic status in midlactation Holstein and Montbéliarde cows¹. 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 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	-0.72	**	-0.17	*	0.64	**	0.34	***	0.51	***	0.48	**	0.63	**	-0.31	**	0.61	**	-0.45	**	0.30	**	0.41	***
	(306)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)	(231)
Plasma NEFA		1.00	0.17	*	-0.53	**	0.45	***	-0.44	**	-0.67	**	0.36	**	-0.67	**	0.48	**	-0.44	**	-0.46	***			
		(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)
Plasma BHB			1.00	-0.33	**	0.28	***	0.21	***	0.17	**	-0.27	**	-0.09	ns	-0.11	†	0.31	**	-0.31	**	-0.06	ns		
			(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)
Plasma glucose				1.00	-	***	0.46	***	0.35	**	0.61	**	-0.25	**	0.56	**	-0.30	**	0.31	**	0.46	***			
				(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)
Plasma glutamate					1.00	-	***	0.17	**	-0.14	*	-0.36	**	0.19	**	-0.39	**	0.33	**	-0.22	**	-0.25	***		
					(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)
Plasma NH ₂ ¹						1.00	0.26	**	0.50	**	-0.20	**	0.59	**	-0.34	**	0.50	**	0.44	***					
						(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)	(249)

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 Billa, P.-A., Faulconnier, Y., Larsen, T., Leroux, C., Pires, J. (2020). Milk metabolites as noninvasive indicators of nutritional status of mid-lactation Holstein and Montbéliarde cows. Journal of Dairy Science, 103 (4), 3133–3146.

	(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***
Milk glucose		1.00	-0.18**	0.70**	-0.50**	0.51**	0.51**	0.51***	
Milk glucose-6-phosphate			1.00	-0.28**	0.34**	0.05	ns	-0.29***	
Milk glutamate				1.00	-0.36**	0.38**	0.72**	0.72***	
Milk isocitrate					1.00	-0.17	**	-0.26***	
Milk uric acid						1.00	0.28	0.28***	
Milk NH₂²							1.00	1.00	

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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 (NH₂): Estimation of free amino acid concentrations (Larsen and Fernández, 2017).

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Billa, P.-A., Faulconnier, Y., Larsen, T., Leroux, C., Pires, J. (2020). Milk metabolites as noninvasive indicators of nutritional status of mid-lactation Holstein and Montbéliarde cows. Journal of Dairy Science, 103 (4), 3133–3146.

Table 7. Spearman rank correlations among milk fatty acids and milk metabolites and classic indicators of metabolic status in midlactation Holstein and Montbéliarde cows¹. 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	<i>Cis</i> -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

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	(162)	(162)	(162)	(162)	(162)	(162)
Cis-9 18:1		1.00	0.97 ***	-0.85 ***	-0.23 **	
		(162)	(162)	(162)	(162)	
Σ >C16³			1.00	-0.83 ***	-0.16 *	
			(162)	(162)	(162)	
Σ OBCFA <C16⁴				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).

²Sum of FA with between 10 and 15 carbons.

³Sum of FA with more than 16 carbons.

⁴Sum of OBCFA with less than 16 carbons.

⁵ Free amino groups (NH₂): Estimation of amino acid concentration (Larsen and Fernández, 2017).

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630 **Figure 1:** Sampling timeline. Control period (CONT; d -3 to -1); restriction period (REST; d 1
 631 to 6, thick line), when feed intake was restricted to meet 50% of NE_L requirements calculated
 632 during the CONT period; week 1 after refeeding (WEEK1; d 7 to 13); week 2 after refeeding
 633 (WEEK2: d 14 to 18). \diamond Milk yield and composition each milking. ∞ Milk sampling for milk
 634 metabolite analyses (d 1 corresponds to samples collected at 24 h of feed restriction). \circ Blood
 635 sampling for plasma metabolite and insulin analyses (d 1 corresponds to samples collected at
 636 24 h of feed restriction). Δ Milk sampling for milk fatty acid composition analyses (pooled
 637 PM and AM samples).

638 **Figure 2.** Effects of feed restriction on milk concentrations of (A) glucose, (B) glucose-6-
 639 phosphate, (C) isocitrate and (D) β -hydroxybutyrate (BHB) in midlactation Holstein (HOLS;
 640 \bullet , solid lines) and Montbéliarde (MONT; \blacktriangle , dashed lines) cows. Intake was limited to meet
 641 50% of NE_L requirements during restriction period (d 1 to 6). Cows were allowed ad libitum
 642 intake during control period (d -3 to -1) and after restriction (d 7 to 18). Values are LSM \pm
 643 SEM.

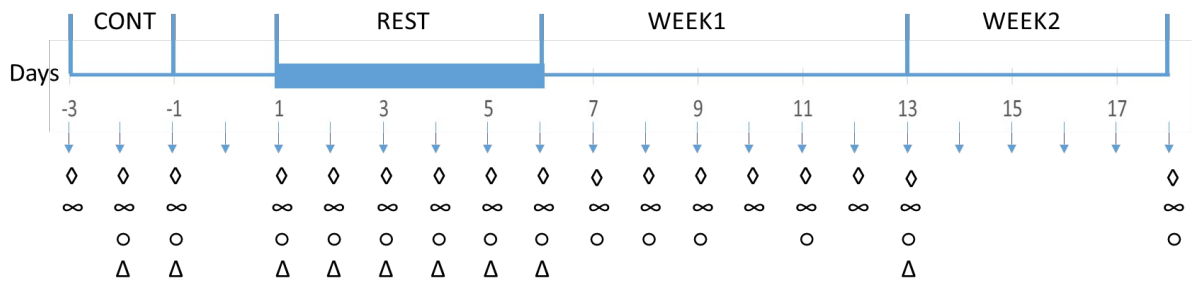
644
 645 **Figure 3.** Effects of feed restriction on milk concentrations of (A) free amino group (NH_2),
 646 (B) glutamate and (C) uric acid in midlactation Holstein (HOLS; \bullet , solid lines) and
 647 Montbéliarde (MONT; \blacktriangle , dashed lines) cows. Intake was limited to meet 50% of NE_L
 648 requirements during restriction period (d 1 to 6). Cows were allowed ad libitum intake during
 649 control period (d -3 to -1) and after restriction (d 7 to 18). Values are LSM \pm SEM.

650
 651 **Figure 4.** Relationship between energy balance and (A) milk glucose ($y = 171.0x - 62.1, r^2 =$
 652 0.49), (B) natural log of milk glutamate ($y = 42.7x + 62.7, r^2 = 0.46$), (C) natural log of milk
 653 glucose-6-phosphate ($y = -9.5x - 37.8, r^2 = 0.10$) and (D) milk isocitrate ($y = -458.4 + 70.7, r^2$
 654 $= 0.26$) in midlactation Holstein (HOLS; \bullet) and Montbéliarde (MONT; \blacktriangle) cows ($P < 0.001$).
 655 Intake was limited to meet 50% of NE_L requirements during restriction period (d 1 to 6). Cows
 656 were allowed ad libitum intake during control period (d -3 to -1) and after restriction (d 7 to
 657 18).

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660 Billa et al. Figure 1

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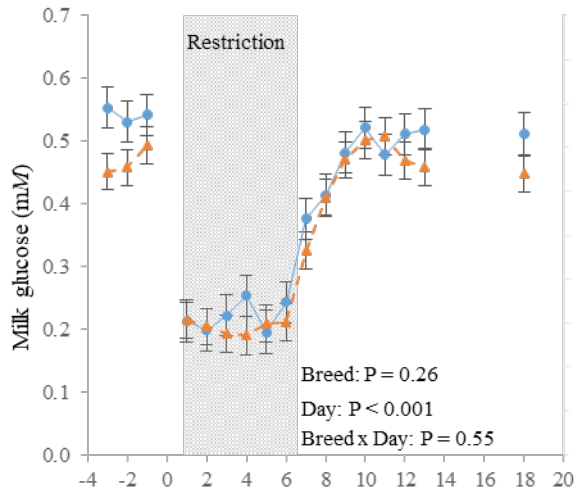
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663 Billa et al. Figure 2

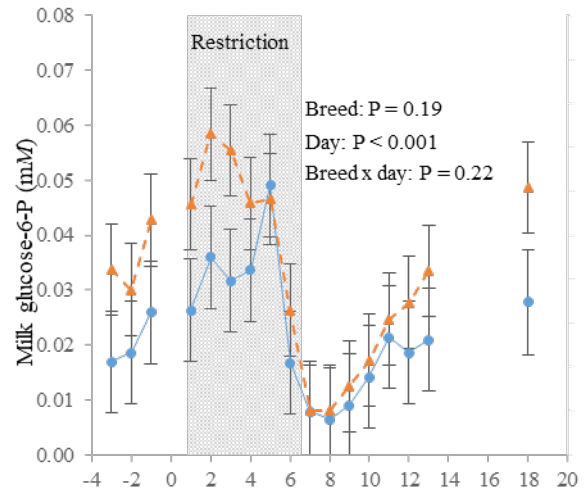
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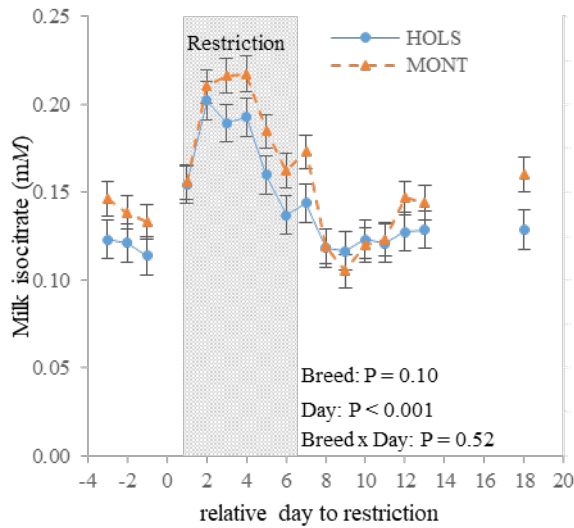
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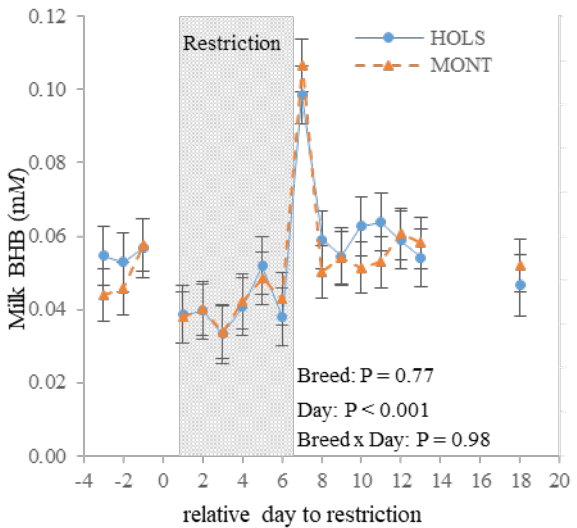
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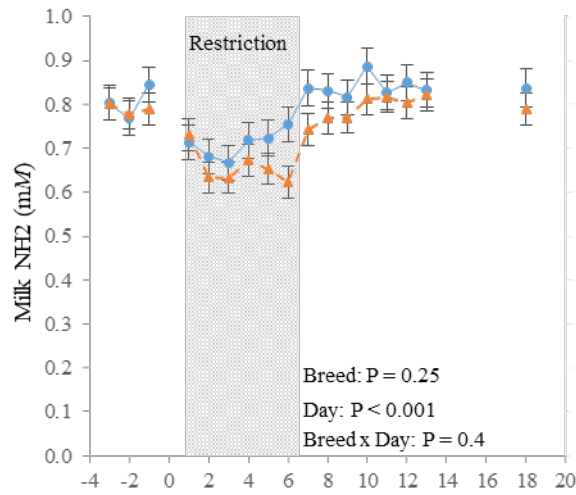
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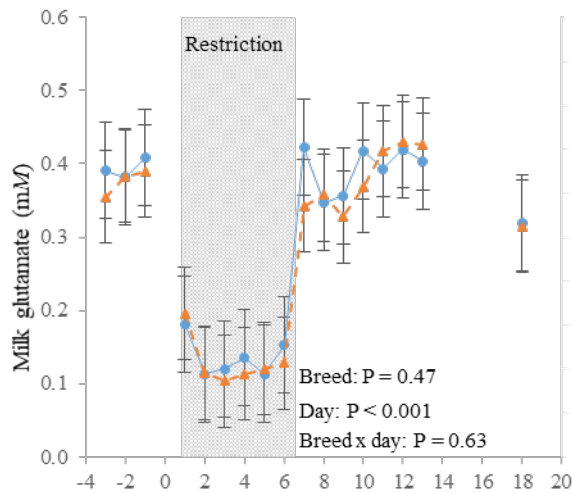
671 Billa et al. Figure 3

672 **A**



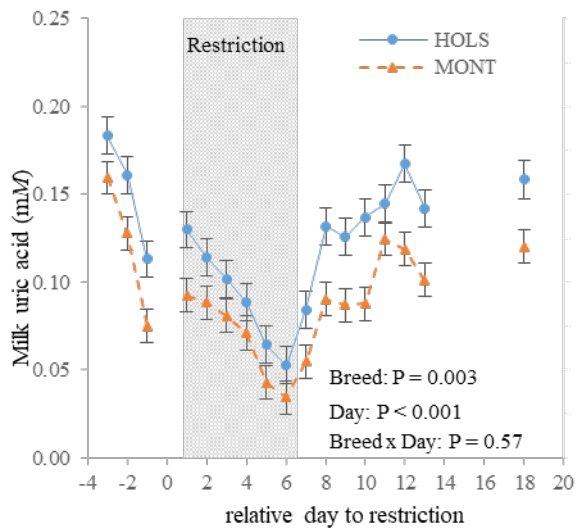
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674 **B**



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676 **C**

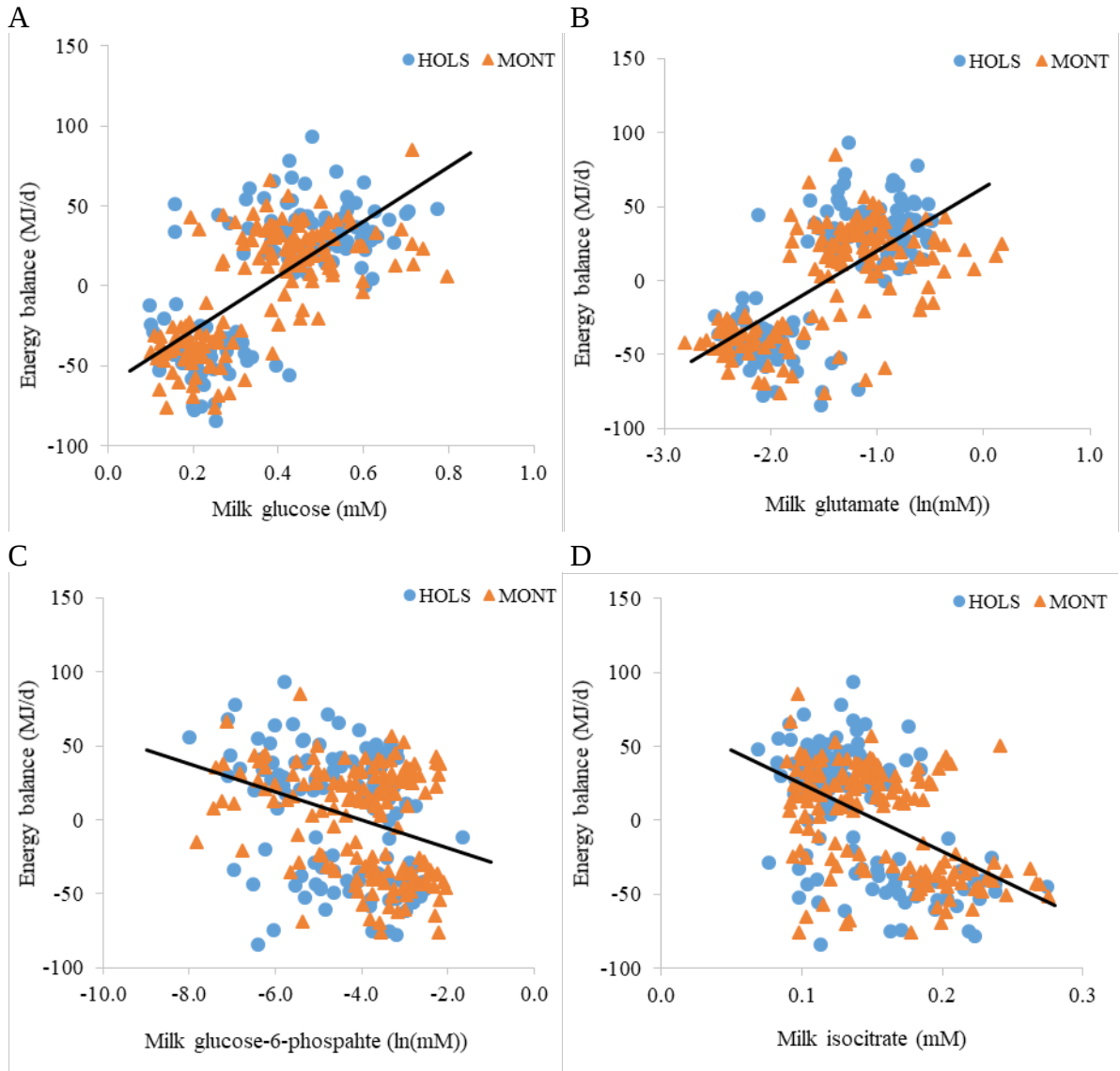


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678 Billa et al. Figure 4

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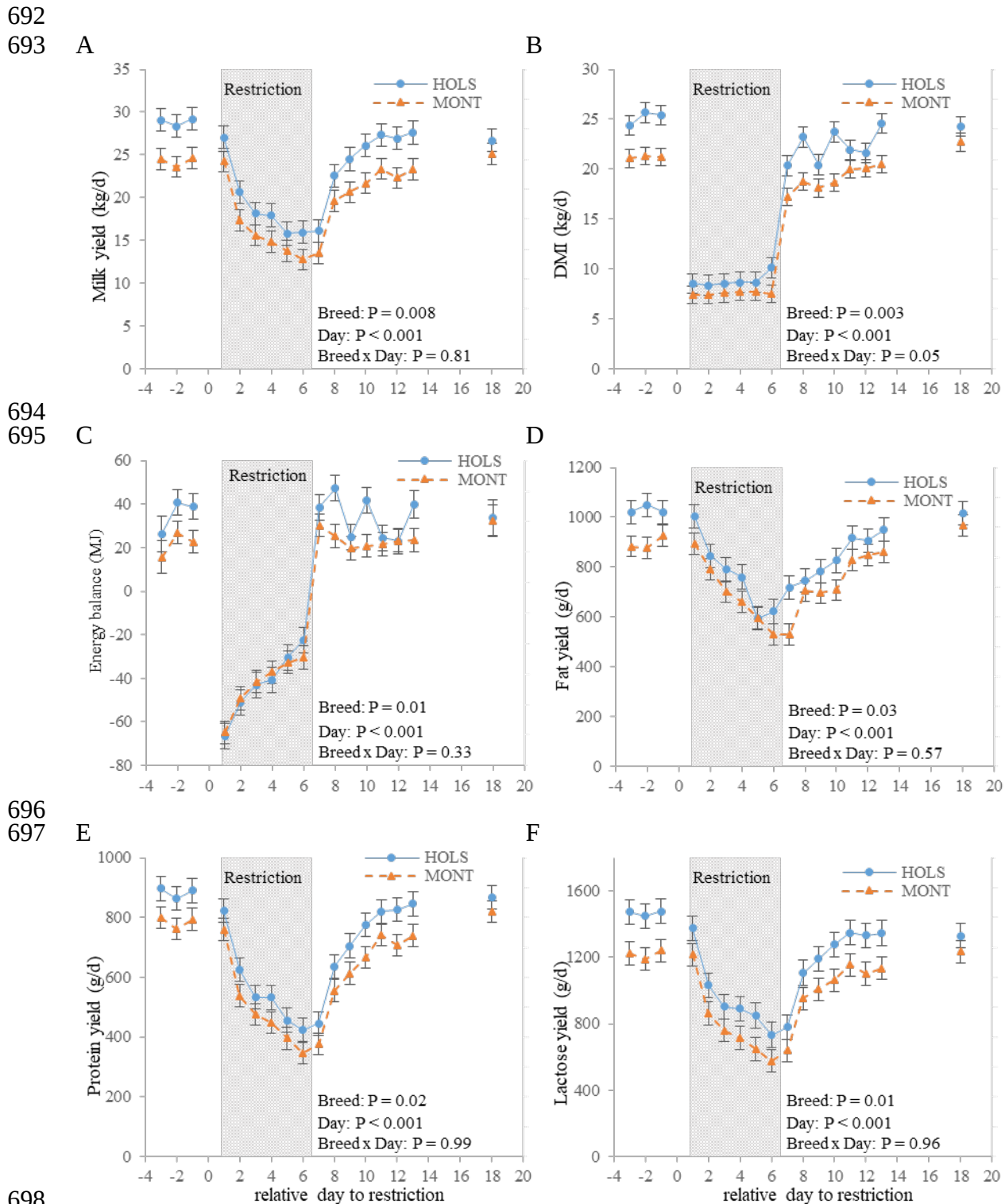
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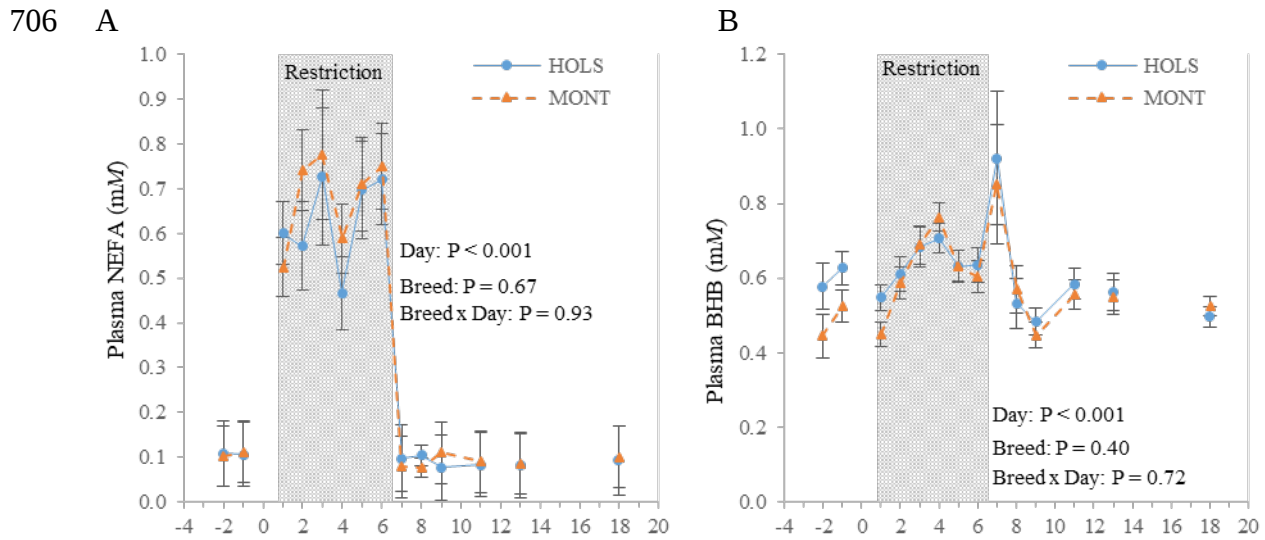
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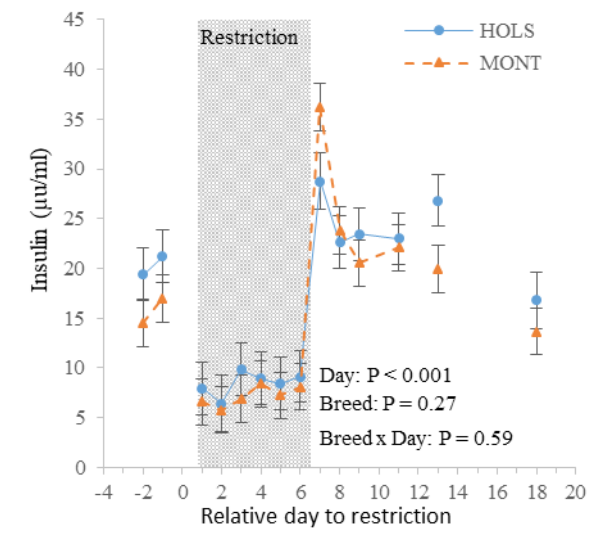
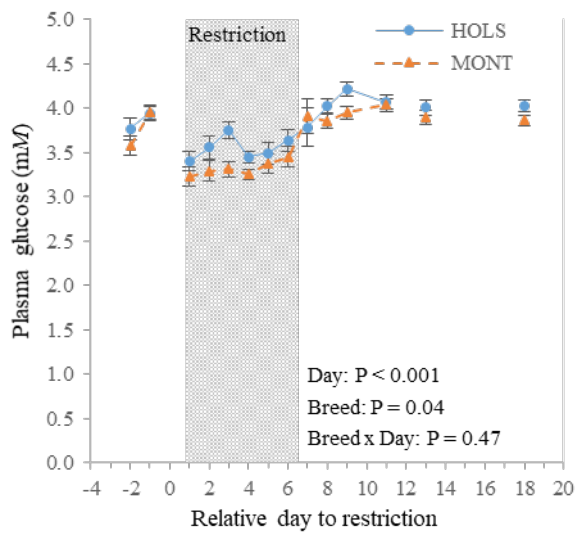
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686 **Supplemental Figure 1.** Effects of feed restriction on milk yield (A), DMI (B), **energy**
 687 **balance (C)**, fat (D), protein (E) and lactose (F) yield in midlactation Holstein (HOLS; ●,
 688 solid lines) and Montbéliarde (MONT; ▲, dashed lines) cows. Intake was limited to meet
 689 50% of NE_L requirements during restriction period (d 1 to 6). Cows were allowed *ad libitum*
 690 intake during control period (d -3 to -1) and after restriction (d 7 to 18). Values are LSM \pm
 691 SEM.



699 **Supplemental Figure 2.** Effects of feed restriction on plasma concentrations of non-esterified
 700 fatty acid (NEFA) (A), BHB (B), glucose (C) and insulin (D) in midlactation Holstein (HOLS;
 701 ●, solid lines) and Montbéliarde (MONT; ▲, dashed lines) cows. Intake was limited to meet
 702 50% of NE_L requirements during restriction period (d 1 to 6). Cows were allowed *ad libitum*
 703 intake during control period (d -3 to -1) and after restriction (d 7 to 18). Values are LSM \pm
 704 SEM.
 705

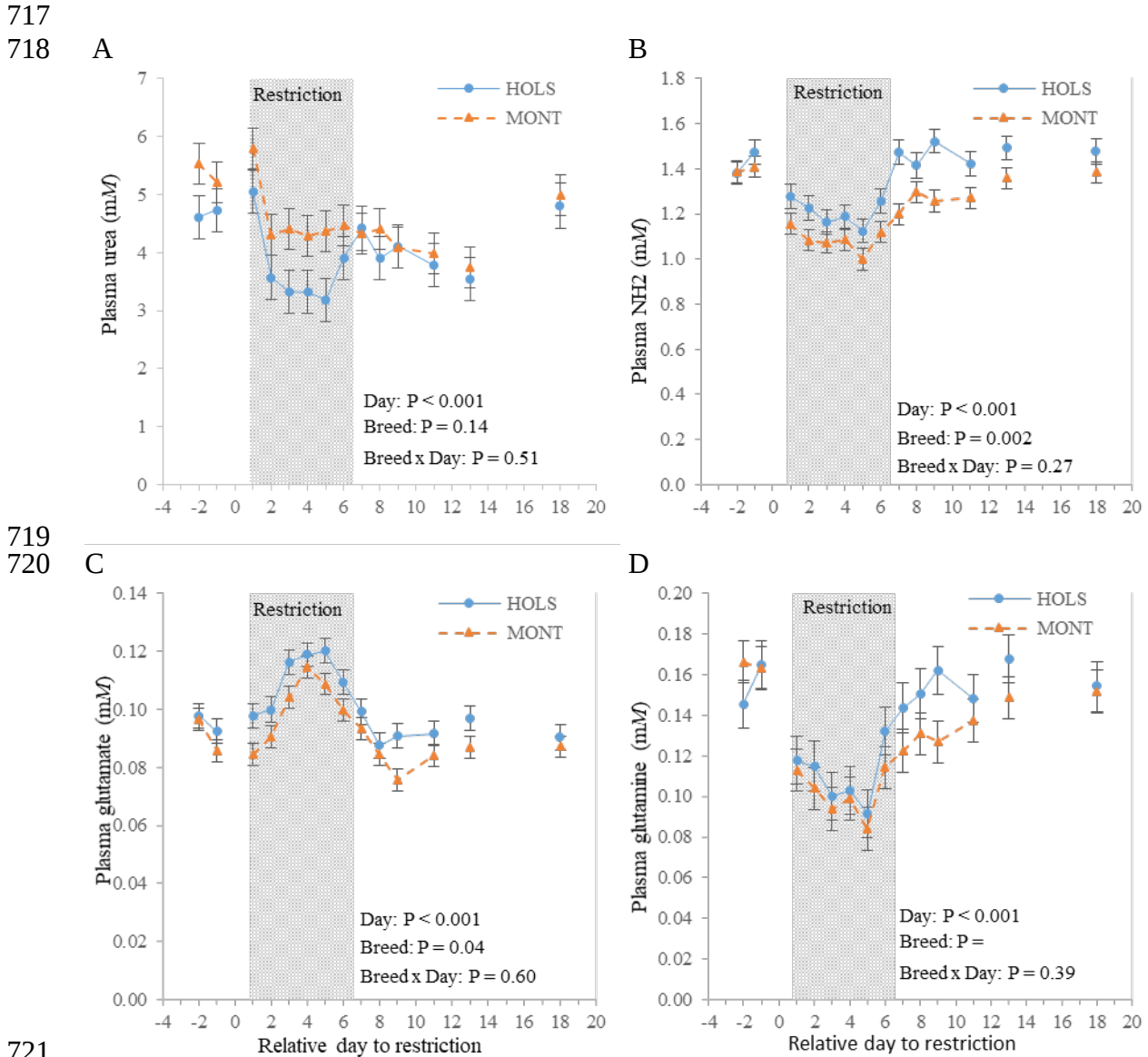




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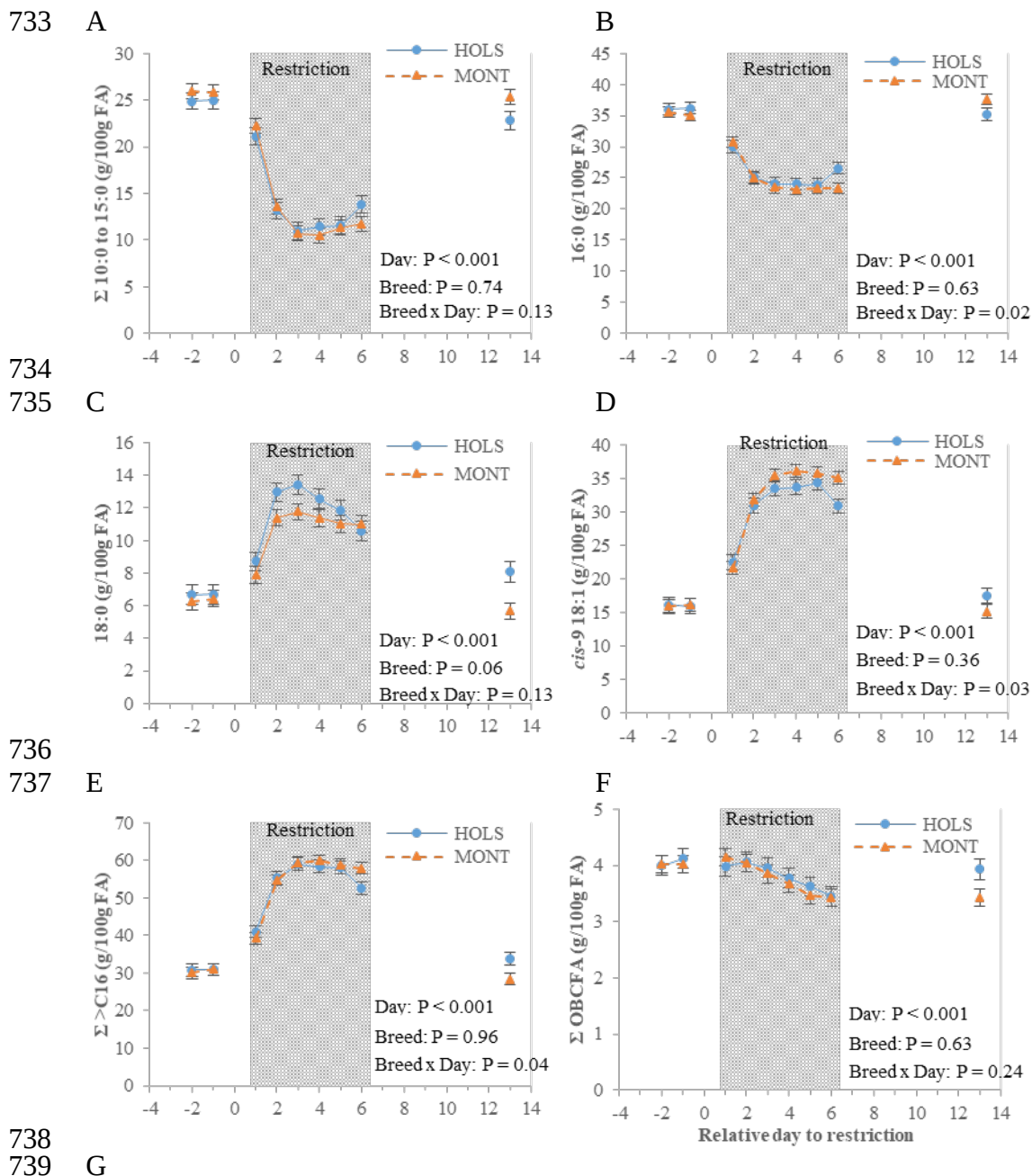
711 **Supplemental Figure 3.** Effects of feed restriction on plasma concentrations of urea (A), free
 712 amino groups (NH₂) (B), glutamate (C) and glutamine (D) in midlactation Holstein (HOLS; ●,
 713 solid lines) and Montbéliarde (MONT; ▲, dashed lines) cows. Intake was limited to meet
 714 50% of NE_L requirements during restriction period (d 1 to 6). Cows were allowed ad libitum
 715 intake during control period (d -3 to -1) and after restriction (d 7 to 18). Values are LSM ±
 716 SEM.

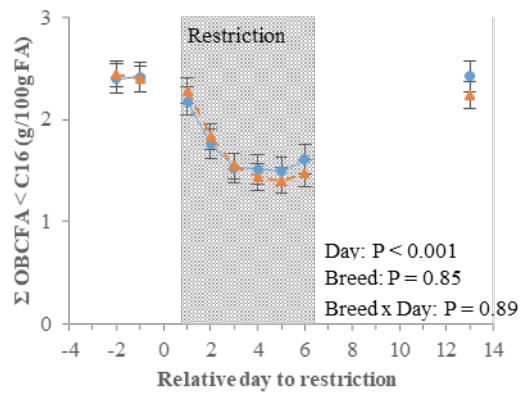


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724 **Supplemental Figure 4.** Effects of feed restriction on fatty acid concentrations of sum of FA
 725 with 10 to 15 carbons (Σ 10:0 to 15:0) (A), 16:0 (B), 18:0 (C), *cis*-9 18:1 (D), sum greater
 726 than C16 carbon ($\Sigma >C16$) (E), sum odd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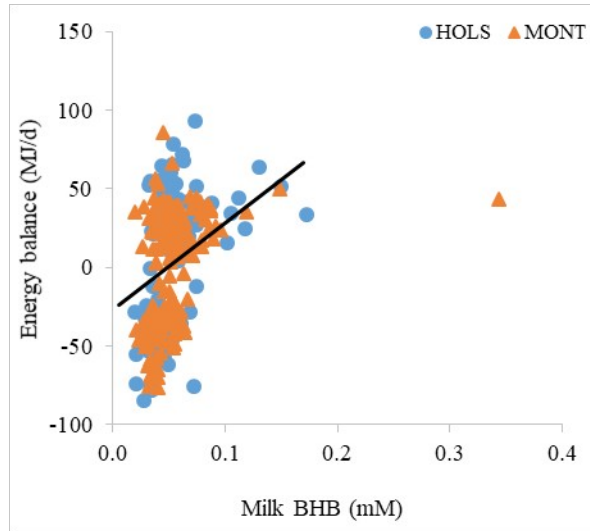


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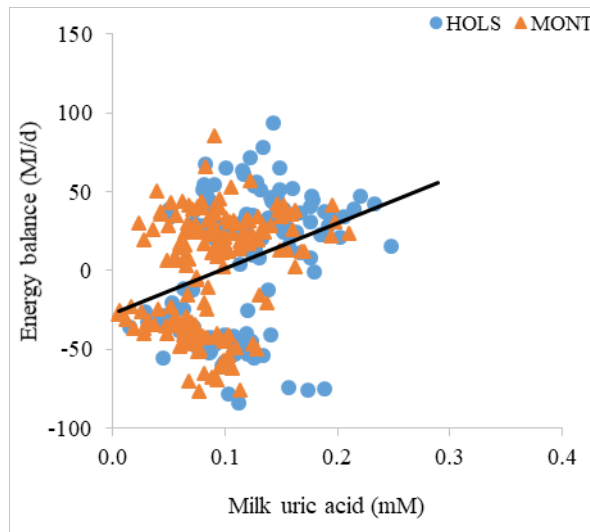
743 **Supplemental Figure 5.** Relationship between energy balance and (A) milk BHB ($y = 549.5x$
744 $- 26.8, r^2 = 0.14$), (B) milk uric acid ($y = 288.4x - 27.5, r^2 = 0.11$) and (C) milk total free
745 amino groups ($\text{NH}_2; y = 129.3x - 96.5, r^2 = 0.17$) in midlactation Holstein (HOLS; ●) and
746 Montbéliarde (MONT; ▲) cows ($P < 0.001$). Intake was limited to meet 50% of NE_L
747 requirements during restriction period (d 1 to 6). Cows were allowed ad libitum intake during
748 control period (d -3 to -1) and after restriction (d 7 to 18).
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A



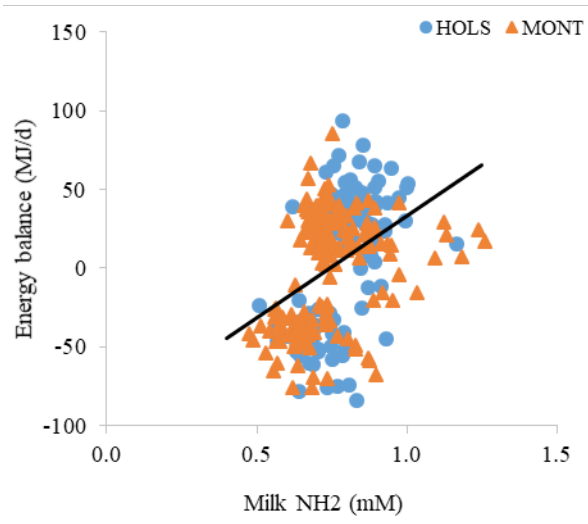
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B



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C



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