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Undernutrition modified metabolic responses to intramammary lipopolysaccharide but had limited effects on selected inflammation indicators in early-lactation cows

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1 **Interpretive Summary**, *Pires et al.*, page XX. After calving, dairy cows often experience
2 nutritional deficits and depend on body reserves to support milk production. Suboptimal nutrition
3 may explain increased occurrence of inflammatory diseases such as mastitis that impact animal
4 welfare and profitability of dairy farms. We studied the effects of insufficient nutrition on the
5 ability of cows to respond to inflammation. Modifications in blood composition during
6 inflammation differed, and suggest that underfed cows elicit metabolic adaptations in order to
7 support short-term inflammation response. This response may impose a burden to the dairy cow and
8 impact their adaptive capacity to early lactation.

9
10 RUNNING HEAD: INFLAMMATION RESPONSE DURING UNDERNUTRITION

11
12 Title

13 **Undernutrition modified metabolic responses to intramammary lipopolysaccharide but had**
14 **limited effects on selected inflammation indicators in early lactation cows.**

15
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30

ABSTRACT

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The objective was to assess effects of experimentally-induced undernutrition on responses to an intramammary lipopolysaccharide (LPS) challenge in early lactation cows. Starting at 24 ± 3 d in milk, multiparous Holstein cows either received a ration containing 48% of straw for 96 h to restrict nutrient intake (REST, $n = 8$), or were allowed ad libitum intake of a lactation diet (CONT, $n = 9$). After 72 h on diet, or at an equivalent period for CONT, 50 μ g of LPS (*E. coli* 0111:B4) were injected into one healthy rear mammary quarter in order to induce an acute inflammation response. Blood samples were collected weekly until 7 weeks of lactation, daily during feed restriction (or control), before and at 1, 2, 4, 6, 10 and 24 h relative to LPS injection. Foremilk quarter samples were collected before and at 4, 6, and 10 and 24 h after LPS injection. Dry matter intake, milk yield, energy balance, plasma glucose, nonesterified fatty acids (NEFA) and BHB (β -hydroxybutyrate) concentrations did not differ between CONT and REST immediately before nutrient restriction in REST (least square means at day -1 were 21.8, 39.0 kg/d, -2.5 MJ/d, 3.78, 0.415 and 0.66 mM, respectively), but were significantly altered at 72 h of nutrient restriction (9.8, 28.3 kg/d, and -81.6 MJ/d, 2.77, 1.672 and 2.98 mM, respectively), when the LPS challenge was performed. Rectal temperature increment from baseline values in response to LPS did not differ, but cortisol increment was greater, and cortisol response area under the curve (AUC) tended to be greater (202 vs 122 (ng/mL) \times 10 h) for REST than CONT. No treatment differences were observed in foremilk milk IL-8, IL-1 β , TNF- α and CXCL3 concentrations in response to LPS injection. Composite milk somatic cell count per kg of milk produced per day (6.919E6 vs 1.956E6 cells/mL) and total somatic cell secretion per day were greater for REST than CONT during the day following LPS. Plasma glucose, urea and insulin concentrations increased after the LPS challenge, suggesting establishment of insulin resistance and modifications of glucose metabolism to support acute inflammation in both CONT and REST. Nonetheless, nutrient-restricted cows had delayed plasma insulin and glucose responses to LPS, smaller insulin AUC, but greater glucose AUC compared to

56 CONT, despite the limited nutrient availability to sustain inflammation response. Undernutrition
57 altered peripheral metabolic responses to an intramammary LPS challenge, but had limited effects
58 on selected indicators of inflammation response in early lactation cows.

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60 **Key words:** dairy cow, negative energy balance, inflammation, LPS

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INTRODUCTION

64 Dairy cows experience profound shifts in hormonal and nutritional status during the
65 periparturient period, rely on extensive mobilization of body fat, proteins, and bone minerals to
66 support the onset of lactation, and often present altered plasma metabolite and mineral profiles
67 (Pires et al., 2013, Valdecabres et al., 2018). Concomitantly, multiple aspects of immune function
68 are modified and the occurrence of metabolic disorders and inflammatory diseases peaks during this
69 period. For instance, leucocyte function is impaired in early lactation compared to midlactation
70 (Shuster et al., 1996), and in cows with increased markers of negative energy balance (Kremer et
71 al., 1993, Hammon et al., 2006). Leucocyte dysfunction is associated with development of metritis
72 and severity of experimental mastitis (Kremer et al., 1993, Shuster et al., 1996, Hammon et al.,
73 2006). Furthermore, clinical mastitis during early lactation may impact reproduction and functional
74 longevity (Albaaj et al., 2017, Hertl et al., 2018).

75 Complex homeorhetic and homeostatic adaptations need to take place during early lactation
76 to orchestrate the partitioning of limiting nutrients towards the mammary gland. These include the
77 establishment of insulin resistance in peripheral tissues and enhanced hepatic glucose output via
78 glycogenolysis and gluconeogenesis (Bell and Bauman, 1997, Doepel et al., 2009). Experimental
79 acute immune activation imposes a glucose drain that may overwhelm the adaptive capacity of the
80 animal and disrupt homeostasis (Kvidera et al., 2017). Accordingly, ovine genetic lines selected for

81 divergent milk SCC also present divergent metabolic profiles in response to negative energy
82 balance in early lactation. The high SCC sheep strain experience a greater metabolic burden during
83 experimental energy restriction than the low SCC strain, further suggesting a link between immune
84 function and metabolic robustness (Bouvier-Muller et al., 2016).

85 Feed restriction models have been used extensively to study the ramifications of nutrient
86 deficit, lipomobilization and related metabolic deviations on biological functions at various
87 lactation stages (Veenhuizen et al., 1991, Perkins et al., 2002, Moyes et al., 2009, Gross et al., 2011,
88 Bjerre-Harpøth et al., 2012). On the other hand, lipopolysaccharide (LPS) challenges have been
89 employed as a model to study the mechanisms and factors modulating Gram-negative bacteria-
90 associated mastitis in dairy cows (Paape et al., 2003, Schukken et al., 2011), including the
91 interactions with nutritional status (Perkins et al., 2002), diet fatty acid composition (Greco et al.,
92 2015) and effects of immune activation on metabolic and mineral homeostasis (Waldron et al.,
93 2003a, Waldron et al., 2003b, Kvidera et al., 2017). Challenges with LPS have the advantage of
94 inducing repeatable, self-resolved inflammatory responses, with both local and systemic effects,
95 while avoiding the adverse consequences of an infection (Hoeben et al., 2001, Vernay et al., 2012).
96 Relatively few studies have focused on experimentally-induced undernutrition, the resulting
97 negative energy balance, and responses to intramammary immune challenges in vivo (Perkins et al.,
98 2002, Moyes et al., 2009, Bouvier-Muller et al., 2016). Nonetheless, research employing feed-
99 restricted midlactation cows as an experimental model have failed to show unequivocal associations
100 between undernutrition and alterations of the immune response at whole animal level (Perkins et al.,
101 2002, Moyes et al., 2009).

102 Immune dysfunction in early lactation cows is driven in part by the prioritization of nutrient
103 partitioning towards milk secretion and altered metabolic milieu resulting from intense
104 lipomobilization and ketosis, and potential deficiencies in key nutrients such as glucose, calcium
105 and specific amino acids (Kremer et al., 1993, Hammon et al., 2006, Martinez et al., 2014, Moyes,

106 2015). However, large nutrient imbalances typical of early lactation are difficult to reproduce later
107 in lactation via feed restriction. For instance, the metabolic and hormonal responses to severe
108 nutrient restriction are of lesser amplitude after peak compared to early lactation (Gross et al., 2011,
109 Bjerre-Harpøth et al., 2012). Plasma BHB increased when nutrient intake was restricted in early
110 lactation cows by feeding a high-straw diet, but was not affected in mid and late lactation, despite
111 marked negative energy balance and corresponding shifts in plasma glucose and NEFA (Bjerre-
112 Harpøth et al., 2012). We hypothesized that experimentally-induced undernutrition would modify
113 animal responses to mammary inflammation in early lactation cows. A high-straw diet was
114 employed as a restriction model because its low nutrient density and digestibility decrease nutrient
115 intake without the need to limit the quantity of feed offered. The experiment was performed during
116 early lactation because the metabolic deviations in response to nutritional challenges are greatest
117 during this period. The objective was to assess the effects of nutrient restriction on whole-animal
118 responses to an intramammary LPS challenge in early lactation cows.

120 MATERIALS AND METHODS

121 *Animals, Diets, LPS Challenge and Housing*

122 All procedures were approved by the regional ethics committee on animal experimentation
123 (APAFIS #2018062913565518).

124 Seventeen multiparous Holstein-Friesian cows were studied from the last 3 weeks of
125 gestation until 7 weeks post-partum. From 24 to 27 ± 3 DIM (mean ± SD), cows were either
126 allowed continuous ad libitum intake of a lactation TMR (CONT, n = 9), or were fed a TMR
127 containing 48% of chopped barley straw (DM-basis) during 96 h (REST, n = 8). Diet ingredient and
128 nutrient composition are presented in Table 1. Cows were selected based on previous lactation
129 performance, health records and expected calving date, and randomly allocated to treatments before
130 the initiation of the experiment.

131 At 72 h of differential diet between CONT and REST, one healthy rear mammary quarter
132 was injected with 50 µg of LPS (*E. coli* O111:B4, Ultrapure LPS-EB, InvivoGen, Toulouse,
133 France) diluted in 10 ml of sterile saline (CDM Lavoisier, Paris, France) containing 0.5 mg/mL of
134 BSA (cell culture grade, endotoxin free, A9576, Sigma-Aldrich, Saint-Quentin-Fallavier, France).
135 After morning milking, teats were cleaned and the tips swabbed with cotton containing 70%
136 ethanol. The LPS solution prepared extemporaneously in a sterile environment was injected into the
137 teat cistern via the teat canal, using a sterile disposable syringe fitted with a teat cannula, and the
138 udder was massaged briefly.

139 Cows were housed in free-stalls equipped with individual feed bunks and automatic gates,
140 except from one week before and until 3 days after the LPS challenge, when they were housed in a
141 tie-stall barn to acclimate, facilitate frequent sampling and animal care. Cows were milked twice
142 daily at approximately 0900 and 1600 h, had fresh feed offered once daily after morning milking
143 and free access to drinking water.

144

145 ***Phenotyping Early Lactation and Responses to Undernutrition***

146 Offered feed and refusals were weighted, and subsamples were collected four days per week
147 to determine DM content after 48 h at 80 °C, and calculate DMI (Lerch et al., 2012). Pre- and
148 postpartum TMR samples were collected weekly, pooled, and analyzed for nutrient composition by
149 wet chemistry (Table 1). Energy balance was estimated as energy intake minus requirements for
150 maintenance and production, calculated according to the INRA system (INRA, 2007). Milk yield
151 was recorded daily, milk samples were collected at 4 consecutive milkings each week and analyzed
152 for composition and SCC by near-infrared spectrometry and Fossomatic FC method (Foss Electric,
153 Hillerød, Denmark), respectively. Weighted milk component means were computed according to
154 PM/AM production. In order to characterize the responses to feed-restriction, DMI and milk
155 composition were measured daily in both groups from 2 d prior to restriction until 7 d after

156 refeeding the lactation TMR to cows in the REST group. Body weight and body condition score
157 (BCS, 6-point, 0 to 5 scale) were recorded weekly, one day before diet change, and on the last day
158 of restriction.

159 Blood samples were collected from coccygeal vessels before the morning feeding on
160 Wednesday of week -3, -2, -1, 1, 2, 3, 5, 6 and 7 relative to calving, corresponding to -18, -11, -4, 8,
161 15, 21, 35, 42 and 48 DIM, respectively. The first postpartum blood sample was collected after 3
162 DIM. Samples were collected from jugular veins at -24, 24, 48 h relative to initiation of restriction
163 and at equivalent period for CONT.

164

165 ***Phenotyping Responses to LPS challenge***

166 Cows were screened for mastitis one week prior and before the milking preceding the LPS
167 challenge using the California mastitis test (Neodis, Rambouillet, France), and SCC analyses of
168 foremilk samples collected from the rear quarters (Galilait, Theix, 63122 Saint Genès-Champanelle,
169 France). Only cows with SCC lower than 100 000 cells/mL in a rear quarter were included in the
170 study. Foremilk samples were collected from the LPS challenged quarters immediately before the
171 morning milking that preceded the LPS injection (time 0), and at 4, 6, 10 and 24 h after LPS
172 injection. These quarter milk samples were analyzed by ELISA for IL-8, TNF- α , CXCL3 and IL1-
173 β . For the ELISA bCXCL3 and bIL-8 assays, the sequence of incubation steps, all performed at
174 room temperature, was: affinity-purified Ab to C-terminal peptide of bCXCL3 or bIL-8 (2 μ g/mL)
175 in PBS overnight; blocking of the plate with 0.5% gelatin in PBS for 1 h; incubation at the
176 appropriate dilution or a series of twofold dilutions of standard bCXCL3 or bIL-8 for 2h;
177 biotinylated IgG fraction of rabbit antiserum to recombinant bCXCL3 (1 μ g/mL) or mouse
178 monoclonal Ab to ovine IL-8 (0.5 μ g/mL; clone 8M6; Bio-Rad Laboratories, Hercules, CA) for 1h;
179 avidin-peroxidase conjugate (Molecular Probes, Thermo Fisher Scientific, Rockford, IL) or
180 peroxidase-conjugated goat Ab to mouse IgG (Jackson Immunoresearch Laboratories, West Grove,

181 PA) diluted 1:20000 for 1 h and finally TMB ELISA substrate. Commercial ELISA were used for
182 TNF- α (R&D Systems, Minneapolis, MN) and IL1- β (Thermo Fisher Scientific, Rockford, IL)
183 according to the manufacturer's instructions. A number of foremilk samples could not be analyzed
184 for SCC for technical reasons, therefore, quarter milk SCC data were excluded to prevent potential
185 bias. The whole-udder composite milk SCC and total number of somatic cells secreted per day were
186 analyzed instead. Total number of somatic cells secreted in milk per day was calculated by
187 converting milk yield from Kg to liters per day (considering a standard density of 1.033), and
188 multiplying the volume of milk by SCC per mL.

189 Jugular blood samples were collected at -1, -0.5, 1, 2, 4, 6, 10 and 24 h relative to LPS
190 injection. Plasma concentrations at 72 h relative to initiation of feed restriction were calculated by
191 averaging values at 1 and 0.5 h before LPS administration. Rectal temperature was recorded at the
192 time of each blood sampling on the day of LPS injection using an electronic thermometer
193 (Veterinär-Thermometer SC 12, SCALA Electronic GmbH, Stahnsdorf, Germany).

194 All blood samples were drawn into evacuated tubes containing EDTA (1.95 mg/mL;
195 Terumo Europe NV, Leuven, Belgium) and centrifuged at 1,400 \times g for 15 min at 4°C. Plasma was
196 conserved at -20°C until analysis for glucose (glucose oxidase method), BHB (D-Beta-
197 Hydroxybutyrate-Dehydrogenase method), urea (glutamate dehydrogenase method; Thermo
198 Electron SAS, France) and NEFA (Acyl-CoA synthase method; Wako, Sodioda, France) using an
199 automatic analyzer (ARENA 20XT, Thermo Fisher Scientific, Cergy Pontoise, France), insulin
200 using a RIA, as previously described (Lerch et al., 2012), and cortisol (Boissy and Bouissou, 1994).
201 Intra-and inter-assay coefficients of variation were 1.4 and 3.1 % for glucose, 2.1 and 3.0% for
202 NEFA, 4.5 and 5.5% for BHB, 5.9 and 8.5% for urea, and 6.9 and 11.8% for insulin, respectively.

203 Biopsies of liver and mammary gland (LPS-infused rear quarter) were performed 24 h after
204 LPS injection, after plasma and quarter milk collection, reported elsewhere.

205

206 *Statistical Analyses and Calculations*

207 Statistical analyses were performed using SAS (version 9.4; SAS Institute Inc., Cary, NC),
208 separately for data collected prepartum, postpartum, and during the time of LPS challenge.
209 Repeated measures data were analyzed by mixed models that included the fixed effects of Diet,
210 Time, and Diet by Time interaction, the random effect of Cow, and Kenward-Rogers adjustment for
211 calculation of denominator degrees of freedom. The Schwarz's Bayesian criterion was used to
212 compare the fitting of different covariance structures, including spatial power, AR(1), ARH(1) and
213 CS. Heterogeneous variance for each Diet was tested whenever suggested by residual plots.
214 Significant interactions of Diet and Time were explored using the SLICE and PDIFF options of the
215 LSMEANS statement. Areas under the curve (AUC) during the 10 and 24 h post LPS challenge
216 were calculated by the incremental (positive and negative) method for plasma metabolites and
217 insulin, and by the positive incremental method for cortisol, after discounting for baseline
218 concentrations (Cardoso et al., 2011) using Microsoft Excel (2013), and analyzed as non-repeated
219 variables. Basal plasma metabolite and insulin concentrations were calculated by averaging values
220 from samples collected at 1 h and 0.5 h prior to LPS injection. Logarithmic transformation of
221 response variables was used whenever needed to comply with the assumptions of normality and
222 homoscedasticity of residuals. When transformation was necessary, least squares means
223 (LSMEANS) and standard error of the mean (SEM) were estimated from untransformed values,
224 whereas *P*-values reflect statistical analysis of transformed data. Values reported are LSMEANS
225 and SEM, unless otherwise stated. The significance level was predefined as $P \leq 0.05$, and trends
226 toward significance at $0.05 < P \leq 0.10$.

227
228 **RESULTS**

229 *Prepartum*

230 Cows in REST group tended ($P = 0.10$) to have greater BCS (2.84 vs 2.47 ± 0.16 ;
231 Supplementary Figure 1A) and plasma BHB (0.56 vs 0.49 ± 0.03) compared to CONT during the 3
232 weeks prepartum. Plasma NEFA concentration increased during late gestation (Time effect, $P =$
233 0.01) and was 0.092 , 0.124 and 0.183 ± 0.021 mM on weeks - 3, -2 and -1 relative to calving,
234 respectively. Dry matter intake was 15.2 , 14.8 and 13.7 ± 0.53 kg (Time effect, $P = 0.11$) during the
235 same period.

236

237 ***Early Lactation and Responses to Undernutrition***

238 Postpartum DMI, milk yield, fat and protein content and yield, NE_L balance, BCS and BW
239 did not differ between the groups before diet change (Figures 1A, 1B and Supplementary Figures
240 1A, 1B, 2A, 2B, 2C and 2D). Feeding a TMR containing 48% straw induced a sudden decrease of
241 DMI and negative energy balance, with milk yield decreasing significantly at the second day of
242 restriction and thereafter (Figures 1A, 1C and 1B).

243 Plasma NEFA, glucose, urea and insulin concentrations did not differ until diet change at 24
244 ± 3 DIM (Figures 2A, 2C, 2D and 2E). Nonetheless, a trend for greater BHB was observed on the
245 first week of lactation for REST compared to CONT cows (Figure 2B). Per design, after nutrient
246 restriction, the REST cows presented significantly greater concentrations of plasma NEFA, BHB
247 and urea, and lower concentrations of glucose and insulin, compared to CONT cows (Figure 2).
248 Therefore, the metabolic profile was profoundly altered at 72 h after dietary treatments (Table 2,
249 Figure 2). Plasma cortisol did not differ between REST and CONT groups during the first 72 h of
250 dietary treatments (data not shown).

251 Milk composition and component yield are presented in Supplementary Figure 2. Milk fat
252 percentage increased during feed restriction and returned to pre-restriction concentrations on the
253 same day of refeeding the regular lactation diet (Supplementary Figure 2A). Lactose content was
254 depressed during the last 3 days of restriction (Supplementary Figure 2E). Milk, fat, protein and

255 lactose yields decreased in REST and returned to pre-restriction values within 7 days after refeeding
256 (Supplementary Figures 2B, 2D and 2F).

257 Body condition score decreased postpartum but did not differ significantly between
258 treatments throughout the study (Supplementary Figure 1A). Nonetheless, BW was lower at the end
259 of restriction period for REST than control ($P = 0.01$; Supplementary Figure 1B), but BW
260 differences were not observed thereafter.

261

262 ***Responses to Intramammary LPS challenge***

263 The inflammatory challenge modified some production variables during the 24 h following
264 the intramammary LPS injection in CONT cows. Control cows decreased DMI (-2.5 kg DM; $P <$
265 0.01; Figure 1A), milk protein yield ($P < 0.01$), lactose content ($P = 0.02$), and tended to decrease
266 protein content ($P = 0.07$) on the day of the LPS challenge (Supplementary Figures 2C, 2D and 2E).
267 In CONT cows, milk fat content was greatest during the 48 h following LPS injection ($P < 0.05$)
268 compared all other DIM, and milk fat yield was increased ($P < 0.05$) during the same period
269 (Supplementary Figures 2A and 2B). In REST cows, milk production tended to decrease (-5.9 kg; P
270 = 0.07) on the day following the LPS challenge (Figure 1B). Milk fat content increased ($P = 0.04$),
271 whereas lactose concentration and protein yield decreased ($P < 0.001$) on the day of LPS challenge,
272 as observed for CONT (Supplementary Figures 2A, 2E, and 2D, respectively).

273 Composite milk SCC increased sharply on the day of LPS challenge in both CONT and
274 REST cows (Figure 1D), but SCC was greater for REST ($6.91E6$ vs $1.91E6 \pm 337E3$ cells/mL, $P =$
275 0.01; Diet \times Time interaction: $P = 0.04$). Furthermore, REST cows secreted a greater total number
276 somatic cells in milk than CONT on the day of LPS injection ($1.35E11$ vs $0.67E11 \pm 0.203E11$
277 cells; $P = 0.03$). Composite milk SCC returned to pre-challenge values after 31 and 34 DIM for
278 REST and CONT, respectively.

279 The increment in rectal temperature above pre-LPS challenge values and measured rectal
280 temperature are presented in Figure 3A and Supplementary Figure 3A, respectively. Rectal
281 temperature after 72 h of diet treatments, measured before LPS injection, did not differ between
282 groups (38.3 vs 37.8 ± 0.2 °C for CONT and REST, respectively; Supplementary Figure 3A). For
283 both treatments, temperature increased significantly at 4, 6 and 10 h after LPS injection (Figure 3A,
284 Supplementary Figure 3A). The average temperature during the day of LPS challenge (measured at
285 0, 1, 2, 4, 6 10 and 24 h) was lower for REST than CONT (38.4 vs 38.9 ± 0.14 °C, *P* = 0.03;
286 Supplementary Figure 3A). Nonetheless, restriction did not alter temperature increment relative to
287 pre-LPS challenge values (Figure 3A). Maximal temperature increment was +2.1 ± 0.15 °C, at 6 h
288 after LPS injection.

289 Plasma cortisol increment from baseline in response to LPS and plasma cortisol
290 concentrations are presented in Figure 3B and Supplementary Figure 3B, respectively. Plasma
291 cortisol concentrations did not differ between REST and CONT before the LPS challenge (19.1 vs
292 24.5 ± 7.3 ng/mL, respectively), peaked in samples collected 4 h after LPS injection (64.5 ± 5.0
293 ng/mL) and returned to baseline at 10 h post-injection (Supplementary Figure 3B). Nonetheless,
294 REST had a greater cortisol increment above baseline in response to LPS than CONT (*P* = 0.05;
295 Figure 3B), and cortisol response AUC was greater when samples collected at 24 h were considered
296 (344 vs 175 ng/mL × 24 h, *P* = 0.02; Table 2). Milk IL-8, IL-1β, TNF-α and CXCL3 concentrations
297 did not differ between CONT and REST in foremilk samples collected from the LPS injected
298 quarters immediately before and at 4, 6, 10 and 24 h relative to LPS challenge (Figures 3C, 3D, 3E
299 and F3). Milk CXCL3 AUC in response to LPS challenge tended to be lower for REST than CONT
300 during the 10 h after the challenge (*P* = 0.08; 9.05E3 vs 11.87E3 ± 1.33E3 ng/mL × 10 h,
301 respectively). Plasma insulin and metabolite responses to LPS challenge are presented in Table 2
302 and Figures 4A, 4B, 4C and 4D. Plasma insulin concentration increased for both CONT and REST
303 after LPS challenge (Time effect, *P* < 0.001; Figure 4A). Nonetheless, for CONT, insulin

304 concentration was significantly greater than basal at 1 h after LPS challenge and reached maximal
305 concentration after 6 h, with a 2.7-fold increase compared to pre-challenge values. For REST, the
306 maximal insulin concentration was reached after 10 h, with a 1.9-fold increase compared to pre-
307 challenge concentrations. Therefore, insulin AUC in response to LPS challenge was smaller in
308 REST compared to CONT cows ($P < 0.001$, Table 2). Nonetheless, absolute changes in NEFA,
309 BHB and glucose were greater in the REST group, as shown by AUC in Table 2. Plasma glucose
310 was lower for REST than CONT on the day of LPS challenge (Diet effect: $P = 0.02$; Figure 4B).
311 When looking at Time effects within each dietary treatment, plasma glucose concentrations
312 increased overtime for REST cows, becoming greater than baseline values at 10 h post LPS
313 injection ($P < 0.01$; Figure 4A). Meanwhile, in the CONT group, glucose concentration decreased
314 initially below baseline at 4 h ($P < 0.01$) and rebounded above baseline at 10 h ($P < 0.05$; Figure
315 4A). Plasma NEFA concentration decreased sharply after LPS, from 1.672 and 0.371 ($P < 0.001$) to
316 a nadir of 0.599 ± 0.12 and 0.101 ± 0.10 mM at 4 h after injection, for REST and CONT,
317 respectively, corresponding to 36 and 22% of pre-challenge concentrations (Figure 4C; Diet and
318 Diet \times Time interaction, $P < 0.001$). In the REST group, plasma BHB was 2.98 mM before the LPS
319 challenge, and decreased significantly between 1 to 10 h after LPS injection, reaching a nadir of
320 1.78 ± 0.43 mM at 10 h. In contrast, BHB was 0.69 mM before LPS injection in CONT, increased
321 significantly above baseline between 1 to 10 h after LPS, reaching 1.23 ± 0.22 mM at 6 h post-
322 challenge (Diet and Diet \times Time interaction, $P \leq 0.001$; Figure 4D). Plasma urea concentration
323 increased over time after LPS injection ($P < 0.01$) for both CONT and REST groups, and was
324 significantly greater at 1, 2, and 4 h post-challenge compared to before LPS challenge. A Diet \times
325 Time interaction was not observed during this period ($P = 0.71$; data not shown). At 24 h after LPS
326 challenge (corresponding to 96 h of dietary treatments), plasma insulin, BHB, and glucose had
327 returned to pre-LPS challenge concentrations for both CONT and REST, whereas in REST, plasma
328 NEFA and urea tended to decrease below the pre-LPS challenge concentrations.

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DISCUSSION

This study assessed whether undernutrition and resulting metabolic imbalance modify the inflammatory response in early lactation cows, as previous research involving midlactation cows showed limited effects of undernutrition on animal-level responses to LPS and *S. uberis* challenges (Perkins et al., 2002, Moyes et al., 2009). Despite the extreme negative energy balance induced in early lactation cows, few of the inflammation indicators measured in this study differed between normal-fed and restricted cows. Nonetheless, metabolic responses to LPS differed between the two treatments, and suggest a prioritization of nutrient partition to sustain immune response during an acute intramammary inflammation.

Early Lactation and Responses to Nutrient Restriction

Production and most indicators of metabolic status did not differ between CONT and REST during the first 3 weeks of lactation before restriction. Exceptions were a trend for greater plasma BHB on week 1 observed in REST, and increased milk content in cis-9 18:1, which is potentially derived from adipose tissue, and decreased in FA synthesized de novo (Σ 10:0 to 15:0) during the first two weeks of lactation (data not shown). These differences may be explained by the trend for greater prepartum BCS observed in REST cows.

The experimental undernutrition model employed in the present study (diet containing 48% straw, DM basis) induced repeatable decreases in DMI, energy balance, milk production, and rapid modifications of metabolite and hormonal profiles, and is in agreement with previous research employing a diet containing 60% straw (Bjerre-Harpøth et al., 2012). Per design, the metabolic status was profoundly altered in REST before initiation of LPS challenge (i.e., elevated NEFA, BHB, and low insulin and glucose concentrations in plasma). For these reasons, the nutritional challenge during early lactation may be better suited to assess the ramifications of undernutrition

354 and metabolic imbalance on immune activation at whole animal level, compared to later in lactation
355 (Perkins et al., 2002, Moyes et al., 2009).

356

357 *Responses to Intramammary LPS*

358 **Immune Activation.** Immune activation following the intramammary LPS injection was confirmed
359 by increased rectal temperature, composite SCC, foremilk milk IL-8, IL-1 β , TNF- α and CXCL3
360 concentrations, and anecdotal observation of mammary gland symptoms (e.g., swelling, hardness,
361 redness, and soreness) and modified quarter milk appearance (clots and yellowish color).

362 Concerning the effect of diet on indicators of inflammatory response, only cortisol response AUC
363 and composite milk SCC differed between CONT and REST. These findings in early lactation cows
364 are in agreement with previous research during midlactation involving partial feed restriction and
365 experimental inflammation by LPS or *Streptococcus uberis* infection (Perkins et al., 2002, Moyes et
366 al., 2009).

367 Restricted cows had greater composite milk SCC the day following LPS injection than
368 CONT, but the biological implications of this observation are uncertain. The SCC difference is not
369 explained by a concentration effect due to differences in milk volume because the total number of
370 secreted somatic cells was higher in REST than in CONT cows on the same day, despite their lower
371 milk yield. Our results contrast with those obtained in midlactation cows restricted to 80% of
372 energy requirements for 2 weeks, followed by an intramammary LPS challenge, where no
373 difference in quarter milk SCC was observed (Perkins et al., 2002), and to decreased SCC response
374 to LPS when hyperketonemia was induced in midlactation cows via prolonged i.v. BHB perfusion
375 (Zarrin et al., 2014a). Several factors may explain the enhanced SCC and total SC secretion in
376 REST cows. Mammary epithelial integrity may be altered in early lactation cows undergoing
377 extreme feed-restriction, potentially facilitating the transfer of plasma constituents and PMN into
378 alveolar milk. **Moderate feed restriction promotes exfoliation of epithelial cells in milk, and**

379 mammary epithelium permeability increases during severe restriction (Stumpf et al., 2013, Herve et
380 al., Accepted). Intramammary LPS reduces the alveoli blood-milk barrier integrity (Wellnitz et al.,
381 2013), via the opening of tight junctions and potential cell damage (Wellnitz et al., 2016).
382 Restricted cows may have been in a proinflammatory state due to extremely low DMI, high plasma
383 NEFA and ketosis. In fact, previous research has shown that selected plasma markers of
384 inflammation are increased before the onset and during ketosis in periparturient cows (Abuajamieh
385 et al., 2016). Some fatty acids at high concentrations are agonists of TLR4, the pathogen
386 recognition receptor on host cells that interacts with LPS to elicit the inflammatory response (Shi et
387 al., 2006, Sordillo and Mavangira, 2014).

388 Nonetheless, the greater milk SCC observed in REST cows after LPS injection does not
389 imply an enhanced immune response, because leucocyte function was probably modified in REST.
390 For instance, leucocyte phagocytic activity is impaired in early lactation cows experiencing
391 negative energy balance and elevated plasma NEFA (Hammon et al., 2006) and the severity of
392 experimental *E. coli* mammary infection seems more pronounced in ketotic cows (Kremer et al.,
393 1993), as leucocyte chemotaxis (Suriyasathaporn et al., 1999) and respiratory burst (Hoeben et al.,
394 1997) are impacted by BHB at concentrations lower than those observed in REST cows at the time
395 of LPS challenge.

396
397 ***Metabolic Responses to Immune Activation.*** The systemic effects of LPS challenge on fat and
398 carbohydrate metabolism are potentially mediated by transient increases in circulating cortisol,
399 TNF-alpha and other proinflammatory cytokines, that modify secretion of insulin, glucagon and
400 other hormones (Steiger et al., 1999, Hoeben et al., 2001, Waldron et al., 2003a, Kushibiki, 2011),
401 as well as the response of target tissues to hormones such as insulin (Zarrin et al., 2014b). Direct
402 effects of intramammary LPS on whole body metabolism should have been minimal in our study,
403 because previous research using greater LPS doses did not detect LPS in the systemic circulation

404 after its administration into the mammary gland cistern, and report only short-term increases in
405 plasma LPS after i.v. injection (Hoeben et al., 2001).

406 The increment in cortisol concentrations above pre-challenge baseline and cortisol response
407 AUC were greater for REST than CONT cows (Figure 3B and Table 2). It is unknown whether
408 these differences in cortisol response have contributed to the metabolic effects of LPS observed in
409 our study. Cortisol concentrations *per se* did not differ between CONT and REST cows
410 (Supplementary Figure 3B), only the cortisol increment above pre-challenge baseline. The increase
411 in plasma insulin concentration was delayed and insulin AUC in response to LPS was attenuated in
412 **nutrient-restricted** early lactation dairy cows. This effect may be explained by the hypoglycemic
413 state of REST cows.

414 Plasma glucose increased gradually after LPS in REST, leading to a positive glucose
415 response AUC, despite a concomitant insulin increase. A transient increase in plasma glucose
416 concentration has been observed in response to LPS injection (Zarrin et al., 2014b, Kvidera et al.,
417 2017). Because REST cows were in early lactation, nutrient-restricted and ketotic at the time of
418 LPS challenge, glycogenolysis could not have been a source of glucose, as depletion of liver
419 glycogen reserves precedes experimentally induced ketosis (Veenhuizen et al., 1991). Previous
420 research suggests that hepatic conversion of propionate to glucose may be upregulated after LPS
421 challenge in fed mid-lactation cows (Waldron et al., 2003a). The increase in plasma urea
422 concentration observed until 4 h after LPS injection in our study probably reflects enhanced
423 reliance on amino acids to sustain inflammatory response and gluconeogenesis (Gifford et al., 2012,
424 Greco et al., 2015).

425 Plasma NEFA decreased sharply after LPS injection for both CONT and REST cows. It has
426 been suggested that plasma NEFA changes post LPS challenge are due to hyperinsulinemia
427 (Kvidera et al., 2017), but a NEFA decrease after LPS injection preceded the plasma insulin
428 increment in REST cows. As a consequence, the typical inverse diurnal relationship between

429 plasma NEFA and insulin (Allen, 2014) is not observed in REST cows challenged with
430 intramammary LPS. Restricted cows were ketotic at the time of LPS injection. The decrease in
431 plasma BHB post LPS challenge is in agreement with the concomitant reduction in plasma NEFA,
432 may result from downregulated liver ketogenesis, increased ketone utilization or a combination of
433 both, and may constitute a glucose-sparing strategy (Zarrin et al., 2014b, Kvidera et al., 2017). The
434 reduction of plasma BHB preceded significant time effects in plasma insulin and glucose, as
435 observed for plasma NEFA. Other factors must have mediated early changes in plasma NEFA and
436 BHB concentrations after LPS challenge in REST cows.

437 In CONT cows, plasma BHB concentrations increased after LPS, despite decreased NEFA
438 and increased insulin, together with a transient decrease in glucose. In CONT cows, these profiles
439 share common patterns with diurnal variations related to morning feed intake (Allen, 2014, Piantoni
440 et al., 2015), but not in REST cows. Therefore, postprandial effects may have played a role in
441 plasma metabolite and insulin profiles observed in CONT cows during the first hours after LPS
442 challenge because fresh TMR was offered after morning milking. Nonetheless, previous research
443 suggests that metabolic effects of high LPS doses are rather independent of feed intake (Steiger et
444 al., 1999). The potential intake effects should be minor in REST cows due to the low DMI, low
445 concentrate content, and low fermentability of the high-straw TMR. Time-changes in plasma
446 metabolite and insulin concentrations in REST cows should have been driven by LPS-induced
447 inflammation.

448 The increase in plasma glucose and insulin concentrations concomitant with inflammatory response
449 (i.e., fever, increased cortisol, milk IL-8, IL-1 β , TNF- α and CXCL3) indicate the establishment of
450 insulin resistance in both CONT and REST cows. Insulin resistance constitutes a homeorhetic
451 adaptation to support the massive glucose requirements of an acute inflammation response, and is
452 probably mediated by sharp increases in systemic cortisol and inflammation cytokines, such as
453 TNF-alpha (Waldron et al., 2003a, Kushibiki, 2011, Vernay et al., 2012, Moyes et al., 2014, Zarrin

454 et al., 2014b, Kvidera et al., 2017). Restricted cows were probably in an insulin resistant state
455 before the LPS challenge, due to hormonal changes, elevated circulating NEFA (Pires et al., 2007)
456 and ceramides (Davis et al., 2017). Acute adaptations may have allowed REST cows to direct
457 glucose and other nutrients for immune activation, as suggested by plasma metabolite and insulin
458 profiles during the hours following intramammary LPS injection. Nonetheless, the ability to cope
459 with prolonged or repeated challenges and resulting metabolic modifications may be limited
460 (Waldron et al., 2003a, Bradford et al., 2015, Kvidera et al., 2017), for instance in the case of
461 chronic inflammation and intake depression during early lactation, which may disrupt homeostatic
462 capacity of the dairy cow.

464 CONCLUSIONS

465 Experimentally-induced undernutrition to early lactation Holstein cows had limited effects
466 on animal-level indicators of acute inflammation measured in this study, as previously observed in
467 midlactation cows. The short-term response following intramammary LPS injection suggests
468 modifications of nutrient partitioning to support immune activation via **an apparent** establishment of
469 insulin resistance in both CON and REST cows, despite intense lipomobilization, ketosis, and
470 limited availability of precursors for glucose synthesis in REST cows. Inflammation during early
471 lactation (acute or chronic) may impose a metabolic burden due to aggravated insulin resistance,
472 utilization of glucose and other limiting nutrients to support immune activation, and further
473 challenge the homeostasis mechanisms of early lactation cows.

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636 **Table 1.** Diet ingredient, nutrient and fatty acid composition. Multiparous Holstein cows were
 637 either allowed ad libitum intake throughout the study (Control, n = 9), or underwent 4 days of
 638 nutrient restriction (Restricted, n = 8) by receiving a ration composed of 48% (DM-basis) straw
 639 from 24 to 27 ± 3 DIM (mean ± SD).

	Dry-period ¹	Lactation Control	Lactation Restriction
Ingredient (% DM)			
Corn silage	43.5	29.0	16.1
Grass silage	40.0	25.5	14.9
Barley straw	5.5	3.5	48.2
Corn grain	8.2	24.2	4.0
Soybean meal	2.8	16.9	15.4
Mineral and vitamin mix ²	-	0.9	1.4
Forage/Concentrate ratio	89/11	58/42	79.2/20.8
Nutrient composition (% DM)			
Net energy (MJ/kg DM)	6.49	7.10	5.16
PDI ³ (g/kg DM)	76	106	93
CP	12.7	17.4	12.2
NDF	42.0	33.5	57.5
ADF	23.7	15.3	33.9
Fat	2.2	2.1	1.2
Starch	19.0	27.5	8.6
Ash	7.3	6.5	8.2
Fatty acid composition (g/100 g FA)			
C16:0	16.49	15.98	20.76
C18:0	1.94	2.27	3.01
<i>cis</i> -9 C18:1	14.36	19.60	17.2
C18:2n6	33.56	40.58	29.60
C18:3n3	20.00	12.26	11.92
Other	13.65	9.31	17.7

¹ During 4 weeks prior to expected calving.

² Mineral and vitamin mix contained 2.5 % P, 20% Ca, 4.5% Mg, 3.5% Na, 1% S, 400,000 IU/kg of vitamin A, 120,000 IU/kg of vitamin D3, 1,600 IU/kg of vitamin E, 1.3 g/kg of Cu, 5 g/kg of Zn, 3.5 g/kg of Mn, 90 mg/kg of I, 36 mg/kg of Co, and 20 mg/kg of Se; Galaphos Midi duo granule, CCPA, Aurillac, France.

³ Protein truly digestible in the small intestine (INRA, 2007).

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Table 2. Effects of nutrient restriction on plasma cortisol, insulin and metabolite responses to LPS challenge in early lactation cows. Multiparous Holstein cows were either allowed ad libitum intake of a regular diet throughout the study (Control, n = 9), or underwent 96 h of nutrient restriction (Restricted, n = 8) by receiving a ration composed of 48% (DM-basis) straw from 24 to 27 ± 3 DIM (mean ± SD). One healthy rear mammary quarter was injected with 50 µg of LPS (E. coli 0111:B4) 72 h after initiation of dietary treatments. Blood samples were collected at -1, -0.5, 1, 2, 4, 6, 10 and 24 h relative to LPS injection.

	Treatments		SEM	P
	Control	Restricted		
Cortisol				
72 h (ng/mL) ¹	24.5	19.1	5.8	0.51
AUC _{10h} ²	122	202	32	0.09
AUC _{24h} ³	175	344	48	0.02
Insulin				
72 h ¹ (µIU/mL)	17	11	1.6	0.009
AUC _{10h} ²	174	42	21	<0.001
AUC _{24h} ³	339	120	44	0.002
Glucose				
72 h (mM) ²	3.83	2.78	0.23	0.005
AUC _{10h} ³	-0.94	3.56	0.94	0.003
AUC _{24h}	1.22	11.78	3.00	0.02
NEFA				
72 h (mM) ¹	0.37	1.67	0.05 to 0.17	<0.001
AUC _{10h} ²	-1.96	-9.05	0.36 to 9.05	0.002
AUC _{24h} ³	-2.76	-19.31	0.88 to 3.86	<0.001
BHB				
72 h (mM) ¹	0.69	2.98	0.12 to 0.47	<0.001
AUC _{10h} ²	3.68	-6.05	1.51	<0.001
AUC _{24h} ³	6.37	-14.68	3.95	0.002
Urea				
72 h (mM) ¹	4.44	5.77	0.19 to 0.54	0.03
AUC _{10h} ²	2.26	-1.23	1.26	0.06
AUC _{24h} ³	0.15	-12.63	3.35	0.01

¹ Average concentration at 1 and 0.5 h prior to LPS challenge.

² Area under the curve during first 10 h post LPS (concentration units × 10 h).

³ Area under the curve during first 24 h post LPS (concentration units × 24 h).

655

656

657 Figure 1: Dry matter intake (DMI, A), milk yield (B), energy balance (C), and somatic cell count
658 (SCC, D). Multiparous Holstein cows were either allowed ad libitum intake of a regular diet
659 throughout the study (Control, n = 9), or underwent 4 days of nutrient restriction (Restricted, n = 8)
660 by receiving a ration composed of 48% (DM-basis) straw from 24 to 27 ± 3 DIM (mean ± SD). One
661 healthy rear mammary quarter was injected with 50 µg of LPS (*E. coli* 0111:B4) 72 h after
662 initiation of dietary treatments. *P*-values for SCC reflect statistical analysis with log-transformed
663 data. Values are LSM ± SEM.

664

665 Figure 2. Plasma NEFA (A), BHB (B), glucose (C), urea (D) and insulin (E) concentrations.
666 Multiparous Holstein cows were either allowed ad libitum intake of a regular diet throughout the
667 study (Control, n = 9), or underwent 4 days of nutrient restriction (Restricted, n = 8) by receiving a
668 ration composed of 48% (DM-basis) straw from 24 to 27 ± 3 DIM (mean ± SD). One healthy rear
669 mammary quarter was injected with 50 µg of LPS (*E. coli* 0111:B4) 72 h after initiation of dietary
670 treatments. Values are LSM ± SEM.

671

672 Figure 3. Effects of nutrient restriction on rectal temperature increment (A), plasma cortisol
673 increment (B), milk IL-8 (C), IL-1β (D), TNF-α (E) and CXCL3 (F) concentrations in response to
674 LPS challenge in early lactation cows. Multiparous Holstein cows were either allowed ad libitum
675 intake of a regular diet throughout the study (Control, n = 9), or underwent 96 h of nutrient
676 restriction (Restricted, n = 8) by receiving a ration composed of 48% (DM-basis) straw from 24 to
677 27 ± 3 DIM (mean ± SD). One healthy rear mammary quarter was injected with 50 µg of LPS (*E.*
678 *coli* 0111:B4) 72 h after initiation of dietary treatments. Rectal temperature was recorded and blood
679 samples collected before and at 1, 2, 4, 6, 10 and 24 h; foremilk milk samples were collected from

680 the injected mammary quarter before and at 4, 6, 10 and 24 h relative to LPS administration. Values
681 are LSM \pm SEM.

682

683 Figure 4. Effects of nutrient restriction on plasma insulin and metabolite concentration in response
684 to LPS challenge in early lactation cows. Multiparous Holstein cows were either allowed ad libitum
685 intake of a regular diet throughout the study (Control, n = 9), or underwent 96 h of nutrient
686 restriction (Restricted, n = 8) by receiving a ration composed of 48% (DM-basis) straw from 24 to
687 27 ± 3 DIM (mean \pm SD). One healthy rear mammary quarter was injected with 50 μ g of LPS (*E.*
688 *coli* 0111:B4) 72 h after initiation of dietary treatments. Blood samples collected before and at 1, 2,
689 4, 6, 10 and 24 h relative to LPS injection. Values are LSM \pm SEM.

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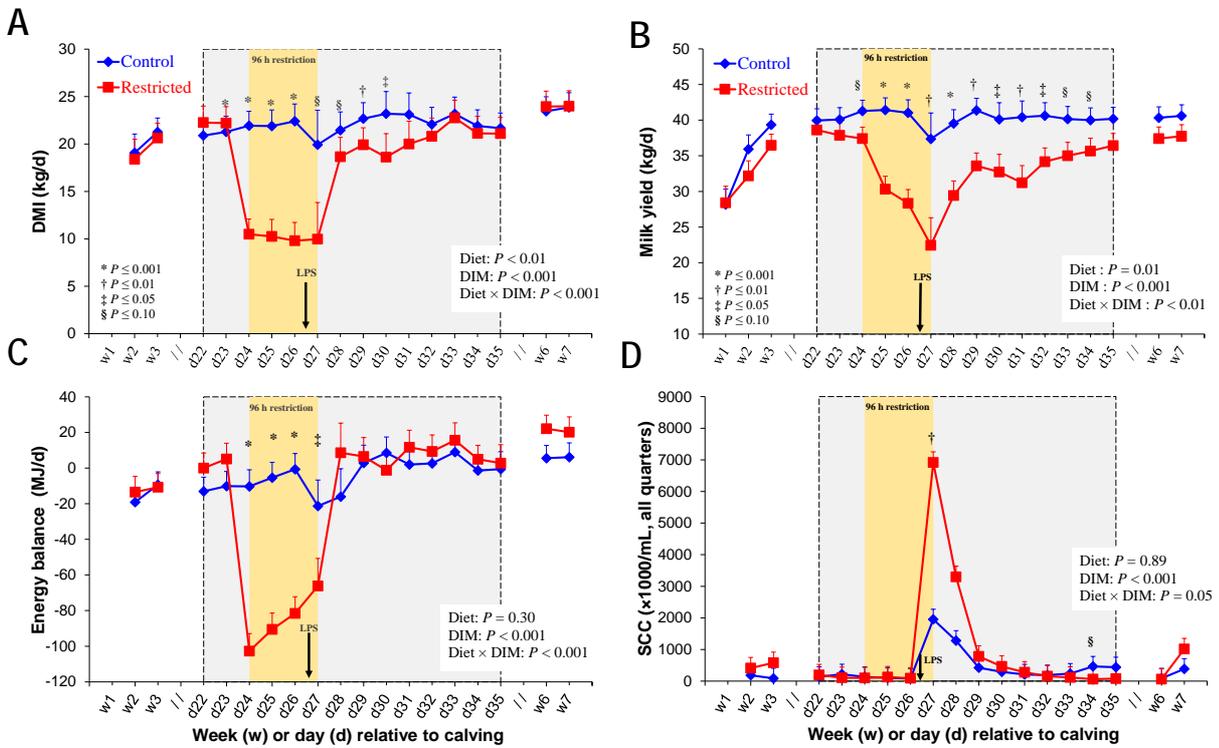
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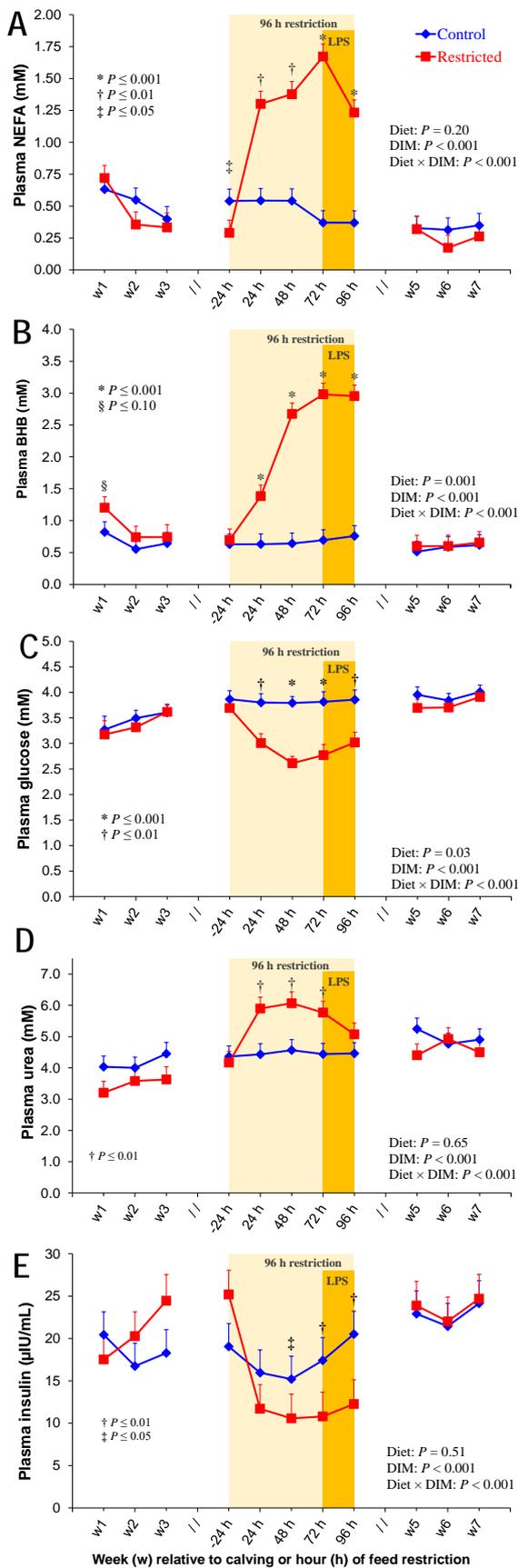
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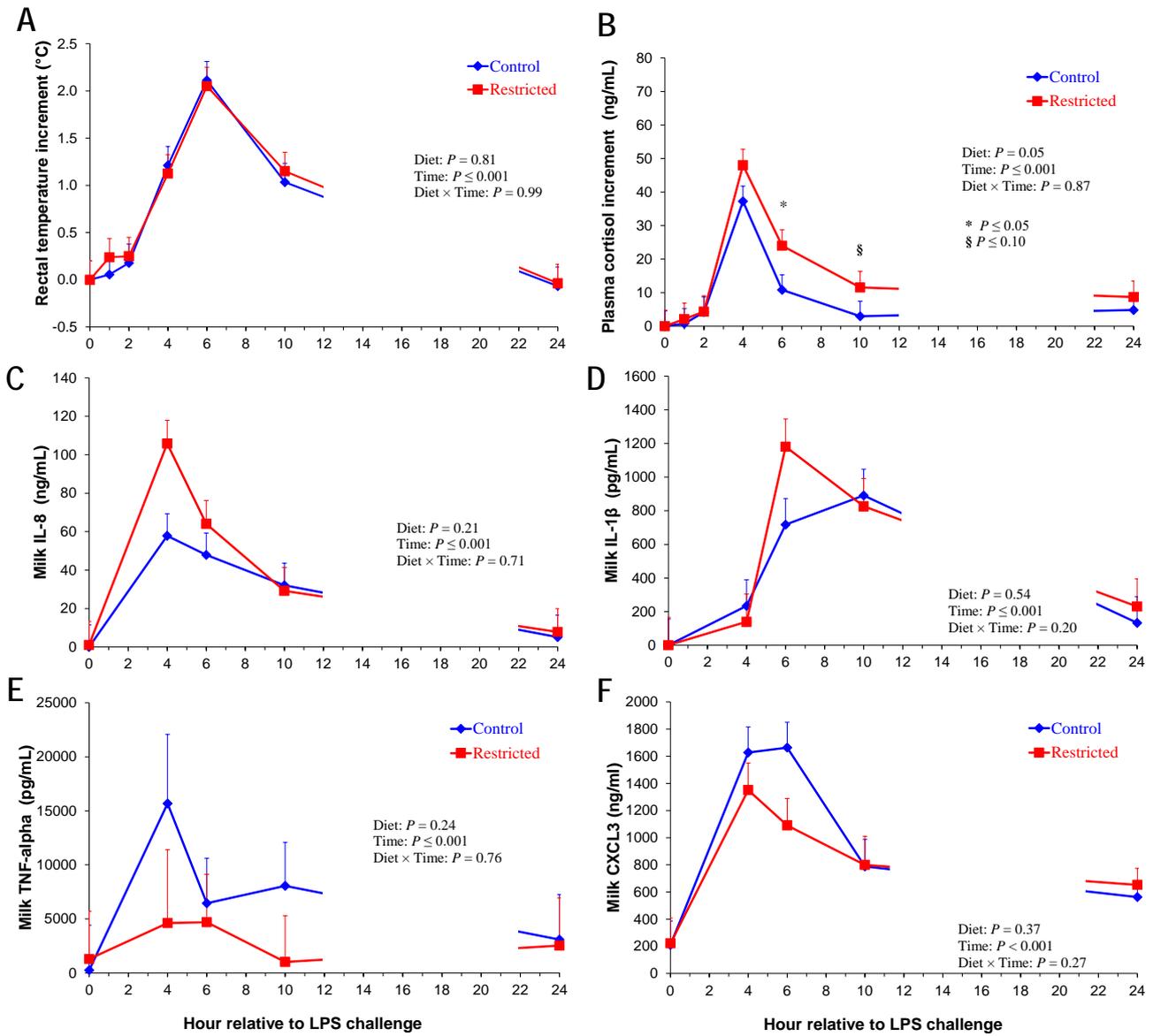
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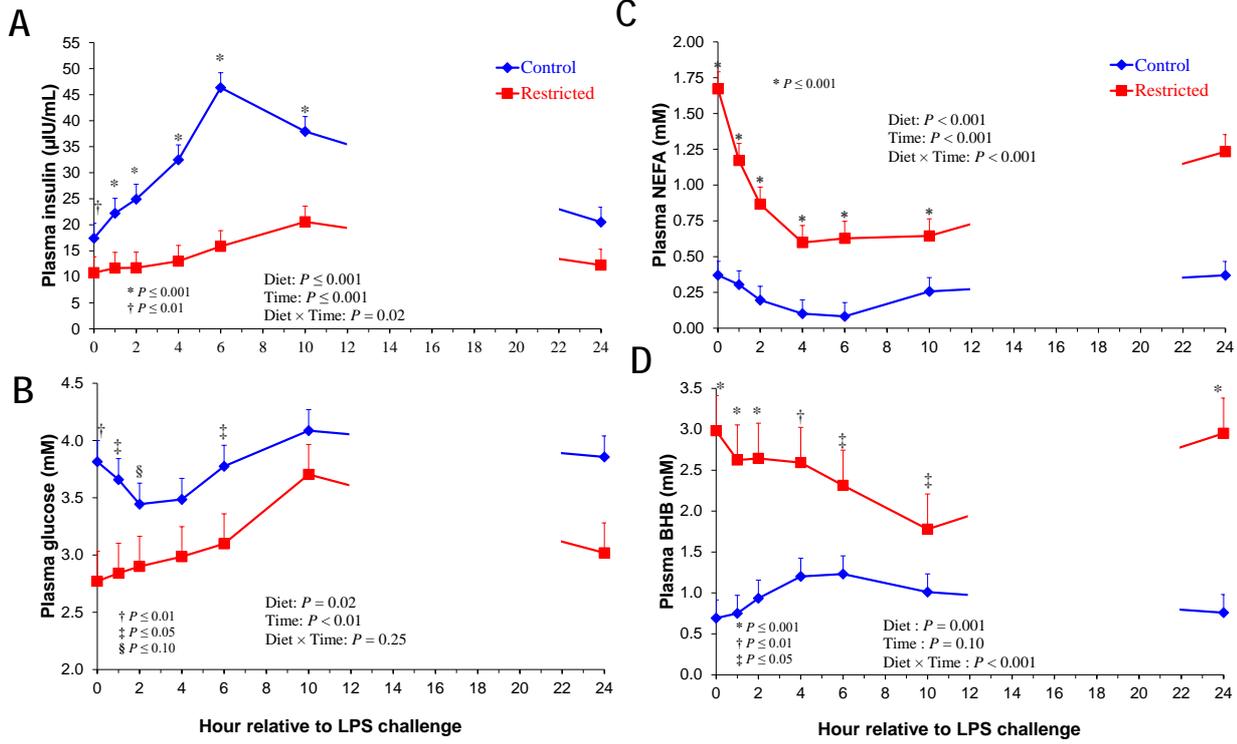
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704 Pires et al. Figure 4.

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Pires, J., Pawlowski, K., Rouel, J., Delavaud, C., Foucras, G., Germon, P., Leroux, C. (2019). Undernutrition modified metabolic responses to intramammary lipopolysaccharide but had limited effects on selected inflammation indicators in early-lactation cows. Journal of Dairy Science, 102 (6). 5347-5360. . DOI : 10.3168/jds.2018-15446