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

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

RUNNING HEAD: INFLAMMATION RESPONSE DURING UNDERNUTRITION

Title

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

J.A.A. Pires* [1], K. Pawlowski[*2], J. Rouel*, C. Delavaud*, G. Foucras[†], P. Germon[‡] and C. Leroux*[§]

* INRA, Université Clermont Auvergne, VetAgro Sup, UMR Herbivores, F-63122 Saint-Genès-Champanelle, France
† UMR1225 IHAP, INRA, Toulouse, France
‡ UMR1282 ISP, INRA, Nouzilly, France
§ University of California Davis, Department of Food Science and Technology, Davis, CA 95616, USA

[1] Corresponding author: jose.pires@inra.fr

[2] Current address : Warsaw University of Life Sciences, Faculty of Veterinary Medicine, Department of Pathology and Veterinary Diagnostics, Warsaw, Poland

ABSTRACT

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 µg 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) ×10 h) for REST than CONT. No treatment differences were observed in foremilk milk IL-8, IL-1β, 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

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

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

INTRODUCTION

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

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

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

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

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

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

MATERIALS AND METHODS

Animals, Diets, LPS Challenge and Housing

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

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

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

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

Phenotyping Early Lactation and Responses to Undernutrition

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

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

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

Phenotyping Responses to LPS challenge

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

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

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

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

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

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***

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

Early Lactation and Responses to Undernutrition

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

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

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

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

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

Responses to Intramammary LPS challenge

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

Composite milk SCC increased sharply on the day of LPS challenge in both CONT and REST cows (Figure 1D), but SCC was greater for REST (6. 91E6 vs 1.91E6 ± 337E3 cells/mL, $P = 0.01$; Diet × Time interaction: $P = 0.04$). Furthermore, REST cows secreted a greater total number somatic cells in milk than CONT on the day of LPS injection (1.35E11 vs 0.67E11 ± 0.203E11 cells; $P = 0.03$). Composite milk SCC returned to pre-challenge values after 31 and 34 DIM for 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

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

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 to 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 synthetized 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

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

Responses to Intramammary LPS

Immune Activation. Immune activation following the intramammary LPS injection was confirmed by increased rectal temperature, composite SCC, foremilk milk IL-8, IL-1β, TNF-α and CXCL3 concentrations, and anecdotal observation of mammary gland symptoms (e.g., swelling, hardness, redness, and soreness) and modified quarter milk appearance (clots and yellowish color). Concerning the effect of diet on indicators of inflammatory response, only cortisol response AUC and composite milk SCC differed between CONT and REST. These findings in early lactation cows are in agreement with previous research during midlactation involving partial feed restriction and experimental inflammation by LPS or *Streptococcus uberis* infection (Perkins et al., 2002, Moyes et al., 2009).

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

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

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

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

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

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

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

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

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

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

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

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

CONCLUSIONS

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

ACKNOWLEDGEMENTS

The authors thank the staff at Herbipôle Research Unit (INRA, UE1414, Theix, France) for animal care and sampling; D. Bany, S. Bes, C. Labonne, E. Tixier, M. Tourret (INRA, UMR1213, Saint-Genès-Champanelle, France) for sample collection and laboratory analyses; P. Rainard

(INRA, UMR1282 ISP, Nouzilly, France) for valuable discussions on experimental design; M. Faure and C. Ravel (INRA, UMR1213, Saint-Genès-Champanelle, France) for proposing cortisol sampling schedule and cortisol analyses, respectively. The authors are grateful to the Galilait laboratory (Clermont-Ferrand, France) for milk component, SCC and microbiological analyses. This research was funded by GISA meta-program of INRA (Ruminflame and Longhealth projects).

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Table 1. Diet ingredient, nutrient and fatty acid composition. Multiparous Holstein cows were either allowed ad libitum intake throughout the study (Control, n = 9), or underwent 4 days of nutrient restriction (Restricted, n = 8) by receiving a ration composed of 48% (DM-basis) straw 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
cis-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).

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).

Figure 1: Dry matter intake (DMI, A), milk yield (B), energy balance (C), and somatic cell count (SCC, D). Multiparous Holstein cows were either allowed ad libitum intake of a regular diet throughout the study (Control, n = 9), or underwent 4 days 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. *P*-values for SCC reflect statistical analysis with log-transformed data. Values are LSM ± SEM.

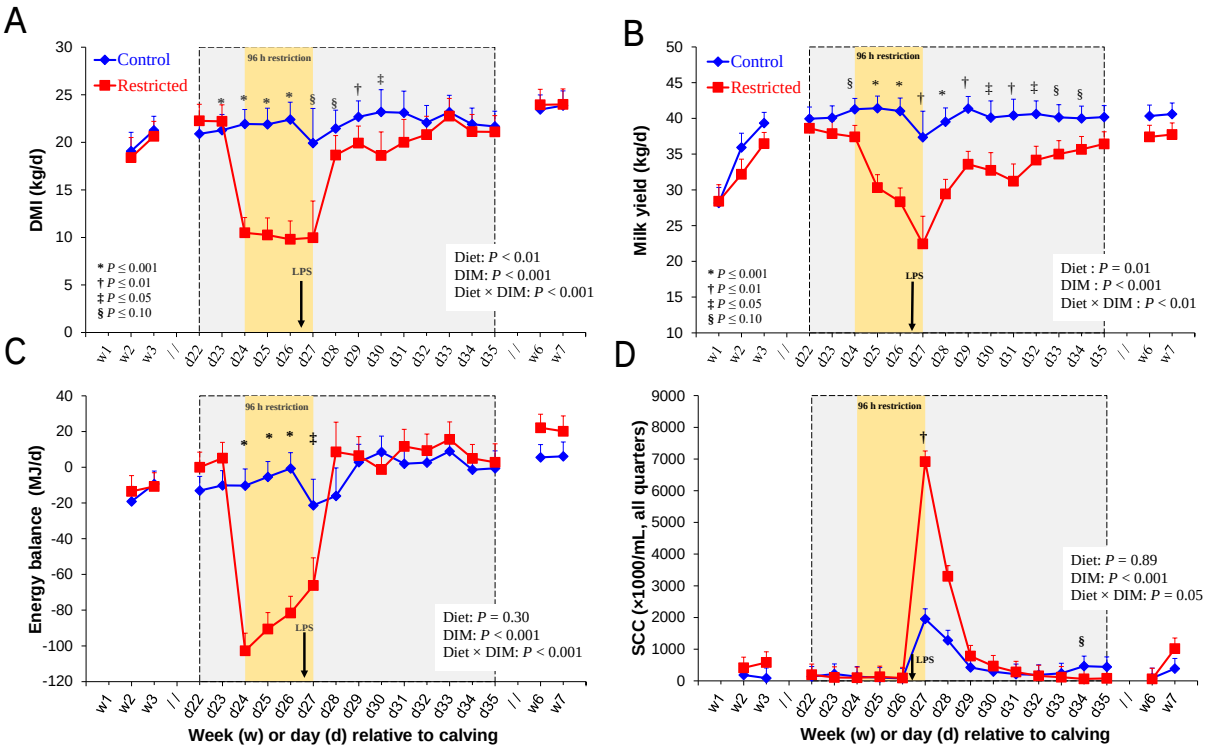
Figure 2. Plasma NEFA (A), BHB (B), glucose (C), urea (D) and insulin (E) concentrations. Multiparous Holstein cows were either allowed ad libitum intake of a regular diet throughout the study (Control, n = 9), or underwent 4 days of nutrient restriction (Restricted, n = 8) by receiving a ration composed 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. Values are LSM ± SEM.

Figure 3. Effects of nutrient restriction on rectal temperature increment (A), plasma cortisol increment (B), milk Il-8 (C), IL-1β (D), TNF-α (E) and CXCL3 (F) concentrations in response 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. Rectal temperature was recorded and blood samples collected before and at 1, 2, 4, 6, 10 and 24 h; foremilk milk samples were collected from

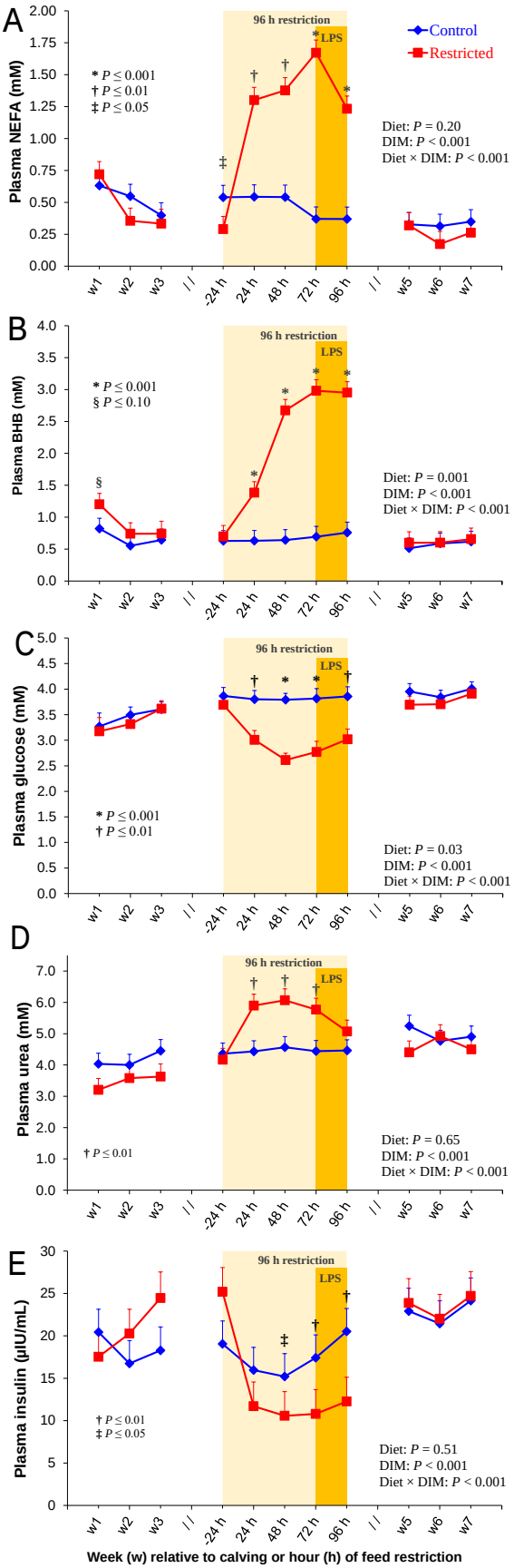
the injected mammary quarter before and at 4, 6, 10 and 24 h relative to LPS administration. Values are LSM ± SEM.

Figure 4. Effects of nutrient restriction on plasma insulin and metabolite concentration in response 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 collected before and at 1, 2, 4, 6, 10 and 24 h relative to LPS injection. Values are LSM ± SEM.

Pires et al. Figure 1:



697 *Pires et al.* Figure 2.



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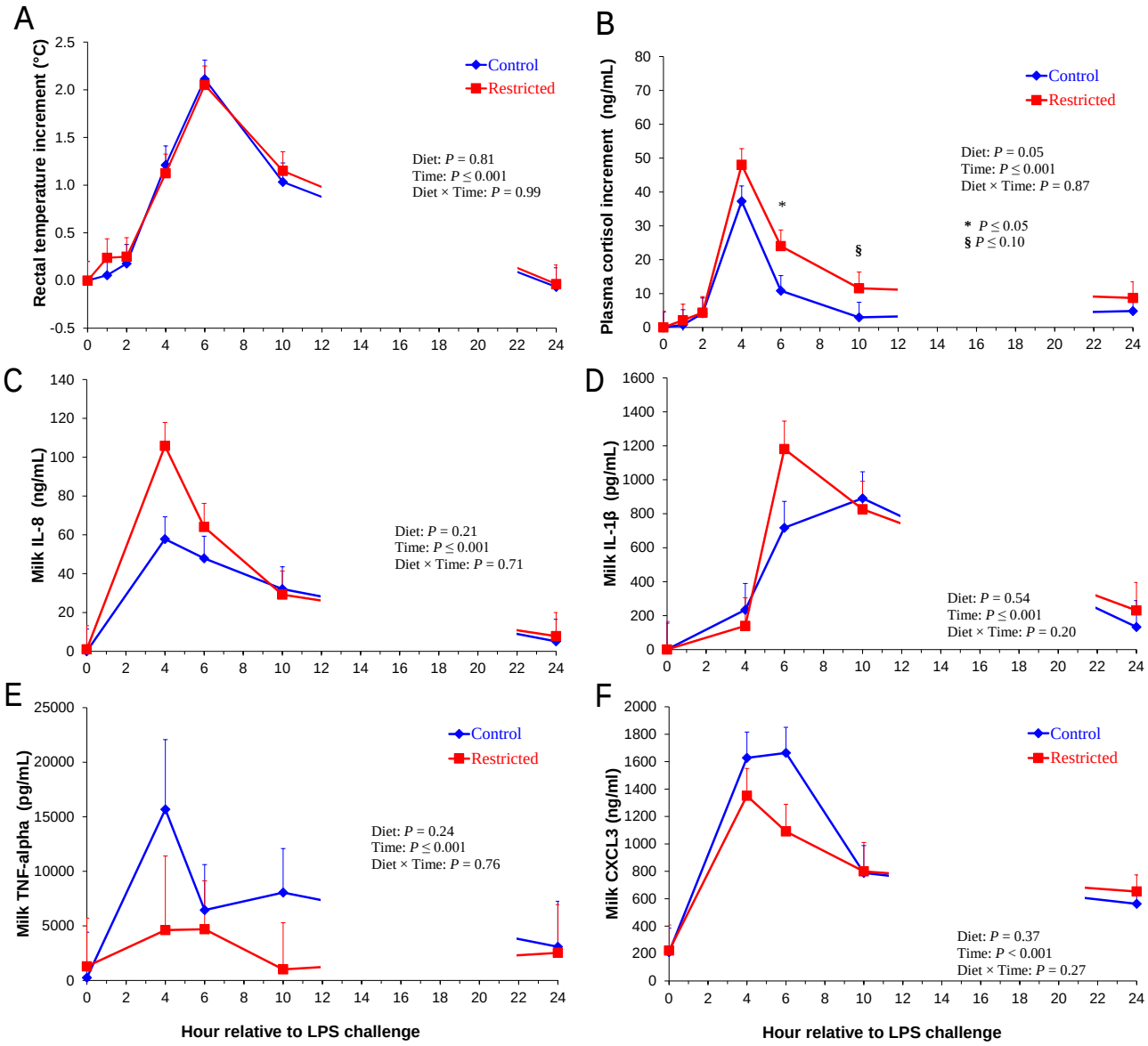

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Pires et al. Figure 4.