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Association of intramammary infection status with energy balance and immune function during peripartum in dairy cows

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ABSTRACT: This observational study evaluated whether intramammary infection (IMI) status was associated with energy balance and immune system indicators during the peripartum period in clinically healthy grazing dairy cows ($n = 24$). Body condition score (BCS) was assessed and blood samples were collected at 52 and 15 days prepartum and at 3, 10, 26, and 47 days postpartum to determine serum concentrations of non-esterified fatty acids (NEFA), β -hydroxybutyrate (BHB), total protein, albumin, globulins, cholesterol, haptoglobin and total and differential white blood cell (WBC) counts in whole blood. Gene expression of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) in peripheral leukocytes were also measured at 51 and 15 days prepartum and at 12 days postpartum. Cows were classified as IMI-positive ($n = 15$) if at least one pathogen was isolated from any quarter at 7 days postpartum, and as IMI-negative ($n = 9$) if no bacterial growth was observed in any of the four quarter samples. Associations between IMI status and response variables were evaluated using mixed linear regression models with repeated measures. Cows with IMI showed a more pronounced and prolonged BCS loss, along with higher NEFA concentrations at 10 days postpartum, suggesting greater lipid mobilization. At the same point, the IMI-positive group had significantly higher serum total protein and albumin concentrations. Haptoglobin concentration was higher in the IMI-positive group, indicating an acute phase response. The IMI-positive group exhibited greater total WBC counts and a trend for increased neutrophil and lymphocyte counts. No significant differences were observed for the remaining metabolic variables and cytokine gene expression markers evaluated. In conclusion, differences in lactational adaptation and immune-related parameters were observed between clinically healthy cows with and without IMI during the peripartum period.

Key words: dairy cows, peripartum period, intramammary infection, energy balance, immune system.

Associação do status de infecção intramamária com o balanço energético e a função imunológica durante o período periparto em vacas leiteiras

RESUMO: Este estudo observacional avaliou se o status de infecção intramamária (IMI) estava associado ao balanço energético e a indicadores do sistema imune durante o período periparto em vacas leiteiras, em pastejo clinicamente saudáveis ($n = 24$). Para realizar a avaliação, o escore de condição corporal (BCS) foi avaliado e amostras de sangue foram coletadas aos 52 e 15 dias antes do parto e 3, 10, 26 e 47 dias após o parto para determinar as concentrações séricas de ácidos graxos não esterificados (NEFA), β -hidroxibutirato (BHB), proteína total, albumina, globulinas, colesterol, haptoglobina e contagens total e diferencial de leucócitos (WBC) em sangue fresco. Além disso, a expressão gênica do fator de necrose tumoral- α (TNF- α) e da interleucina-6 (IL-6) em leucócitos periféricos também foi determinada em 51 e 15 dias antes do parto e 12 dias após o parto. As vacas foram classificadas como IMI-positivas ($n = 15$) quando pelo menos um patógeno foi isolado em qualquer quarto aos 7 dias após o parto, e como IMI-negativas ($n = 9$) quando não foi observado crescimento bacteriano em nenhuma das quatro amostras de quartos. Adicionalmente, as associações entre o status de IMI e as variáveis-resposta foram avaliadas por meio de modelos de regressão linear mista com medidas repetidas. Os resultados mostram que vacas com IMI apresentaram perda de BCS mais acentuada e prolongada, juntamente com maiores concentrações de NEFA aos 10 dias pós-parto, sugerindo maior mobilização lipídica. No mesmo momento, o grupo IMI-positivo apresentou concentrações séricas significativamente mais elevadas de proteína total e albumina. A concentração de haptoglobina foi maior no grupo IMI-positivo, indicando uma resposta de fase aguda. Igualmente, o grupo IMI-positivo também apresentou maiores contagens totais de leucócitos e uma tendência a maiores contagens de neutrófilos e linfócitos. Não foram observadas diferenças significativas para as demais variáveis metabólicas e marcadores de expressão gênica de citocinas avaliados. Em conclusão, foram observadas diferenças na adaptação à lactação e em parâmetros relacionados ao sistema imune entre vacas clinicamente saudáveis com e sem IMI durante o período periparto.

Palavras-chave: vacas leiteiras, período periparto, infecção intramamária, balanço energético, sistema imune.

1 INTRODUCTION

2
3 During the transition from late gestation to
4 lactation, dairy cows experience a period of negative

energy balance (NEB) due to decreased feed intake
and increased energy demand for milk production.
This results in adipose tissue mobilization, and the
consequent loss of body condition score (BCS) and

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increasing concentrations of metabolites related to the NEB such as non-esterified fatty acids (NEFA) and β -hydroxybutyrate (BHB; OVERTON et al., 2017). Decreased feed intake has been negatively associated with serum cholesterol concentrations, a metabolite regarded as an indicator of energy balance (HERDT, 2000). Moreover, the reduction in feed intake and lipomobilization has also been associated with decreased serum concentrations of nitrogenous metabolites (*i.e.* urea, total protein, albumin and globulins; CAVESTANY et al., 2005).

In addition, KIMURA et al. (2014) reported a negative association between blood energy metabolite concentration and immune cell counts and their functionality. BARCA et al. (2021) reported a negative association between prepartum NEFA concentrations and postpartum white blood cell (WBC), neutrophil, and lymphocyte counts after calving. Concurrently, the metabolic adaptation to lactation, which involves NEB, mobilization of body reserves, alterations in nutrient partitioning, and an impaired immune response, has been linked to a high risk of inflammatory diseases such as mastitis around parturition (COMPTON et al., 2007; LEBLANC, 2020).

Bovine mastitis is mostly caused by bacterial intramammary infections (IMI; BANNERMAN, 2009). The pattern and severity of the immune response depend on the bacterial species and on cow factors, including energy balance, parity, and innate host resistance (BURVENICH et al., 2003; SCHUKKEN et al., 2011). The immune response can range from a mild subclinical reaction to severe systemic illness. In subclinical mastitis, this is typically reflected by an increase in somatic cell count (SCC) in the milk, whereas more severe infections are associated with elevated circulating immune cells and upregulation of inflammatory markers, indicating a pronounced host immune response (SCHUKKEN et al., 2011). SADEK et al. (2017) showed that pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), as well as positive acute phase proteins (APPs), such as haptoglobin, were elevated in mastitis. Moreover, studies have analyzed the association between gene expression related to the functionality of WBC and the presence of subclinical (YANG et al., 2022) and clinical mastitis in dairy cows (McCONNEL et al., 2020). However, to our knowledge, there are no reports on gene expressions in WBC of healthy cows with IMI.

Although the inflammatory response is a critical component of the immune defense, it can also significantly impact energy balance and nutrient

metabolism (BRADFORD & SWARTZ, 2020). Accordingly, clinical and subclinical mastitis are associated with alterations in blood biochemical parameters, including changes in serum protein concentrations (MATEI et al., 2010; SADEK et al., 2017). In a recent study, YU et al. (2025) showed that cows with subclinical mastitis had lower cholesterol concentrations than healthy cows, while PEGOLO et al. (2023) reported that IMI was associated with elevated serum concentrations of total protein, globulins, and haptoglobin. However, these studies analyzed metabolic and immune system indicators at a single time point, while there is no data available on energy metabolites, WBC counts, and gene expression profiles during multiple time points in the peripartum period in healthy cows with or without IMI.

Based on these data, we hypothesized that clinically healthy cows with an IMI after calving would exhibit a differential metabolic profile and altered immune activation, reflected in increased WBC counts and the gene expression of TNF- α and IL-6, both prepartum and during early lactation, compared to animals without IMI. Thus, this study described the energy balance, WBC counts, and the expression of TNF- α and IL-6 during the peripartum period in clinically healthy grazing dairy cows with or without an IMI after calving.

MATERIALS AND METHODS

Animals and study design

An observational cohort study was conducted in a pasture-based dairy system using Holstein-Friesian cows from a 400-cow commercial herd located in Colonia, Uruguay (34°S-57°W). Cows were selected according to the expected calving date ($n = 50$). Approximately two months before the expected calving date, all multiparous cows (an animal with one or more previous calvings [mean $\pm$ sd = 3.6 $\pm$ 1.5 calvings]) received antibiotic dry cow therapy and were moved to outdoor close-up paddocks with a low supply of grass (<10 kg dry matter intake/cow/day), where they were supplemented twice a day with total mixed ration with anionic salts, according to close-up requirements. No vaccination programs against mastitis pathogens were implemented in the herd. Primiparous cows (an animal approaching her first calving) were managed in the prepartum period under the same environmental conditions, including feeding management. Calving occurred in a subdivision of the same paddock under the same conditions. Calving for cows monitored in the study occurred between August 5 and October 7, 2015. The calving system

was a split calving period with a concentration in autumn and spring. After calving, cows were kept on pasture only between the two daily milkings, and at least 40% of the dry matter intake came directly from the grazing sessions, supplemented with a partial mixed ration before milking. Cows were milked twice daily with an average milk production of around 25 L/cow/day and the bulk tank somatic cell count during the trial was ~ 350.000 SCC/mL. Only clinically healthy animals remained in the final analysis. Clinical status was assessed by the veterinarian in charge of the research (first author) and was verified regularly throughout the entire study period. Animals with clinical signs of retained fetal membranes, metritis, clinical mastitis (abnormal milk, local symptoms, and/or signs of systemic illness), abomasal displacement, milk fever, and clinical ketosis (KELTON et al., 1998; SHELDON et al., 2006; PINZÓN-SANCHEZ & RUEGG, 2011) were excluded from the study. After excluding clinically diseased cows, only 24 (n = 15 and n = 9; multiparous and primiparous cows, respectively) clinically healthy cows remained in the study.

Body condition score was assessed using a 5-point scale (FERGUSON et al., 1994) at 52 (SD = 7.9) and 15 (SD = 5.2) days before calving and at 3 (SD = 1.6), 10 (SD = 3.1), 26 (SD = 3.8) and 47 (SD = 11.8) days after calving. During each of these visits, blood samples were collected from the coccygeal vessels into 8.5-mL clot accelerator tubes and 6.5-mL sterile K2-EDTA tubes (Becton Dickson, Franklin Lakes, NJ). The clot accelerator tubes were centrifuged at 3,000 x g for 20 min and serum was stored frozen (-20 °C) until further laboratory analyses.

Bacteriology

Quarter milk samples of each cow were collected at 7 (SD = 4.6) days after calving, stored immediately on ice, and delivered the same day to Lactodiagnóstico Sur laboratory, Uruguay, for isolation and identification of bovine mastitis pathogens. Isolation and identification of mastitis pathogens was performed according to the standards of the National Mastitis Council (NMC, 1999). Briefly, *Staphylococcus aureus* (*S. aureus*) and non-*uberis* esculin-positive *Streptococcus* (EPS) were considered to cause IMI if one (100 CFU/mL) was isolated. Growth of 2 colony-forming units (200 CFU/mL) of *Escherichia coli* (*E. coli*) and *Prototheca* spp., or 10 CFU (1000 CFU/mL) of non-*aureus* staphylococci and mammaliicocci (NASM) or *Corynebacterium bovis* (*C. bovis*) were considered a positive isolation (NMC, 1999). If three or more bacterial species were cultured from a sample, the

sample was considered contaminated. Based on these IMI definitions, the results of the cultures were used to classify cows as IMI-negative: cows with no IMI in any of the four quarter samples, and IMI-positive: cows with an IMI in at least one quarter sample.

Metabolite determinations

Serum metabolites were determined at the Animal Endocrinology and Metabolism Laboratory, Veterinary Faculty, Montevideo, Uruguay and measured by colorimetric assays on a Vitalab Selectra II autoanalyzer (Vital Scientific, Dieren, The Netherlands) using commercial kits: NEFA (Wako NEFA-HR (2), Wako Pure Chemical Industries Ltd., Osaka, Japan); BHB (Randox Laboratories Limited, 55 Diamond Road, Crumlin, County Antrim, BT29 4QY, United Kingdom); cholesterol, total protein, and albumin (Wiener Laboratories S.A.I.C. Riobamba, Rosario, Argentina). Globulin concentration was calculated by subtracting albumin from total protein concentration. Haptoglobin concentrations were measured using a commercial ELISA kit (Thermo, Multiskan EX, USA). The inter-assay coefficients of variation (CV) for all commercial serum controls were ≤ 10 %.

White blood cell counts

Blood samples taken in K2-EDTA tubes were used for total (WBC) and differential leukocyte (*i.e.* neutrophil, lymphocyte, and monocyte) counts on the same day of extraction. The total count was determined using an automated hemocytometer (Sysmex XT1000, Roche Diagnostic, CA, USA), while cell morphology (differential blood count) was assessed by microscopic examination of blood smears. The neutrophil-to-lymphocyte ratio (N/L) was calculated as the ratio between absolute neutrophil and lymphocyte counts. Blood samples taken in K2-EDTA tubes were centrifuged at 3,000 x g for 20 min and the resulting white buffy coat was aspirated, suspended in TRIzol reagent, and frozen (-80 °C) for gene expression.

Gene expression

Gene expression was evaluated at 51 (SD = 8.4) and 15 (SD = 5.2) days before, and 12 (SD = 6.4) days after calving. Gene expression analysis was performed on a reduced number of sampling time points compared with metabolic variables due to logistical and analytical constraints associated with mRNA processing. One multiparous cow from the IMI-positive group was excluded because the 12 day postpartum sample was identified as an outlier with respect to sampling time. Total RNA from white

blood cells was extracted using TRIzol (Invitrogen), followed by lithium chloride precipitation to remove cDNA synthesis inhibitors and DNase treatment (DNA-Free™ Kit, Ambion) to eliminate contaminating DNA. The RNA concentration and purity were assessed via absorbance at 260 nm and 260/280 ratio, respectively. The cDNA was synthesized using SuperScript III (Invitrogen) with oligo-dT primers and 1 µg of total RNA. Primer sequences, amplicon length, and amplification efficiency were determined for the genes TNF-α (NM_173966; 103 bp; efficiency 1.30), IL-6 (NM_173923.2; 69 bp; efficiency 1.19), ACTB (NM_173979.3; 54 bp; efficiency 1.18; forward: GCGTGGCTACAGCTTCACC; reverse: TTGATGTACACGGACGATTTC), and GAPDH (BC102589; 67 bp; efficiency 1.10). Primer sequences for TNF-α, IL-6, and GAPDH were obtained from GALVÃO et al. (2012), whereas ACTB primers were designed in the present study. Real-time PCR was performed using SYBR®Green master-mix (Quantimix EASY SYG, Biotools), equimolar primers, 2 µL of cDNA, and RNase/DNase-free water in a 15 µL reaction. Each sample was analyzed in duplicate using a Rotor-Gene™ 6000 (Corbett Life Sciences). PCR conditions were 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 45 s at 60 °C, and 20 s at 72 °C. Dissociation curves and No Template Controls ensured specificity and absence of contamination. Pooled cDNA from six cows (three per group) served as an exogenous control for standard curves using five dilutions. Assay efficiency (E) was calculated as $E = (10^{-1/\text{slope}} - 1)$ following RUTLEDGE & COTE (2003). Relative quantification (PFAFFL, 2001) was used for gene expression analysis, normalized to the geometric mean of ACTB and GAPDH.

Statistical analysis

Data were analyzed using SAS software (SAS Institute Inc., 2018. SAS® University Edition, Cary, North Carolina, USA). Univariate analyses (PROC UNIVARIATE) were performed for all variables to identify potential outliers, inconsistencies, and to assess normality. Haptoglobin concentrations and gene expression were $\log_{10}$ -transformed to achieve normality; the back log-transformed data are presented. The association of BCS, serum metabolite concentrations, WBC counts, and gene expression with the IMI status were evaluated with mixed linear regression models (PROC MIXED). Fixed effects in the model included IMI-status group (positive vs. negative), time (days relative to calving), and the interaction IMI status group by time. Calving date was also included as a covariate in the model. The

individual cow was included as a random effect. The IMI status group, time, and interaction were always forced into the models. Calving date was retained when $P \leq 0.10$. Degrees of freedom were adjusted using the Kenward-Roger approximation. Data were analyzed using repeated measures (time within cow). To account for the correlation between repeated measurements within subjects, a spatial power covariance structure was used. Model assumptions of normality and homoscedasticity of the residuals were visually assessed. When relevant, data are reported as LSM $\pm$ SEM or back-transformed LSM and 95% confidence interval. Differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered a tendency.

RESULTS

Cow-level milk bacteriology

Quarter milk samples from nine cows ($n = 6$ and $n = 3$; multiparous and primiparous cows, respectively) yielded no growth in any of the four quarters and were thus classified as IMI-negative. Fifteen cows ($n = 9$ and $n = 6$; multiparous and primiparous cows, respectively) showed growth of at least one pathogen in one or more quarters (Table 1) and were classified as IMI-positive. There were no contaminated samples.

Body condition score and metabolite concentrations

Variables that remained in the final regression models for each response variable are presented in table 2.

Body condition score began to decline after 15 days before calving until 3 days after calving in both groups ($P = 0.002$ and $P = 0.04$, in IMI-positive group and IMI-negative group, respectively). Body condition score in the IMI-negative group reached the nadir at 3 days after calving, while in the IMI-positive group, the BCS continued to decline until 10 days after calving ($P = 0.01$), whereas no significant changes were observed in the IMI-negative group ($P = 0.83$). At 10 days after calving, the IMI-positive group showed lower BCS than the IMI-negative group ($P = 0.04$; Figure 1A). Non-esterified fatty acids concentration started to increase after 15 days before calving until 3 days after calving in both groups ($P < 0.0001$ and $P = 0.002$, in IMI-positive group and IMI-negative group, respectively). At 10 days after calving, the IMI-positive group showed higher NEFA concentrations than the IMI-negative group ($P = 0.05$; Figure 1B). β -hydroxybutyrate concentrations were not associated with any effect (Table 2).

Table 1 - Quarter-level bacterial isolation results of milk samples taken after calving in 15 healthy cows under grazing conditions.

-----Quarter milk bacteriological results-----				
Cow ID	LF	RF	LH	RH
1	ng	ng	ng	<i>C. bovis</i>
2	ng	ng	EPS	ng
3	ng	NASM	ng	ng
4	ng	<i>C. bovis</i>	ng	<i>C. bovis</i>
5	ng	ng	<i>C. bovis</i>	ng
6	ng	<i>Prototheca</i> spp.	ng	ng
7	ng	ng	<i>E. coli</i>	ng
8	ng	NASM	NASM	ng
9	ng	ng	EPS	ng
10	NASM	ng	NASM	ng
11	<i>S. aureus</i>	ng	ng	<i>S. aureus</i>
12	NASM	ng	ng	NASM
13	ng	<i>C. bovis</i>	NASM	ng
14	ng	ng	ng	NASM
15	ng	<i>C. bovis</i>	NASM	ng

LF, RF, LH and RH: left front, right front, left hind and right hind, respectively; EPS: non-*uberis* esculin-positive *Streptococcus*; NASM: non-*aureus* staphylococci and mammaliicocci. ng: no growth. Milk samples for bacteriological analysis were collected at 7 ± 4.6 days postpartum.

Cholesterol concentrations in both groups began to decline after 52 days before calving until 15 days before calving (P = 0.04 and P = 0.05, in IMI-positive group and IMI-negative group, respectively), reaching a nadir 3 days after calving and starting to increase 10 days after calving until 26 days after calving in both groups (P = 0.02 and P = 0.01, in IMI-positive group and IMI-negative group, respectively; Figure 1D). Total protein concentrations in both groups began to decline 15 days before calving and reached their nadir 3 days after calving (P = 0.009 and P = 0.02, in IMI-positive group and IMI-negative group, respectively). At 10 days after calving, total protein concentrations were greater in the IMI-positive group than in the IMI-negative group (P = 0.03; Figure 1E). Albumin concentrations in both groups showed a nadir at 10 days after calving; however, the IMI-positive group presented higher concentrations than the IMI-negative group (P = 0.01; Figure 1F). Globulin concentrations in both groups started to decrease from 15 days before calving and reached a nadir at 3 days after calving (P = 0.01 and P = 0.009, in IMI-positive group and IMI-negative group, respectively; Figure 1G). Haptoglobin concentrations were associated with IMI status, as the IMI-positive group showed greater concentrations than the IMI-negative group [0.14 (95% CI: 0.08-0.25) vs. 0.09

(95% CI: 0.05-0.19) g/L, P = 0.05). Haptoglobin concentrations in both groups started to increase at 15 days before calving and peaked at 3 days after calving (P = 0.002 and P = 0.009, in IMI-positive group and IMI-negative group, respectively). At 26 days after calving, haptoglobin concentrations still tended to be greater in the IMI-positive group than in the IMI-negative group (P = 0.09; Figure 1H).

White blood cell counts and gene expression

White blood cell counts according to IMI status and time are shown in figure 2. White blood cell counts showed an association with the IMI status: the IMI-positive group had greater WBC counts than the IMI-negative group (14.9 ± 1.6 vs. 12.9 ± 1.8 x 10³ cells/μL, respectively; P = 0.04). In the IMI-positive group, WBC counts decreased from 15 days before calving until 3 days after calving (P = 0.02), whereas no significant changes were observed in the IMI-negative group (P = 0.45; Figure 2A). Neutrophil counts tended to be associated with the IMI status group: the IMI-positive group tended to show greater counts than the IMI-negative group (6.1 ± 0.09 vs. 4.9 ± 1.1 x 10³ cells/μL, respectively, P = 0.08). Lymphocyte counts tended to be associated with the IMI status: the IMI-positive group tended to present greater lymphocyte counts than the IMI-

Table 2 - F-tests ($P > F$) of fixed effects included in the final regression models for measured parameters in cows under grazing conditions. Fixed effects are the effects of group, time and group by time interaction.

Variable	Group	Time	Group x Time
BCS	0.12	< 0.0001	0.62
NEFA	0.31	< 0.0001	0.36
BHB	0.71	0.11	0.86
Cholesterol	0.18	< 0.0001	0.92
Total protein	0.25	< 0.0001	0.51
Albumin	0.39	< 0.0001	0.21
Globulins	0.65	< 0.0001	0.94
Haptoglobin	0.05	0.02	0.81
WBC	0.04	0.17	0.97
Neutrophil	0.08	0.38	0.81
Lymphocyte	0.09	0.52	0.91
Monocyte	0.69	0.72	0.64
N/L ratio	0.51	0.72	0.66
TNF- α mRNA	0.24	0.36	0.96
IL-6 mRNA	0.87	0.42	0.58

Group: intramammary infection status group (positive vs. negative); Time: days relative to calving; BCS: body condition score; NEFA: non-esterified fatty acids; BHB: β -hydroxybutyrate; WBC: white blood cell; TNF- α : tumor necrosis factor- α ; IL-6: interleukin-6. Calving date not presented because no associations were detected for any variables ($P > 0.10$).

negative group (7.2 ± 1.1 vs. $6.1 \pm 1.1 \times 10^3$ cells / μL , respectively, $P = 0.09$). In the IMI-positive group, lymphocyte counts decreased from 15 days before calving until 3 days after calving ($P = 0.05$), whereas no significant changes were observed in the IMI-negative group ($P = 0.83$; Fig. 2-C). Monocyte counts were not associated with IMI status (Table 2). The N/L ratio were not associated with IMI status (LSM = 0.91 vs. 0.99, from IMI-positive group and IMI-negative group; $P = 0.72$). Gene expression was not associated with IMI status group or time (Table 2). Tumor necrosis factor- α gene expression (LSM, 95% CI) was 0.25 (0.09-0.65), 0.89 (0.69-1.39), and 0.23 (0.05-0.63) in IMI-positive group and 0.09 (0.008-0.59), 0.27 (0.11-0.87), and 0.08 (0.009-0.58) in IMI-negative group at 51 and 15 days before calving and 12 days after calving, respectively. Interleukin-6 gene expression was 0.25 (0.05-0.75), 0.52 (0.31-1.12), and 0.28 (0.11-0.88) in IMI-positive group and 0.09 (0.005-0.79), 1.17 (0.81-1.67), and 0.38 (0.22-1.08) in IMI-negative group at 51 and 15 days before calving and 12 days after calving, respectively.

DISCUSSION

To our knowledge, this is the first study that investigates energy balance, WBC counts,

and their TNF- α and IL-6 gene expression profiles during the peripartum period in clinically healthy grazing dairy cows with or without an IMI after calving. Body condition score decreased around calving in both groups, reflecting the typical body fat mobilization, a homeorhetic mechanism to cope with NEB and ensure energy substrate availability. This mobilization was also evident in the increase of serum NEFA concentrations, consistent with previous findings (MEIKLE et al., 2004). Interestingly, while in the IMI-negative group BCS reached its nadir at 3 days postpartum, consistent with the peak in NEFA concentration, in the IMI-positive group, the BCS nadir and the maximum NEFA concentration reached 10 days after calving. These data suggested that lipid mobilization in IMI-positive cows was more pronounced or longer lasting than in the IMI-negative group. These findings may be explained by a lower feed intake and greater catabolic metabolism in the IMI-positive group. DERVISHI et al. (2015) showed that cows with naturally occurring subclinical mastitis had a lower feed intake. Moreover, the immune response during infection increases the energy expenditure to produce immunoglobulins and activate defense cells (BRADFORD & YLIOJA, 2018), which potentially could have contributed to the longer-lasting BCS loss.

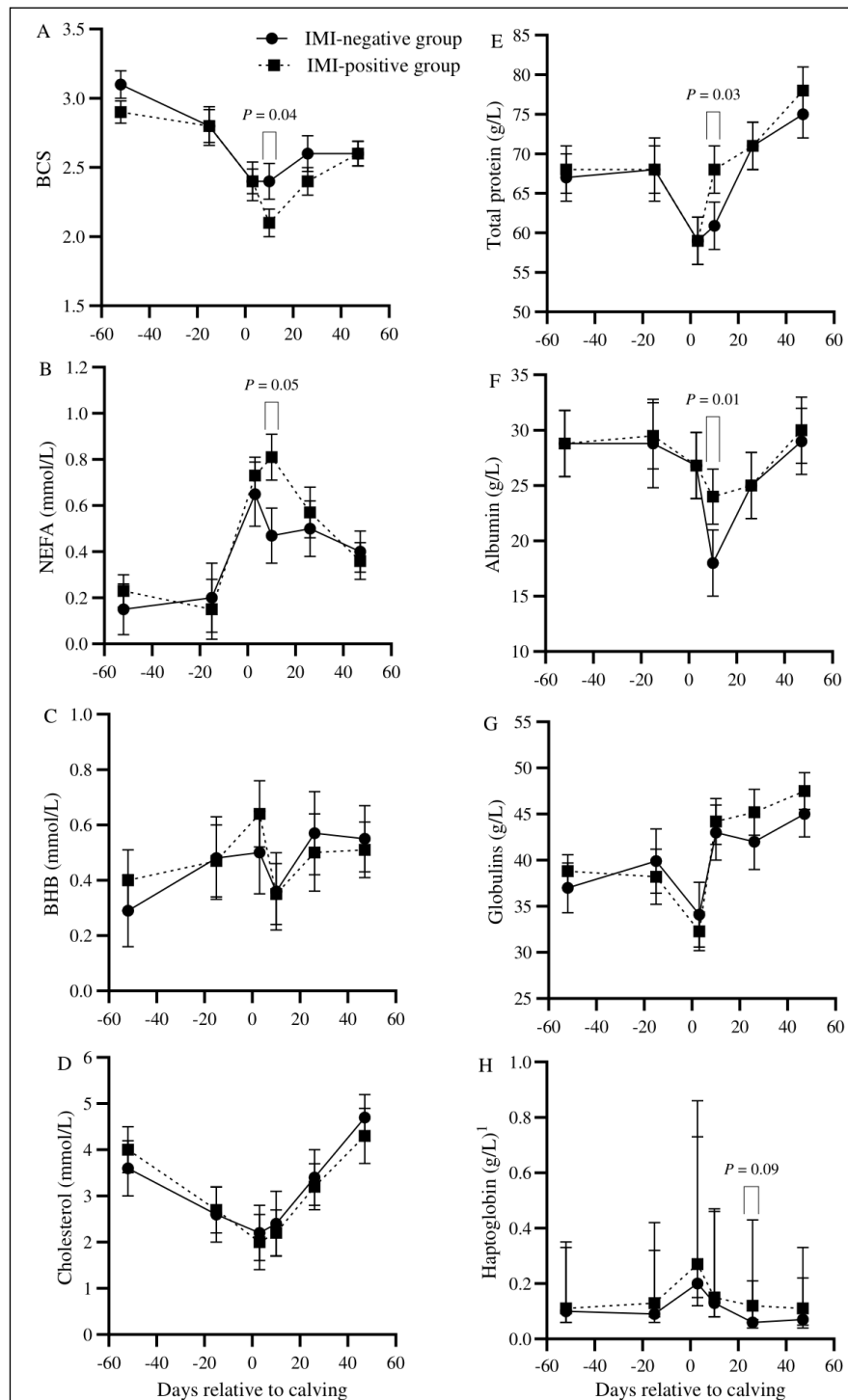


Figure 1 - Least square means of (A) body condition score (BCS), (B) non-esterified fatty acids (NEFA), (C) β -hydroxybutyrate (BHB), (D) cholesterol, (E) total protein, (F) albumin, (G) globulins and (H) haptoglobin concentrations in the intramammary infection positive group (dashed line) compared with the intramammary infection negative group (solid line). Error bars represent standard error. ¹Back log-transformed least square means and 95 % confidence interval presented. Brackets and associated P-values indicate between-group differences or trends at the corresponding sampling time.

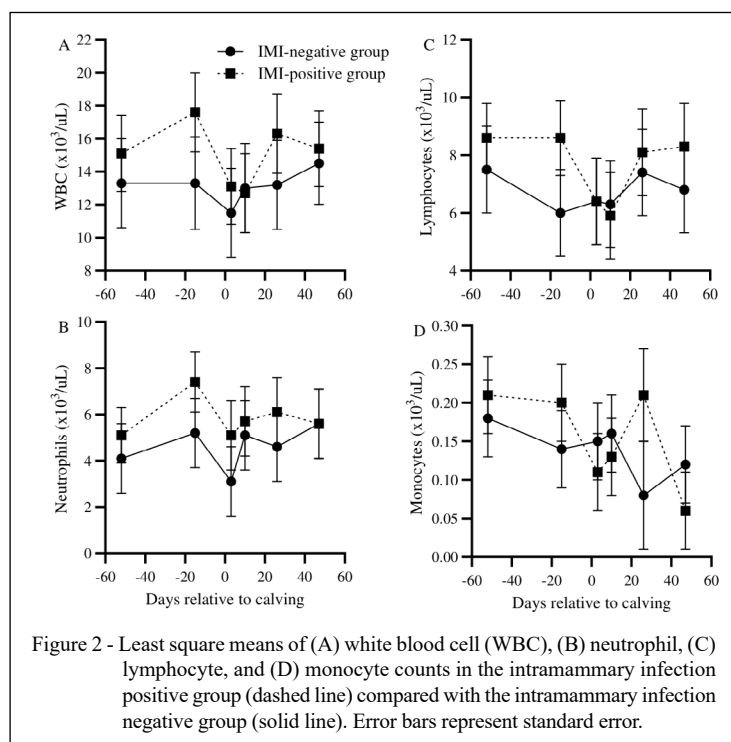


Figure 2 - Least square means of (A) white blood cell (WBC), (B) neutrophil, (C) lymphocyte, and (D) monocyte counts in the intramammary infection positive group (dashed line) compared with the intramammary infection negative group (solid line). Error bars represent standard error.

In our experiment, we reported no differences in total protein concentrations between IMI status groups. However, at 10 days after calving, the IMI-positive cows showed a higher total protein concentration, similar to previous studies that reported increased total protein concentrations in cows with subclinical mastitis (MATEI et al., 2010). Interestingly, PEGOLO et al. (2023) reported that IMI-positive cows had higher total protein and globulin concentration at one-time point in late lactation (220 ± 125 days postpartum), which has been associated with the activation of the acute phase response with increases in APP and immunoglobulins (STANOJEVIĆ et al., 2023). Unexpectedly, albumin concentrations at 10 days after calving were greater in the IMI-positive group and we have no obvious explanation for this finding. Indeed, previous studies have reported lower serum albumin levels in cows affected by subclinical mastitis (MATEI et al., 2010; SADEK et al., 2017; PEGOLO et al., 2023), which may be explained by a prioritization of the synthesis of positive APPs, such as haptoglobin, in the liver (TOTHOVA et al., 2014). Haptoglobin data supports the idea that the IMI-positive group experienced a mild acute phase response, since the concentrations were higher in the IMI-positive group and tended to be still higher at 28 days after calving. Increased

haptoglobin serum concentrations were observed in clinical and subclinical mastitis, as well as in IMI-positive cows (GRÖNLUND et al., 2003; PEGOLO et al., 2023), compatible with the activation of the immune system. Haptoglobin actively participates in the recruitment of neutrophils, tissue regeneration, and limits oxidative stress resulting from the inflammatory process (TOTHOVA et al., 2014).

In this study, the presence of intramammary infection resulted in higher WBC counts and a tendency to increased neutrophil and lymphocyte counts, as reported in cows with subclinical mastitis (SADEK et al., 2017). As far as we know this is the first study reporting an association between WBC and IMI during the peripartum period using repeated measurements over time. The presence of infection within the udder activates local macrophages and epithelial cells that release inflammatory mediators, which cause a systemic increase in the proliferation, differentiation and release of mature forms of leukocytes (SCHUKKEN et al., 2011). Interestingly, we observed that in the IMI-positive group, the WBC and lymphocyte counts decreased from 15 days before calving to 3 days after calving, while in the IMI-negative group, the counts remained constant. This could be explained by an influx of immune cells into the infected mammary gland (SCHUKKEN et al.,

2011). Unlike BRAUN et al. (2021), who reported an increased N/L ratio in cows with acute toxic mastitis, no differences in the N/L ratio were observed in IMI-positive group compared to IMI-negative group. This may suggest that this index primarily reflects systemic responses in severe clinical disease rather than subclinical intramammary infection during the peripartum period.

Despite the lack of statistical significance, which might be attributed to the small sample size, gene expression of TNF- α before calving was approximately three times higher in the IMI-positive group than in the IMI-negative group. Although, this numerical difference should be interpreted with caution, it may suggest that the IMI could already be present during the dry period - widely recognized as one of the highest risk phases for the establishment of IMI (BALLOU, 2012) -, as demonstrated by GREEN et al. (2002). Previous transcriptomic studies in dairy cows with clinical and subclinical mastitis did not show significant differential expression of the TNF- α gene in circulating leukocytes compared to healthy cows (MCCONNEL et al., 2020; CHENG et al., 2021; YANG et al., 2022). However, YANG et al. (2022) demonstrated increases in cell signaling pathways activated by TNF- α .

In the infected udder tissue, TNF- α is synthesized by activated mammary epithelial cells and is involved in neutrophil recruitment (SCHUKKEN et al., 2011). Elevated TNF- α expression in blood leukocytes could be consistent with disruption of the blood-milk barrier and systemic inflammation, suggesting that the response extends beyond the udder. DERVISHI et al. (2015) showed that TNF- α concentrations were higher in the serum of cows with subclinical mastitis at four weeks before calving compared to their healthy counterparts. Once in circulation, this pro-inflammatory cytokine can induce systemic effects, including appetite suppression through hypothalamic anorexigenic signaling pathways (BURVENICH et al., 2003). This mechanism may partly explain the greater loss of BCS observed in the IMI-positive group, as well as the higher circulating concentrations of NEFA at 10 days after calving. Tumor necrosis factor- α is known to induce positive APP synthesis in hepatocytes and to activate genes that promote cell survival and proliferation (MINUTI et al., 2015). Therefore, the elevated haptoglobin concentrations and the higher WBC counts in the IMI-positive group may be associated with the numerically higher TNF- α gene expression observed in our study.

Contrary to expectations, IL-6 gene expression was numerically higher in the IMI-negative

group compared to the IMI-positive group during the second prepartum sampling; however, this difference was not statistically significant. Interleukin-6 has been recognized as an early but non-specific indicator of mastitis (BOCHNIARZ et al., 2024). Besides, IL-6 is known to have both pro- and anti-inflammatory properties, depending on the context and stage of the immune response (SCHELLER et al., 2011). In this regard, some studies have reported elevated serum IL-6 levels in subclinical mastitis (SADEK et al., 2017; BOCHNIARZ et al., 2017), while others have found lower levels (HAGIWARA et al., 2001), and yet another study found no significant differences between mastitic and healthy cows (BOCHNIARZ et al., 2024). Similarly, previous transcriptomic studies have not shown significant differences in IL-6 expression in circulating leukocytes between cows with or without clinical mastitis (MCCONNEL et al., 2020). These inconsistencies suggested a variable systemic IL-6 response during mastitis. Interestingly, a recent study reported higher IL-6 gene expression in the blood of healthy cows compared to those with subclinical mastitis (YANTHI et al., 2024). This suggested that the reduced expression of IL-6 in cows with subclinical mastitis may indicate an attenuated immune response or a downregulation of this cytokine in response to infection.

This study provided preliminary findings that may be valuable for generating hypotheses about the relationship between IMI, metabolic challenges, and immune function. However, because the study is observational in nature, causal relationships cannot be inferred. The results may reflect either a metabolic response to immune activation triggered by IMI, or a metabolic challenge associated with reduced immune competence that predisposes cows to IMI, but the current study design does not allow to disentangle these pathways. Accordingly, future studies may test the hypothesis that IMI occurring during the peripartum or dry period precedes and induces systemic immune activation and metabolic alterations in dairy cows.

Before drawing conclusions from this small-scale study, some limitations have to be considered: the sample size is limited, primiparous and multiparous cows were analyzed as a single group and an unequal distribution of parity among the groups, the detection of an IMI was performed in a single milk sample, and no previous history of the cows was included, as well as the lack of data on milk production and SCC count. In addition, IMI status was determined based on the bacteriological findings obtained at 7 days after calving. Although

this approach allowed the identification of cows with or without IMI at the time of classification, repeated bacteriological sampling throughout the transition period would have provided a more comprehensive characterization of infection dynamics and persistence. Furthermore, cows classified as IMI-positive were grouped regardless of the bacterial species isolated. Since mastitis pathogens differ in their virulence and in the host immune response they elicit (RAINARD & RIOLLET, 2006; SCHUKKEN et al., 2011), pathogen-specific effects on metabolic and inflammatory variables may have been obscured. It should also be noted that these results were obtained from a single dairy farm, which limits their extrapolation to other animals and production systems. Also, while significant differences were observed between groups, it should be noted that no correction for multiple mean comparisons, such as Tukey's adjustment, was applied. This may limit the robustness of the statistical inferences. Moreover, the limited sample size restricted the statistical power to detect potential correlations between variables. Future studies including larger populations, repeated bacteriological assessments throughout the peripartum period, and pathogen-specific analyses are warranted to confirm and expand upon the present findings.

In conclusion, differences in lactational adaptation and immune-related parameters were observed between clinically healthy cows with and without IMI during the peripartum period. These findings suggest that IMI may influence both immune and metabolic responses around calving, even in the absence of clinical mastitis.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available upon reasonable request to the corresponding author.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTION

All authors contributed equally to the conception and writing of the manuscript. All authors critically revised the manuscript and approved the final version.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

We declare that the project that gave rise to this data was submitted and performed in compliance with regulations set by Honorary Committee for Animal Experimentation, University of the Republic (project number: CEUAFVET-PI-40).

DECLARATION OF USE OF ARTIFICIAL INTELLIGENCE

The authors did not employ any artificial intelligence in this study.

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