Characterization of metabolic and inflammatory profiles of transition dairy cows fed an energy-restricted diet

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Abstract

Periparturient diseases of dairy cows are caused by disproportionate energy metabolism, mineral imbalance, and perturbed immune function. The aim of the present study was to characterize metabolism, innate immune endometrial gene expression, and uterine microbial populations of transition animals receiving normal or restricted energy diets. Pregnant multiparous Holstein cows (n = 14) were randomly assigned to one of the two dietary treatments from 20 d prepartum until 35 d postpartum (**DPP**). One group was fed a diet providing 100% energy requirements (NE), whereas the other received an energy-restricted diet providing 80% energy requirements (RE). Feed intake, milk yield, body weight, body condition score, temperature, respiratory, and pulse rate were recorded. After calving, blood was collected weekly to analyze nonesterified fatty acids (NEFAs), β-hydroxybutyrate (**BHB**), and total cholesterol (**TC**). Endometrial cytobrushes were collected for gene expression analysis of inflammatory markers, microbial populations determination, and cytological evaluation. The restricted energy diet did not alter feed intake or milk yield but changed energy balance and metabolites levels ($P < 0.05$). In fact, RE animals had high NEFA and BHB levels, and low TC concentrations ($P < 0.05$). Moreover, RE animals had upregulated gene expression of serum amyloid A3 (**SAA3**) at 35 DPP ($P < 0.05$) and CXC chemokine receptor 2 (**CXCR2**) at 14 DPP ($P < 0.01$). Interleukin (IL) 1 and IL8 genes were downregulated 14 DPP but upregulated 35 DPP in RE animals, whereas IL6 and lipopolysaccharide-binding protein (**LBP**) genes were upregulated at 14 DPP ($P \leq 0.05$). The most abundant phyla in RE animals (n = 3) were *Bacteroidetes* and *Fusobacteria*, whereas *Proteobacteria* was the least abundant at both 14 and 35 DPP. In conclusion, it can be speculated that energy balance is one of the main drivers for uterine inflammation by affecting metabolism, immune function, and uterine microbiota. However, these findings should be validated in a larger sample size.

Key words: energy restricted diet, gene expression, inflammatory markers, metabolites, transition cow

Introduction

In dairy cattle, the main challenge during the transition period [3 wk prepartum to 3 wk postpartum (Drackley, 1999)], is the sudden increase in nutrient demand for milk production at a time when feed intake lags behind (Little et al., 2017; Dänicke et al., 2018) resulting in negative energy balance (**NEB**). During NEB, metabolic imbalances arise from low insulin uncoupling the growth hormone-insulin-like growth factor (GH-IGF) axis and promoting direct action of GH on lipolysis and gluconeogenesis, thus releasing nonesterified fatty acids (NEFA) in the bloodstream (Butler et al., 2003; Lucy, 2007). Elevated NEFAs impair lymphocytes from proliferating and secreting immunoglobulin M and interferon-γ (Lacetera et al., 2004). In addition, acute phase proteins haptoglobin (**HP**) and cytokine interleukin (IL) 8 are elevated around calving (Bionaz et al., 2007; Bertoni et al., 2009; Huzzey et al., 2009). Furthermore, oxidative burst activity is disrupted (Scalia et al., 2006), thus increasing the risk of endometritis (Adnane et al., 2017a). In fact, cows that develop endometritis have increased proinflammatory expression of IL1a and 1b and toll-like receptor (**TLR**)-4 genes in the first week postpartum (Herath et al., 2009). Dysregulation of the immune response in these animals has also an implication on uterine bacterial clearance and thus fertility postpartum (Kawashima et al., 2007). In particular, virulent bacteria play a key role in disease pathogenesis.

Microbiota profiling to date has shown an association between the diverse bacterial influx into the uterus postpartum, endometrial health, and reproductive performance of cattle (Bicalho et al., 2016; Knudsen et al., 2016). Given that physiological endometrial inflammation in postpartum cows is driven by efficient energy metabolism, microbial diversity, and robust immune function, this study used a comprehensive approach to characterize metabolic, molecular, and microbiota profiles of transition cows fed energy-restricted diet.

Materials and Methods

The animals were handled in accordance with the South African National Council of Societies for the Prevention of Cruelty (NSPCA), and all experimental procedures were approved by the Animal Use and Care Committee of the University Pretoria (project number EC045-12).

Herd identification and experimental design

Gravid (247 ± 7 d in gestation) multiparous Holstein cows (n = 14), dried off 60 d prior the expected day of calving, were selected according to their previous parity (2 to 4) and milk yield (expressed in mature equivalent—ME305), body weight (**BW**: 830 ± 50 kg), and body condition score (**BCS**: 3 to 3.5). During an adaptation period of 15 d, in which the animals were allocated to individual pens (120 ×180 cm) with sawdust bedding and free access to water, the animals were clinically evaluated, and blood samples were collected to determine their baseline parameters. Information about previous lactation, clinical data, and blood metabolites profile were used for the selection of the animals and their grouping to the respective treatment diets. Baseline data are reported in Table 1. The study was conducted from 20 ± 7 prepartum until 35 d postpartum (**DPP**).

The animals were randomly assigned to two diets: normal energy (**NE**) and restricted energy (**RE**). Isonitrogenous diets were formulated using the Nutritional Dynamic System (NDS professional; RUM&N, Reggio Emilia, Italy), a software based on the Cornell Net Carbohydrate and Protein System (Van Amburgh et al., 2015), to provide 100% (NE) or 80% (RE) energy requirements according to the National Research Council (NRC) recommendations (National Research Council, 2001). Daily, at 0800 hours, the cows were offered a total mixed ratio (**TMR**) for ad libitum intake to allow approximately 5% orts. Samples of TMR were collected weekly and combined into monthly composite samples, dried, and grounded in a Wiley Mill (Arthur H. Thomas, Philadelphia, PA) with a 2-mm screen. All composite samples were analyzed for dry matter (DM; method 930.15; AOAC International, 2012), crude protein (CP; method 2003.05; AOAC International, 2012), fat (method 2003.05; AOAC International, 2012), acid detergent fiber (ADF; method 973.18; AOAC International, 2012), neutral detergent fiber (NDF; Mertens, 2002), starch (Hall, 2009), and macro- and microminerals (Sirois et al., 1994).

Table 1. Baseline data (current parity, previous milk production, clinical data, and blood metabolites at -20DPP) expressed as least square means ± SE for the NE and RE cows

Parameter	NE	RE	P-value
Parity	4.14 ± 0.34	4.14 ± 0.34	1.000
ME305, Kg	7980.83 ± 1275.97	8377.33 ± 1275.97	0.8305
BW, Kg	887.33 ± 23.84	832.00 ± 23.84	0.1891
BCS	3.43 ± 0.27	3.39 ± 0.27	0.2546
Temperature, °C	38.55 ± 2.25	38.12 ± 2.25	0.3182
Respiration, brpm[1]	34.00 ± 1.118	33.25 ± 1.118	0.6337
Pulse, bpm[2]	79.33 ± 5.95	78.00 ± 5.95	0.8721
NEFA, mmol/L	0.36 ± 0.23	0.45 ± 0.23	0.7830
BHB, mmol/L	0.49 ± 0.11	0.46 ± 0.11	0.8376
TC, mmol/L	4.39 ± 0.33	3.57 ± 0.33	0.1641

[1]brpm, breath per minute.
[2]bpm, beats per minute.

Energy balance

Dietary energy values of each diet were calculated according to NRC (National Research Council, 2001). Net energy intake (**NEI**) was determined by multiplying the weekly dry matter intake (DMI) by the calculated energy value of the diet. Energy required for body maintenance (**NEM**) was computed using the equation NEM = $BW^{0.75}$ × 0.08 (National Research Council, 2001). Pregnancy requirements (**NEP**) were computed using the equation NEP = [((2 × 0.00159 × days pregnant – 0.0353) × (calf birth weight / 45)) / 0.14] × 0.64 (National Research Council, 2001). The average weight of newborn calves was 41×3.2 kg. Estimated energy balance (**EB**) prepartum was computed on a daily basis using the equation EB = NEI – (NEM + NEP). Milk energy was calculated using the equation NEL = MP × [(0.0929 × Fat) + (0.0547 × Prot) + (0.0395 × Lact)], where MP, Fat, Prot, and Lact are milk production (kg), fat, CP, and lactose percentage in milk, respectively (National Research Council, 2001). The estimated EB postpartum was calculated on a weekly basis using the equation EB = NEI – (NEM + NEL).

Data recording and samples collection

DMI was recorded daily, whereas BCS, BW, and vital parameters were recorded weekly. Blood samples were collected at day 20 prepartum, and once a week after calving. The samples were collected by coccygeal venipuncture in 10-mL vacutainer tubes and stored at 4°C for 4 h to allow coagulation. Serum was obtained by centrifuging whole blood at 2,800 × g for 15 min. The serum was then snap-frozen in liquid nitrogen and stored at –20°C for analyses of total cholesterol (**TC**), NEFA, and β-hydroxybutirate

(**BHB**). After calving, cows were milked twice a day (at 0600 hours and 1700 hours). Milk production and chemical composition were recorded (AfiMilk MPC Milk Meter & AfiLab Milk Analyzer, Afimilk Ltd, Kibbutz Afikim, Israel).

Endometrial samples were collected using a cytobrush as previously described (Foley et al., 2015). Briefly, vulva was cleaned after which a sterile lubricated speculum covered by a sterile sanitary sleeve was passed into the vagina. A sterile cytobrush was then introduced through the speculum to the external *cervical os* and the sanitary sleeve punctured, the cytobrush was then advanced through the cervix into the base of previously gravid horn to obtain the endometrial sample. For cytological evaluation, endometrial samples were collected weekly; the cytobrushes were rolled onto clean frosted microscope slides, fixed, and then stained with Diff-Quick. From a subset of animals (five cows per group), duplicate endometrial cytobrushes collected at 14 and 35 DPP were stored in RNAlater at –20°C for quantification and analysis of innate immune gene expression. The other cytobrush was snap frozen and stored at –80°C until analysis for microbial populations.

Metabolite analysis

Serum was analyzed using an enzymatic colorimetric method assay to measure levels of NEFA and BHB (Radox Laboratories, Ltd, Midrand South Africa), and TC (Cobas Integra cholesterol gene, Roche Diagnostic, Ltd, Sandton, South Africa). All assays were performed on a Cobas Integra 400 plus analyzer using test protocol, according to the manufacturer guidelines.

Uterine cytology

Endometrial cytology was evaluated in duplicate by a single examiner according to Kasimanickam et al. (2004). Briefly, using light microscopy at 100× magnification, 10 fields per slide (a minimum of 100 cells) were counted, and cells classified as endometrial epithelial, polymorphonuclear (neutrophils), large mononuclear (macrophages), and small mononuclear (lymphocytes).

DNA and RNA extraction

One milliliter of phosphate-buffered saline (PBS) was added to each uterine cytobrush and vortexed for 3 min (Adnane et al., 2017b). Samples were homogenized and DNA was extracted using a QIAamp DNA Micro Kit (Qiagen, Milan, Italy) as previously described (Sanjay et al., 2019) and according to manufacturer instructions. DNA concentration and quality were measured using a spectrophotometer (Eppendorf Biophotometer ® 6131, Eppendorf, Randburg, South Africa) and agarose gel, respectively. Samples stored in RNA later were thawed, vortexed for 3 min, and transferred to 1.5-mL microcentrifuge tubes and centrifuged at 2800 × *g* for 10 min at 4°C. The nucleotide pellet was homogenized with Qiazol (Qiagen, Milan, Italy) and RNA extracted using the Mini Kit (Qiagen, Milan, Italy). RNA yield and quality were determined using the Nanodrop and Bioanalyzer instruments (Illumina Genome Analyzer II [GAII] at TrinSeq), the Trinity Genome Sequencing Laboratory, in the Molecular Medicine Institute, Trinity College Dublin (http://www. medicine. tcd.ie/sequencing), respectively.

Gene expression and microbiota

Reverse transcription

Reverse transcription was performed using QRT Kit (www.Qiagen. com). An optimized blend of oligo-dT and random hexamer primers was used to synthesize the cDNA. One microgram of total RNA from each sample was converted to cDNA in a total volume of 20 µL. A reverse transcriptase (**RT**) minus control of each sample was created by performing the same reaction without RT to be able to subsequently screen each sample for residual DNA contamination in the gene-specific quantitative real-time polymerase chain reaction (*qRT*-PCR) assay.

Primer design, qRT-PCR, and data analysis

Intron-spanning gene-specific primers were designed using primer 3 to amplify products from regions of exon 2 containing different nucleotides to maximize the chances of differentiating between the closely related sequences. Primer specificity was confirmed by Primer Blast before commercial synthesis (Sigma-Aldrich Ireland Ltd, Wicklow, Ireland). Quantitative RT-PCR was performed on Applied Biosystems 7500 Fast Real-Time PCR System, using SYBR Green intercalating DNA dye to amplify primer target genes: alpha (1)-acid glycoprotein (*AGP*), *HP*, *IL1*, *IL2*, *IL4*, *IL6*, *IL8*, *IL10*, lipopolysaccharide-binding protein (***LBP***), ceruloplasmin (***CPN***), serum amyloid A3 (***SAA3***), C-X-C motif chemokine receptor 2 (***CXCR2***), and reference glyceraldehyde 3-phosphate dehydrogenase (***GAP***) gene, β-actin (***Act***) gene, peptidyl prolyl isomerase A (***PPI***). Efficiency for each primer pair was calculated using a 2-fold dilution series on a pooled cDNA sample, obtained by pooling 2 µl of cDNA from each sample in a single microcentrifuge tube. All genes were normalized to the expression of *PPI* reference gene. The software package GenEx 5.2.1.3 (MultiD Analyses, Gothenburg, Sweden) was used for qPCR data analyses (Schmittgen and Livak, 2008). Melt curve analysis was performed to ensure that a single product is formed with few primer-dimer artifacts. The amount of target, normalized to an endogenous reference and relative to a calibrator, was: $2^{-\Delta\Delta Ct}$, where ΔCt is the difference in Ct between target and reference and ΔΔCt is the difference in ΔCt between all the samples and calibrator.

PCR amplification of the bacterial 16S rRNA genes

Uterine microbiota were compared between NE and RE animals at 14 and 35 DPP. DNA isolated from uterine cytobrush samples from 6 animals out of 10 (3 NE and 3 RE) were selected based on DNA quantity and quality to run the bacterial 16S rRNA gene sequencing and generate sequence reads. The V1-V3 variable regions of 16R rRNA genes in the individual samples were amplified using the primer pair 27F (AGAGTTTGATCCTGGCTCAG) and 534R (ATTACCGCGGCTGCTGG). The reverse primers (543R) included sample-specific Golay sequence bar codes (Caporaso et al., 2012). PCR reactions were performed in 50 µL volumes using 50 ng of DNA template, 5U of DNA free MTP Taq DNA polymerase (Sigma), and 0.5 µM of each primer. Previously described touch-down thermal cycling PCR conditions were used for amplification (Santos and Bicalho, 2012). The 16S rRNA PCR amplicons were gel purified, quantified using NanoDrop, and pooled at equimolar ratios. Pooled 16S rDNA library was subject to Illumina sequencing at Eurofins GATC Biotech GmbH (Konstanz, Germany; https://www.eurofinsgenomics.eu/de/custom-dna-sequencing/gatc-services/).

Sequence analysis

A combination of de novo and reference-based operational taxonomic units (**OTU**s) identification was carried out using the open reference calling method implemented within Quantitative Insights Into Microbial Ecology (QIIME) software package. A default similarity level of 97 % was used to cluster sequences into individual OTUs and a single representative sequence from each clustered OTU was used to align to the Green genes

database (version: gg_13_5) (Caporaso et al., 2010). Taxonomic classification for each OTU was determined with Ribosomal Database Project (RDP) Classifier using a minimum confidence cutoff of 0.8. The OTUs with fewer than 100 sequences across all samples were excluded from further analysis. Estimates of distance matrices for both alpha and beta diversity calculation and a per-sample summary of OTU representation at various taxonomic levels were also calculated.

Statistical analyses

Milk production, milk components, DMI, BCS, BW, EB, blood metabolites, and percentage of polymorphonuclear cells (**PMNs**) were evaluated using ANOVA for repeated measures, with the PROC MIXED procedure (SAS 9.3) with a factorial arrangement of treatments with covariate adjustment corresponding to the response variable in the prepartum period, when available. Clinical parameters were evaluated separately for the prepartum and postpartum period as well as for the calving day. The model included the fixed effects of diet, parity, weekly DPP interval, and the respective two-way interactions. The three-way interaction was removed from the model because it was nonsignificant. Weekly DPP interval was included in the model as a repeated measure using the compound symmetry covariance structure which provided the best fit based on the Akaike's information criterion (Littell et al., 1998). Degrees of freedom were calculated using the Kenward–Roger method (Kenward and Roger, 1997). Least squares means were determined, and treatment means within weekly intervals compared using the SLICE option. Progression of PMNs over days postpartum were evaluated by regression against time (PROC REG, SAS 9.3). Slopes were compared with the *F*-test. The Pearson correlation coefficients between intake, milk production and composition, and blood metabolites at different DPP were evaluated using the partial CORR procedure (SAS 9.3). Differences in gene expression levels between groups and DPP were analyzed by ANOVA and Tukey's post hoc test as implemented in GraphPad Prism (Version 6). The partial CORR procedure was also used to evaluate the Pearson correlation coefficients between innate immune genes and intake, milk production and composition, blood metabolites, and **PMNs** at 14 and 35 DPP.

The stepwise multiple linear regression with backward elimination of each least significant input until all remaining variables had a $P < 0.05$ (Neter et al., 1996) was applied to determine the variables which best predicted variation in uterine health and metabolic responses. The variance inflation factor was taken into account to avoid collinearity (Dohoo et al., 2009).

Venn diagrams were built using the online Venn diagram construction tool Venny (Oliveros, 2007; https://bioinfogp.cnb.csic.es/tools/venny/). To determine if there were statistically significant differences between OTU relative abundances between RE and NE cows, the DEseq2 in the Phyloseq package was used (McMurdie and Holmes, 2013; Love et al., 2014) in R (https://www.r-project.org). Genera and Phylla were selected from phyloseq object using the command "tax_glom" with the option NArm = FALSE. OTUs were considered differentially expressed when they had an adjusted *P*-value < 0.05 and a differential abundance of at least 2-fold. Alpha diversity was determined using the option estimate_richness in phyloseq. Beta diversity was calculated using the option distance in phyloseq with the method "Bray." Weighted and unweighted UniFrac were calculated using the option UniFrac with the options "(normalized = TRUE, parallel = FALSE, fast = TRUE)".

Statistical significance and trends were considered at $P \leq 0.05$ and $P > 0.05$ to $P \leq 0.10$, respectively.

Results and Discussion

Dietary characteristics

The isonitrogenous diets were designed to provide 100% (NE diet) or 80% (RE diet) of the energy requirements of dry and early lactation dairy cows. Although the diets provided different percentages of CP between groups, the amount of metabolizable protein (MP) offered did not change between dietary treatments (1,092.2 and 1,087.3 in the close-up, and 2,793.3 and 2,793.1 in the lactation diets for NE and RE animals, respectively). The diets provided 100.7% and 81.7% of the energy requirements prepartum, and 100.4% and 80.6% postpartum for the NE and RE animals, respectively (Table 2).

Energy balance can be altered either by changing output, such as increasing the number of milking times per day, or by manipulating intake (Dänicke et al., 2018). Although some studies use quantitative restriction of feed intake to limit energy intake (Burke et al., 2010; Contreras et al., 2016; McDougall et al., 2017; Salin et al., 2017), manipulating the diet composition to reduce energy density was chosen in this study. Whereas intake restriction downregulates the pentose phosphate pathway in the mammary gland (Guinard-Flament et al., 2006), energy reduction causes an increase in lactose synthesis thus favoring milk yield and consequently, NEB (Dänicke et al., 2018). Furthermore, feed

Table 2. Nutrients and chemical composition (% of DM) of the NE and RE close-up and lactating diets

	Close-up diet		Lactating diet	
Ingredient, %DM	NE	RE	NE	RE
Alfa alfa hay	3.44	3.45	10.11	15.36
Eragrostis hay	30.93	15.86	36.33	26.22
Corn grain ground	11.00	1.38	26.22	2.25
Wheat straw	37.80	53.79	3.75	24.34
Canola meal	2.75	7.59	4.87	7.12
MinVit	2.06	2.76	1.12	1.12
Soybean meal	6.88	8.28	10.48	14.24
Soybean hulls ground	3.44	3.45	5.24	7.12
Molasses dried	—	—	1.12	0.75
Fish meal	—	—	0.37	0.75
Limestone ground	1.03	2.07	—	—
Urea	0.69	1.38	0.37	0.75
Nutrient (DM)				
Forage, %	72.2	73.1	50.20	65.90
CP, %	12.00	15.80	15.50	19.1
Sol P, %	4.30	6.90	4.31	6.21
RDP, %	7.70	10.40	9.39	11.72
NDF, %	57.79	60.56	41.34	53.05
fNDF, %[1]	53.05	55.13	33.04	44.62
peNDF, Kg	7.52	7.82	33.2	44.1
Starch, %	9.70	3.00	20.90	3.8
NFC, %	19.40	12.30	33.50	18.1
EE, %	1.70	1.50	2.50	0.30
Ca, %	0.72	1.08	0.46	0.60
P, %	0.24	0.25	0.34	0.35
K, %	1.54	1.56	1.52	1.81
Mg, %	0.20	0.24		0.24
Biotin, mg/Kg	0.03	0.08	0.05	1.81
ME, Mcal/Kg	1.94	1.76	2.32	1.96
NEL, Mcal /Kg	1.25	1.13	1.50	1.26
MP, g/d	1092.2	1087.3	2793.3	2793.1
DCAD, mEq/100g	−7.2	−5.8	16	14

restriction may alter the eating behavior (Patterson et al.,1998) thus inducing additional metabolic stress (Dänicke et al., 2018).

Dry matter intake, energy balance, body weight, and condition score

Table 3 summarizes the effect of energy restriction on DMI, EB, BW, BCS, milk yield and composition. The effect of DPP, parity, and the relative interactions were also evaluated; however, these are not reported in the table being mostly nonsignificant. For those variables where interaction was significant, it was specified in the text. Feed intake (calculated both as DMI and as %DMI of BW) was not affected by the energy-restricted diet, probably because ad libitum feeding allowed normal eating behavior (Dänicke et al., 2018). However, the limited number of multiparous cows enrolled in the study resulted in large SEs (4.5 kg prepartum and 5 kg postpartum); therefore, it cannot be excluded that with a larger sample size, differences in intake would be noted.

The animals fed the restricted energy diet had a trend for lower EB prepartum and a significant lower EB postpartum. These results are in line with the findings of Contreras et al. (2016) and Janovick and Drackley (2011). In their studies, in which four cows per dietary group and eight cows per treatment were enrolled respectively, the restricted diet was applied only during the close-up period. Nonetheless, in their studies, restricting energy intake lowered the calculated energy balance postpartum.

BW and BCS were significantly lower in the RE cows compared to the NE group ($P \leq 0.05$; Table 3). Most of the studies investigating the relationship between BCS and energy balance focused on body condition after parturition. Only recently, Barletta et al. (2017) investigated the role of BCS prepartum and its changes during the transition period on fat mobilization, fertility, milk yield, and health of lactating dairy cows. Contrary to previous reports of an inverse relationship between the lactation curve and BCS postpartum (Berry et al., 2006; Roche et al., 2009), Barletta et al. observed that the BCS 21 d before parturition determined the BCS loss postpartum (Barletta et al., 2017). In fact, the heaviest cows 21 d prepartum suffered severe BCS loss before calving and continued up to 21 DPP, whereas cows with the lowest BCS 21 d prepartum gained body condition prior to calving and after parturition (Barletta et al., 2017). In addition, Carvalho et al., (2014) observed that cows gaining or maintaining BCS postpartum had lower BCS at calving compared to cows that lost BCS after calving. In our study, the starting BCS (at 247 ± 7 d in gestation) for the two groups was similar (3.43 ± 0.27 and 3.39 ± 0.27 for the NE and RE group, respectively; $P \geq 0.1$); therefore, the greater loss from prepartum to postpartum in BCS in the RE group compared to the NE group can be addressed entirely to the restricted energy intake. Although milk yield was numerically lower for the cows in the RE group, it was not affected by the treatment (27.14 ± 2.08 kg and 24.53 ± 2.08 kg for the NE and RE group; $P = 0.4029$). Therefore, we could speculate that the magnitude of BCS loss seems to be independent of the level of milk production. In accordance with our results, Barletta et al. (2017) did not observe any correlation between BCS loss and milk production, but they did observe a correlation with DMI and speculated that BCS may be primarily driven by prepartum and postpartum DMI. Researchers have observed that BCS ≥. 3 is associated with a reduced DMI shortly after calving (Roche et al., 2009) and to an increased susceptibility to metabolic diseases (Lacetera et al., 2005; Roche et al., 2009). In our study, no differences were observed in DMI between the two groups, probably because none of the cows had a BCS ≥.

3. Therefore, BCS loss seems to be independent of the level of milk production and driven by energy intake rather than DMI. However, further studies with larger sample size are needed to confirm these results.

Milk production

Similar to previous studies (Mann et al., 2015; van Hoeij et al., 2017; Djoković et al., 2017) although milk production was numerically lower in the RE group compared to the cows in NE group, energy-corrected milk (**ECM**), yield, and milk composition were not affected by the diets (Table 3). However, the interaction treatment × days postpartum showed that there was a trend for higher levels of fat percentage (trt × DPP, $P \leq 0.1$) and a significantly higher fat-to-protein ratio (trt × DPP, $P \leq 0.05$) in the RE group compared to NE cows at 14 DPP (Table 3). When considering milk production for the first 60 DPP, there was a trend for higher production in the NE group compared to the RE cows. However, at the end of the study, milk production of the energy-restricted cows fully recovered to the level of the control animals (36.889 ± 2.318 vs. 30.218± 2.318 in the NE and RE cows, respectively; $P = 0.0777$; Figure S1 in supplementary material). Surprisingly, the decline in milk yield observed in the first 35 DPP was of only about 5 kg compared to what reported in other studies where the milk production was reduced of about 20% (Velez and Donkin, 2005; Carlson et al., 2006). The authors can speculate that besides the level of NEB induced by feed restriction, its initiation and duration may play an

Table 3. Effect of NE and RE on DMI, EB, BW, BCS, and milk yield and composition (least-square means ± SE)

	Prepartum			Postpartum		
Parameter	NE	RE	*P*-value	NE	RE	*P*-value
DMI, Kg	17.31 ± 4.51	20.82 ± 4.51	0.6037	24.31 ± 5.05	22.64. ± 5.05	0.8271
DMI, % of BW	2.02 ± 0.55	2.52 ± 0.55	0.5470	3.69 ± 0.70	3.24 ± 0.60	0.6771
BW, Kg	890.57 ± 15.13	866.85 ± 15.13	0.0893	731.19 ± 16.59	654.28 ± 16.59	0.0092
BW loss, Kg	−88.00 ± 9.29	−126.33 ± 9.29	0.0596	−71.00 ± 20.69	−128.500 ± 20.69	0.0970
BCS	3.53 ± 0.21	2.71 ± 0.21	0.0498	3.094 ± 0.13	2.59 ± 0.13	0.0104
BCS loss	−0.31 ± 0.07	−0.62 ± 0.07	0.0414	−0.2500 ± 0.10	−0.7875 ± 0.10	0.0113
EB, Mcal/kg	34.92 ± 3.53	25.01 ± 3.53	0.0947	−30.42 ± 3.20	−42.67 ± 3.20	0.0486
Milk yield, Kg	—	—	—	27.14 ± 2.08	25.53 ± 2.08	0.4029
ECM, Kg	—	—	—	41.44 ± 3.20	36.64 ± 3.20	0.2581
Milk fat, %	—	—	—	5.29 ± 0.28	5.55 ± 0.28	0.5388
Milk protein, %	—	—	—	3.67 ± 0.07	3.73 ± 0.07	0.5781
Lactose, %	—	—	—	4.32 ± 0.13	4.32 ± 0.13	0.9747

important role in affecting milk production. In this study the energy restriction started at -20 DPP and continued until 35 DPP, therefore it can be speculated that the homeorhetic adaptations were accentuated in the RE cows to compensate for the limited availability of nutrients. However, this hypothesis could not be confirmed because metabolites such as IGF I, growth factor, and insulin were not measured. Furthermore, especially with a limited number of animals as in this study, factors such as age and genetic merit of the dairy cows may also have influenced the cows' response to a deliberately induced energy deficiency.

Higher milk fat content is typical during the first days after calving due to the unavoidable status of negative energy balance of the animals which causes increased mobilization of fat deposit (Palmquist et al., 1993), with active utilization of plasma NEFA by the mammary gland for milk fat synthesis (Pullen et al., 1989). The intensity and duration of the lipid mobilization in response to the NEB affect milk fat percentage (Knegsel et al., 2007; Mann et al., 2015) and, therefore, the fat-to-protein ratio. With the energy-restricted diet, a more intense and longer lipid mobilization was induced, resulting in higher NEFA mobilization and thus higher milk fat percentage and fat-to-protein ratio. According to Mann et al. (2015) and Agenäs et al. (2003), the higher supply of fatty acids from body origin leads to an increased milk fat percentage by increasing the concentration of the preformed fatty acids.

Furthermore, an interaction treatment × DPP was also observed for the ECM which was significantly lower in the RE group compared to the NE group at 21 DPP.

Clinical parameters and blood metabolites

Clinical parameters (body temperature, heart rate, and respiratory rate) before during and after parturition are summarized in Table 4. All the parameters were within normal ranges in both groups and, at calving, no differences were observed between the two animal groups for any of the clinical parameters. However, the RE cows had a trend for lower body temperature and heart rate ($P \leq 0.1$) compared to the NE cows prepartum and postpartum. This observation can be explained by the restriction of caloric intake which reduced the animals' energy expenditures by altering the cardiovascular homeostasis (Herlihy et al., 1992). As reported by Trevisi et al. (2012), cows show a transient inflammatory status around parturition which leads to a temporary increase in clinical parameters. In this study, both groups presented with increased values on the calving day, but similar between groups ($P > 0.5$). However, the clinical parameters pre and postpartum were lower in the RE cows compared to the NE animals. Therefore, it could be hypothesized either that the changes in clinical parameters from prepartum to parturition were higher in the RE group due to a higher degree of transient inflammatory status caused by the energy restriction, or that a certain degree of inflammatory status at parturition, clinically expressed by the changes in body temperature, heart and respiratory rate, is unavoidable and unrelated to the clinical status prior to calving. Treatment did not affect the average blood NEFA levels. However, the interaction diet × DPP showed significantly higher blood levels of NEFA (at 14 DPP) and BHB (at 14 and 21 DPP) in the RE group compared to NE animals ($P \leq 0.05$; Figure 1). Regardless of the diet, the animals mobilized fatty acids from adipose tissue to meet increased energy demands postpartum thus elevating blood NEFA levels. During the transition phase, a certain degree of NEB is considered physiological (Ingvartsen et al., 2003), resulting in enhanced gluconeogenesis and elevated blood NEFA (Gordon, 2013). For animals experiencing prepartum NEB, blood NEFA levels rise, starting at 48 h before parturition (Oetzel (2004). According to Zhang et al. (2016), NEFA concentration above 0.7 mmol/L is indicative of risk for subclinical ketosis, whereas NEFA level above 1.0 mmol/L is indicative of risk for clinical ketosis. Ospina et al. (2010) found that prepartum blood NEFA levels greater than 0.6 mmol/L were indicators of higher risk of retained fetal membranes, metritis, or both. In our study, although both groups experienced an increase in blood NEFA levels at 0 and 7 DPP, we did not observe any case of clinical ketosis. Thus, in accordance with Herdt (2000), we can speculate that the development of ketosis or of reproductive diseases linked to higher blood NEFA levels is not due to the NEB itself, but to inadequate metabolic adaptation to it. In fact, NEFAs can be exported from the liver as lipoproteins, or they can partially be oxidized into BHB and other ketone bodies (Reynolds et al., 2003; von Soosten et al., 2011). Only when the liver is overloaded with NEFA, their partial oxidation by the liver increases, thus causing the accumulation of BHB and other ketone bodies (Herdt, 2000). Blood BHB has been used as a rapid diagnostic biomarker for ketosis in dairy cows, with levels of 1.2 to 1.4 mmol/L considered to be indicators of ketosis (Zhang et al., 2016). In this study, both groups experienced BHB concentration well above 1.2 mmol/L (means ± SD: 2.28 ± 1.429 and 2.76 ± 1.445 for the NE and RE group, respectively) from 7 DPP; however, at 14DPP the RE cows still had high BHB blood levels (means ± SD: 3.74 ± 1.913), whereas the NE group had BHB concentration just above the suggested threshold (means ± SD: 1.23 ± 0.628). These results prove that although both groups experienced a degree of NEB, the cows in the RE group experienced a more severe failure in metabolic adaptation due to a more severe NEB. Zhang et al. (2016) observed that cows that developed ketosis had greater BHB blood concentrations from 4 wk before parturition, but did not have any change in DMI during the prepartum. Based on these results, they speculated that NEB is not satisfactory to explain the greater BHB. However, the authors do not report any information about the initial BCS of the animals which could have played a role in adipose tissue mobilization (Reist et al., 2003). In fact, NEFA and BHB concentration during the early

Table 4. Effect of dietary energy restriction on clinical parameters prepartum, at day of calving, and postpartum[1]

	Prepartum			Calving day			Postpartum		
	NE	RE	P-value	NE	RE	P-value	NE	RE	P-value
Temperature, °C	38.52 ± 0.19	37.94 ± 0.19	0.0558	38.91 ± 0.26	39.14 ± 0.26	0.5296	38.55 ± 0.12	38.25 ± 0.12	0.0803
Respiration, brpm[2]	36.4 ± 3.03	30.8 ± 3.03	0.2108	48 ± 6.59	43 ± 6.59	0.5913	41.17 ± 2.82	33.16 ± 2.82	0.0423
Pulse, bpm[3]	89.06 ± 3.66	78.2 ± 3.66	0.0610	93.3 ± 4.55	93 ± 4.55	0.958	79.77 ± 2.40	73.68 ± 2.40	0.0898

[1]Results are presented as least square means ± SE.
[2]brpm, breath per minute.
[3]bpm, beats per minute.

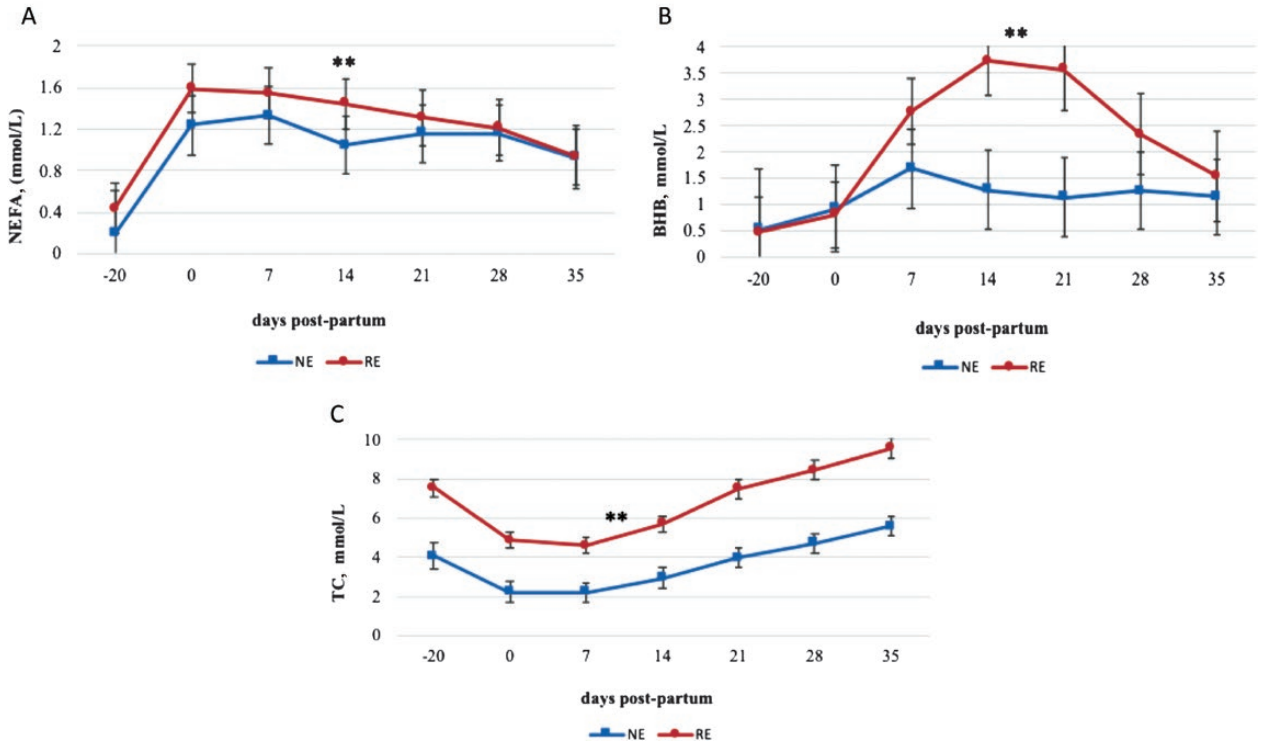

Figure 1. Blood plasma levels of NEFA (A), BHB (B), and TC (C) levels in cows receiving RE diet and NE diet. Results are presented as least square means ± SE, and significant differences between groups are calculated. ** Denotes significance at $P < 0.001$.

postpartum period are associated with BCS at calving and losses in BCS after calving (Carter et al., 2008; Roche et al., 2015). For example, high BCS at calving or a rapid loss in BCS after calving, or both, have been associated with high postpartum circulating NEFA and BHB concentrations regardless of the animals' intake (Reist et al., 2003) and poor cow health (Adrien et al., 2012; Pires et al., 2013). Thus, we can explain our findings with the fact that the animals in our study were blocked by BW and body condition and that none of them had a BCS greater than 3.5. Therefore, the difference in blood concentration of NEFA and BHB between the NE and RE cows can be explained by the different energy intake of the two groups.

Previous studies have reported milk production to be inversely related to blood NEFA and BHB (Ospina et al., 2010; McArt et al., 2013). However, in our study, milk production did not differ between groups and no significant correlation was observed between milk and NEFA or BHB levels. Although these results are contradicting previous studies (Ospina et al., 2010; McArt et al., 2013), they are in accordance with what reported by Bicalho et al. (2017) which observed no correlation between NEFA and BHB levels with milk loss.

It has been speculated that the degree of NEB, identifiable by increased levels of NEFA and BHB, and excessive NEB have detrimental effects on health (Ospina et al., 2010), potentially due to a relationship between postpartum energy deficits and immunosuppression (Hammon et al., 2006; Scalia et al., 2006). Although we did not observe any correlation between NEFA or BHB levels and fat-to-protein ratio or BCS, we did observe a negative correlation with respiratory rate ($r = -35.00$; $P \leq 0.1$), whereas BHB levels were positively correlated with percentage of PMNs ($r = 37.29$; $P \leq 0.05$) thus confirming their association with the animals poor health status and increased risk for metritis as reported in previous studies (Burke et al., 2010; Dubuc et al., 2010). However, Bicalho et al. (2017) did not find a significant link between plasma BHB or NEFA and the occurrence of any uterine disease.

Cholesterol has been lately associated with energy status and diseases. In dairy cows, its concentration normally decreases in the periparturient period, increasing soon after (Quiroz-Rocha et al., 2009). During the status of NEB, characterized by severe BCS loss, Kim and Suh (2003) have reported a lower concentration of cholesterol suggesting its possible use as a predictor of energy status during the transition period. When examining the relationship between cholesterol concentration and the risk of diseases, it has been observed an increased risk of retained placenta in cows with low cholesterol levels prepartum (Kaneene et al., 1997); Kaneene et al. (1997) and Kim and Suh (2003) also reported that cows with low cholesterol levels are at higher risk of metritis after parturition. These findings were also confirmed by Sepulveda-Varas et al. (2015) which showed that low concentration of cholesterol postpartum in grazing dairy cattle was associated with health disorders, especially metritis and combination of more than one disorder. In our study, the average blood TC levels did not differ between the two groups being 3.76 ± 0.43 and 3.12 ± 0.43 for NE and RE cows, respectively ($P = 0.187$). However, the interaction diet × DPP showed significantly lower TC levels in the RE group compared to NE at 7 and 14 DPP ($P < 0.001$; Figure 1). The mechanism of action, regulating the association between cholesterol concentration and diseases, has been explained by several research approaches which have shown that lipoproteins' metabolism is markedly altered during the acute phase response in an inflammatory condition. Some studies, in fact, have shown that experimental induction of inflammation and, therefore, of the acute phase response resulted in increased circulation of triglycerides and very low-density lipoproteins and reduced concentration of

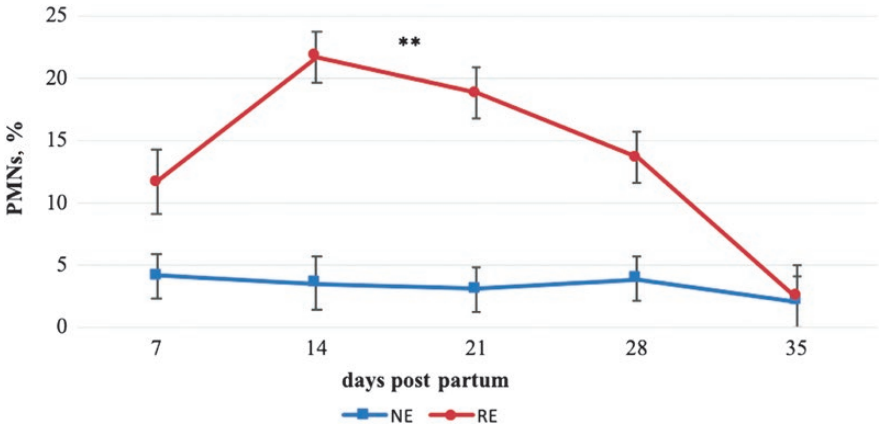

Figure 2. Effect of dietary energy restriction on the percentage of PMNs. Results are presented as least square means ± SE. ** Denotes significance at $P < 0.001$.

high-density lipoproteins in several mammal species (Cabana et al., 1996). In this study, we only analyzed TC; therefore, we were unable to verify if the lower concentration of TC was due to a dramatic reduction of high-density lipoproteins from an inflammatory status or, as speculated by Guretzky et al. (2006) and Cavestany et al. (2005), if the decrease in cholesterol levels could be solely related to energy balance status after calving which are both linked to illness in dairy cows (Huzzey et al., 2007; Goldhawk et al., 2009). Severe NEB has been associated with decreased cholesterol levels (Kim and Suh, 2003) and Sepúlveda-Varas et al. (2015) suggested that lipid mobilization, NEFA, and cholesterol concentrations in serum are interrelated. The study from Sepúlveda-Varas et al. (2015) suggests a relationship between health status and concentration of cholesterol, speculating that low serum cholesterol is a better indicator of EB during early lactation and a better predictor of postpartum diseases. In our study, TC was significantly correlated with BCS ($r = 50.708$; $P \leq 0.05$), milk yield, and ECM ($r = 51.197$, $P \leq 0.01$ and $r = 53.451$, $P \leq 0.01$, respectively) confirming the hypothesis of TC being a better indicator of the EB during early lactation. Furthermore, the Pearson correlation coefficient showed a positive correlation between TC levels and body temperature ($r = 40.8$, $P \leq 0.05$).

Uterine cytology

During endometrial cytology assessment, the proportion of PMNs decreased with increasing days postpartum in both animal groups, but the interaction trt × DPP showed a significant higher percentage of PMNs in the RE group compared to the NE at 14 and 21 DPP ($P \leq 0.001$; Figure 2). Only from 28 DPP, percentage of PMNs was not significantly different between groups. Although early neutrophil infiltration into the uterus is fundamental for killing internalized bacteria and for contributing to the formation of pus when the phagocytes die, their function is often reduced after parturition in many cattle (Zerbe et al., 2000), and this may predispose to the establishment of uterine disease. The suggested threshold value for PMNs as diagnostic for subclinical endometritis depends on the time postpartum and varies from 5% to 18%. However, it has been shown that a general threshold of 5% PMNs is eligible for all cows between 21 and 62 d postpartum (Madoz et al., 2013). In our study, the animals in the RE group presented values between 21.656 ±2.033 at 14 DPP and 13.607± 2.033 at 28 DPP compared with 3.563±2.033 at 14 DPP and 3.860±2.033 at 28 DPP for the cows in the NE group, thus suggesting that the cows fed a restricted energy diet suffered from a cytological subclinical endometritis.

Table 5. Best-fit responses of dependent variables related to uterine involution and innate immune response

Dependent variable	Parameter	Estimate	P-value	R^2
PMNs	Fat/Prot	20.977 ± 3.898	0.0015	0.6498
	Resp	0.0163 ± 0.007	0.0323	0.7953
	BCS	5.931 ± 2.930	0.0409	0.8646
IL1	Lactose	10.706 ± 0.489	0.0021	0.9719
	PMNs	−0.129 ± 0.032	0.0552	0.9970
SAA3	NEFA	3.440 ± 0.130	0.0240	0.9986

The results from the stepwise multiple linear regression showed that variation in milk fat-to-protein ratio explains 65% of this variability ($P \leq 0.01$); detailed information about the multilinear stepwise regression are reported in Table 5.

Endometrial inflammatory profile

At the molecular level, energy restriction did not affect the expression of the acute phase proteins genes AGP, HP, and CP. HP is expressed by endometrial epithelial cells stimulated by LPS in vitro (Chapwanya et al., 2014; Zhang et al., 2016), and it is likely to be elevated in cattle with endometritis (Chapwanya et al., 2012). Expression of AGP is activated via the NFkB pathway and extracellular stimulants that are NFkB pathway inducers, such as LPS, cytokines (IL-1, IL-6) (Liou and Baltimore, 1993; Thanos and Maniatis, 1995). Growth factors and free oxygen radicals activate gene expression of AGP in the liver and many cell lines (Baird, 1982; Reynolds et al., 2003). Levels of AGP increase early postpartum during uterine involution and decrease gradually by 21 DPP (Reist et al., 2003). However, AGP secretion is regulated by the uterine health status, whereby cows with clinical endometritis or *Escherichia coli* infection or fetid mucus discharge have high AGP levels in peripheral blood (McArt et al., 2013). Here, there were no differences between RE and NE animals, maybe because energy restriction ought to be severe before the effects become evident.

SAA3 gene expression was not significantly different between the animals at 14 DPP. However, RE animals had higher SAA3 gene expression at 35 DPP compared to NE animals ($P < 0.05$, Figure 3). SAA plays an important host defense role (Ingvartsen et al., 2003) through opsonization and affinity for gram-negative bacteria ligand OmpA (Oetzel, 2004; Gordon, 2013). In fact, SAA is increased in cows at risk of developing endometritis 7 d postpartum (Foley et al., 2015).

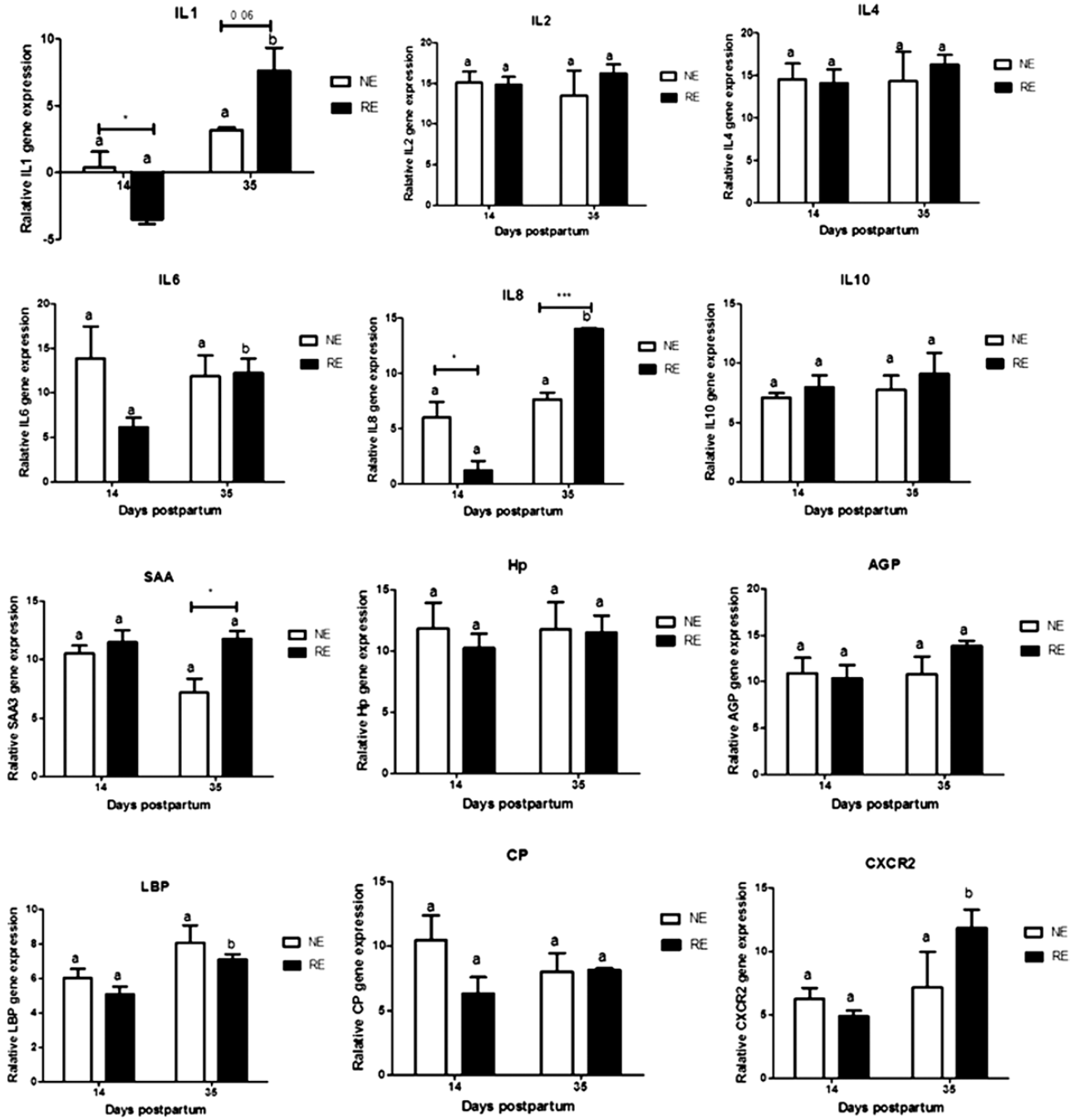

Figure 3. Relative gene expression of *IL1*, *IL2*, *IL4*, *IL6*, *IL8*, *IL10*, *SAA*, *HP*, *AGP*, *LBP*, *ceruloplasmin (CPN)*, and *CXCR2* in cows receiving RE diet and NE diet at 14 and 35 DPP. Results are presented as mean ± SEM. a and b denote significant differences between the results of each group at different time points at the probability, $P < 0.05$.

Expression of CXCR2 was not significantly different between the animal groups at 14 or 35 DPP. RE animals had significantly increased IL1 gene expression ($P \leq 0.01$) (Figure 3). IL-1 is produced by endometrial epithelial and stromal cells and modulates physiological involution after calving (Foley et al., 2015). Moreover, in the presence of pathogenic bacteria, endometrial cells secrete IL-1β and other cytokines as part of the proinflammatory immune response (Herdt, 2000). At the protein level, IL-1b is secreted at high con-centrations in cervicovaginal mucus of cows with endometritis at 7 and 21 DPP, indicating persistent inflammation (Adnane et al., 2017b).

There were no significant differences in IL2, IL4, IL6, and IL10 gene expression between animal groups (Figure 3). Although important for endometritis (Foley et al., 2015; Bicalho et al., 2017), here, these cytokines were not altered by energy diet.

The chemokine IL8 gene expression was significantly downregulated in RE animals at 14 DPP ($P \leq 0.05$) and upregulated at 35 DPP ($P \leq 0.001$, Figure 3). In cows, endometritis is significantly associated with increased expression of IL8 mRNA in the endometrium (Hammon et al., 2006; Jenkins et al., 2015). In the present study, we found that IL8 gene expression was decreased in RE animals at 14 DPP but increased at 35 DPP.

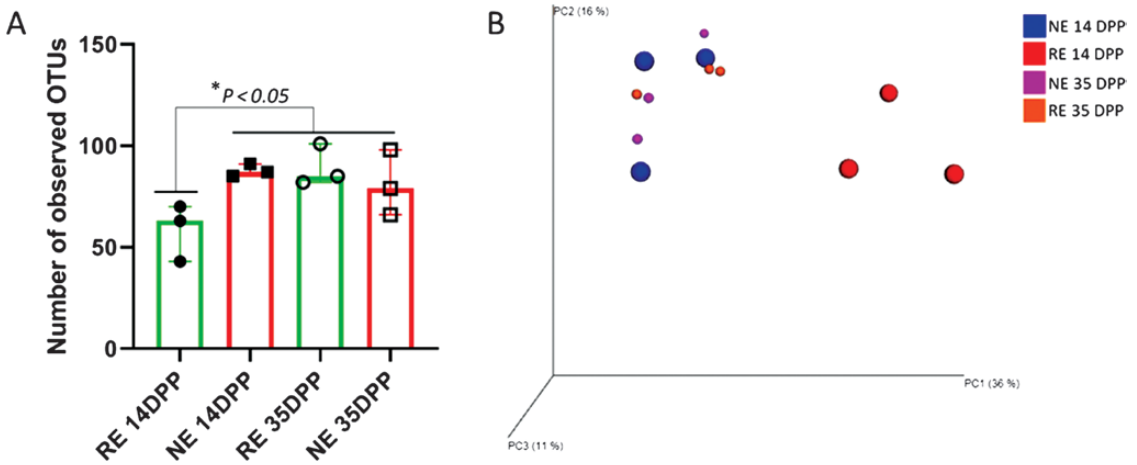

Figure 4. Genus level comparison of bacterial species alpha and beta diversity between the RE and NE uterine microbiota at 14 and 35 DPP. (A) Alpha diversity (species richness) based on the number of observed genus OTUs and (B) Beta diversity patterns based on principal coordinate analysis of weighted UniFrac distances between the NE and RE uterine microbiota at 14 DPP and 35 DPP. Data presented as medians and 95% confidence intervals are based on the analysis of 3 dairy cows per diet group. *Denotes statistically significant differences in bacterial species richness (alpha diversity).

Likewise, RE cows had an upregulation of IL8 gene expression indicating persistent endometrial inflammation. There were no significant differences in LBP gene expression between the groups.

Uterine microbiota comparisons at 14 and 35 DPP

A total of 446 897 16S rRNA sequence reads with an average of 37,241 reads per sample were generated after quality filtering. Taxonomic assignment based on a 97% similarity definition of an OTU identified 235 OTU's with an average of 180 OTU's per animal. Assessment of alpha diversity based on the number of observed OTU's at genus level revealed that there were no significant differences in uterine microbiota in NE animals at 14 or 35 DPP. For RE animals, the 14 DPP uterine microbiota showed significantly lower species diversity ($P < 0.05$) compared to 35 DPP (Figure 4A). There were, however, no statistically significant differences in microbiota bacterial species diversity between the two groups based on the number of observed bacterial taxa at 35 DPP. The beta diversity patterns between RE and NE uterine microbiota at 14 and 35 DPP were explored using principal coordinate analysis (**PcoA**) plots. The first (PC1), second (PC2), and third (PC3) principal coordinates captured 36%, 16%, and 11% of the variation respectively, in a weighted UniFrac PcoA plot (Figure 4). Although our sample size was not large enough to conduct a thorough statistical analysis of the clustering patterns formed, all three RE 14 DPP uterine microbiota samples appeared to group together away from the NE 14 DPP samples as well as the 35 DPP RE and NE samples. In contrast, the NE 14 DPP as well as the 35 DPP RE and NE uterine microbiota did not show any sample-specific grouping, suggesting that their uterine microbial communities might be more similar to each other than to the 14 DPP RE uterine bacterial community. Our observations thus indicate the RE 14 DPP uterine microbial com-munity differed in phylogenetic composition from that detected in NE cows at 14 as well as from the 35 DPP NE and RE uterine microbial communities.

Comparing taxonomic composition at the phylum level also revealed the differences in microbial community composition within as well as between the RE and NE uterine microbiota (Figure 5A and B). An increase in the relative abundances of *Bacteroidetes*, *Cyanobacteria*, and *Actinobacteria* relative abundances were detected between the 14 DPP and 35 DPP uterine microbial communities within both diet groups. Comparing the two groups revealed that *Proteobacteria* (median = 0.2% vs. 23%; adj $P < 0.05$), *Firmicutes* (median = 3.4% vs. 65%; adj $P < 0.05$), and *Cyanobacteria* (median = 0.05% vs. 0.5%; adj $P < 0.05$) were all significantly less abundant in RE compared to NE uterine microbiota at 14 DPP. Although both *Bacteroidetes* (median = 83% vs. 4.5%; adj $P > 0.05$) and *Fusobacteria* (median = 3.6% vs. 2.1%; adj $P > 0.05$) were more abundant in RE than the NE cows at 14 DPP, the differences were not statistically significant. Meanwhile at 35 DPP, the RE uterine microbiota showed lower *Proteobacteria* (median = 2.5% vs. 9.8%; adj $P < 0.05$) but higher *Firmicutes* (median = 54.6% vs. 5.6%; adj $P < 0.05$), *Bacteroidetes* (median = 25.3% vs. 5.9%; adj $P < 0.05$), *Cyanobacteria* (median = 4.4% vs. 0.8%; adj $P < 0.05$), and *Fusobacteria* (median = 1.8% vs 0.6%; adj $P < 0.05$) relative abundances compared to the NE cows.

In addition, a comparison of microbial core genera, defined here as genera detected in all dairy cows per group, showed that there were differences in the distribution of bacterial genera within as well as between the 14 and 35 DPP NE and RE uterine microbiota (Figure 5B). Overall there was a group of 26 genera that were shared between the NE and RE groups at both 14 and 35 DPP. Within the NE uterine microbiota, 27 genera found at 14 DPP were not detected at 35 DPP, whereas 6 genera detected at 35 DPP were not found at 14 DPP. In the RE group, 8 genera found at 14 DPP were not detected at 35 DPP, whereas 21 genera detected on 35 DPP were not present at 14 DPP. Between groups, 21 and 7 genera at 14 DPP, as well as 8 and 28 genera at 35 DPP, were only detected in the NE and RE uterine microbiota, respectively.

We found that the taxonomic composition of bacterial communities differs between RE and NE in postpartum uterine microbiota determined at 14 and 35 DPP suggesting that negative energy balance diet among other effects is also associated with alterations in the uterine microbial community composition (Figures 4 and 5). The uterine microbiota of the RE group at 14 DPP was less diverse and differed in phylogenetic composition compared to the NE group. In addition, differences in bacterial species composition as well as in the relative abundances at phylum level of shared bacterial sequences were detected between the RE and NE groups (Figure 5). Notably, the RE uterine microbiota were characterized by lower relative abundance of Proteobacteria at both 14 and 35 DPP as

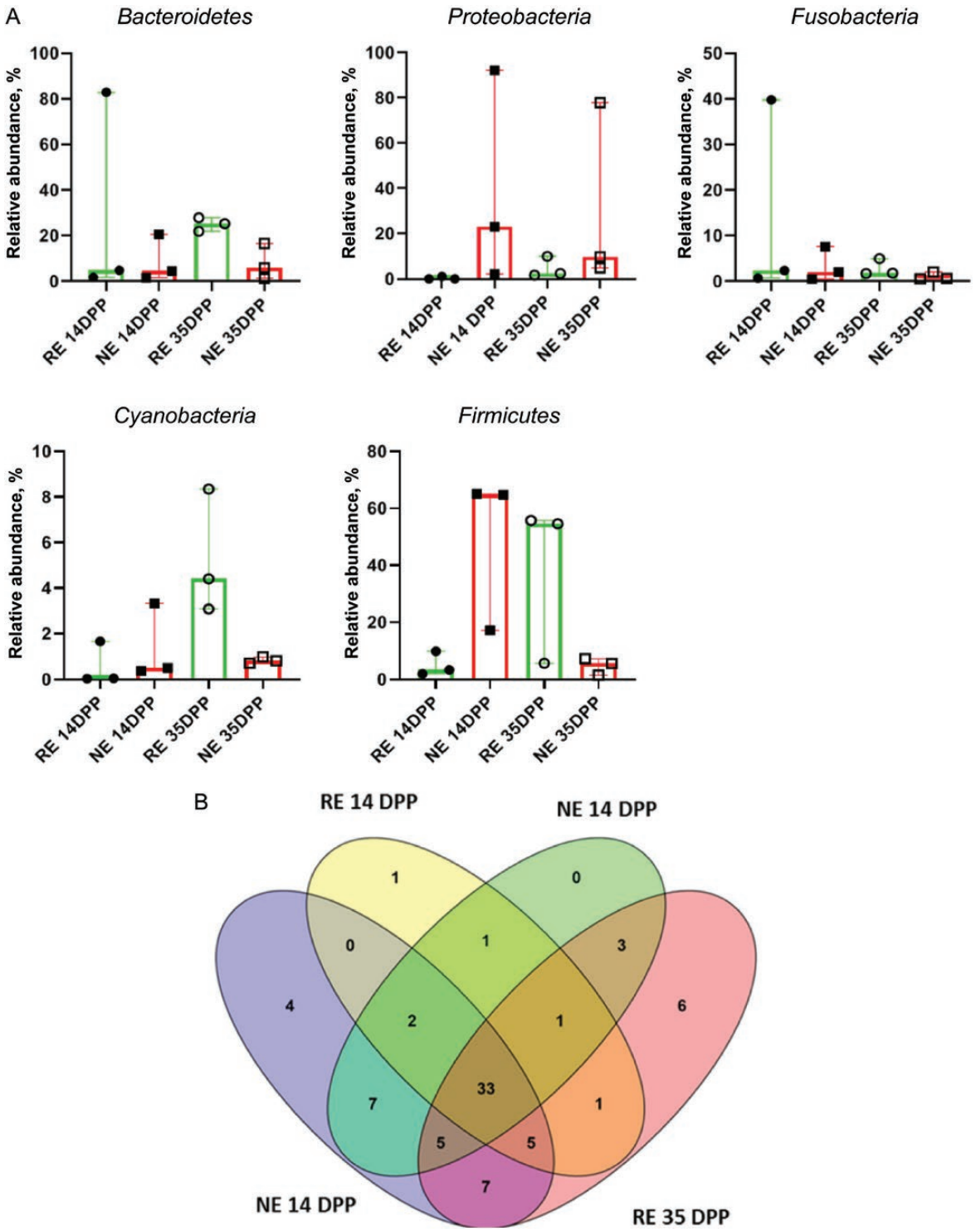

Figure 5. (A) Relative abundances of the five dominant phyla in RE and NE uterine microbiota determined at 14 and 35 DPP. The medians and 95% confidence intervals based on data of 3 dairy cows per diet group are presented. *Denotes statistically significant differences in relative abundances of bacterial phyla in uterine microbiota between the RE and NE cows. (B) Venn diagrams showing the numbers of unique and shared core (genera detected in all dairy cows per group) bacterial genera among the RE and NE uterine microbial communities at 14 DPP and 35 DPP.

well as higher abundances of *Bacteroidetes*, *Fusobacteria*, and *Cyanobacteria* at 35 DPP compared to the NE cows. On the other hand, the relative abundance of *Firmicutes* was higher at 14 DPP and lower at 35 DPP in RE compared to the NE uterine microbiota. Although the uterine microbiota comparison between groups shows interesting results, it is necessary to analyze a larger sample size and to include the bead-beating step during the DNA extraction process to exclude any possible bias especially from a gram-positive bacterial perspective.

NEB postpartum predisposes dairy cows to uterine health disorders including metritis (Toni et al., 2011; Carvalho et al., 2014). However, although it is recognized as a risk factor for uterine health disorders, there is still limited knowledge on the impact of NEB on the prevalence of bacterial groups and links to uterine health or disease in dairy cows post calving. Among the uterine microbiota dysbiosis reported in metritis cows to date, there are increased abundance of *Bacteroidetes* and *Fusobacteria* phyla and low abundance of *Proteobacteria* when compared to healthy cows (Carter et al., 2008; Santos and Bicalho, 2012; Pires et al., 2013; Roche et al., 2015; Bicalho et al., 2016). As such, our findings here indicate that the taxonomic composition of the endometrial microbiota in RE cows is shifting toward a composition that might predispose cows to metritis.

Conclusion

The results of this study show that the causes of uterine diseases during the transition period are multifactorial, and that energy balance plays a key role. Restricted energy intake in transition cattle alters metabolism and increases NEFA and ketone bodies which dysregulate the innate immune response, thus increasing the risk of reproductive diseases. Furthermore, the energy-restricted diet has also influenced the uterine microbial populations further confirming the influence that nutrition has on animal health. Although the objective of this study was not to identify possible markers for cows at risk of metabolic and/or reproductive diseases, correlations between metabolism and immune functions were observed, thus posing the base for more in-depth and bigger studies on this field aiming at identify possible multifactorial markers for early detection of risks for disease to improve the animals health and fertility by implementing precision prevention strategies.

Supplementary Data

Supplementary data are available.

Acknowledgments

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Conflict of interest

The authors G.E., E.R., S.D.L, M. A., P.C.I., P.C., T.T., and A.C. have no conflicts of interest.

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