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RESEARCH ARTICLE

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Associations between systemic inflammatory blood biomarkers and intramammary infection caused by *Staphylococcus aureus* in susceptible and resistant Holstein cows

Gabriele Giannotta[a], Vittoria Bisutti[a], Diana Giannuzzi[a], Antonio Barberio[b], Margherita Soncin[b], Lara Biasio[b], Giulia Secchi[a], Carlo Rovetta[a], Martina Zappaterra[c], Alice Vanzin[a], Martino Cassandro[a,d,e], Maurizio Marusi[d], Raffaella Finocchiaro[d], Alessio Cecchinato[a] and Sara Pegolo[a]

[a]Department of Agronomy, Food, Natural Resources, Animals, and Environment (DAFNAE), University of Padova, Legnaro, Italy; [b]Istituto zooprofilattico Sperimentale delle Venezie (IZSVe), Legnaro, Italy; [c]Department of Agri-Food Science and Technology (DISTAL), University of Bologna, Bologna, Italy; [d]Associazione Nazionale Allevatori della Razza Frisona, Bruna e Jersey Italiana (ANAFIBJ), Cremona, Italy; [e]Federazione delle Associazioni Nazionali di Razza e Specie (FedANA), Roma, Italy

ABSTRACT

Intramammary infections (IMI), especially in their subclinical form, are a major concern in dairy herds due to their impact on udder health and productivity. This study assessed the associations between IMI induced by *Staphylococcus aureus* and 33 hematobiochemical parameters (inflammation, liver function, metabolism, and oxidative stress) in Holstein cows without clinical signs of disease. Four hundred multiparous cows were selected from a commercial herd based on genetic (estimated breeding values for somatic cell count [SCC]) and phenotypic data (SCC and mastitis history), and classified as resistant (TOP, $n = 200$) or susceptible (BOTTOM, $n = 200$) to mastitis. At time 0 (T0), milk cultures identified 37 cows positive for IMI pathogens (27 for *S. aureus*, 4 for *S. dysgalactiae*, and 3 for *S. uberis*) that were bacteriologically monitored also at T1 and T2 (2 and 4 weeks after T0, respectively) and sampled for the hematochemical parameters. Statistical analyses focused on cows infected with *S. aureus*, the only group with adequate case numbers. Infected cows showed evidence of systemic inflammatory and immune responses, such as increase in serum amyloid A (T1; $p = 0.0496$) and neutrophil counts (T2; $p = 0.0337$). Markers of hepatic function were also altered, with higher gamma-glutamyl transferase (T2; $p = 0.0125$) and aspartate aminotransferase (T2; $p = 0.0304$) concentrations, suggesting liver involvement during infection. Beta-hydroxybutyrate (T1; $p = 0.0172$) levels were elevated in infected cows, reflecting energy imbalance. Overall, these findings underscored the value of blood biomarkers for the early detection of affected animals.

HIGHLIGHTS

- Subclinical mastitis induced systemic alterations in energy metabolism, liver function and inflammatory blood traits
- At time 1, susceptible animals displayed higher levels of β-hydroxybutyrate and neutrophils, suggesting a more active systemic inflammatory state
- Oxidative stress markers showed no significant changes, likely reflecting the nature of the infection.

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Subclinical mastitis; blood parameters; *Staphylococcus aureus*; somatic cell count

Introduction

Bovine mastitis is one of the most impactful diseases in dairy farms, particularly those with high milk production (Ruegg 2017). It can present itself in two forms: the clinical one, characterised by visible changes in milk or the udder, and the subclinical form, which poses a greater challenge to the dairy sector (Qayyum et al. 2018). Subclinical mastitis is 15 to 40 times more frequent than its counterpart and being asymptomatic it is more difficult to detect, therefore representing a huge source of infection for the animals within the herd (Stanojević et al. 2023). Mastitis is primarily caused by the invasion of the udder by a variety of pathogenic and environmental microorganisms, such as *Staphylococcus aureus*

CONTACT Vittoria Bisutti vittoria.bisutti@unipd.it

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(S. aureus), *Streptococcus agalactiae (S. agalactiae)*, *Mycoplasma bovis*, *Escherichia coli (E. coli)*, *Streptococcus uberis (S. uberis)* and *Streptococcus dysgalactiae (S. dysgalactiae)* (Bianchi et al. 2019). Confirming intramammary infections (IMI) still relies on bacteriological testing (NMC 2020). However, somatic cell count (SCC), and more recently differential somatic cell count (DSCC), are widely used at the herd level to screen for subclinical mastitis and monitor milk quality (Bobbo et al. 2020; Pegolo et al. 2022; Visentin et al. 2024), with a typical threshold of 200,000 cells/ml for SCC (Middleton et al. 2014) and 60% for DSCC (Bobbo et al. 2019). However, SCC is only an indirect marker of inflammation and does not provide a definitive diagnosis. Discrepancies can occur, such as high SCC with negative culture results or low SCC with positive ones (Sharma et al. 2011). Recently, there has been growing interest in blood-based biomarkers, which may provide more specific information on systemic stress responses associated with infection and offer complementary insights that could aid in the understanding and early detection of subclinical mastitis. Mastitis has been associated with metabolic changes driven by the energy demands of the immune response. Indeed, blood levels of beta-hydroxybutyrate (BHB), nonesterified fatty acids (NEFA), and glucose can shift during infection. Inflammation can reduce BHB *via* increased glucose concentrations, reduced rumen motility, or impaired hepatic ketogenesis (Moyes et al. 2014; Pegolo et al. 2023). Liver injury biomarkers such as aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT) are also relevant, though most practical attention has focused on their milk concentrations. Finally, antioxidant status is important in managing udder health, with parameters such as glutathione peroxidase activity and albumin (ALB) levels serving as indicators of oxidative stress during mastitis (Kleczkowski 2017; Tufarelli et al. 2023). Until recently, research has focused on alterations in blood parameters mostly in clinical and/or experimentally induced IMI (Hur et al. 2013; Moyes et al. 2014) which, while providing valuable insights, may not fully capture the complexity of naturally occurring infections on farms. Consequently, there has been growing interest in evaluating the effects also of subclinical forms, given their impact on the dairy system (Pegolo et al. 2023). Furthermore, these studies presented the limitation of not evaluating the effects of mastitis over time warranting for the need of longitudinal studies (Babaei et al. 2007; Moyes et al. 2014; Stanojević et al. 2023). To deeper address this topic, the present study examined the association between naturally occurring *S. aureus* IMI and a comprehensive panel of 33 hematobiochemical parameters related to immune response, inflammation, metabolism, liver function, and oxidative stress in Holstein cows that showed no clinical signs of disease. Animals were classified as resistant or susceptible to mastitis based on a combination of genetic merit for SCC and historical phenotypic data, allowing a framework to explore how individual predisposition to mastitis may influence systemic response to the disease. Understanding the physiological responses involved in *S. aureus* infections is also particularly important for improving diagnostic accuracy and guiding therapeutic interventions, given the pathogen's chronic nature, intermittent shedding, and ability to evade host defences.

Material and methods

Ethical statement

The study was carried out within the MASTITOMIC project that has the overall aim of deeply dissecting the risk factors linked to the development of subclinical mastitis. Experimental procedures have been reviewed and authorised by the University of Padua Ethical Committee for the Care and Use of Experimental Animals (OPBA, Organismo Preposto al Benessere degli Animali), according to the national legislation (D. Lgs 26/2014), which transposes the rules defined in the Directive 2010/63/EU (authorization with protocol n. 23578, 07/02/2024).

Animal data and experimental design

The study was conducted in a commercial dairy herd located in the Veneto region (northern part of Italy). The herd size was on average 1.200 lactating cows milked thrice on a rotatory parlour. Milking routine included pre and post dipping and every case of clinical mastitis was tested with an on-farm test before treatment decision. The cows were fed with a conventional diet designed for high-producing dairy cows, primarily based on silage. This diet contained approximately 16% crude protein, 4% extract, and 2.6 Mcal/kg of metabolisable energy, as reported in Table S1. Additionally, the diet included supplementation with sodium bicarbonate, calcium carbonate, and magnesium oxide. For recruitment in the study, cows were *a priori* selected based on their official estimated breeding value (EBV) for SCC and previous phenotypic information (average SCC from previous and current lactations at the time of sampling) provided by the National Association of Holstein, Bruna and Jersey Breeders (ANAFIBJ), and clinical mastitis history. With this information two groups of animals were created:

i) animals with low susceptibility to develop mastitis (top of the ranked animals for SCC EBV and SCC phenotypic data; TOP, $n = 200$) and ii) animals at risk for the development of mastitis (bottom of the ranked animals for SCC EBV and SCC phenotypic data; BOTTOM, $n = 200$). In addition, to further reduce sources of variations, cows were required to meet the following criteria: i) being multiparous, ii) having > 60 days in milk and with an average of 210 (DIM), iii) absence of clinical signs of disease (mastitis, metritis, retained placenta, ketosis, diarrhoea, abomasal dislocation, respiratory problems), and iv) no antibiotic treatment or anti-inflammatory medications for 6 months prior enrolment, following a previously adopted selection protocol (Pegolo et al. 2022; Bisutti et al. 2023). Initially, 400 cows were selected for the study. However, by the first sampling time (time 0, T0), the number of animals actually enrolled had decreased to 352 mostly due to culling, development of the above-mentioned clinical diseases, and/or treatment with antibiotics. Additionally, a qualified veterinarian performed clinical examinations throughout the study to assess the overall health of the cows and to identify any clinical signs of mastitis. Therefore, at T0, we performed a first bacteriological screening at the single quarter level on a final number of 352 cows ($n = 177$ TOP, 175 BOTTOM, respectively) to differentiate between healthy and infected animals. Each individual quarter was subsequently examined, and the first streams of milk were assessed for abnormalities, such as clots, blood, or a watery appearance. Following the bacteriological results obtained at T0, we created two experimental groups: i) healthy animals ($n = 49$) with no microbial growth for all the four mammary quarters, and animals with IMI ($n = 37$; 27 *S. aureus,* 5 *S. dysgalactiae,* 3 *S. uberis*) from mastitis pathogens with at least one positive mammary quarter and no clinical signs of the disease. At T1, two weeks after T0, and at T2 (two weeks after T1), clinical examination of the udder and bacteriological testing were repeated at the single quarter levels to confirm the experimental groups and to follow the progression of disease. Samples with coinfections or other microbial contaminations were excluded from the study. This longitudinal design allowed us to monitor the infection status and related physiological changes over time within the same animals. Detailed information on the number of cows and mammary quarters sampled at each time point, as well as the different pathogen prevalence, are reported in Table 1 and Table S2. Milk and blood samples were collected both at T1 and T2 and aliquots were adopted for a wide range of analysis. The detailed flow chart of the experimental design is reported in Figure 1.

Table 1. Prevalence of pathogens and their percentages at different time points.

	T0		T1		T2	
Microbiological result	n	%[1]	n	%[1]	n	%[1]
Positive	37	10.51	27	31.40	28	35.00
Staphylococcus aureus	27	7.67	23	26.74	23	28.75
Streptococcus dysgalactiae	5	1.42	1	1.16	3	3.75
Streptococcus uberis	3	0.85	3	3.49	2	2.50
Co-infections	1	0.28	–	–	–	–
Negative	92	26.14	27	31.40	33	41.25
Environmental microrganisms[2]	223	63.35	32	37.21	19	23.75
Total animals	353		86		80	

[1]Percentage of samples on the total animal samples analysed according to the bacteriological results.
[2](Streptococcus spp., Staphylococcus spp., Acinetobacter spp. Staphylococcus chromogenes, Aerococcus viridans, Proteus spp., Serratia spp., Enterococcus, Klebsiella spp., Serratia marcescens, Bacillus, Staphylococcus coagulasi negativo, Corynebacterium spp., Enterobatteriacea, Lactococcus, E.coli).

Milk sampling

Approximately 10 mL of milk from each quarter (quarter level) were aseptically collected for bacteriological culture from each of the preselected cows following the National Mastitis Council (NMC) recommendations (Adkins and Middleton, 2018). Briefly, the teat ends were cleaned externally using a commercial pre-milking disinfectant, dried with individual towels, and then disinfected again with a 1% solution of Virkon (Rely + On Virkon™, Lanxess AG, Cologne DE). The milk from each quarter was collected after discarding the first streams of milk. Milk samples, immediately after sampling, were transported to the microbiological laboratory of the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), and stored at −20 °C.

For further analysis, an aliquot of milk (∼50 mL pool milk) containing bronopol preservative, was collected from each cow using a lactometer. This aliquot was used for the evaluation of: i) milk composition, ii) SCC. All samples were immediately refrigerated at 4 °C to minimise the metabolic activity of cells and enzymes, ensuring that the bacterial composition remained as stable as possible and then transported to the various laboratories for analysis.

Milk microbiological analysis

Milk samples were thawed, gently mixed and cultured following the NMC guidelines (Adkins and Middleton, 2018). In brief, 10 µL of milk was streaked on each of the following selective media: Baird- Parker agar with rabbit plasma fibrinogen (Biokar Diagnostic) and tallium kristalviolette tossin agar (IZSVe internal

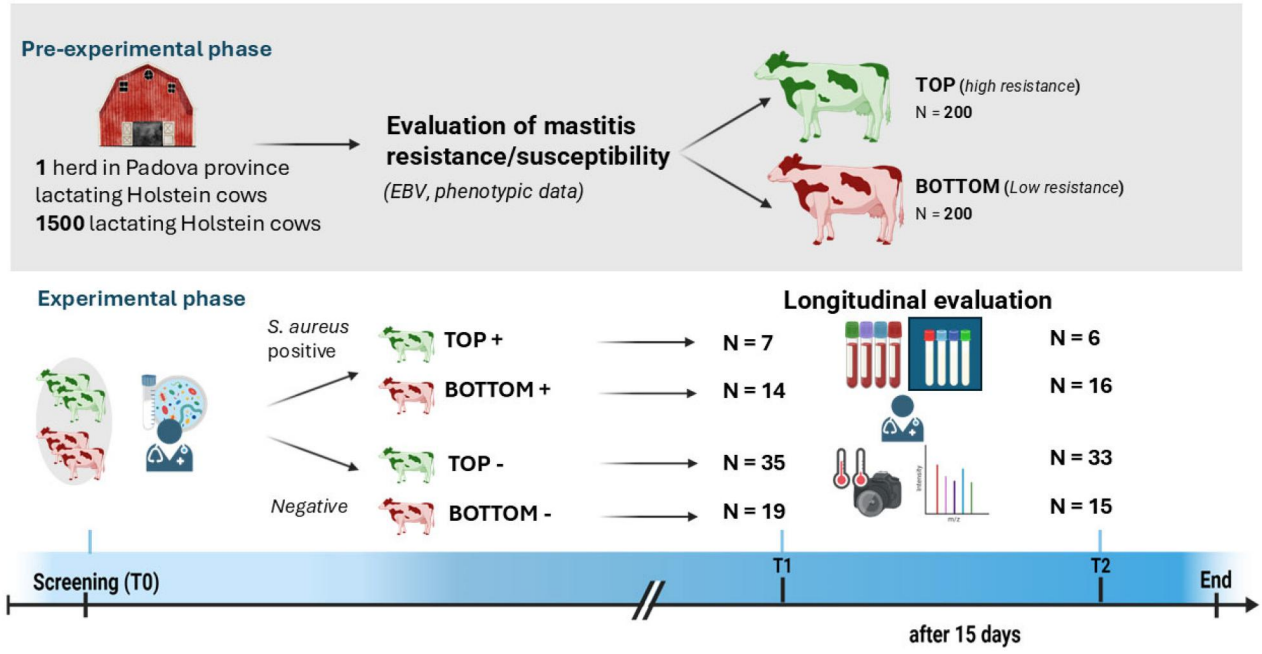

Figure 1. The detailed flow chart of the experimental design.

production). Baird-Parker agar with rabbit plasma fibrinogen plates were prepared according to standard 6888–2 (ISO, 1999), tallium kristalviolette tossin agar to the method described by Hauge and Ellingsen (1953). Inoculated media were incubated at 37 °C. Baird-Parker agar base plates were observed for growth at 24 and 48 h of incubation. Suspected *S. aureus* colonies were confirmed by tube coagulase test (NMC, 2017). Thallium sulfate-crystal violet-B toxin blood agar plates were observed at 24 h, and suspected colonies of *S. agalactiae* were confirmed using the Christie–Atkins–Munch-Peterson test (NMC, 2017).

Moreover, 10 µL of milk was streaked onto blood esculin agar and then incubated at 37 °C for 16 to 18 h. After this incubation period, the isolated colonies were selected, sub-cultured, and identified using both morphological analysis and additional NMC tests (Adkins and Middleton, 2018). Presumptive identification of the colonies was confirmed using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) with the Microflex Biotyper LT system (Bruker Daltonics GmbH, Bremen, Germany). A score $\geq$ 2.3 was used as the threshold for species-level identification. Samples containing more than three distinct types of colonies were considered 'contaminated.'

SCC, milk composition and quality traits

SCC, milk composition (protein, casein, lactose, fat, and urea content), milk conductivity (mS/cm), milk pH analysis were carried out on fresh samples. This analysis was performed in the dairy herd improvement (DHI) laboratory of the local dairy breeder association A.R.A.V. (Associazione Regionale Allevatori del Veneto) using an FT6000 Milkoscan infra-red analyser (Foss A/S, Hillerød, Denmark) and for SCC the Fossomatic 7 DC analyser (Foss A/S, Hillerød, Denmark).

Blood collection and analysis

Blood samples were collected from the jugular vein in the morning between 08:00 and 12:00, before Total Mixed Ration (TMR) distribution. A total of four 4 mL tubes were used: one containing K3EDTA, one with a coagulase activator, one with lithium heparin, and one with sodium citrate. All the tubes were gently agitated to ensure the sample was thoroughly mixed with the preservative (if present) and were immediately placed in refrigeration at 4 °C. Samples were transported within 2 h to the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), that performed the blood analysis. Detailed specifics of the analytic methods used to measure the blood traits, as well as the analytical sensitivity and linearity intervals, are reported in Table S3. Briefly, hematochemical profile (i.e. total proteins [PT], albumin [ALB], urea, glucose [GLU], cholesterol, HDL [High-Density Lipoprotein], total bilirubin, AST, GGT, creatine kinase [CK], calcium, phosphorus, and magnesium [Mg]) was performed on bovine serum obtained from blood collected in tubes without anticoagulant, after centrifugation at 3076 x g for 10 min at 20 °C using the Cobas Pure e303 analyser (Roche

Diagnostics, Mannheim, Germany). The following traits were also additionally measured in serum: i) reactive oxygen metabolites (ROM's); ii) NEFA (non-esterified fatty acids) and BHB (beta-hydroxybutyrate); iii) serum amyloid A (SAA) and; iv) the concentration of IL-6 (interleukin 6) (Table S3).

Moreover, on the whole bovine blood samples in lithium heparin, the concentration of GPX (glutathione peroxidase) was determined with a commercial kit using UV absorbance (Randox Laboratories Ltd., Ardmore, United Kingdom) applied to the Cobas Pure e303 analyser; the value obtained was corrected with the total haemoglobin value, measured with the XN 1000Vet haematology analyser (Sysmex Corporation, Kobe, Japan).

The fibrinogen value was determined on bovine plasma obtained from whole blood collected in Na-citrate tubes as anticoagulant, following centrifugation at 1201 x g for 15 min at 4 °C. The fibrinogen concentration was obtained using the CA650 analyser (Sysmex Corporation, Kobe, Japan) with a commercial kit from Sclavo Diagnostics International S.r.l., using the Clauss method (Clauss 1957).

Finally, the complete blood count with leukocyte formula was performed using the automatic analyser XN 1000Vet (Sysmex Corporation, Kobe, Japan) on whole bovine blood samples in K3EDTA.

Of the 33 blood parameters analysed, most of them (29) were validated. GPX and ROM's assays are currently under validation, while SAA and IL-6 were the only two parameters for which the analyses had not been formally validated, although linearity and sensitivity were measured. All analyses were performed using commercially available kits (Table S3).

Statistical analysis

All variables were analysed separately using ANOVA and including infection status, mastitis risk class, SCC class as fixed effects. Variables that did not meet the normality assumption were log-transformed. No additional pre-processing or editing of the data was performed, as the study aimed to characterise hematobiochemical parameters as they naturally occur in the context of subclinical mastitis. Two separate analyses were conducted for T1 and T2 due to heterogeneity of the variances. The following model was run:

$$y_{ijklmn} = \mu + DIM_i + Parity_j + BACT_k + SCC_l + MR_m + e_{ijklmn},$$

where y_{ijklmn} is the selected blood analyte; μ is the overall mean; DIM_i is the fixed effect of the ith class of DIM ($i = 3$ classes; $60 <$ class $1 \leq 120$; $120 <$ class $2 \leq 240$; class $3 > 240$); $Parity_j$ is the fixed effect of the jth parity ($j = 3$ classes; class $1 \leq 2$; class $2 = 3$; class $3 > 3$); $BACT_k$ is the fixed effect of the lth class of bacteriological status (k = positive or negative); SCC_l is the fixed effect of the lth class of SCC ($l = 4$ classes; class $1 <$ 50,000 cells/mL; 50,000 cells/mL $\leq$ class $2 <$ 200,000 cells/mL; 200,000 cells/mL $\leq$ class $3 <$ 400,000 cells/mL; class $4 \geq$ 400,000 cells/mL); MR_m is the fixed effect of the mth class of MASTITIS RISK ($m = 2$ classes; class 1 TOP; class 2 BOTTOM); $e_{ijklmn,}$ is the random residual that was assumed to be normally distributed with a mean of zero and variances of σ^2_e. Preliminary models also included the interaction between infection status (BACT) and mastitis risk class (MR) to test whether to the effect of *Staphylococcus aureus* infection on systemic biomarkers differed between resistant and susceptible cows. However, this interaction was not statistically significant for any of the investigated traits and was therefore removed from the final model. The lack of significance likely reflects the unbalanced distribution of infected animals between risk groups (only seven resistant cows were infected), which limited the power of the test and model convergence. The interaction between BACT and SCC could not be tested because some combinations of factor levels were not represented in the dataset. In addition, the effect of the number of infected quarters was previously assessed and it was not significant. Therefore, it was not included in the model.

Polynomial contrasts ($p < 0.05$) were estimated between the least squares means (LSM) of the effects of SCC (first-order comparisons measured linear relationships, whereas the second- and third-order comparisons measured quadratic and cubic relationships, respectively).

Results

Characteristics of cows and microbiological analyses

The 352 cows sampled at T0 had average days in milk (DIM) of 210 days (SD = 135.67) and an average parity of 2.5 calvings (SD = 1.07). Based on the results of bacteriological examination, 86 were followed up at T1 and T2. Overall, 37 cows were positive at the T0 bacteriological examination for either one of the following microorganisms: *S. aureus* ($n = 27$), *S. dysgalactiae* ($n = 6$), and *S. uberis* ($n = 4$). Among the animals positive at the bacteriological examination at T1, 23 animals were positive for *S. aureus* (26.74%), one for *S. dysgalactiae* (1.16%), and three for *S. uberis* (3.49%). At T2, 23 animals were positive for *S. aureus* (28.75%),

Table 2. Changes in pathogen status over time in animals.

Animals	T0	T1	T2
23	+	+	+
45	−	−	−
1	+	+	−
5	+	−	−
3	+	−	+
2	−	+	+
1	−	+	−

+ = POSITIVE if at least one quarter tested positive for S. aureus, S. dysgalactiae, S. uberis, or co-infections; − = NEGATIVE only if all four quarters were negative for the mastitis pathogens of interest (S. aureus, S. dysgalactiae, S. uberis), although they could be positive for environmental microorganisms.

Table 3. Descriptive statistics of milk composition and udder -health traits in holstein cows T0 ($n = 352$).

Item[1]	n	Mean	SD	P1[1]	P99[2]
Milk yield, kg/d	352	45.54	11.62	17.83	70.20
Milk composition					
Fat, %	352	4.13	0.68	2.59	5.97
Protein, %	352	3.46	0.36	2.72	4.52
Casein, %	352	2.70	0.33	2.06	3.55
Urea, mg/dL	352	21.60	3.81	13.05	32.64
Udder health traits					
Lactose, %	352	4.73	0.18	4.17	0.27
Casein Index, %	352	77.92	4.26	75.74	79.87
F/P, ratio	352	1.20	0.18	0.79	1.65
SCC,10^3 cells/mL	352	232.67	566.15	10.00	2399.23

[1]SCC = somatic cells count; F/P = fat/protein; P1 = first percentile; P99 = 99th percentile.

three for *S. dysgalactiae* (3.75%), and two for *S. uberis* (2.5%) (Table 1). Quarter-level results are reported in Supplementary Tables S2.

During the experimental phase, 23 animals were positive for *S. aureus* at all sampling times, 45 always remained bacteriologically negative, while 12 animals changed their bacteriological status at different time points (Table 2). Among these 12, the most interesting cases are those that showed a positive-negative-positive pattern, as this trend demonstrates how *S. aureus* behaves as an intermittent pathogen. One cow from this group exhibited a change in pathogen, shifting from *S. aureus* at T0 to *S. dysgalactiae* at T2.

Since the number of positive cases for *S. uberis* and *S. dysgalactiae* were not representative, the study focused on IMI from *S. aureus*, hence the following groups were considered: healthy animals ($n = 59$ at T1 and $n = 52$ at T2) and *S. aureus* infected animals ($n = 23$ at T1 and $n = 23$ at T2).

Milk composition

Table 3 reports the descriptive statistics of milk composition and SCC of the enrolled cows at T0. Animals had an average milk yield of 45.54 ± 11.62 kg/day (T0). The mean values of protein, fat and lactose were 34.6 ± 0.36 g/kg, 41.13 ± 0.68 g/kg, 47.3 ± 0.18 g/kg, respectively. Concerning

Table 4. Descriptive statistics of milk composition and udder -health traits in holstein cows T1 ($n = 81$).

Item[1]	n	Mean	SD	P[11]	P99[2]
Milk yield, kg/d	83	43.17	10.92	18.14	64.80
Milk composition					
Fat, %	81	4.10	0.94	2.04	6.06
Protein, %	81	3.43	0.38	2.57	4.56
Casein, %	81	2.67	0.32	1.90	3.61
Urea, mg/dL	81	26.43	5.29	15.00	39.74
Udder health traits					
Lactose, %	81	4.69	0.28	3.86	5.08
Casein Index, %	81	77.86	1.37	73.69	79.81
F/P, ratio	81	1.19	0.23	0.63	1.74
SCC,10^3 cells/mL	81	571.94	2060.26	8.60	8443.80

[1]SCC = somatic cells count; F/P = fat/protein; P1 = first percentile; P99 = 99th percentile.

Table 5. Descriptive statistics of milk composition and udder -health traits in holstein cows T2 ($n = 80$).

Item[1]	n	Mean	SD	P1[1]	P99[2]
Milk yield, kg/d	80	42.86	10.13	22.60	67.93
Milk composition					
Fat, %	80	3.83	0.82	1.97	5.68
Protein, %	80	3.34	0.33	2.64	4.15
Casein, %	80	2.58	0.28	1.96	3.29
Urea, mg/dL	80	26.91	4.57	17.19	37.16
Udder health traits					
Lactose, %	80	4.62	0.33	3.43	5.03
Casein Index, %	80	77.27	1.36	73.35	79.69
F/P, ratio	80	1.14	0.21	0.68	1.64
SCC, 10^3 cells/mL	80	306.75	670.51	8.32	3414.82

[1]SCC = somatic cells count; F/P = fat/protein; P1 = first percentile; P99 = 99th percentile.

the SCC, the results show that the average content was 232.67×10^3 cells/mL, with the range varying from 10×10^3 cells/mL to 2399.23×10^3 cells/mL. Table 4 and 5 reports the descriptive statistics of milk composition and SCC for T1 and T2.

Hematochemical parameters

Table 6 presents the descriptive statistics for the investigated 33 hematochemical parameters measured in the enrolled cows at T1 and T2. The mean values of these parameters, which reflect the overall health status of the animals, show noteworthy deviations from the normal reference ranges for several key indicators. Regarding energy-related metabolites, cholesterol levels showed some variability. One *S. aureus* positive animal had values above the upper limit of the reference range of 2 – 5.5 mmol/L L (Bertoni and Trevisi 2013) at both T1 and T2, while eleven *S. aureus* positive animals showed values below the lower limit of the reference range at either T1 or T2. Glucose levels were elevated at T1, with eight *S. aureus* positive animals showing values above the reference range of 2.2–3.3 mmol/L (Ruoff et al. 2017). This number decreased to only one animal at T2. NEFA levels revealed that at T1, three *S.*

Table 6. Descriptive statistics of blood major constituents time point 1 and 2 (T1 and T2).

		T1				T2			
Blood metabolite[1]	n	Mean	SD	P1[1]	P99[2]	Mean	SD	P1[1]	P99[2]
Energy-related metabolite									
BHB, mmol/L	66	0.56	0.21	0.31	1.34	0.57	0.21	0.28	1.18
GLU, mmol/L	66	3.37	0.375	2.49	4.07	3.02	0.338	2.27	3.6
Cholesterol, mmol/L	66	5.71	1.26	3.24	8.29	5.72	1.19	3.7	7.97
NEFA, meq/L	66	0.13	0.02	0.1	0.23	0.16	0.03	0.12	0.24
Urea, mg/dL	66	5.04	1.03	3.19	7.58	5.5	0.98	3.46	7.51
HDL, mmol/L	66	5.08	1.06	2.92	7.43	4.96	1.11	2.79	7.09
LDL, mmol/L	66	2.7	0.907	0.855	4.42	2.52	0.8411	1.07	4.27
CK, U/L	66	180.83	57.9	119.25	420.5	1.57	0.14	1.32	1.94
Liver function/hepatic damage									
ALB, g/L	66	38.64	2.89	29.65	43.35	39.76	2.4	33.83	44.39
AST, U/L	66	127.55	45.41	72.56	259	122.15	47.07	65.8	245.35
GGT, U/L	66	36.7	12.29	19.6	73.6	38.73	13.74	20.95	86.35
Red blood cell indices									
MCH, pg	66	16.63	1.21	14.33	19.58	16.82	1.32	14.23	19.88
MCHC, g/dL	66	33.6	0.78	31.69	35.21	34.06	0.94	32.26	36.4
MCV, fL	66	49.44	3.13	43.01	57.16	49.38	3.25	42.58	57.33
RBC, M/μL	66	6.41	0.57	5.38	8.01	6.32	0.57	5.22	5.22
RDW, %	66	19.6	1.19	17.77	22.42	19.52	1.27	17.47	22.79
Platelets									
PLT, K/μL	66	294.03	122.01	6.3	524.05	317.17	86.86	7.13	433.44
MPV, fL	66	7.97	0.47	7.13	9.2	7.76	0.47	6.76	9.24
White blood cell indices									
Neutrophils, % of neutrophils	66	46.09	7.2	31.98	64.32	45.15	6.84	32.89	63.41
Monocytes, % of monocytes	66	10.53	2.29	5.15	14.9	10.24	1.63	7.7	13.7
WBC, K/μL	66	8.08	1.49	4.82	11.24	8.08	1.48	4.82	11.91
Lymphocytes, % of lymphocytes	66	39.01	6.35	24.02	52.88	40.72	7.22	24.44	55.44
Inflammation/innate immunity									
IL-6, pg/mL	66	39.35	30.68	12.46	135.58	40.71	17.73	11.03	77.93
SAA, mg/L	66	0.38	0.24	0.04	0.96	0.5	0.26	0.1	1.19
GLO, g/dL	66	38.41	6.72	27.65	58.75	37.92	6.61	27	58.35
Haptoglobin, mg/dL	66	23.55	8.4	15.49	54.07	25.82	15.74	12.86	81.48
Oxidative stress metabolite									
GPx, U/g Hb	66	761.91	99.69	533.56	990.04	708.56	82.3	564.35	894.6
ROM's (UCARR)	66	72.53	26.49	20.85	133.85	95.5	26.86	40	168.8
Tissue injury									
TP, g/L	66	77.05	5.15	65.3	91	77.26	5.24	65.95	91.75
Fibrogen, mg/dL	66	310.2	85.31	123.55	492.5	346.52	94.87	198.6	570.6
Minerals									
Ca, mmol/L	66	2.47	0.11	2.27	2.68	2.48	0.12	2.24	2.73
Mg mmol/L	66	1.11	0.1	0.92	1.37	1.09	0.08	0.89	1.28
P, mmol/L	66	1.61	0.26	1.06	2.14	1.54	0.22	1.14	2.01

[1]Mean Corpuscular Haemoglobin = MCH, Mean Corpuscular Haemoglobin Concentration = MCHC, Mean Corpuscular Volume = MCV, Mean Platelet Volume = MPV, Platelet Count = PLT, Red Blood Cell Count = RBC, Red Cell Distribution Width = RDW, White Blood Cell Count = WBC, Albumin = ALB, Aspartate Aminotransferase = AST, Beta-Hydroxybutyrate = BHB, Creatine Kinase = CK, Gamma-Glutamyl Transferase = GGT, Globulin = GLO, Glucose = GLU, Magnesium = MG, Non-Esterified Fatty Acids = NEFA, Phosphorus = P, Total Protein = TP, Glutathione Peroxidase = GPx, Haptoglobin, Reactive Oxygen Metabolites = ROM, High-Density Lipoprotein = HDL, Interleukin-6 = IL-6, Serum Amyloid A = SAA.

aureus positive animals had values outside the physiological threshold of 0.4 mmol/L (Ospina et al. 2010), while at T2 this number increased to fifteen, with consistently elevated values above the reference range. BHB levels showed that at T1, out of fifteen animals exceeding the reference range for healthy individuals, four were *S. aureus* positive, with values above the range < 0.6 mmol/L (Ospina et al. 2010). No substantial difference was observed at T2. With respect to liver function and hepatic damage markers, ALB levels showed that at T1, eighteen animals had values outside the reference range of 30–40 g/L proposed by Macrae et al. (2006) for healthy individuals, including four *S. aureus*-positive animals. The same was observed at T2.

Similarly, AST levels, whose reference values range from 80–120 U/L (Sattler and Fürll 2004; Trevisi et al. 2012), were altered, with twenty-nine animals presenting values outside the normal range at T1, eleven of which were *S. aureus* positive. No significant variation was observed at T2. For GGT, a comparable trend was observed: thirty animals had values outside the reference range at T1, including ten *S. aureus* positive animals. No significant difference was noted at T2. The reference range was 15–35 U/L (Engelking, 2015). Regarding white blood cell indices, lymphocyte levels showed a slight increase, with two animals exceeding the reference range at T1 and four animals at T2, in both cases above the normal range of 45–75% (Kaneko et al. 2008).

Table 7. P-Values (T1) of classes of days in milk (DIM_c), classes of parity, bacteriological status (BACT), mastitis rick (MR) and classes of SCC, effects for blood metabolites.

	DIM		Parity		BACT		MR		SCC	
Blood metabolite[1]	*F-value*	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*
Energy-related metabolite										
BHB, (log_{10})	0.63	0.539	0.296	0.745	0.162	0.689	1.943	0.168	0.792	0.503
GLU, mmol/L	0.69	0.506	5.401	0.007	1.977	0.164	0.045	0.832	0.74	0.532
Cholesterol, mmol/L	1.71	0.19	1.943	0.152	0.253	0.617	0.62	0.434	0.631	0.598
NEFA, (log_{10})	1.13	0.331	0.417	0.661	0.24	0.626	0.027	0.87	1.923	0.135
Urea, mg/dL	3.83	0.027	0.986	0.379	0.204	0.653	0.151	0.699	0.643	0.59
HDL, mmol/L	1.78	0.177	1.647	0.201	0.16	0.691	0.506	0.48	0.548	0.651
LDL, mmol/L	1.062	0.35	4.334	0.017	0.004	0.949	1.119	0.35	0.35	0.551
CK, (log_{10})	0.27	0.763	2.068	0.135	4.599	0.036	0.079	0.78	1.407	0.249
Liver function/hepatic damage										
ALB, g/L	0.69	0.507	0.56	0.574	1.082	0.302	0.741	0.392	1.197	0.318
AST, (log_{10})	3.02	0.056	0.731	0.485	5.893	0.018	5.739	0.02	1.1	0.356
GGT, (log_{10})	1.52	0.227	0.901	0.411	0.102	0.751	0.853	0.359	0.792	0.503
Red blood cell indices										
MCH, pg	4.04	0.022	3.648	0.032	0.105	0.747	3.092	0.083	0.128	0.943
MCHC, g/dL	1.83	0.169	0.203	0.817	2.516	0.118	0.859	0.357	1.024	0.388
MCV, fL	5.12	0.009	5.107	0.009	1.129	0.292	6.122	0.016	0.105	0.957
RBC, M/μL	2.63	0.08	4.663	0.013	0.04	0.843	0.052	0.821	0.187	0.905
RDW, %	0.79	0.46	4.664	0.013	0.036	0.851	1.027	0.315	0.23	0.875
Platelets										
PLT, K/μL	1.22	0.302	0.525	0.594	0.119	0.732	0.288	0.593	0.306	0.821
MPV, fL	1.37	0.261	1.716	0.188	0.05	0.824	1.602	0.21	4.338	0.008
White blood cell indices										
Neutrophils, % of neutrophils	1.12	0.332	0.491	0.614	0.125	0.725	4.709	0.034	0.315	0.814
Monocytes, % of monocytes	0.62	0.541	0.346	0.709	0.066	0.798	0.984	0.325	1.002	0.398
WBC, K/μL	0.43	0.655	2.695	0.075	0.564	0.455	0.667	0.417	0.613	0.609
Lymphocytes, % of lymphocytes	1.67	0.196	0.28	0.757	0.133	0.716	1.866	0.177	0.738	0.533
Inflammation/innate immunity										
IL-6, (log_{10})	1.23	0.298	0.469	0.628	1.33	0.253	0.282	0.597	1.705	0.175
SAA, mg/L	4.59	0.014	2.156	0.124	0.036	0.85	0.415	0.522	2.757	0.05
GLO, g/dL	0.05	0.953	0.639	0.531	0.006	0.941	0.004	0.95	1.956	0.129
Haptoglobin, (log_{10})	0.39	0.68	0.266	0.767	0.115	0.736	1.68	0.2	0.457	0.713
Oxidative stress metabolite										
GPx, U/g Hb	0.15	0.864	0.313	0.733	0.039	0.843	0.852	0.36	2.912	0.041
ROM's (UCARR)	0.4	0.671	0.779	0.463	0.37	0.545	0.289	0.833	0.051	0.822
Tissue injury										
TP, g/L	0.13	0.875	1.895	0.159	0.484	0.489	0.333	0.566	1.947	0.131
Fibrogen, mg/dL	2.26	0.113	0.335	0.716	0.184	0.669	0.009	0.924	0.141	0.935
Minerals										
Ca, mmol/L	1.3	0.279	0.819	0.446	0.382	0.539	2.579	0.113	0.697	0.557
Mg mmol/L	2.87	0.064	3.572	0.034	5.167	0.026	0.758	0.522	0.832	0.365
P, mmol/L	4.4	0.016	0.809	0.45	0.865	0.356	0.349	0.79	0.634	0.429

[1]Mean Corpuscular Haemoglobin = MCH, Mean Corpuscular Haemoglobin Concentration = MCHC, Mean Corpuscular Volume = MCV, Mean Platelet Volume = MPV, Platelet Count = PLT, Red Blood Cell Count = RBC, Red Cell Distribution Width = RDW, White Blood Cell Count = WBC, Albumin = ALB, Aspartate Aminotransferase = AST, Beta-Hydroxybutyrate = BHB, Creatine Kinase = CK, Gamma-Glutamyl Transferase = GGT, Globulin = GLO, Glucose = GLU, Magnesium = MG, Non-Esterified Fatty Acids = NEFA, Phosphorus = P, Total Protein = TP, Glutathione Peroxidase = GPx, Haptoglobin, Reactive Oxygen Metabolites = ROM, High-Density Lipoprotein = HDL, Interleukin-6 = IL-6, Serum Amyloid A = SAA.

In contrast, neutrophil levels showed a marked increase, with thirty-four animals presenting values above the reference range of 15–45% (Mohri et al. 2007) at T1 and twenty-nine at T2, suggesting a sustained inflammatory response in the animals at both time points.

ANOVA results for hepatic function and liver damage indicators

Results of the ANOVA for the associations between hepatic-related traits and mastitis indicators are reported in Tables 7 and 8, for T1 and T2 respectively. AST levels were significantly higher (35.7%) in cows with IMI caused by *S. aureus* compared to bacteriologically negative animals at T1 ($p < 0.05$; Figure 2(A,B)). Although this difference was not significant at T2, a similar trend was maintained ($p < 0.10$). AST was also significantly higher in TOP animals compared to BOTTOM ones ($p < 0.05$; Figure 2(C,D)) at both time points. TOP animals showed a 22.9% higher AST concentration compared with BOTTOM ones at T1 and a 25.2% higher AST concentration compared with BOTTOM ones at T2. Regarding GGT, a significant quadratic relationship with SCC was found at T2 ($p < 0.01$; Figure 2(E,F)). Specifically, the GGT concentrations decreased by 29.6% for the SCC class 50,000–200,000 cells/mL compared to the SCC class <50,000 cells/mL and increased by 1.3% and 41.8% for the SCC classes 200,000–400,000 cells/mL and SCC class ≥ 400,000 cells/mL, respectively.

Table 8. P-Values (T2) of classes of days in milk (DIM_c), classes of parity, bacteriological status (BACT), mastitis rick (MR) and classes of SCC, effects for blood metabolites.

	DIM		Parity		BACT		MR		SCC	
Blood metabolite[1]	*F-value*	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*
Energy-related metabolite										
BHB, (log_{10})	1.148	0.324	10.027	0.0001	2.355	0.13	4.867	0.031	1.612	0.196
GLU, mmol/L	0.166	0.848	0.544	0.583	0.328	0.569	0.221	0.64	2.923	0.041
Cholesterol, mmol/L	0.636	0.533	0.539	0.586	2.551	0.116	0.017	0.898	0.132	0.94
NEFA, (log_{10})	0.512	0.602	1.434	0.247	3.043	0.086	0.849	0.361	2.288	0.088
Urea, mg/dL	0.424	0.656	0.653	0.524	0.361	0.55	0.302	0.585	1.761	0.164
HDL, mmol/L	3.169	0.049	1.914	0.156	0.85	0.36	0.548	0.462	1.425	0.244
LDL, mmol/L		0.493	1.109	0.336	0.534	0.467	2.018	0.123	2.326	0.132
CK, (log_{10})	1.181	0.314	0.883	0.419	2.958	0.091	0.017	0.897	0.315	0.815
Liver function/hepatic damage										
ALB, g/L	0.097	0.908	0.634	0.534	0.103	0.75	0.412	0.524	2.179	0.101
AST, (log_{10})	2.388	0.1	2.553	0.086	3.181	0.08	3.978	0.051	0.808	0.495
GGT, (log_{10})	2.021	0.142	1.402	0.255	0.082	0.776	0.373	0.544	1.196	0.32
Red blood cell indices										
MCH, pg	4.624	0.014	3.94	0.025	0.001	0.977	4.355	0.041	0.256	0.856
MCHC, g/dL	0.924	0.403	0.295	0.746	0.946	0.335	0.606	0.439	0.706	0.552
MCV, fL	4.981	0.01	5.231	0.008	0.313	0.578	4.883	0.031	0.192	0.901
RBC, M/μL	2.578	0.084	4.895	0.011	0.313	0.578	1.069	0.369	0.014	0.905
RDW, %	1.58	0.215	17.548	0	0.705	0.405	0.723	0.399	0.913	0.44
Platelets										
PLT, K/μL	0.731	0.486	0.115	0.891	0.065	0.801	0.108	0.744	1.454	0.238
MPV, fL	0.255	0.776	0.22	0.804	0.249	0.62	0.001	0.973	2.701	0.054
White blood cell indices										
Neutrophils, % of neutrophils	0.286	0.752	2.139	0.127	0.06	0.808	0.438	0.511	0.188	0.904
Monocytes, % of monocytes	0.089	0.915	0.247	0.782	2.934	0.092	0.193	0.662	0.98	0.409
WBC, K/μL	0.117	0.889	9.103	0	0.133	0.717	1.154	0.287	0.52	0.67
Lymphocytes, % of lymphocytes	0.258	0.774	0.224	0.8	0.487	0.488	0.872	0.354	0.418	0.741
Inflammation/innate immunity										
IL-6, (log_{10})	0.283	0.754	2.998	0.057	0.005	0.942	2.227	0.141	1.918	0.136
SAA, mg/L	0.79	0.459	1.071	0.349	0.429	0.515	0.949	0.334	0.736	0.535
GLO, g/dL	3.804	0.028	1.787	0.176	0.094	0.76	0.193	0.662	4.34	0.008
Haptoglobin, (log_{10})	1.063	0.352	4.024	0.023	0.007	0.933	0.501	0.482	1.628	0.193
Oxidative stress metabolite										
GPx, U/g Hb	1.339	0.27	1.796	0.175	0.333	0.566	0.455	0.503	0.375	0.772
ROM's (UCARR)	1.82	0.171	0.261	0.771	0.002	0.969	0.694	0.408	1.39	0.255
Tissue injury										
TP, g/L	2.46	0.094	0.168	0.846	0.045	0.833	0.437	0.511	0.442	0.724
Fibrogen, mg/dL	0.159	0.854	1.201	0.308	1.263	0.266	0.056	0.813	2.546	0.064
Minerals										
Ca, mmol/L	0.638	0.532	3.72	0.030	3.79	0.050	0.845	0.362	2.486	0.069
Mg mmol/L	2.474	0.093	0.299	0.743	1.002	0.321	0.029	0.866	1.238	0.304
P, mmol/L	0.702	0.5	1.36	0.265	2.983	0.089	0.91	0.344	2.519	0.067

[1]Mean Corpuscular Haemoglobin = MCH, Mean Corpuscular Haemoglobin Concentration = MCHC, Mean Corpuscular Volume = MCV, Mean Platelet Volume = MPV, Platelet Count = PLT, Red Blood Cell Count = RBC, Red Cell Distribution Width = RDW, White Blood Cell Count = WBC, Albumin = ALB, Aspartate Aminotransferase = AST, Beta-Hydroxybutyrate = BHB, Creatine Kinase = CK, Gamma-Glutamyl Transferase = GGT, Globulin = GLO, Glucose = GLU, Magnesium = MG, Non-Esterified Fatty Acids = NEFA, Phosphorus = P, Total Protein = TP, Glutathione Peroxidase = GPx, Haptoglobin, Reactive Oxygen Metabolites = ROM, High-Density Lipoprotein = HDL, Interleukin-6 = IL-6, Serum Amyloid A = SAA.

ANOVA results for inflammatory and immune response markers

Results of the ANOVA for the associations between inflammatory and immune response and blood hematochemical parameters are reported in Table 7 and 8, for T1 and T2 respectively. Serum Amyloid A showed a cubic significant association with SCC at T1 ($p < 0.05$; Figure 3(A,B)). Specifically, SAA increased from class <50,000 to 50,000–200,000 cells/mL, and then decreased from class 50,000–200,000 cells/mL to class 200,000–400,000 cells/mL, with a subsequent increase by 97.8% in the highest class of SCC compared to the first one. Globulin was not significantly affected by SCC at T1. At T2, a quadratic significant increase was observed ($p < 0.05$; Figure 3(C,D)), with the ≥400,000 cells/mL class showing an increase by 20.11% compared to <50,000 cells/mL. Neutrophil percentage was significantly lower (8.6%) in TOP compared to BOTTOM animals at T1 ($p < 0.05$; Figure 3(E,F)).

ANOVA results for metabolic indicators

Results of the ANOVA for the associations between metabolic and blood hematochemical parameters are reported in Table 7 and 8, for T1 and T2 respectively. TOP animals showed higher (22%) BHB concentrations

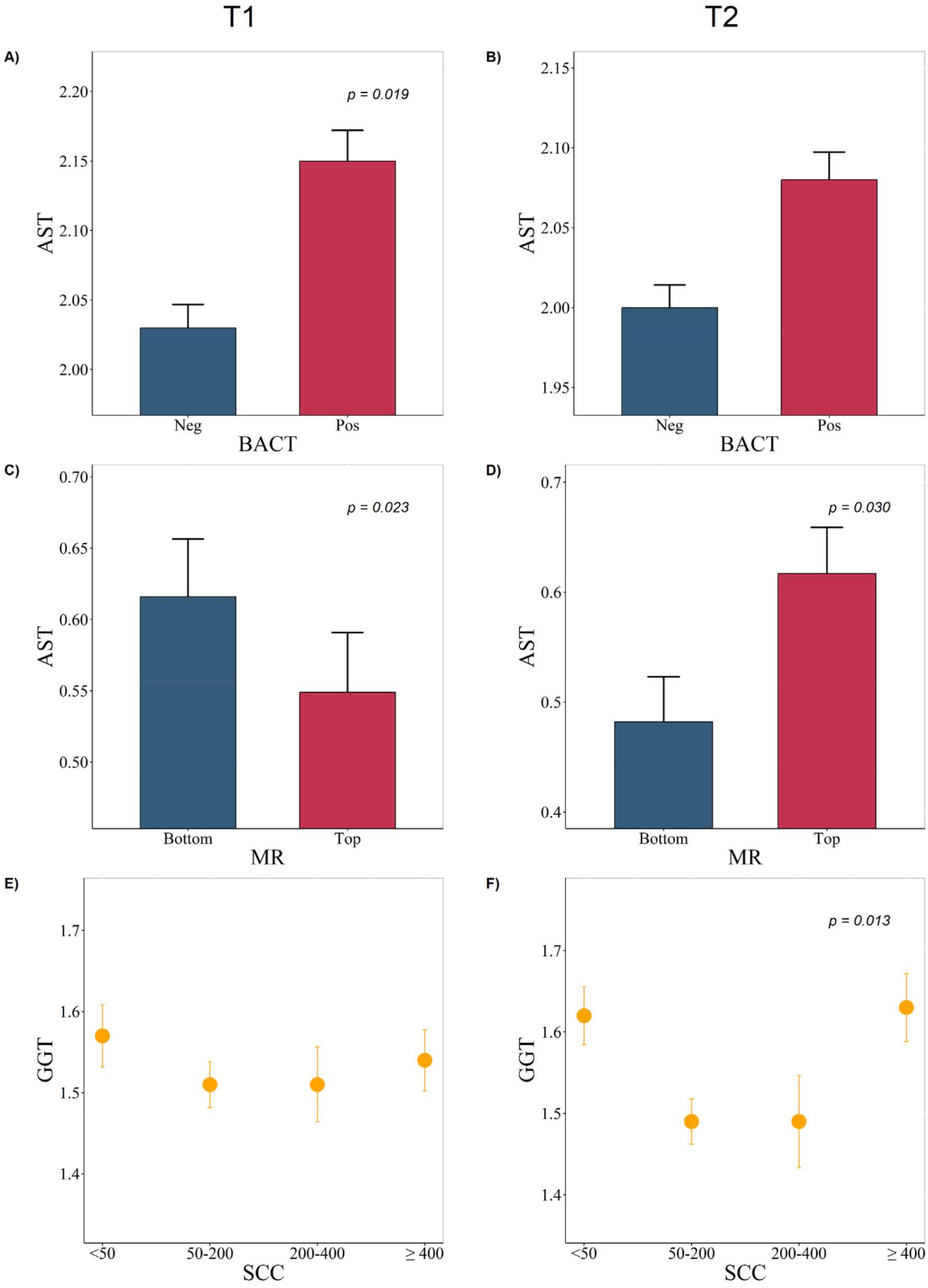

Figure 2. Least squares means ± SE of aspartate aminotransferase (AST-BACT and AST-MR) and gamma-glutamyltransferase (GGT). Bacteriological status (BACT) was defined as follows: POSITIVE = if at least one quarter tested positive for *S. aureus*, and NEGATIVE = only if all four quarters were negative for the mastitis pathogens of interest (*S. aureus*), although they could test positive for environmental microorganisms. T1 is shown on the left, and T2 on the right. Mastitis risk (MR) was categorised as follows: TOP = animals with low susceptibility to developing mastitis, and BOTTOM = animals at risk for developing mastitis. T1 is shown on the left, and T2 on the right. SCC classes were defined as follows: Class 1 < 50,000 cells/mL; Class 2: 50,000 ≤ SCC < 200,000 cells/mL; Class 3: 200,000 ≤ SCC < 400,000 cells/mL; Class 4 ≥ 400,000 cells/mL. T1 is shown on the left, and T2 on the right.

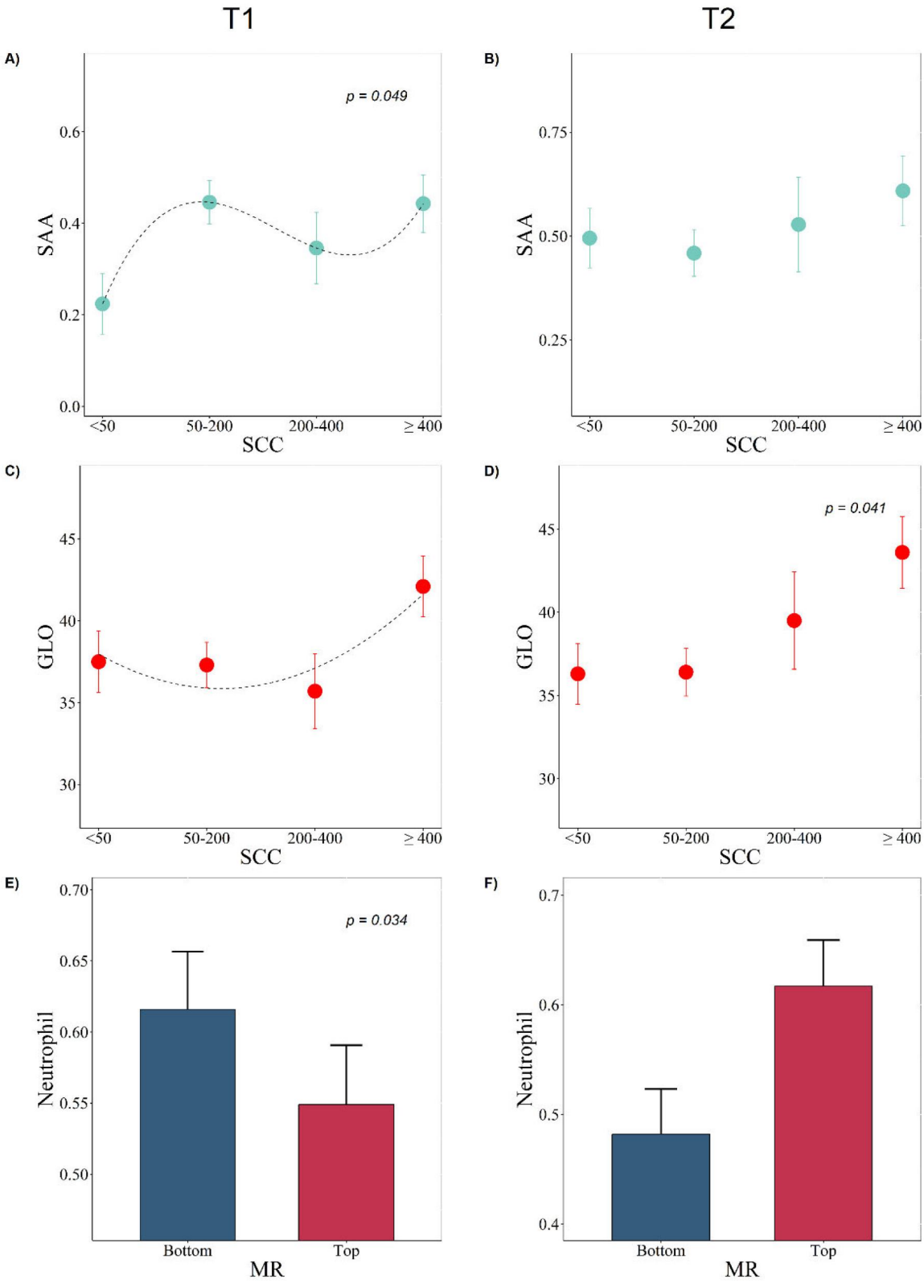

Figure 3. Least squares means ± SE of serum amyloid A (SAA), globulin (GLO) and neutrophil. SCC classes were defined as follows: Class 1 < 50,000 cells/mL; Class 2: 50,000 ≤ SCC < 200,000 cells/mL; Class 3: 200,000 ≤ SCC < 400,000 cells/mL; Class 4 ≥ 400,000 cells/mL. T1 is shown on the left, and T2 on the right). Mastitis risk (MR) was categorised as follows: TOP = animals with low susceptibility to developing mastitis, and BOTTOM = animals at risk for developing mastitis. T1 is shown on the left, and T2 on the right.

compared to BOTTOM ones at T2 ($p < 0.05$; Figure 4(A,B)). Another significant association was found between urea and SCC at T2 ($p < 0.05$) (Figure 4(C,D) respectively), with a decrease of 13.0% going from the <50,000cells/mL class to the ≥400,000 cells/mL class. CK activity was significantly higher (17%) in bacteriologically negative animals compared to positive ones at T1 ($p < 0.05$; Figure 4(E,F)).

ANOVA results for minerals

At T1, magnesium and calcium concentrations were significantly higher (6.7% and 1.2%, respectively) in BACT positive animals in respect to negative cows (Figure 5(A–D)) At T2, Mg concentration was also significantly associated with SCC ($p < 0.001$; Figure 5(E,F)), showing a cubic trend. Cows in the 200,000–400,000 cells/mL class had approximately 10.1% lower Mg values compared with those in the <50,000 cells/mL class.

ANOVA results for red blood cell indices

Mean corpuscular volume (MCV) was significantly higher in TOP compared to BOTTOM animals at both time points, with increases of approximately 4.1% at T1 and 3.7% at T2 ($p < 0.05$; Figure 6(A,B)). Similarly, MCH was higher in TOP animals, showing increases of 3.6% at T1 and 4.2% at T2 ($p < 0.05$; Figure 6(C,D)). The Mean Platelet Volume (MPV) was significantly associated with SCC at T1 ($p < 0.01$), showing a quadratic trend. MPV was approximately 4.8% lower for cows in the 200,000–400,000 cells/mL class compared with those in the <50,000 cells/mL class (Figure 6(E,F)).

Discussion

The identification of subclinical intramammary infection (sIMI) is complex in the absence of obvious clinical signs or milk alterations (Stanojević et al. 2023). This work was conducted with the aim of evaluating changes in blood metabolites related to energy metabolism, indicators of liver function and liver damage, red blood cell indices, white blood cell indices, minerals, inflammation, innate immunity, markers of oxidative stress and tissue injury in Holstein cows affected by sIMI. A limitation of this study is that not all analytical assays were fully validated. In particular, the IL-6 and SAA assays were performed using commercially available kits but did not undergo complete in-house validation. Although linearity and sensitivity were evaluated, other validation parameters were not assessed. Therefore, results related to these analytes should be interpreted with caution, and further studies including fully validated assays are warranted.

It is worth noting that IMI in dairy cows could offer a practical model to investigate systemic responses to localised infections under field-relevant conditions. Cows with clinical signs of disease were excluded from the study; however, the presence of concurrent subclinical pathological conditions associated with IMI cannot be ruled out, and these may have influenced the observed biochemical profiles. Concerning milk traits, no significant differences in milk production and composition were observed between animals without and with *S. aureus* IMI at the bacteriological analysis, which aligns with the subclinical nature of the condition. In contrast, animals with bacteriologically positive samples showed an average significantly higher SCC values (positive= 910.000 cells/mL vs negative= 180.000 cells/mL), supporting the usefulness of this indicator in screening for subclinical mastitis. It is important to note, however, that 26 cows with positive samples had SCC < 200,000 cells/mL (16.88%), and of these, eight had SCC <100,000 cells/mL (27.59%).

Hepatic function and liver damage indicators

In our study, AST levels were significantly higher in cows with sIMI caused by *S. aureus* compared to bacteriologically negative animals at T1. This enzyme is primarily associated with hepatic function but also found in muscle and other tissues, and its elevation is commonly linked to tissue damage or systemic stress. As reported by Golshan et al. (2021), AST levels tend to be higher in cows with persistent subclinical mastitis. Similarly, in our study, we observed higher values in bacteriologically positive animals compared to uninfected ones (130 U/L vs 94.50 U/L), confirming the trend described in the literature, even in the absence of clinical signs. At T2, infected animals continued to exhibit higher concentrations, suggesting ongoing subclinical involvement, potentially indicating chronic or recurrent impairment associated with pathogen persistence. Interestingly, AST levels were also higher (22.88% T1 and 25.24% T2, respectively) in TOP animals compared to the BOTTOM ones. As described by Oliszewski et al. (2002), pathogenic invasion can trigger an inflammatory response, leading to cell damage and the subsequent release of intracellular enzymes into the bloodstream and milk. The significant increase observed at T1 may reflect a systemic inflammatory response, even in the absence of clinical signs, or indicate a more rapid systemic response to the infection challenge, warranting further investigation. GGT

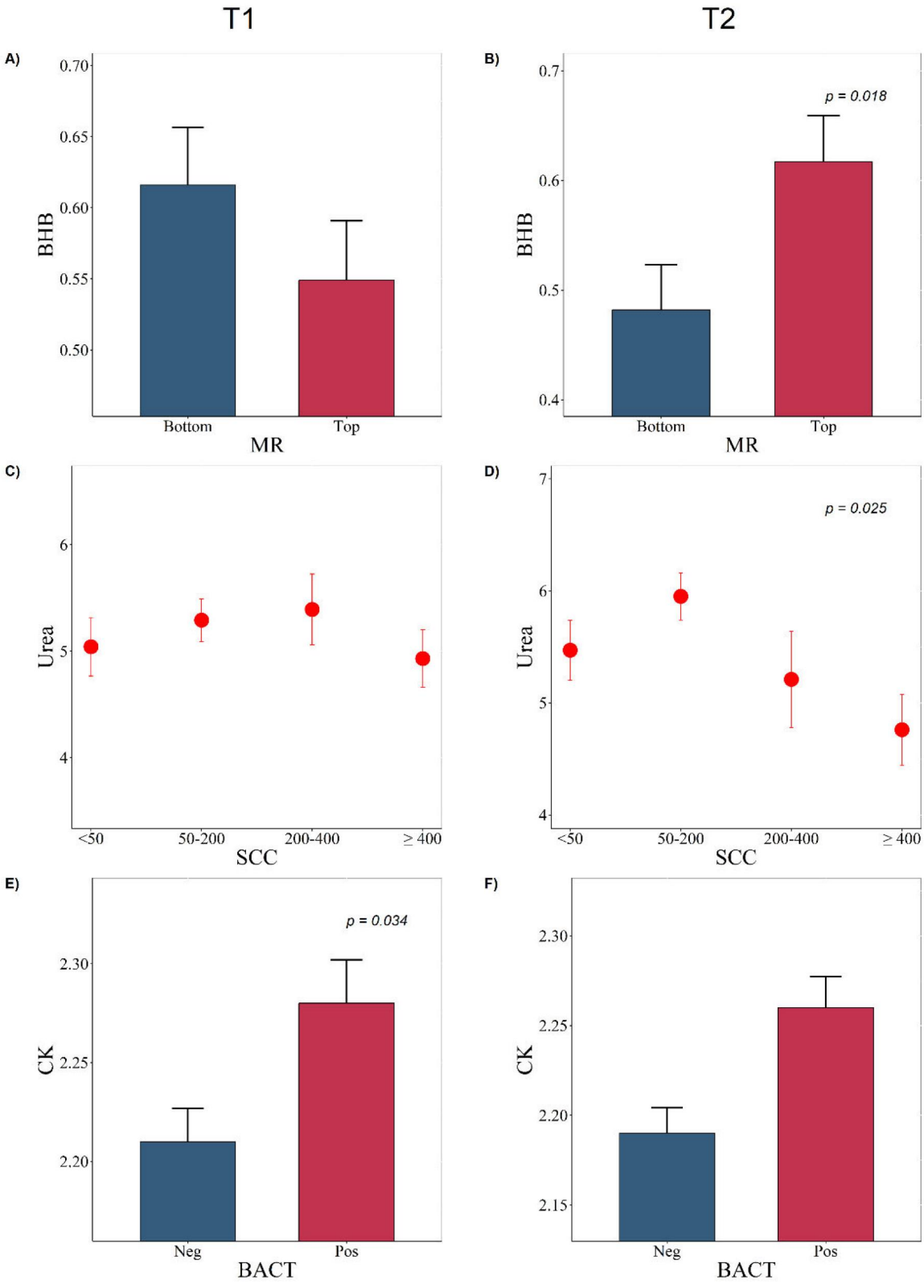

Figure 4. Least squares means ± SE of beta-hydroxybutyrate (BHB), urea and creatine kinase (CK). SCC classes were defined as follows: Class 1 < 50,000 cells/mL; Class 2: 50,000 ≤ SCC < 200,000 cells/mL; Class 3: 200,000 ≤ SCC < 400,000 cells/mL; Class 4 ≥ 400,000 cells/mL. T1 is shown on the left, and T2 on the right). Mastitis risk (MR) was categorised as follows: TOP = animals with low susceptibility to developing mastitis, and BOTTOM = animals at risk for developing mastitis. T1 is shown on the left, and T2 on the right.

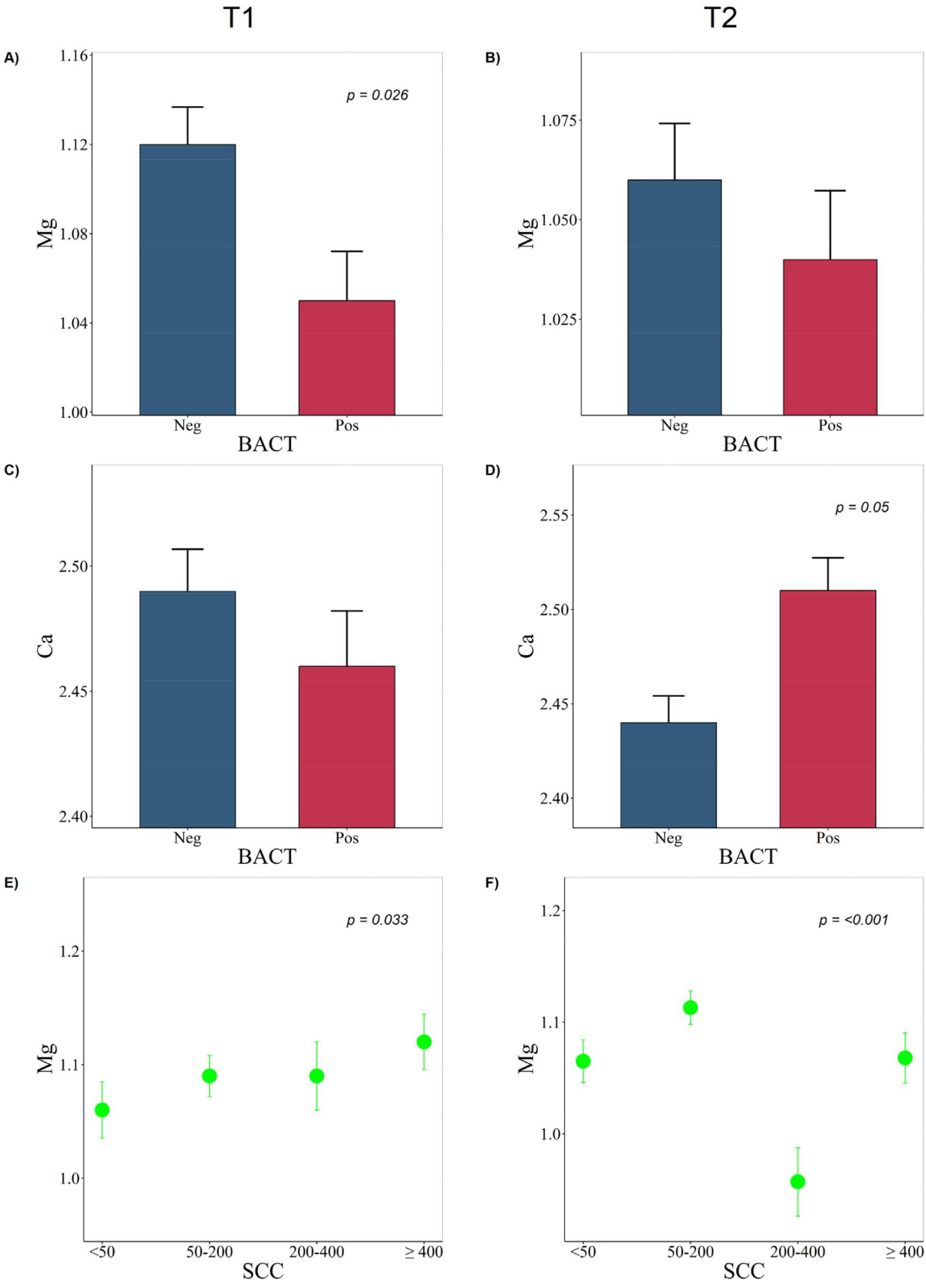

Figure 5. Least squares means ± SE of magnesium (Mg-BACT and Mg-SCC) and calcium (Ca). Bacteriological status (BACT) was defined as follows: POSITIVE = if at least one quarter tested positive for *S. aureus*, and NEGATIVE = only if all four quarters were negative for the mastitis pathogens of interest (*S. aureus*), although they could test positive for environmental microorganisms. T1 is shown on the left, and T2 on the right. SCC classes were defined as follows: Class 1 < 50,000 cells/mL; Class 2: 50,000 ≤ SCC < 200,000 cells/mL; Class 3: 200,000 ≤ SCC < 400,000 cells/mL; Class 4 ≥ 400,000 cells/mL. T1 is shown on the left, and T2 on the right.

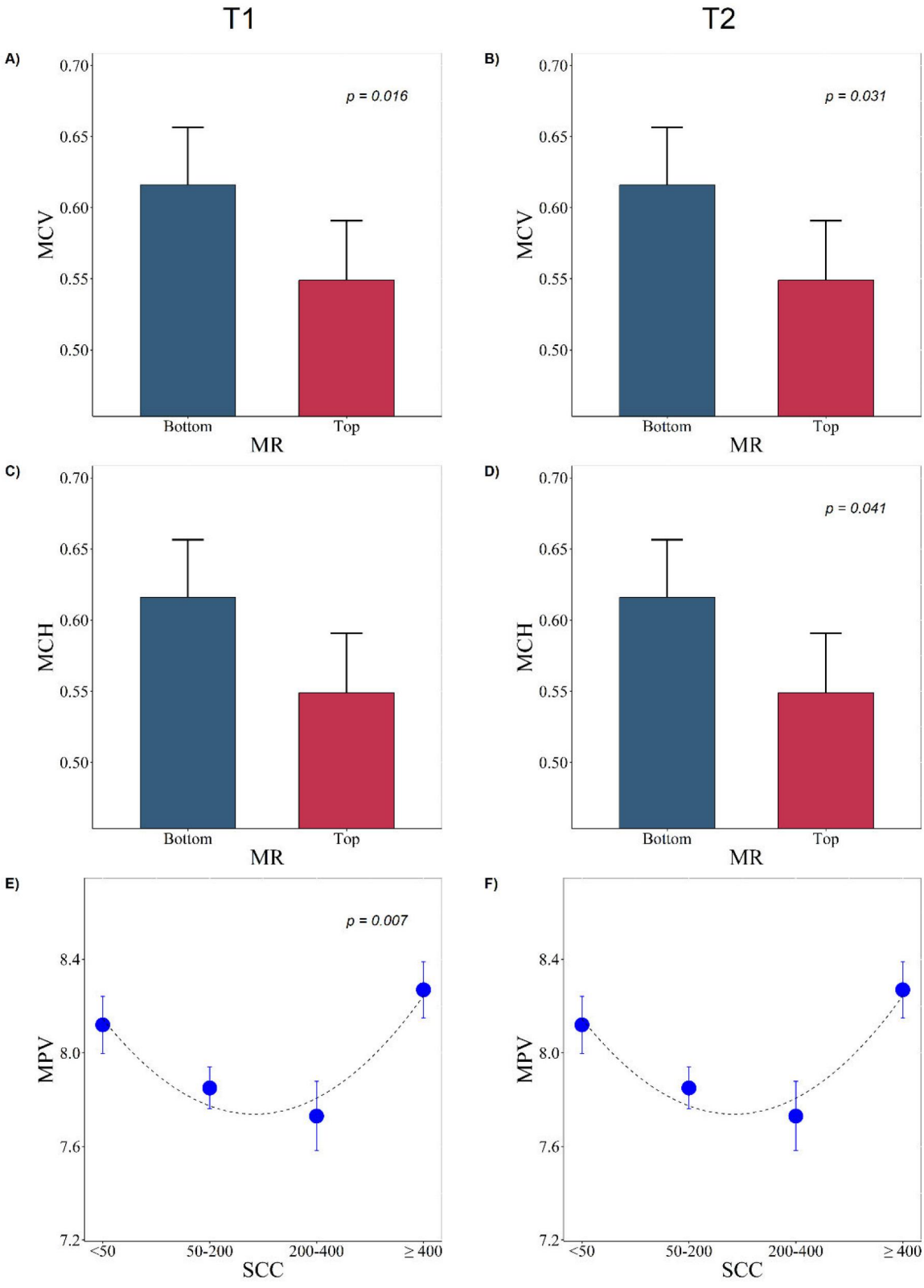

Figure 6. Least squares means ± SE of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean platelet volume (MPV). SCC classes were defined as follows: Class 1 < 50,000 cells/mL; Class 2: 50,000 ≤ SCC < 200,000 cells/mL; Class 3: 200,000 ≤ SCC < 400,000 cells/mL; Class 4 ≥ 400,000 cells/mL. T1 is shown on the left, and T2 on the right. Mastitis risk (MR) was categorised as follows: TOP = animals with low susceptibility to developing mastitis, and BOTTOM = animals at risk for developing mastitis. T1 is shown on the left, and T2 on the right.

showed a significant quadratic relationship with SCC at T2, with the highest levels observed in animals belonging to the highest SCC class ($\geq$ 400,000 cells/mL). GGT is an enzyme involved in oxidative stress responses and liver function, and its concentration may reflect chronic tissue injury or oxidative damage (Kleczkowski 2017). The absence of an association with bacteriological status suggests that GGT is not a sensitive marker of infection per se, but rather an indicator of the severity of inflammation, particularly in cows with longstanding infections or compromised udder health. These findings are consistent with previous observations indicating that GGT concentrations are more informative when measured in milk rather than blood (Babaei et al. 2007). However, our results suggest that blood GGT may still have diagnostic relevance in cases of severe or prolonged inflammation.

Inflammatory and immune response markers

Serum amyloid A proteins are small, highly conserved blood molecules that are prominently induced during the acute phase response, rising dramatically after trauma or infection (Hoeger et al. 2020). SAA showed a significant association with SCC classes at T1, following a cubic pattern suggesting a nonlinear immune activation during infection. Specifically, SAA levels increased in subjects with moderate to high SCC, decreased in intermediate ranges, and rose again in subjects with SCC $\geq$400,000, indicating potential involvement of this protein in both the early and late stages of the inflammatory response. These findings are consistent with Gerardi et al. (2009), who highlighted the utility of SAA as an early biomarker in both blood and milk of cows with sIMI. Similarly, Kovacevic-Filipovic et al. (2012) reported an increased expression of SAA isoforms in response to *S. aureus* infections, reinforcing the hypothesis that this protein plays a role in both systemic and local immune responses. Overall, the data may suggest that SAA could serve as a valuable serological indicator for detecting inflammatory states, even in the absence of clinical sign, supporting its potential as a complementary screening tool to SCC in subclinical mastitis monitoring strategies. The significant increase in neutrophil count observed at T1 in BOTTOM animals may reflect an active systemic inflammatory state, consistent with enhanced stimulation of their innate immune response. Neutrophils represent the first line of defense against bacterial infections of the mammary gland, and their recruitment is indicative of persistent immune activation, even in the absence of overt clinical signs (Riollet et al. 2002). The significant increase in neutrophil count observed in the BOTTOM group at T1, regardless of their infection status, suggests the presence of an underlying systemic inflammatory condition. This could be due to a predisposition in these animals to heightened immune activation, potentially influenced by genetic, metabolic, or environmental factors. Alternatively, the observed differences may partly reflect differential migration of neutrophils between the bloodstream and inflamed tissues, which could alter circulating populations independently of total neutrophil production. Neutrophils are key components of the innate immune system, and their elevated levels indicate a pre-activated immune state, which may reflect an ongoing response to subclinical inflammation or other underlying physiological stressors. Indeed, as highlighted by Leblanc (2020), this heightened baseline inflammatory response may make these animals more susceptible to mastitis, with an immune system that is chronically activated but potentially less effective at clearing infections.

Metabolic indicators

In our study, at T2 BOTTOM animals showed lower blood concentrations of BHB compared to TOP animals, although all values remained within physiological ranges. β-hydroxybutyrate is a ketone body that can be utilised as an energy substrate, and lower concentrations may indicate its consumption to sustain increased energy requirements associated with immune activation (Hillreiner et al. 2016). Conversely, elevated BHB has been reported to exert modulatory effects on immune functions, potentially downregulating genes involved in innate defense mechanisms (Swartz et al. 2021). However, literature on the relationship between BHB and immune competence in dairy cows remains inconsistent, with both positive and negative associations reported. Therefore, while the difference observed between groups was statistically significant, it may not directly reflect differences in mastitis risk. Further studies are needed to clarify the biological significance of BHB fluctuations within physiological ranges and their potential link to immune efficiency.

Mineral indicators

For the bacteriological status, Mg was significantly affected by the cows' inflammatory status, showing a cubic trend at T2. This mineral is known to play an essential role in cell metabolism and acts as a co-factor for numerous enzymes (Libera et al. 2021), and plenty of evidence on rodents' models have demonstrated its importance in the modulation of immune cell functions (Van

Orden et al. 2006). While no specific studies have specifically evaluated the role of Mg in the modulation of dairy cows' immune system during mastitis, it was observed that deficiency in Mg was associated with an increased prevalence of lameness in cattle (Jelinski et al. 2018). The non-linear pattern of Mg observed in this study may reflect phase-dependent changes in mineral utilisation, redistribution, or excretion, as previously hypothesised in chronic inflammatory conditions (Martens et al. 2018), suggesting that its variations during sIMI may be linked to immune modulation. Although further studies are needed to fully understand the role of Mg in the modulation of inflammatory processes, our findings support its potential role of a complementary marker for monitoring systemic responses in intramammary infections.

Conclusions

This study shows that intramammary infections caused by *Staphylococcus aureus*, even in the absence of clinical signs, are associated with measurable systemic changes in blood parameters related to energy metabolism, liver function, and inflammatory response. Observed alterations in BHB, AST, GGT, SAA, and neutrophil levels suggest that localised infection can elicit systemic responses, although markers of oxidative stress remained largely unchanged, possibly reflecting the mild nature of the infection or variability in infection timing among individuals. These findings indicate that IMI can affect systemic physiology and provide insights into host responses to localised infections. However, the potential use of these changes as biomarkers for early detection requires further studies and validation before any practical application can be recommended.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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