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Acute phase reaction to LPS induced mastitis in early lactation dairy cows fed nitrogenic, glucogenic or lipogenic diets

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ABSTRACT

The availability of certain macronutrients is likely to influence the capacity of the immune system. Therefore, we investigated the acute phase response to intramammary (i.mam.) LPS in dairy cows fed either a nitrogenic diet (n = 10) high in crude protein, a glucogenic diet (n = 11) high in carbohydrates and glucogenic precursors, or a lipogenic diet (n = 11) high in lipids. Thirty-two dairy cows were fed one of the dietary concentrates directly after calving until the end of trial at 27 ± 3 DIM (mean ± SD). In wk 3 of lactation, 20 μg of LPS was i.mam. injected in one quarter, and sterile NaCl (0.9%) in the contralateral quarter. Milk samples of the LPS challenged and control quarter were taken hourly from before (0 h) until 9 h after LPS challenge, and analyzed for milk amyloid A (MAA), haptoglobin (Hp), and IL-8. In addition, blood samples were taken in the morning, and composite milk samples at morning and evening milkings from 1 d before until 3 d after LPS challenge, and again on d 9 to determine serum amyloid A (SAA) and Hp in blood, and MAA and Hp in milk. The mRNA abundance of various immunological and metabolic factors in blood leukocytes was quantified by RT-qPCR from samples taken at −18 h, −1 h, 6 h, 9 h and 23 h relative to LPS application. The dietary concentrates did not affect any of the parameters in blood, milk, and leukocytes. The IL-8 was increased from 2 h, Hp from 2 to 3 h, and MAA from 6 h relative to the LPS administration in the milk of the challenged quarter and remained elevated until 9 h. The MAA and Hp were also increased at 9 h after LPS challenge in whole udder composite milk, whereas Hp and SAA in blood were increased only after 23 h. All 4 parameters were decreased again on d 9. Similar for all groups, the mRNA abundance of Hp and the heat shock protein family A (HSP70) increased after the LPS challenge, while the mRNA expression of the tumor necrosis factor α (TNF) and the leukocyte integrin β 2 subunit (CD18) were decreased at 6 h after LPS challenge. The glucose transporter (GLUT)1 mRNA abundance decreased after LPS, whereas that of the GLUT3 increased, and that of the GLUT4 was not detectable. The mRNA abundance of GAPDH was increased at 9 h after LPS and remained elevated. The APP response was detected earlier in milk compared with blood indicating mammary production. However, immunological responses to LPS were not affected by the availability of specific macronutrients provided by the different diets.
Key words: early lactation, diet, mastitis, metabolism, immune system

INTRODUCTION

In the periparturient period dairy cows are at high risk for infectious diseases including mastitis, which is why most veterinary-treated mastitis cases occur during the first few wk after calving (Persson Waller et al., 2009). The infection and inflammation of the mammary gland induces a systemic acute phase reaction, which is orchestrated by pro-inflammatory cytokines. The reaction includes systemic changes represented by hepatically produced acute phase proteins (APP) that support the innate immune response fighting microbes and the restoring homeostasis (Conner et al., 1986; Murata et al., 2004). Increased plasma concentrations of haptoglobin (Hp) and serum amyloid A (SAA) mainly derived from the liver, and elevated milk amyloid A (MAA) derived from mammary tissue, measured in milk of the infected gland have been observed during bovine mastitis (Eckersall et al., 2006; Eckersall and Bell, 2010). Both Hp and SAA have been shown to exert immunomodulatory functions as well as inhibitory effects on parts of the immune system and therefore help controlling the immune response (Petersen et al., 2004). Haptoglobin mRNA was shown to be expressed in bovine leukocytes (Thielen et al., 2005); therefore, it is likely that SAA is also expressed by leukocytes. These APP are of increasing interest as biomarkers for infection and inflammation (Eckersall and Bell, 2010). Both MAA and Hp in milk are used in research as alternative markers to the SCC to detect mastitis, and often also to differentiate between subclinical and clini-

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cal mastitis (Wollowski et al., 2021). A study in rats showed that the acute phase protein response can be altered by nutritional protein deficiency (Jennings et al., 1992). However, studies in cows investigating how the availability of macronutrients affects acute phase proteins are limited. The infusion of amino acids that are usually reduced in early lactation did not alter the systemic immune response (Chandler et al., 2022). Regarding the immune reaction to mastitis, Burton and Erskine (2003) discussed that neutrophils are the key effector cells fighting mastitis causing bacteria, and delayed neutrophil recruitment into the mammary gland seems to be a major determinant for the clinical outcome. Also, social stressors, antioxidant supply, fat mobilization, and reduced calcium and glucose availability can impair neutrophil function (LeBlanc, 2020). Hence, nutrition probably plays an important role in neutrophil function and, therefore, mammary immune response. Glucose is the preferred fuel for immune cells, but its availability vastly decreases during early lactation as the mammary gland′s consumption of glucose for milk synthesis increases tremendously (Bell, 1995; Pithon-Curi et al., 2004; Gross et al., 2011). Apart from the glucose that is scarcely available during early lactation, nonesterified fatty acids (NEFA) and BHB increase during negative energy balance (NEB), and negatively affect leukocyte functions in vitro (Suriyasathaporn et al., 1999; Ster et al., 2012; Djoković et al., 2017). It has been demonstrated that energy balance (EB) and the previously mentioned metabolites can be altered through different dietary compositions in early lactation (van Knegsel et al., 2007). Compared with cows in late lactation, early lactating cows react more severely to an intramammary (i.mam.) endotoxin challenge and show diminished function of blood polymorphonuclear leukocytes (Lehtolainen et al., 2003). Parturition itself, but also dietary energy content during the dry-period and the metabolic profiles during the early lactation were shown to affect blood leukocyte gene expression during early lactation (Moyes et al., 2014; Crookenden et al., 2016; Wathes et al., 2021). However, studies analyzing blood leukocyte gene expression during mammary inflammation after i.mam. LPS challenge incorporating diets varying in macronutrients are to our knowledge not available.

In the present study we tested the hypothesis that feeding diets differing in macronutrients to early lactating cows affects the acute phase response to an i.mam. LPS induced mastitis. Additionally, we investigated the mRNA abundance in blood leukocytes encoding for various factors with potential importance for the immune response to LPS.

MATERIALS AND METHODS

Animal experiments were carried out in accordance with the Swiss law on animal protection and welfare, and approved by the cantonal committee of animal experimentation (approval no. 2018_35_FR). The experimental design was already published by Wagner et al. (2023).

Animals and Husbandry

Thirty-two dairy cows (Holstein Friesian n = 20, Red Holstein n = 9, Red Holstein x Simmental n = 3) in their second or higher lactation were included in the study. All animals were selected from and housed at the Agroscope research station (Posieux, Switzerland). Except for the time around parturition when animals were housed in individual calving pens with straw bedding, they were kept in a tie-stall barn with straw and sawdust bedding throughout the experimental period. The health status of each cow was assessed by a veterinarian before it entered the trial. Immediately after calving, cows were assigned to 1 of 3 dietary groups according to their body weight and milk production in the previous lactation.

Beginning after calving, cows were machine milked twice daily (0530 h and 1600 h).

Feeding Regimen

Diets were formulated according to the national feeding recommendations of Agroscope (2016) for dry and lactating dairy cows.

During the whole trial, hay was fed ad libitum and minerals (50 g/cow) were provided daily. Additionally, a concentrate for dry cows (1.5 kg/d) and lactating cows (1.5 kg/d), respectively, was fed throughout the experimental period.

For the 3 wk before expected calving, all cows were fed the same control diet (1.5 kg/d) consisting of a mixture of each a 1/3 of the 3 specific concentrates. After calving, the control diet was replaced with the group specific concentrate. Cows were distributed into the 3 treatment groups based on similar mean performance, BW, and milk yield in their previous lactation and an equal distribution of different breeds. The concentrates were fed in equal portions thrice daily. The nitrogenic concentrate (10 cows) consisted of components rich in crude protein, the glucogenic one (11 cows) of components rich in carbohydrates, and the lipogenic one (11 cows) of components rich in lipids. The nitrogenic feed was composed of 55% soybean meal, 15% rapeseed meal, 10% barley, 10% corn, 10% beet pulp, the glucogenic of 40% corn, 20% wheat, 10% barley, 10%

soybean meal, 10% rapeseed meal, 10% beet pulp, and the lipogenic feed was composed of 20% barley, 20% corn, 15% beet pulp, 15% rumen protected fat, 10% soybean meal, 10% rapeseed meal, 10% rapeseed press cake. Each cow was fed 3.5 kg/d of the specific concentrate directly after calving for at least 4 d and at most 10 d, before the amount was increased to 6 kg/d for 7 d on what was defined as the start of the second wk of lactation. From the beginning of the third wk of lactation until the end of trial (27 ± 3 DIM, mean ± SD), the amount of the specific concentrate was increased to 8.5 kg/d.

Intramammary LPS challenge

During wk 3 of lactation (18 ± 3 DIM, mean ± SD) after the morning milking (0700 h), cows were treated through the teat canal in one quarter with 20 μg of *E.coli* LPS (O26:B6, Sigma Aldrich) diluted in 10 mL sterile saline solution (0.9%). The contralateral quarter served as control and was injected with 10 mL sterile saline solution.

Milk Sampling and Analyses

Whole udder composite milk samples were collected twice daily during machine milking from 1 d before until 3 d after the LPS challenge and again on d 9. On the day of the LPS challenge, quarter milk (~20mL) from the LPS injected quarter and from the control quarter were taken separately by hand milking directly before the administration of LPS (0 h relative to LPS challenge) and hourly until 9 h after. Milk samples were stored at −20°C until further analysis. Milk amyloid A was measured in all milk samples using a commercially available ELISA kit according to the manufacturer's instruction (Tridelta Development LTD, cat. no. TP-8072). Samples for measurement of Hp and IL-8 were degreased as described by Bannerman et al. (2003). Hp was measured in milk serum, using a commercially available ELISA kit according to the manufacturer's instruction (Immunology Consultants Laboratory, cat. no. E-10HPT). IL-8 was measured in the hourly samples of the LPS challenged quarter with a commercially available human ELISA kit (R&D systems, cat. no. D8000C) that had been shown to cross-react with bovine IL-8 (Shuster et al., 1997). Measurements were performed according to the manufacturers' instructions. The intra- and inter-assay Coefficients of Variability (CV) were 1.5 and 9.4 for MAA, 3.5 and 4.5 for Hp, and 3.7 and 6.2 for IL-8, respectively.

Blood Sampling and Analyses

Plasma samples were taken from the jugular vein at −18 h, −1 h and 9 h relative to LPS challenge and then once daily in the right after morning milking and before feeding (0600 h) until 3 d after the LPS challenge and again on d 9. Blood was drawn through an 18 G canula into evacuated tubes coated with EDTA (Vacuette, 9 mL with K_3EDTA, cat. no. 455036; Greiner Bio-One International GmbH) and immediately put on wet ice. After centrifugation at 2,000 × *g* (+4°C, 20 min), plasma samples were frozen in aliquots at −80°C until measurements with commercially available ELISA kits for Hp (Immunology Consultants Laboratory, cat. no. E-10HPT) and SAA (Tridelta Development LTD, cat. no. TP-802) according to the manufacturers' instructions. The intra- and inter-assay CV were 4.0 and 9.0 for Hp, and 5.7 and 13.8 for SAA, respectively.

Total RNA Extraction, Reverse Transcription, and Quantitative PCR in Blood Cells

Details about the whole procedure to measure mRNA abundance in blood cells have been described recently (Jermann et al., 2022). In short, blood cells were isolated from blood samples harvested at −18 h, −1 h, 6 h, 9 h and 23 h relative to LPS challenge. Total RNA was extracted and the RNA concentration and purity were determined by spectrophotometry. Only samples measuring at OD A260/280: > 1.8 were used for further analyses. Five samples from 4 different cows (3 samples from 2 nitrogenic, one from a glucogenic, and one from a lipogenic fed cow) had to be excluded from this study. Samples were stored at −80°C until further processing. 100 ng RNA was reverse transcribed into complementary DNA and then stored at −21°C. Primers were commercially synthesized (Microsynth, Balgach, Switzerland), details are shown in Table 1. Quantitative mRNA abundance was measured in duplicates and all samples of a cow repeated if duplicates differed in one Cycle threshold (Ct) or more. Average values of qPCR duplicates were normalized with the arithmetic means of 2 reference genes: Ubiquitin B (*UBB*) and Tyrosine 3-monooxygenase (*YWHAZ*). Ct values of target genes were calculated as relative expression, multiplied with −1, so a higher Ct value indicates an increase in mRNA abundance of the respective gene.

Statistical Analyses

Data are presented as mean values ± SEM, except where denoted as standard deviation. Test statistics was calculated with the statistical software SAS (version 9.4; SAS Institute Inc., Cary, NC). Factors measured at

Table 1. Primer sequences for PCR (F = forward, R = reverse), GenBank accession number and product size (bp; base pair) of reference and target genes

Gene[1]	Primer sequence, 5′-3′	GenBank accession no.	Product size (bp)
UBB[1]	F: AGA TCC AGG ATA AGG AAG GCA T R: GCT CCA CCT CCA GGG TGA T	NM_174133.2	426
YWHAZ[2]	F: CAG GCT GAG CGA TAT GAT GAC R: GAC CCT CCA AGA TGA CCT AC	NM_174814.2	141
Hp[3]	F: GTC TCC CAG CAT AAC CTC ATC TC R: AAC CAC CTT CTC CAC CTC TAC AA	AJ_271156.1	174
SAA3[4]	F: GGG CAT CAT TTT CTG CTT CCT R: TTG GTA AGC TCT CCA CAT GTC TTT AG	NM181016.3	106
TNF[2]	F: CCA CGT TGT AGC CGA CAT C R: CCC TGA AGA GGA CCT GTG AG	NM_173966.3	155
HSPA1A[3]	F: ACA TGA AGA GCG CCG TGG AGG R: GTT ACA CAC CTG CTC C	NM_203322.3	170
SLC2A1[5]	F: GCT TCT CCA ACT GGA CTT CG R: ACA GCT CCT CAG GTG TCT TG	NM_174602	225
SLC2A3[5]	F: GGA AAA CTT GCC GCC GAT AG R: CGC CTC AGG AGC ATT GAT GA	NM_174603	223
SLC2A4[5]	F: GAC TGG TAC CCA TGT ACG TG R: CCG GAT GAT GTA GAG GTA GC	NM_174604.1	242
GAPDH[2]	F: GTC TTC ACT ACC ATG GAG AAG G R: TCA TGG ATG ACC TTG GCC AG	NM_001034034.2	197
ITGB2[1]	F: CAG CAA CGA ATT TGA CTA R: GTA GGC ATT CTT GAT AAG C	NM_175781.1	196

[1]*UBB* = Ubiquitin B, *YWHAZ* = tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, *HSPA1A* = heat shock protein family A (HSP70), *Hp* = haptoglobin, *SAA3* = serum amyloid A (SAA), *TNF* = tumor necrosis factor α, *SLC2A* = solute carrier family 2 (GLUT), *ITGB2* = leukocyte integrin β 2 subunit (CD18).

[1]designed with Beacon Designer 8.21 (Premier Biosoft International), [2]Griesbeck-Zilch et al., 2008, [3]Caldeira et al., 2019, [4]Mukesh et al., 2010, [5]Gross et al., 2015.

a protein level were tested for normal distribution, and the individual values were logarithmized (log 2), because none of the parameters was normally distributed.

Mixed model analyses of variance (MIXED procedure) were conducted for all statistical evaluations. The model included time, dietary group and interaction of time and dietary group (nitrogenic, glucogenic, lipogenic) as fixed effects, and the individual cow as repeated subject. For none of tested parameters the dietary group or the interaction of time and dietary group showed signficant effects. Therefore, post hoc testing was only performed to compare time points withing dietary group. For the mRNA expression data, Ct values were compared among each other by Tukey's t-test which adjusted for multiple comparisons but compared among individual time points. Because of the greater number of time points in milk than in blood samples, for all APP and IL-8 data (at a protein level), effects of LPS challenge of the different time points within dietary group were individually compared with the respective baseline value shortly before the LPS challenge (−1 h or 0 h relative to LPS challenge) using the Dunnett test procedure. Effects were considered to be significant if $P < 0.05$.

RESULTS

Milk amyloid A, Haptoglobin and IL-8 in Milk

Milk amyloid A and Hp in the whole udder composite milk did not differ between the 3 dietary groups (Figure 1A and 1D). Both factors were elevated from 9 h until 3 d after LPS compared with 1 h before LPS challenge in all dietary groups, and were decreased again on d 9 ($P < 0.05$; Figure 1A and 1D). In the milk of LPS injected quarters MAA was increased from 6 h after LPS in the nitrogenic and lipogenic groups, and only from 7 h in the glucogenic group, and remained elevated in all groups until the end of quarter milk sampling at 9 h ($P < 0.05$; Figure 1B). Milk Hp was already significantly increased at 2 h after LPS in the glucogenic group, at 3 h after LPS in all groups, and remained elevated until the end of sampling at 9 h ($P < 0.05$; Figure 1E). Both MAA and milk Hp did not increase in the control quarters (Figures 1C; 1F). Milk IL-8 concentration in the LPS injected quarter was increased at 2 h after the LPS challenge in all groups and remained elevated until the last sampling at 9 h after LPS ($P < 0.05$; Figure 2). The IL-8 in the milk of the quarter injected with LPS (Figure 2) did not differ among the 3 dietary groups.

Acute Phase Proteins in Blood

In contrast to Hp and MAA in the LPS-injected quarters, both Hp and SAA blood concentrations were not yet significantly elevated at 6 h after LPS challenge. Elevated systemic concentrations of Hp and SAA were only detected from the subsequent blood sample at 23 h until 3 d after LPS challenge. Both were decreased again in the last sample on d 9 ($P < 0.05$) if compared with 1 h before LPS challenge (Figures 3A and B). The patterns of both Hp and SAA in blood did not differ among the 3 dietary groups.

mRNA Abundance in Blood Leukocytes

When comparing the 3 dietary groups at individual time points, no differences in mRNA abundance were found for any of the measured factors among groups. Additionally, the 2 values before LPS challenge (−18 h vs. −1 h relative to LPS challenge) did not differ for any of the measured genes and in all groups.

The relative mRNA abundance of Hp (coded by *HP*; Table 2) was increased at 6 h and 9 h after LPS challenge, and was decreased again at 23 h in all dietary groups ($P < 0.0001$). The mRNA expression of SAA (coded by *SAA3*) could not be detected in each sample of the present study (data not shown). To exclude potential laboratory issues, a positive control of LPS-stimulated mammary epithelial cells were analyzed for SAA3 under the same PCR conditions. In these cells the Ct values for SAA3 were around 22. In the blood leukocytes of the present study SAA3 mRNA abundance was only detected at a very low level in one individual sample before LPS challenge (Ct value 36), at a similarly low level in 70% of the samples at 6 h and 9 h after LPS, and only in 14% of samples at 23 h. A statistical evaluation of SAA3 results was not possible.

The mRNA expression of TNF decreased 6 h after LPS challenge in all dietary groups ($P < 0.05$; Table 2). TNF increased again from 6 h to 23 h in the lipogenic group ($P < 0.05$) and by tendency also in the nitrogenic group ($P = 0.06$; Table 2), whereas an increase in the was not observed in the glucogenic group ($P = 0.14$). Relative mRNA expression of the heat shock protein (HSP) family A (HSP70; coded by *HSPA1A*) increased short-term 6 h after LPS challenge in all dietary groups, before it decreased to pre-challenge levels 9 h after LPS challenge and remained on this level until 23 h ($P < 0.0001$; Table 2). The mRNA abundance of the glucose transporter (GLUT) 1 (coded by solute carrier family 2; *SLC2A1*) started to decrease 6 h after LPS challenge and was lower at 9 h compared with pre-challenge levels in all groups ($P < 0.05$; Table 2). At 23 h, mRNA expression of GLUT1 was neither different to the values before LPS challenge, nor to 9 h after. The mRNA expression of the GLUT3 (coded by *SLC2A3*; Table 2) increased in the nitrogenic and glucogenic group at 6 h after LPS challenge and remained elevated until 23 h ($P < 0.05$). In the lipogenic group, GLUT3 mRNA was only increased 9 h after LPS challenge and decreased again at 23 h, but was still higher than pre-challenge levels ($P < 0.05$). The mRNA abundance of the GLUT4 was not detectable in any of the tested samples (data not shown). To exclude experimental errors, a positive control was tested under the same laboratory conditions. The mRNA abundance of GAPDH did not differ at 6 h after LPS challenge compared with pre-challenge values, but increased from 6 h to 9 h in all dietary groups and was still elevated at 23 h after LPS ($P < 0.05$; Table 2). The mRNA abundance of the leukocyte integrin β 2 subunit (CD18; coded by leukocyte integrin β 2 subunit; *ITGB2*; Table 2) was not affected throughout the experiment in the nitrogenic group, even though a tendency to decrease from 1 h before to 6 h after LPS challenge ($P = 0.08$) and an increase from 6 h to 23 h ($P = 0.07$) was found. In the glucogenic and lipogenic groups, mRNA expression of CD18 decreased 6 h after LPS challenge ($P < 0.05$). It increased again at 9 h and remained on this level in the lipogenic group ($P < 0.05$), while CD18 expression only slowly increased until 23 h in the glucogenic group ($P < 0.05$; Table 2).

DISCUSSION

The high susceptibility of cows to intramammary infection in early lactation is well documented (Burton and Erskine, 2003; LeBlanc, 2020). Characteristic changes of metabolites during this period are likely key factors of a reduced function of leukocytes and other immune components. The availability of some blood components such as glucose and calcium is reduced, whereas elevated concentrations of NEFA and BHB have been shown to impair neutrophil function (Zarrin et al., 2014; LeBlanc, 2020). Concentrations of these metabolites in blood can be altered through amount and composition of the feed ration (van Knegsel et al., 2007). We have therefore investigated the acute phase response to an LPS-induced mammary inflammation in dairy cows in their third wk of lactation during the intake of diets which varied considerably in macronutrient composition.

Interleukin-8 in Milk

As a representative of key pro-inflammatory cytokines we have measured IL-8 in the milk of the challenged udder quarter. It is known to be released early after

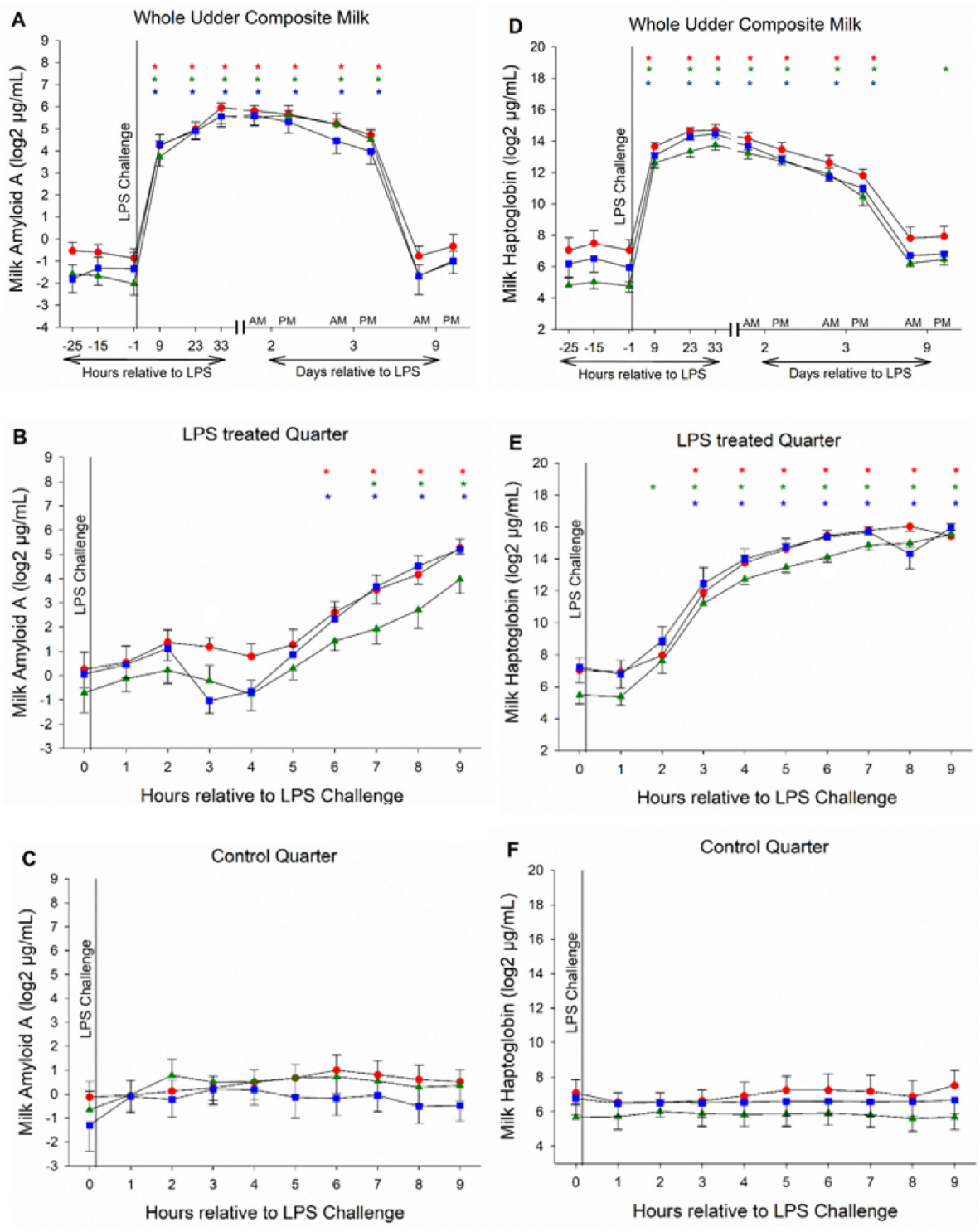

Figure 1. Milk amyloid A (MAA) and Haptoglobin (Hp) in the whole udder composite milk (Figure A and D), in the milk of the LPS challenged quarter (Figure B and E) and in the control quarter (Figure C and F) before and after intramammary LPS challenge in dairy cows during their third wk of lactation. Samples taken during morning milking (0600 h) are marked with AM, those taken in the evening (1600 h) are marked with PM. Cows belonged to one of 3 dietary groups that were fed different concentrates either rich in crude proteins (Nitrogenic; n = 10; ●), carbohydrates (Glucogenic; n = 11; ▲), or lipids (Lipogenic, n = 11; ■). A significant difference ($P < 0.05$) between a specific hour compared with directly before LPS challenge (−1 h or 0 h) within one dietary group is marked with "*" in the color of the respective group. Data is presented as logarithmic mean value (log2) ± SEM.

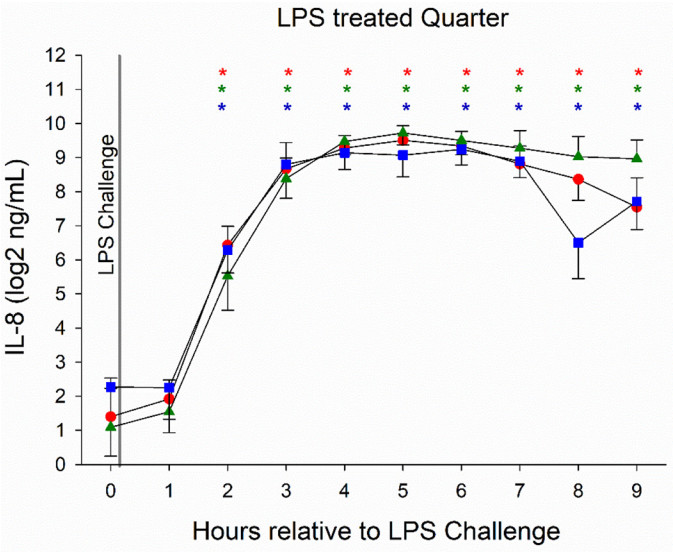

Figure 2. Interleukin 8 (IL-8) in hourly milk samples of LPS challenged quarters before and after intramammary LPS application in dairy cows during their third wk of lactation. Cows belonged to one of 3 dietary groups that were fed different concentrates either rich in crude proteins (Nitrogenic; n = 10; ●), carbohydrates (Glucogenic; n = 11; ▲), or lipids (Lipogenic, n = 11; ■). A significant difference ($P < 0.05$) between a specific hour compared with directly before LPS challenge (0 h) within one dietary group is marked with "*" in the color of the respective group. No significant effect of dietary group was found. Data is presented as logarithmic mean value (log2) ± SEM.

an infection to initiate the response of the innate immune system. As a chemokine IL-8 attracts neutrophils toward the place of infection (Barber and Yang, 1998) which is represented by the characteristic increase of SCC at IMI. In agreement with Persson Waller et al. (2003) the IL-8 concentration in milk of the LPS injected quarter increased within 2 h after LPS challenge, i.e., clearly before the increase of neutrophils in the milk of the same quarter. Both neutrophils and mammary epithelial cells have been shown to be sources of IL-8 in the mammary gland (Wellnitz et al., 2006; Sohn et al., 2007; Wellnitz et al., 2014). The increase of IL-8 in milk in our experiment before the increase of SCC indicates that the mammary epithelial cells are the main source of IL-8 during the early inflammatory response. After the immigration of neutrophils into the milk, they may have also contributed to the production of IL-8. The lacking difference of IL-8 production among the 3 dietary groups indicates that the availability of specific nutrients is not crucial for the chemokine induced neutrophil recruitment into the mammary gland during mastitis.

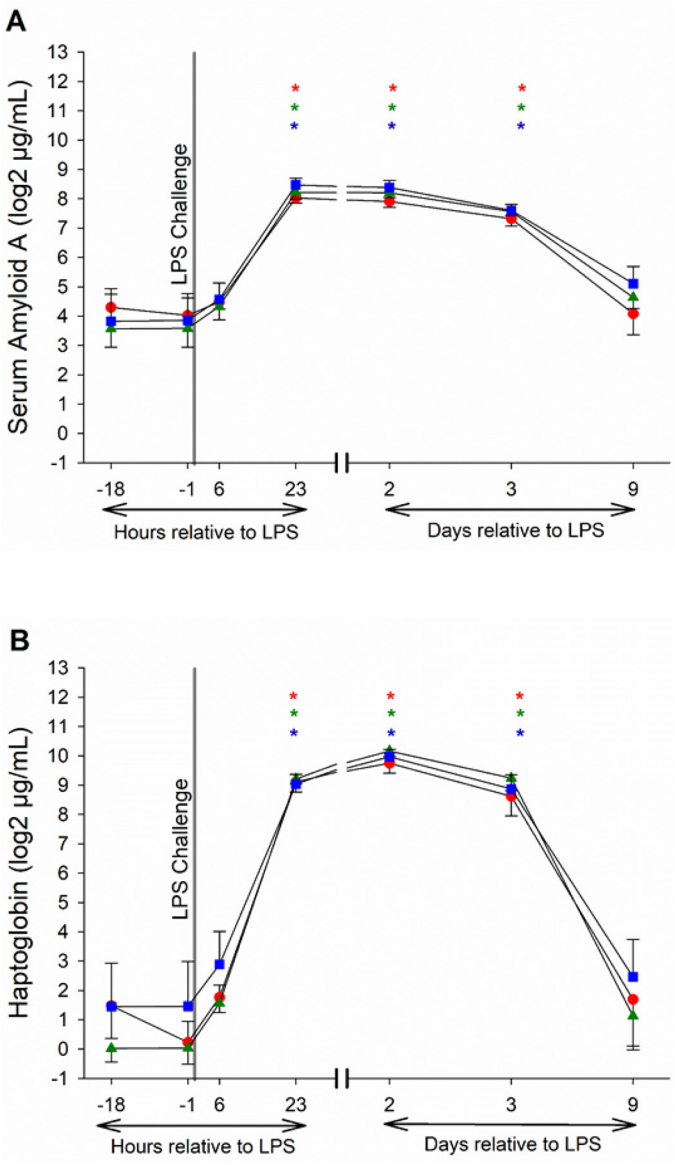

Figure 3. Serum amyloid A (SAA, Figure A) and haptoglobin (Hp, Figure B) in blood before and after intramammary LPS challenge in dairy cows during their third wk of lactation. Cows belonged to one of 3 dietary groups that were fed different concentrates either rich in crude proteins (Nitrogenic; n = 10; ●), carbohydrates (Glucogenic; n = 11; ▲), or lipids (Lipogenic, n = 11; ■). A significant difference ($P < 0.05$) between a specific hour compared with directly before LPS challenge (−1 h) within one dietary group is marked with "*" in the color of the respective group. No significant effect of dietary group was found. Data is presented as logarithmic mean value (log2) ± SEM.

Acute Phase Proteins in Blood and Milk

The increase of SAA and Hp in blood as well as MAA in milk during the distinct LPS-induced mammary inflammation confirms earlier work by Humer et al. (2018). We additionally measured Hp in milk and showed that it increased even earlier than MAA. All

Table 2. Relative mRNA abundance of target genes normalized to two reference genes (*UBB* and *YWHAZ*) in blood leukocytes at specific hours before and after intramammary LPS challenge in early lactating dairy cows fed different concentrates rich in proteins (Nitrogenic; n = 10), carbohydrates (Glucogenic; n = 11) or lipids (Lipogenic; n = 11). Data is presented as mean Ct values ± SEM

		Hours relative to LPS challenge				
Gene[1]	Group	−18 h	−1 h	6 h	9 h	23 h
Hp	Nitrogenic	3.3 ± 0.3^{b}	3.7 ± 0.4^{b}	6.8 ± 0.3^{a}	7.7 ± 0.4^{a}	2.7 ± 0.4^{b}
	Glucogenic	3.6 ± 0.5^{b}	3.7 ± 0.5^{b}	6.6 ± 0.3^{a}	8.1 ± 0.4^{a}	2.7 ± 0.5^{b}
	Lipogenic	3.6 ± 0.3^{b}	3.6 ± 0.3^{b}	6.2 ± 0.6^{a}	7.3 ± 0.5^{a}	3.0 ± 0.5^{b}
TNF	Nitrogenic	6.6 ± 0.1^{a}	6.4 ± 0.1^{ab}	5.6 ± 0.3^{b}	6.0 ± 0.3^{ab}	6.5 ± 0.1^{ab}
	Glucogenic	6.5 ± 0.1^{a}	6.3 ± 0.1^{ab}	5.5 ± 0.3^{b}	5.9 ± 0.2^{ab}	6.2 ± 0.1^{ab}
	Lipogenic	6.4 ± 0.1^{a}	6.0 ± 0.1^{a}	5.1 ± 0.4^{b}	5.8 ± 0.2^{ab}	6.1 ± 0.1^{a}
HSPA1A	Nitrogenic	2.0 ± 0.2^{b}	1.9 ± 0.3^{b}	6.2 ± 1.0^{a}	1.7 ± 0.4^{b}	1.3 ± 0.3^{b}
	Glucogenic	1.9 ± 0.2^{b}	1.8 ± 0.2^{b}	6.2 ± 0.9^{a}	1.8 ± 0.2^{b}	1.4 ± 0.2^{b}
	Lipogenic	2.0 ± 0.1^{b}	2.0 ± 0.2^{b}	5.8 ± 1.1^{a}	2.5 ± 0.6^{b}	1.7 ± 0.2^{b}
SLC2A1	Nitrogenic	5.2 ± 0.2^{a}	5.2 ± 0.3^{a}	4.5 ± 0.2^{ab}	4.0 ± 0.2^{b}	4.4 ± 0.2^{ab}
	Glucogenic	5.1 ± 0.1^{a}	5.3 ± 0.1^{a}	4.8 ± 0.2^{ab}	4.1 ± 0.3^{b}	4.6 ± 0.1^{ab}
	Lipogenic	4.9 ± 0.2^{a}	5.1 ± 0.2^{a}	4.3 ± 0.2^{ab}	3.9 ± 0.2^{b}	4.4 ± 0.2^{ab}
SLC2A3	Nitrogenic	4.1 ± 0.2^{b}	4.1 ± 0.1^{b}	5.0 ± 0.2^{a}	5.6 ± 0.2^{a}	5.1 ± 0.1^{a}
	Glucogenic	4.0 ± 0.2^{bc}	3.8 ± 0.1^{c}	4.9 ± 0.3^{ab}	5.8 ± 0.3^{a}	4.9 ± 0.2^{ab}
	Lipogenic	4.0 ± 0.1^{bc}	3.6 ± 0.3^{c}	4.4 ± 0.3^{bc}	5.9 ± 0.3^{a}	4.8 ± 0.1^{b}
GAPDH	Nitrogenic	5.4 ± 0.1^{b}	5.6 ± 0.2^{b}	5.6 ± 0.1^{b}	6.4 ± 0.2^{a}	7.0 ± 0.2^{a}
	Glucogenic	5.7 ± 0.1^{b}	5.6 ± 0.2^{b}	5.4 ± 0.3^{b}	6.6 ± 0.2^{a}	6.9 ± 0.2^{a}
	Lipogenic	5.6 ± 0.1^{bc}	5.6 ± 0.1^{bc}	5.2 ± 0.3^{c}	6.3 ± 0.3^{ab}	6.5 ± 0.3^{a}
ITGB2	Nitrogenic	6.9 ± 0.1	6.9 ± 0.2	5.7 ± 0.3	6.2 ± 0.5	7.0 ± 0.4
	Glucogenic	6.8 ± 0.1^{ab}	6.8 ± 0.2^{ab}	5.2 ± 0.4^{c}	6.1 ± 0.5^{bc}	7.2 ± 0.2^{b}
	Lipogenic	7.0 ± 0.2^{a}	6.8 ± 0.3^{a}	4.2 ± 0.8^{b}	6.2 ± 0.3^{a}	7.1 ± 0.2^{a}

[a-c] A different letter indicates a significantly different ($P < 0.05$) mRNA expression between the specific hours within the same dietary group. No significant effect of dietary group was found.

[1]*Hp* = haptoglobin, *TNF* = tumor necrosis factor α, *HSPA1A* = heat shock protein family A (HSP70), *SLC2A* = solute carrier family 2 (GLUT), *ITGB2* = leukocyte integrin β 2 subunit (CD18).

measured parameters in the milk of the LPS-treated quarters were recovered at 9 d after the LPS challenge independent of the dietary group. While the liver appears to be the major source of SAA and Hp to induce a systemic response, many tissues including the mammary gland can locally produce APP (Lecchi et al., 2012). The results of the present study confirm that SAA and Hp during mastitis are released systemically, however also in the challenged quarters. The local release of APP appeared to occur earlier than the systemic release. Obviously, locally produced APP, besides those derived from the liver, are considerably involved in the early acute phase reaction during an IMI (Hiss et al., 2004; Eckersall et al., 2006). Thus, APP are potent and sensitive markers for mastitis detection at an early stage already before the increase of SCC. Both SAA and Hp have several important immunomodulatory functions. While Hp binds free hemoglobin and has antioxidant function that might be relevant when reactive oxygen species are produced to kill bacteria in the udder, SAA can directly bind LPS and to reduce cell damage (Tseng et al., 2004; Lai et al., 2009; Cheng et al., 2018).

Although the immune challenge was performed locally in one udder quarter, systemic effects such as the increase of body temperature (shown by Wagner et al., 2023), and the increase of APP in blood were obvious. At present it is not clear if this systemic response is caused by LPS which reaches the blood circulation, or by inflammatory mediators derived from the challenged quarter. In the present study MAA and Hp in milk increased only in the challenged quarter and not in the quarter injected with NaCl. In response to a more severe inflammation caused by a higher LPS dosage compared with that in the present study an increase of MAA was also observed in the non-challenged quarters (Larsen et al., 2010). This indicates the potential transfer of inflammatory mediators only into circulation but also among different quarters of the udder. For detection of subclinical mastitis in quarter milk, it was suggested that MAA is more sensitive than SCC, especially because it is not influenced by factors other than inflammation as it is the case for SCC (Hussein et al., 2018). However, we induced a strong mammary immune response by injecting 20 μg of LPS per quarter, and the SCC and Hp increased earlier than MAA in the hourly measured samples of the LPS challenged quarter (Wagner et al., 2022). In the total composite milk, MAA, Hp and SCC increased at the same time but the sampling frequency was obviously too low to detect any differences. The overall response of APP to the mammary immune challenge in early lactation was

not influenced by the availability of different macronutrients in the feed of the 3 dietary groups.

Immunological and Metabolic Responses of Blood Leukocytes (mRNA)

To assess the immune response of the leukocytes, we measured the mRNA abundance in blood samples containing the whole leukocyte population. For the interpretation of these results we had to consider changes in the leukocyte population in response to the LPS challenge. As shown by Wagner et al. (2023) the leukocyte count in blood decreased transiently from 9.1, 8.4, and 8.9 $\times$ 10^9/L in nitrogenic, glucogenic and lipogenic fed cows to 3.4, 3.9, and 4.0 $\times$ 109/L, respectively. It was fully recovered until 23 h after challenge, i.e., a high percentage of leukocytes were newly built cells. The neutrophil fraction decreased from about 40% before LPS challenge to a nadir of about 30% within 3 h after LPS administration. As key effector cells in the fight against mastitis, leukocytes, mainly neutrophils, migrated to the challenged quarter as described earlier (Burton and Erskine, 2003). Therefore, the measured changes of mRNA expression cannot be interpreted as a change of expression in the same leukocytes over time, but rather as the change of abundance in all leukocytes which are present in blood circulation at a specific time point. Changes of the composition of cell types as well as cells of different levels of maturity derived from the bone marrow are obvious (Paape et al., 2003). This is in agreement with earlier studies were i.mam. LPS lead to a decrease of segmented (mature) neutrophils in blood circulation followed by an increase of immature neutrophils in blood circulation (Paape et al., 1974). A key for successful diapedesis of leukocytes from blood into milk is their adhesion to the vascular endothelium with the adhesion receptor Mac-1, whose β-chain is coded by the CD18 gene (*ITGB2*) (Burton and Erskine, 2003). The decreased mRNA abundance of CD18 in blood leukocytes after the LPS challenge is, therefore, likely due to the migration of CD18 expressing leukocytes from the blood circulation into the udder. The strong response of leukocytes to i.mam. LPS challenge was, however, not influenced by the availability of different macronutrients in the feed.

Serum Amyloid A transcript in leukocytes was obviously very low as mRNA was not detectable in most samples. However, at 6 and 9 h after LPS challenge SAA mRNA abundance could be detected in several samples albeit at a low level. It appears that the SAA gene expression is not active in resting blood leukocytes, and increases only in newly recruited leukocytes.

In response to i.mam. LPS challenge the mRNA abundance of Hp increased in blood leukocytes. Interestingly, this increase occurred already at 6 h and 9 h after LPS application, whereas plasma Hp at a protein level in blood was still low at 6 h after LPS challenge, and was only elevated at 23 h after LPS application. Obviously, the secretion of Hp by leukocytes was not a considerable source of Hp reflected by an increased plasma concentration, and we still consider the liver as the main source of Hp. However, Hp increased earlier in milk than the mRNA expression in the blood leukocytes did. Neutrophils in milk express Hp, but also the mammary tissue may contribute to the increase of Hp in milk during mastitis (Hiss et al., 2004; Lai et al., 2009).

The detected mRNA abundance of TNF in blood leukocytes was very low (CT $\geq$30), and even decreased short time after the LPS challenge. It is well established that TNF is secreted by activated leukocytes and has several functions mediating inflammatory and immune responses (Fitzgerald et al., 2001). During mastitis, macrophages in the mammary gland produce TNF locally and TNF is then involved with recruitment of neutrophils to the udder, but it is also absorbed into the circulation (Hoeben et al., 2000; Elazar et al., 2010). However, our cells were collected from blood samples, and were therefore not from the focus of inflammation. The cells remaining in blood were possibly not sufficiently activated to produce TNF, or the stimulated ones already left the circulation before we took our samples 6 h after LPS challenge. It has to be kept in mind that the Ct values were quite low right from the beginning and the leukocyte count was lowest 6 h after LPS challenge, making it difficult to interpret these results.

The mRNA abundance of HSP70 was increased at 6 h after LPS administration, indicating that there was some kind of stress on the leukocytes, since heat shock proteins have a protective role against different stressors like heat or presence of abnormally folded proteins (Welch, 1992). The peak of the immune response with a fever around 41°C was around 6 h after LPS challenge (Wagner et al., 2022), what was accompanied by an increased mRNA abundance at that time (Urban-Chmiel et al., 2009). Additionally, HSP70 was shown to suppress several inflammatory cytokines, its upregulation during LPS mastitis could therefore be a mechanism to protect against an excessive production of those cytokines in blood what can lead to septic shock (van Miert, 1995; Shi et al., 2006).

We did not find any differences of the recorded inflammatory traits between the 3 dietary treatments. Although rather different in nutrient composition, our diet formulations did not substantially affect the key metabolic traits in blood (glucose, NEFA, BHB and others) (Wagner et al., 2023). Obviously, both mecha-

nisms, the ruminal fermentation as well as intermediary metabolic pathways compensated for potential nutrient imbalances imposed by the diets (Gross, 2022). At the time of the LPS challenge, all cows were still in a catabolic metabolic state likely overwhelming the postabsorptive situation. As a consequence of the similar metabolic status (Wagner et al., 2023), it is not surprising that also inflammatory parameters did not differ among the dietary treatments. The exception was urea in milk and plasma that increased in the nitrogenic fed groups compared with the glucogenic and lipogenic ones (Wagner et al., 2023), and even though excess dietary nitrogen can influence bovine neutrophil functions (Raboisson et al., 2014), no differences were found for our measured immunologic parameters.

A fully activated immune system consumes a lot of glucose, and glucose consumption of cells is facilitated by glucose transporters (GLUT) (Kvidera et al., 2017). The mRNA expression of the insulin-independent GLUT1 decreased after LPS challenge in our experiment, in agreement with Moyes et al. (2014), who discussed that this decrease might be related to the potential increase of immature neutrophils in blood during inflammation. While it is known that functions like phagocytic activity of neutrophils are not identical for different maturation stages (Silva et al., 1989), little is known about the metabolism of immature bovine neutrophils. Opposed to the GLUT1, mRNA expression of the GLUT3 increased after i.mam. LPS challenge. While an increased expression of GLUT3 was shown in bovine polymorphonuclear leukocytes and peripheral blood mononuclear cells after in vitro stimulation with LPS (O'Boyle et al., 2012; Garcia et al., 2015), these findings in blood leukocytes after i.mam. LPS challenge in vivo are new. An insulin resistance was present after the i.mam. LPS challenge (Wagner et al., 2023). In human leukocytes it was shown that the expression of the GLUT3 to insulin was dependent on the cell type and activation state (Maratou et al., 2007). Studies about the bovine GLUT3 are rare and it is therefore difficult to conclude if this insulin resistance influenced the GLUT3 mRNA expression. However, because GLUT3 is a high affinity transporter, its increased mRNA abundance can be interpreted as a compensation to allow sufficient glucose uptake by leukocytes despite reduced availability caused by the glucose uptake in the mammary gland (Zhao and Keating 2007; Eger et al., 2016). Therefore, an increase in this high affinity transporter after activation of the immune system might be favorable for the immune cells to increase their glucose consumption. The GLUT4 is the main insulin-sensitive transporter, and its mRNA abundance was not detectable in blood leukocytes in the present study as well as during the dry off period (Jermann et al., 2022). The GLUT4 mRNA has been previously shown to be only be detected in non-stimulated macrophages and an insulin effect on the glucose uptake via GLUT4 has not been observed (Eger et al., 2016). Extremely low insulin concentrations in early lactating dairy cows are likely the reason that the GLUT4 was not important during the experimental period. Therefore the GLUT4 mRNA remained below the detection limit whereas insulin-independent glucose transporters could provide sufficient nutrients for the immune cells to maintain their immunological activity.

Glyceraldehyde-3-phosphate dehydrogenase, known as GAPDH, is often used as an internal reference gene (housekeeping gene) in transcriptomic studies. In our experiment, it became obvious that its mRNA abundance was systemically influenced by our treatments. It was increased in all dietary groups at 9 h after the LPS challenge, making it unsuitable as a reference gene for our experiment. After getting this clear result we recommend that GAPDH should not be used as a reference gene in metabolic and immune studies. However, GAPDH was an interesting target gene in the present study. Because GAPDH is an important enzyme during glycolysis, and immune cells can switch from oxidative phosphorylation toward aerobic glycolysis when activated, its upregulation after i.mam. LPS stimulation seems to be a sign for activation of the immune cells (Palsson-McDermott and O'Neill, 2013; Kornberg et al., 2018; Eder et al., 2020).

CONCLUSIONS

The measured inflammatory parameters in milk increased before the APP in blood, which indicates their local production in the mammary gland. Leukocytes in blood showed specific alterations in their mRNA expression after the i.mam. LPS challenge, and may contribute to the production of APP in blood. Their glucose consumption seems to increase after activation, but a definitive interpretation is difficult, due to a possible shift in the cell population. However, feeding early lactating dairy cows with dietary concentrates rich in either crude protein, carbohydrates, or lipids did not affect the local or systemic immune response to an i.mam. LPS challenge.

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