Effects of peripartal energy supply and nicotinic acid supplementation on functional activity and gene expression of blood leukocytes and on serum antioxidant variables of periparturient dairy cows

Dissertation
zur Erlangung des
Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

der

Naturwissenschaftlichen Fakultät III
Agrar- und Ernährungswissenschaften,
Geowissenschaften und Informatik

der Martin-Luther-Universität Halle-Wittenberg

vorgelegt von

Frau Diplom Biologin Susanne Bühler

geb. am 04.11.1970 in Schwäbisch-Hall

verteidigt am 14.01.2019, Halle (Saale)

Gutachter:
Prof. Dr. Annette Zeyner
Prof. Dr. Dr. Sven Dänicke
PD Dr. Björn Kuhla

Contents

I Abbreviations ... iii

II Figures ... v

III Tables ... vi

1. Introduction ... 1

2. Background ... 2

2.1. The transition period phenomena ... 2

2.1.1. The transition period phenomena: negative energy balance resulting in adipose tissue mobilization ... 2

2.1.2. The transition period phenomena: reduction of immune competence – immunosuppression – immune dysfunction ... 3

2.1.3. The transition period phenomena: oxidative stress ... 5

2.1.4. The transition period phenomena: systemic inflammation ... 5

2.1.5. The transition period phenomena: hypocalcaemia ... 6

2.2. The bovine blood immune system – a brief overview ... 6

2.2.1. Polymorphonuclear leukocytes ... 8

2.2.2. Peripheral blood mononuclear cells ... 8

2.3. Functional capacities examined in the blood leukocytes ... 9

2.3.1. Evaluation with flow cytometry ... 9

2.3.1.1. Apoptosis ... 9

2.3.1.2. Phagocytosis ... 10

2.3.1.3. ROS production and oxidative burst capacity ... 10

2.3.2. Gene expression analysis ... 11

2.4. Anti-oxidative serum parameters ... 12

2.5. Niacin ... 12

3. Scope of the thesis ... 14

4. Paper I ... 16

5. Paper II ... 27

6. Paper III ... 40

7. General Discussion ... 51

7.1. The influences of the feeding regimen ... 51

7.1.1. NA supplementation effects ... 51

7.1.2. Effects of energy supply ... 53

7.2. Influences over the course of time ... 53

7.3. The differences between primiparous and pluriparous animals ... 56

7.4. Principal component analysis 59
8. Conclusion 65
9. Summary 66
10. Zusammenfassung 69
11. References 72
12. Appendix 80

I Abbreviations

ADP	adenosine diphosphate
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BCL-xL	BCL2-like 1
BCS	body condition score
BHB	β-hydroxybutyrate
Ca	calcium
CASP3	caspase 3
CD4/CD8	cluster of differentiation 4/8
Cu	copper
DHR 123	dihydrorhodamine 123
DIM	days in milk
DM	dry matter
DMI	dry matter intake
DNA	deoxyribonucleic acid
EB	energy balance
FITC	fluorescein isothiocyanate
FSC	forward-scattered light
γGT	gamma glutamyl transferase
GPX/GPX1	glutathione peroxidase/glutathione peroxidase 1 (cytosolic)
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
Mn	manganese
NA	nicotinic acid
NAD^+/NADH	nicotinamide adenine dinucleotide oxidized/reduced
$NADP^+$/NADPH	nicotinamide adenine dinucleotide phosphate oxidized/reduced
NAM	nicotinamide
NEFA	non-esterified fatty acids

NET	neutrophil extracellular trap
NFκB	nuclear factor kappa B
NRF2	nuclear factor (erythroid-derived 2)-like 2
PAMP	pathogen-associated molecular pattern
PARP1	poly (ADP-ribose) polymerase 1
PBMC	peripheral blood mononuclear cells
PC	principal component
PCA	principal component analysis
PI	propidium iodide
PMN	polymorphonuclear leukocytes
PS	phosphatidylserine
qRT-PCR	quantitative real time polymerase chain reaction
R 123	rhodamine 123
RELA	v-rel avian reticuloendotheliosis viral oncogene homolog A
ROS	reactive oxygen species
Se	selenium
SOD/SOD2	superoxide dismutase/superoxide dismutase 2 (mitochondrial)
SSC	side-scattered light
TG	triglycerides
TLR4	toll-like receptor 4
TNFα	tumor necrosis factor alpha
TPA	12-*O*-tetradecanoylphorbol-13-acetate
VLDL	very low-density lipoproteins
XDH	xanthine dehydrogenase, xanthine:NAD^+ oxidoreductase
Zn	zinc

II Figures

Background, General Discussion

Figure 1 Nutritional and metabolic changes in the transition dairy cow that lead to increased NEFA and BHB concentrations in the blood.

Figure 2 Factors inducing dysfunctional immune responses during the periparturient period.

Figure 3 Haematopoiesis of blood cellular components with the emphasis on blood immune cells and the classification in polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC).

Figure 4 Exemplary scatterblot of a flow cytometry analysis with bovine blood with the according gate setting for PMN and PBMC.

Figure 5 Feeding conditions during the animal experiment.

Figure 6 Parameters that differ significantly between primiparous and pluriparous dairy cows during the periparturient period.

Figure 7 Principal component analysis for the two-dimensional visualization of the relationships between 63 variables collected from the experiment.

Figure 8 Principal component analysis projection of the individual cows of the four feeding groups at the different time points of the experiment.

Figure 9 Principal component analysis projection of the individual cows for the individual time points of the experiment.

III Tables

Background

Table 1 Apoptosis and oxidative stress related genes of interest examined with quantitative real-time polymerase chain reaction (qRT PCR) in blood leukocytes of periparturient dairy cows.

1. Introduction

The periparturient or more precise the transition period (3 weeks before until 3 weeks after parturition) is a most challenging time in the life of dairy cows. It is marked by massive nutritional, physiological, metabolic, hormonal and immunological changes and these changes have an impact on the incidence and severity of infectious and metabolic diseases (Drackley, 1999; Goff and Horst, 1997; Mallard et al., 1998). The high yielding dairy cow, that was genetically favoured in the last decades, is especially prone to develop metabolic and infectious diseases like mastitis and metritis during the transition phase. Approximately 75% of disease incidence occur during the first month of lactation (LeBlanc et al., 2006) and about 25% of cows drop out of productive life during this time because of e.g. mastitis, lameness and low fertility (McGrath et al., 2017). These numbers illustrate the great importance of the transition period for cow's health but also as a considerable economic factor for dairy farms. Before parturition and in the first weeks of lactation, the strong increase in the energy and nutrient requirement because of the growing foetus and the beginning milk production is no longer met by an adjusted feed intake. Hence, the animals experience a negative energy balance during this period, which they try to overcome by increased body fat mobilization (Drackley, 1999). The immunosuppression in late gestation and early lactation is well established in the literature and available data suggests that the immune system is dysregulated around parturition (Aleri et al., 2016; Ingvartsen and Moyes, 2013; Kimura et al., 2006). The nutritional status of dairy cows and the metabolism of specific nutrients are critical regulators of immune cell function. With clever feeding regimens and nutritional supplements like vitamins, minerals and other feed additives, the nutritional deficit of the cow in the transition phase might partly be overcome and the immune system and, therefore, cow's health could be positively influenced (McGrath et al., 2017; Sordillo, 2016). Since the management of diet-composition is the easiest available strategy on the farm level to help the animals to get over the difficult periparturient period, many feeding experiments are undertaken worldwide to help the cow adapt to the transition phase and to lessen the effects of a negative energy balance and to support animal's health. Since it is easily accessible throughout a feeding experiment by bleeding the animals, the blood immune system can be monitored and gives information about its functionality. In this respect, the present study evaluated the influence of different energy supplies and a nicotinic acid supplementation throughout the periparturient period of dairy cows on the functional activity and gene expression of blood leukocytes as well as on antioxidant parameters in the blood serum.

2. Background

2.1. The transition period phenomena

Dairy cows experience in the transition period dramatic metabolic, hormonal, immunological and physiological changes as an adaptation from a pregnant non-lactating state to a non-pregnant lactating condition. Hereby, this period is characterized by a negative energy balance associated with transient immunosuppression and a higher incidence of metabolic and infectious diseases in dairy cows. According to Trevisi and Minuti (2018), five critical points of the transition period can be summarized.

2.1.1. The transition period phenomena: negative energy balance resulting in adipose tissue mobilization

Intensive foetal growth before parturition, morphological and endocrine changes related to mammary gland development, the tremendous increase in energy and nutrient demand with colostrum production and the onset of lactation, as well as the hormonal changes with the actual parturition are the most substantial challenges the dairy cow has to cope with during the transition phase. The degree of meeting the energy and nutrient requirement plays hereby a crucial role. Before calving, the dry matter intake (DMI) (Grummer, 1995) and consequently the blood glucose concentration decreases (Figure 1), although the nutritional demands still increase because of foetus growth and the onset of lactation. The cow enters a temporary state of negative energy balance. To meet the energy requirements the cow mobilises body mass and here foremost adipose tissue (Bell, 1995; Drackley, 1999). The increase in lipolysis leads to an elevated blood concentration of non-esterified fatty acids (NEFA). Circulating NEFA enter the liver in proportion to blood concentrations and have three fates: a) complete oxidation for energy in the tricarboxylic acid cycle; b) conversion to ketone bodies like beta-hydroxybutyrate (BHB); and c) resynthesizing to triglycerides (TG) with either subsequent export via very low-density lipoproteins (VLDL) into the blood or storage in the liver. However, with the rapid and vast invasion of NEFA into the liver because of massive lipolysis during early lactation and the very limited capacity of the liver for export via VLDL, increased amounts of BHB are produced and exported into circulation and TG can build up in the liver impairing its function. Metabolic diseases like ketosis and fatty liver can consequently become apparent. Additionally, NEFA and BHB are known to impair immune cell functions and are said to be partly responsible for the immune suppression in transition dairy cows (Ingvartsen and Moyes, 2013). Furthermore, the rate and extent of tissue mobilisation has been linked to immunosuppression and risk of diseases during the transition period (Cai et al., 1994) and high blood concentrations of NEFA and BHB are

identified as risk factors for mastitis (Moyes et al., 2009) and uterine diseases (Hammon et al., 2006). Therefore, management approaches that reduce the negative energy balance and the increase in NEFA at the beginning of lactation might improve cow's health during the periparturient period.

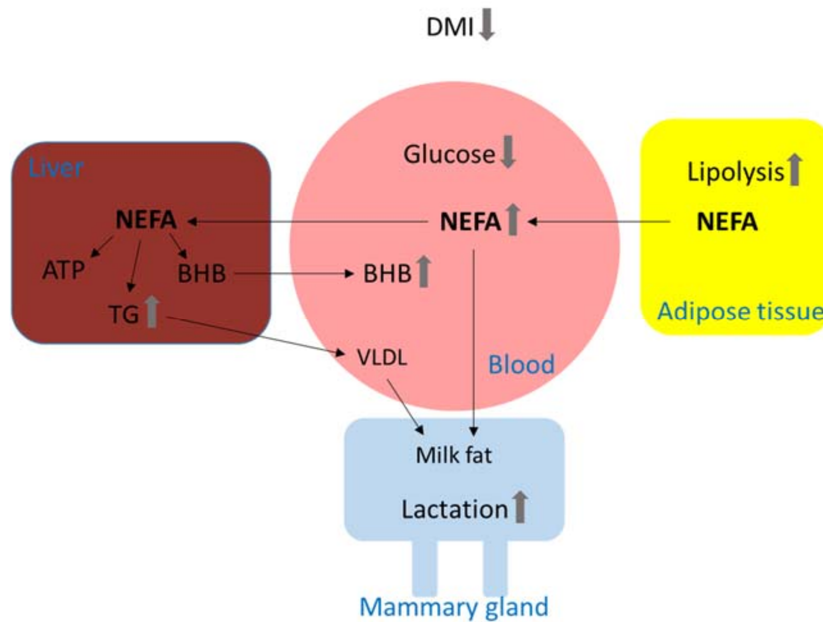


Figure 1: Nutritional and metabolic changes in the transition dairy cow that lead to increased NEFA and BHB concentrations in the blood. Adapted from Drackley (1999).

2.1.2. The transition period phenomena: reduction of immune competence – immunosuppression – immune dysfunction

It is well established, that during the transition period most dairy cows experience a natural state of immunosuppression that goes strongly together with the increased disease incidence in this phase (Goff and Horst, 1997; Mallard et al., 1998). Reasons for this immunosuppression and altered immune cell function are diverse and cannot be attributed to just one factor but are rather the outcome of an interplay of different incidences (Ingvartsen and Moyes, 2013; Mallard et al., 1998; Sordillo, 2016):

a) Steroid hormones like the glucocorticoid cortisol, that expresses increased blood concentrations around the time of calving, have immunosuppressive functions. They impair for example proper activation and migration of blood neutrophils to the sites of tissue injury (Burton et al., 1995). Furthermore, changes in estradiol and progesterone concentrations just before calving are reported to have effects on the functional capabilities of lymphocytes and neutrophils (Lamote et al., 2006; Roth et al., 1982). However, the changes of these hormones around parturition are rather short-lived and do not overlap with the entire transition phase. Yet, other hormones including prolactin and growth hormone that also

fluctuate considerably during the transition period may also influence immune system responses (Kelley et al., 2007).

b) Glucose is the preferred metabolic fuel for immune cells (Ingvartsen and Moyes, 2013). It is for example required by phagocytic cells for proliferation, survival and differentiation. An efficient glucose uptake by immune cells is critical for maintaining cellular functions and eliciting an optimal host response to invading microorganisms, as was shown in studies with murine macrophages (Barghouthi et al., 1995). During early lactation, blood glucose concentrations are significantly decreased because of reduced DMI and increased utilization in milk production. This reduced glucose availability may limit the functional capabilities of the immune cells and, therefore, increase the risk for infectious diseases in transition dairy cows.

c) The negative energy balance and the subsequent forced lipolysis with increased NEFA and BHB blood concentrations in consequence of the increased metabolic demands of early lactation also have a negative impact on blood immune cells (Lacetera et al., 2005). This became evident from experiments with mastectomized pregnant cows. In these experiments, the influence of milk production on immune parameters was examined while maintaining the endocrine changes associated with late pregnancy and parturition (Kimura et al., 1999; Kimura et al., 2002; Nonnecke et al., 2003). Immune function was briefly adversely affected around parturition in mastectomized cows. However, lymphocyte and neutrophil functions were impaired longer in cows with mammary glands (Kimura et al., 1999; Kimura et al., 2002; Nonnecke et al., 2003). The NEFA concentration in the mastectomized cows increased only moderately compared to the intact cows. NEFA and BHB are reported to adversely affect the functional capacity of polymorphonuclear leukocytes (PMN) (Hammon et al., 2006; Hoeben et al., 1997; Sartorelli et al., 1999; Scalia et al., 2006; Suriyasathaporn et al., 1999) and peripheral blood mononuclear cells (PBMC) (Brassard et al., 2007; Lacetera et al., 2004; Renner et al., 2012; Schulz et al., 2015; Ster et al., 2012). NEFA have additionally been shown to induce inflammatory responses via toll-like receptor 4 (TLR4) pathways (Lee et al., 2001; Sordillo et al., 2009). Additionally, fatty acids can modify immune cell functions by altering the physical nature of cellular membranes and thereby changing their immune cell capabilities (Raphael and Sordillo, 2013).

d) The oxidative stress that is developed in the transition phase is also a significant underlying factor for dysfunctional host immune and inflammatory responses (Sordillo and Aitken, 2009). Especially the oxidation of lipids impair the functional capacity of blood immune cells.

2.1.3. The transition period phenomena: oxidative stress

Because of the increased energy requirement at the onset of lactation, increased oxygen consumption through cellular respiration takes place. This increased metabolic activity leads to an enhanced accumulation of reactive oxygen species (ROS) as a normal by-product of the respiratory chain in mitochondria (Valko et al., 2007). However, because of diminished DMI and the export of minerals, vitamins and trace elements into colostrum and milk the antioxidant defences are decreased and not able to get rid of the accumulating ROS (Sordillo and Mavangira, 2014; Spears and Weiss, 2008). The most important antioxidant enzymes for example are glutathione peroxidase (GPX) and superoxide dismutase (SOD) that contain selenium (Se), copper (Cu) and zinc (Zn) or manganese (Mn) in their catalytic site, trace elements that can exhibit reduced plasma concentrations around calving (Meglia et al., 2001; Miller et al., 1995). Therefore, the equilibrium between ROS and antioxidants gets disturbed and oxidative stress develops in the transition dairy cow. Further enhanced is this oxidative stress, by present stress hormones at parturition and inflammatory processes that accompany parturition (Bionaz et al., 2007; Trevisi et al., 2009). ROS, however, are also potent signalling molecules in signal transduction and are essential for the regulation of normal cellular processes (Franchina et al., 2018). They activate for example the transcription factors nuclear factor kappa B (NFκB) and E2-related factor 2 (Nrf2). Hence, ROS promote inflammatory processes and initiate antioxidant defences.

2.1.4. The transition period phenomena: systemic inflammation

Even without signs of a microbial infection, dairy cows show a systemic inflammatory response around the time of calving (Bionaz et al., 2007; Sordillo et al., 2009; Trevisi et al., 2012) which can be confirmed by a peak of acute phase proteins a few hours after calving. The reasons for these inflammatory occurrences are manifold and are highly interconnected with the above mentioned critical events during the transition period (Sordillo and Mavangira, 2014). For example, fatty acids are known to induce inflammatory responses and the expression of pro-inflammatory mediators via TLR4 and the NFκB pathway (Sordillo et al., 2009). Oxidative stress and ROS also increase inflammation and the expression of pro-inflammatory mediators by activating redox-sensitive transcription factors like NFκB (Sordillo et al., 2009). Although during pregnancy, an immunosuppressed state is advantageous in order to tolerate the foetus (Oliveira et al., 2012), after parturition, the foetal placenta needs to be rejected which is also achieved by the means of inflammatory processes (Mordak and Stewart, 2015). In addition, the lesions of the uterine and vaginal tissue that accompany the process of calving induce inflammatory responses (Trevisi and

Minuti, 2018). Inflammation occurs furthermore because of invading microorganisms into the mammary gland with the onset of lactation (Burvenich et al., 1999) and, additionally, by invading bacteria into the uterus and birth canal at parturition (LeBlanc, 2010).

2.1.5. The transition period phenomena: hypocalcaemia

Because of foetus skeletal growth and milk production associated with a calcium (Ca) loss for the cow, the transition dairy cow needs enormous amounts of Ca. Therefore, the animals can enter a state of hypocalcaemia that can lead to milk fever after parturition. Ca however, is also an important signalling molecule in immune cell function and hence hypocalcaemia can increase the disease rate during parturition (McGrath et al., 2017). A low concentration of calcium in the first two weeks postpartum is associated with decreased neutrophil functions (Martinez et al., 2014).

Increasing evidence suggests that nutritional and metabolic changes, oxidative stress, inflammation and dysregulated immune response are closely linked and influence each other (Aleri et al., 2016; McGrath et al., 2017; Sordillo and Mavangira, 2014; Trevisi and Minuti, 2018). They are the underlying factors of increased disease incidence in transition dairy cows and greatly influence health and performance of the periparturient animal. Figure 2 summarizes the factors that influence immune response in the periparturient dairy cow and are concertedly responsible for the compromised immune system.

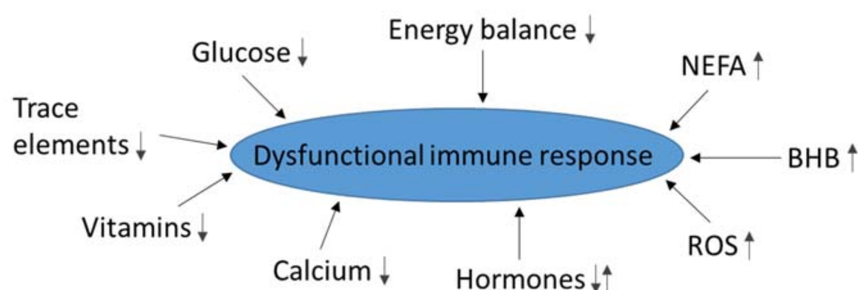


Figure 2: Factors inducing dysfunctional immune responses during the periparturient period.

2.2. The bovine blood immune system – a brief overview

The primary roles of the immune system are to prevent microbial invasion of the body, to eliminate existing infections and other sources of cellular injury, and to restore tissues to normal function. In dairy cattle, the immune system utilizes, hereby, an interactive network of physical, cellular and soluble factors that facilitate the defence against a diverse array of microbial challenges. The immune system can be divided into two categories based on

speed and specificity of the reactions, namely the innate immune system and the adaptive immune system, although, the two parts are highly integrated (Daha, 2011; Lippolis, 2008).

The innate immune system is characterized by an early and rapid response. Constituents of innate immunity represent the first line of defence against invading pathogens because they are already present or are activated quickly at the site of pathogen exposure (Sordillo, 2016). Cells that comprise the innate immune system are neutrophils, monocytes and macrophages, as well as natural killer cells. These cells contain pattern recognition receptors like the toll-like receptors in their cell membranes, thereby, recognizing and binding a range of microbial products and endogenous ligands. These receptors induce two types of responses: inflammation and phagocytosis (Trevisi and Minuti, 2018).

Adaptive immunity is a more customized or specific response to infectious pathogens and can be augmented by repeated exposure to the same microorganism (Sordillo, 2016). It is characterized by the generation of antigen-specific lymphocytes and memory cells with the ability to recognize specific antigens of a pathogen. The cells that carry out the adaptive immune response are the B and T lymphocytes.

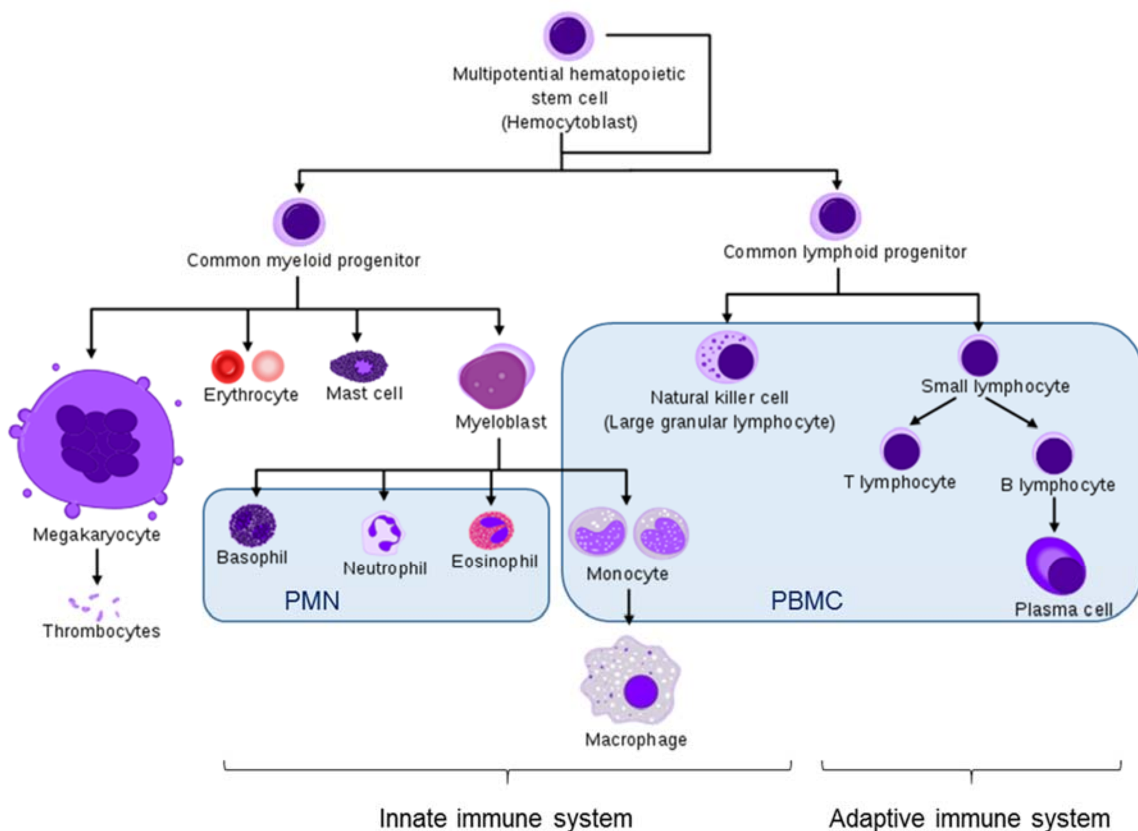


Figure 3: Haematopoiesis of blood cellular components with the emphasis on blood immune cells and the classification in polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC). Adapted from Mikael Haggström, used with permission.

2.2.1. *Polymorphonuclear leukocytes*

The polymorphonuclear leukocytes (PMN) are blood immune cells characterized by granules in their cytoplasm and are also called granulocytes. The nucleus is existing in varying shapes usually lobed into multiple segments. The granulocytes consist of neutrophils, eosinophils and basophils distinguished by size, nucleus segments, and staining of the granules. The majority of the PMN are comprised of neutrophils (Paape et al., 2003). These cells belong to the innate immune system and are rapidly activated and recruited to the site of infection. They migrate from the blood into the infected tissue by chemotaxis. Here, they engulf the invading microorganisms by phagocytosis with subsequent killing these microorganisms with hydrolytic enzymes and bactericidal ROS. The ROS are formed by respiratory burst activity that involves the activation of NADPH oxidase and subsequent production of superoxide radicals and hydrogen peroxide (Fialkow et al., 2007). In addition to phagocytosis, neutrophils can kill bacteria through an extracellular mechanism, the neutrophil extracellular trap (NET) and by release of soluble antimicrobials.

2.2.2. *Peripheral blood mononuclear cells*

The peripheral blood mononuclear cells (PBMC) have a round nucleus. These blood immune cells are comprised of monocytes and lymphocytes (B cells, T cells and natural killer cells). The monocytes belong to the innate immune system and are the blood progenitor cells of the later tissue macrophages. Monocytes are also able to engulf and kill invading microorganisms by phagocytosis. They release in addition immune regulatory cytokines and oxylipids. Natural killer cells also belong to the innate immune system. They target and help to eliminate infected host cells. B and T lymphocytes belong to the adaptive immune system. The B-lymphocytes are hereby the antibody producing cells and play a major role in the humoral immune response. In contrast, the T-lymphocytes are involved in cell-mediated immune responses. The cytotoxic T-cells (CD8+) induce the death of cells that are infected with viruses and other pathogens, or are otherwise damaged or dysfunctional. The helper T-cells (CD4+) are immune response mediators, and play an important role in establishing and maximizing the capabilities of the adaptive immune response by directing other cells to perform their cytotoxic or phagocytic activity (Janeway, 2001).

2.3. Functional capacities examined in the blood leukocytes

2.3.1. Evaluation with flow cytometry

With flow cytometry, single fluorescence labelled cells can be analysed and the blood leukocytes can be separated according to their size and structures in the cell in terms of forward-scattered light (FSC) and side-scattered light (SSC) (Figure 4).

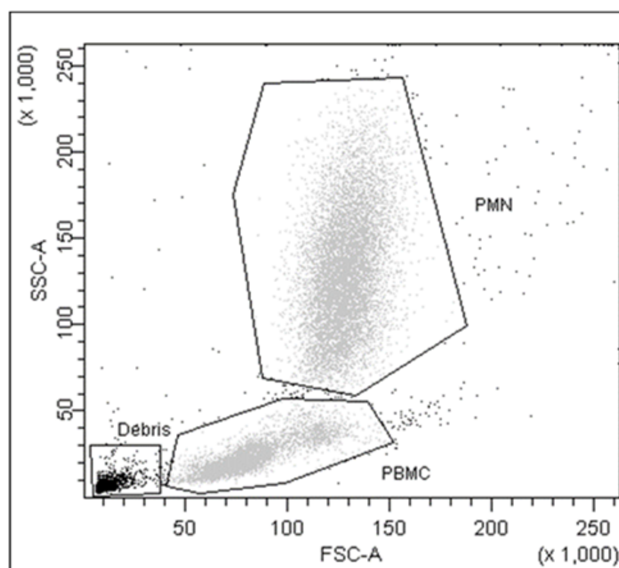


Figure 4: Exemplary scatterblot of a flow cytometry analysis with bovine blood with the according gate setting for polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC).

2.3.1.1. Apoptosis

Apoptosis is a process of programmed cell death that plays an important role in tissue homeostasis and embryonic development in all organisms (Hotchkiss et al., 2009). It is a highly regulated and controlled process and once apoptosis is started it cannot be stopped. The advantage of apoptosis in contrast to necrosis, a form of traumatic cell death after acute cellular injury, is that during apoptosis membrane bound cell fragments, called apoptotic bodies, are build that are removed by phagocytic cells before the contents of the dying cell can spill out and cause damage to surrounding cells and trigger inflammatory responses. Apoptosis can be initiated by two pathways. The intrinsic pathway, also called the mitochondrial pathway, is activated when the cell senses cell stress like e.g. oxidative stress (Sinha et al., 2013). The extrinsic pathway is activated when the cell recognizes signals from other cells via membrane receptors. The cytokine TNFα, that is mainly produced by activated macrophages, is a major extrinsic mediator of apoptosis (Sinha et al., 2013). There is strong evidence that PMN apoptosis plays a key role in resolution of inflammatory

responses (Paape et al., 2003; Sladek and Rysanek, 2001). Under physiological conditions, bovine PMN have a relatively short half live and spontaneously undergo apoptosis. It was shown that PMN apoptosis is influenced by stage of parturition (Van Oostveldt et al., 2001). The apoptotic process is characterized by certain morphological features. These include loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment at an early stage of apoptosis. Annexin V is a phospholipid-binding protein that has a high affinity for PS and, thus, serves as a sensitive probe for flow cytometry analysis of cells that are undergoing apoptosis. The staining with fluorescein isothiocyanate (FITC) labelled Annexin V detects early apoptotic cells and in combination with the vital dye propidium iodide (PI), that only permeates membranes of damaged or dead cells, one can distinguish between early apoptotic (FITC Annexin V positive and PI negative) and late apoptotic (FITC Annexin V positive and PI positive) cells.

2.3.1.2. Phagocytosis

Phagocytosis is a complex process for the ingestion and elimination of pathogens. It is also important for eliminating phagocytic cells, and for maintaining tissue homeostasis (Vernon and Tang, 2013). Monocytes, eosinophils and neutrophils are the professional phagocytes in the blood. Phagocytosis initiates with recognition of the microbial pathogen. This is achieved either by receptors that recognize pathogen-associated molecular patterns (PAMPs) on the surface of the microorganism or by opsonic receptors that bind to opsonins. Opsonins, like antibodies or complement components, are host-produced molecules that bind to microorganisms, thereby, marking them for ingestion. After the receptors recognize a pathogen, the microorganism is internalized in a membrane bound vacuole, the phagosome. Phagosomes develop an oxidative and degradative milieu that finally kills the microorganism. With flow cytometry the phagocytic activity of blood leukocytes can be measured by evaluating the internalization of FITC- labelled opsonized *E. coli* bacteria. It was shown that the phagocytic activity and capacity of blood leukocytes varies during the periparturient period (do Amaral et al., 2011; Kehrli et al., 1989; Ster et al., 2012).

2.3.1.3. ROS production and oxidative burst capacity

ROS are produced in cells as normal by-products of the respiratory chain in mitochondria (Valko et al., 2007). They play an increasingly recognized role as signal molecules in signal transduction (Fialkow et al., 2007). However, phagocytes produce large amounts of ROS

for the killing of internalized microorganism. Upon activation, NADPH oxidase, a membrane bound enzyme complex, generates superoxide that rapidly dismutates into hydrogen peroxide and consequentially other ROS arise that together are efficient antimicrobial agents because they damage proteins, lipids, and DNA of microorganisms in the phagosome. Dihydrorhodamine (DHR) 123 is a non-fluorescent dye that is cell membrane permeable. Intracellular hydrogen peroxide oxidises DHR 123 to the fluorescent rhodamine (R) 123 that is no longer cell membrane permeable and is the quantitative output of existing ROS in cells measurable by flow cytometry. With 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C activation reagent, a maximum NADPH oxidase activity can be induced and, therefore, the ability to perform an oxidative burst can be assessed. There is evidence that the amount of ROS formation and the ability of PMN to perform an oxidative burst changes during the periparturient period (Detilleux et al., 1995; Kehrli et al., 1989; Mehrzad et al., 2001).

2.3.2. Gene expression analysis

The transcription level of genes of interest can be evaluated with quantitative real time polymerase chain reaction (qRT-PCR) utilizing the detection of double stranded DNA with a fluorescence dye and the amplification of this double stranded DNA by PCR. Listed in Table 1 are the apoptosis and oxidative stress related genes examined in the present study with their names and functions.

Table 1: Apoptosis and oxidative stress related genes of interest examined with quantitative real-time polymerase chain reaction (qRT PCR) in blood leukocytes of periparturient dairy cows.

Gene	**Name**	**Function**
BAX	BCL2-associated X protein	apoptosis regulator - pro-apoptotic
BCL2	B-cell CLL/lymphoma 2	apoptosis regulator - anti-apoptotic
BCL-xL/BCL2L1	BCL2-like 1	anti-apoptotic
CASP3	caspase 3	cysteine-aspartic acid protease, executioner caspase - pro-apoptotic
GPX1	glutathione peroxidase 1	antioxidant enzyme, protects cells from oxidative stress by detoxification of hydrogen peroxide (H_2O_2)
NRF2	nuclear factor (erythroid-derived 2)-like 2	redox sensitive transcription factor that regulates the expression of anti-oxidative and cyto-protective genes

Gene	Name	Function
PARP1	poly (ADP-ribose) polymerase 1	modifies nuclear proteins by poly ADP ribosylation; consumes NAD^+ for its activity; gets induced by DNA strand breaks (e.g. caused by ROS) and takes part in the regulation of oxidative stress induced cell death
RELA	v-rel avian reticuloendotheliosis viral oncogene homolog A	NF-kB transcription factor p65 subunit; involved in cell survival (anti-apoptotic) and cytokine production
SOD2	superoxide dismutase 2, mitochondrial	antioxidant enzyme, protects cells from oxidative stress by dismutation of the superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2)
XDH	xanthine dehydrogenase, xanthine:NAD^+ oxidoreductase	generates ROS like hydrogen peroxide (H_2O_2) and superoxide (O_2^-)
Abbreviations: ADP, adenosine diphosphate; NAD^+, nicotinamide adenine dinucleotide; ROS, reactive oxygen species		

2.4. Anti-oxidative serum parameters

To protect the organism from oxidative damage several anti-oxidative defence mechanisms have been developed. Important antioxidant enzymes are hereby superoxide dismutase (SOD) and glutathione peroxidase (GPX). SOD catalyses the dismutation of the superoxide (O_2^-) radical that is produced as a by-product in oxygen metabolism or during an oxidative burst, either, in oxygen (O_2) or hydrogen peroxide (H_2O_2). GPX reduces H_2O_2 to water by oxidising reduced monomeric glutathione (GSH) to glutathione disulfide (GS-SG). Subsequently, GS-SG is recycled to GSH by a reduction reaction of glutathione reductase (GR) with the participation of the cofactor NADPH. SOD and GPX enzyme activity can both be measured with established spectrophotometric enzyme assays. Ferric reducing ability (FRA) assesses the non-enzymatic antioxidants, like e.g. α-tocopherol, ascorbic acid, retinol and uric acid in biological fluids by measuring the conversion of $Fe3^+$-tripyridyltriazine to $Fe2^+$-tripyridyltriazine and the concomitant development of blue colour (Benzie and Strain, 1996).

2.5. Niacin

Niacin, also known as vitamin B3, exists as nicotinic acid (NA) and nicotinamide (NAM) in the vertebrates' body and is a precursor of the coenzymes nicotinamide adenosine dinucleotide ($NAD^{+)}$ and nicotinamide adenosine dinucleotide phosphate ($NADP^+$). As such,

it is involved in many redox reactions including catabolic and anabolic pathways. NA is well known for its anti-lipolytic effect and in the bovine it downregulates lipolysis *in vitro* (Kenez et al., 2014) and *in vivo* with consequentially decreased NEFA concentrations in NA treated animals (Morey et al., 2011; Pires and Grummer, 2007; Yuan et al., 2012), although, results are inconsistent (Niehoff et al., 2009; Schwab et al., 2005).

In addition, niacin is reported to influence the immune system via anti-inflammatory and immune modulating properties (Yu and Zhao, 2007). Further, niacin is involved in oxidative stress and apoptotic processes (Maiese et al., 2009).

With the aforementioned effects of niacin on lipolysis, inflammation and oxidative stress, it is an ideal supplementation to test in the periparturient period, where all these factors are increased and lead to dysfunctional immune responses in the transition dairy cow. Niacin may therefore have the ability to ameliorate the known immunosuppression in these animals.

3. Scope of the thesis

The transition period of dairy cows is accompanied by oxidative stress, inflammation and a dysfunctional immune response that is associated with increased metabolic and infectious diseases during this time. NEFA and BHB that show increased blood concentrations in the two weeks after parturition because of increased lipolysis to meet the energy requirements of the animal are discussed as important factors for the well-known immunosuppression during the periparturient period. In addition the immune system depends on energy to perform its' necessary functions and in times of negative energy balance this may not be warranted. Niacin, with the potential to inhibit lipolysis as well as to exert anti-inflammatory and anti-oxidative effects, could ameliorate the dysfunctional immune responses in the transition period.

Following working hypothesis were deduced: A) Niacin inhibits lipolysis in the periparturient dairy cow, decreases NEFA and BHB blood concentrations in niacin treated animals and therefore amends the functional capacities of blood leukocytes like apoptosis, phagocytosis and ROS formation in these animals. B) Niacin inhibits lipolysis in the transition dairy cow, decreases NEFA blood concentration in niacin treated animals and therefore changes gene expression of apoptosis and oxidative stress related genes in blood leukocytes since fatty acids are known to activate the transcription factor NFκB via the TLR4 signal transduction pathway. C) Niacin influences directly gene expression of apoptosis and oxidative stress related genes in blood leukocytes. D) Niacin influences antioxidant blood parameters. E) A higher energy supply is beneficial for the functional capacities of blood leukocytes.

Therefore, the aim of the present study was to investigate the functional capacity of blood immune cells and selected antioxidant blood parameters of periparturient dairy cows under the feeding conditions of differing energy supplies antepartum, differing energy escalation strategies after parturition to trigger different degrees of lipolysis and a nicotinic acid supplementation during the periparturient period. In Figure 5. the schematic diagram of the feeding conditions of the animal trial from 42 days before expected parturition until 100 days in milk (DIM) is depicted according to Tienken et al. (2015b). The concentrate proportions represent values on dry matter (DM) basis the remaining percentage being composed of roughage. The niacin supplementation of 24g powdered and non-rumen protected NA per day and animal was applied to half of the animals from 42 days antepartum until 24 DIM. The four generated feeding groups were: LC-NA (4 primiparous and 6 pluriparous) and HC-NA (5 primiparous and 7 pluriparous) as well as LC-CON (5 primiparous and 7 pluriparous) and HC-CON (4 primiparous and 9 pluriparous).

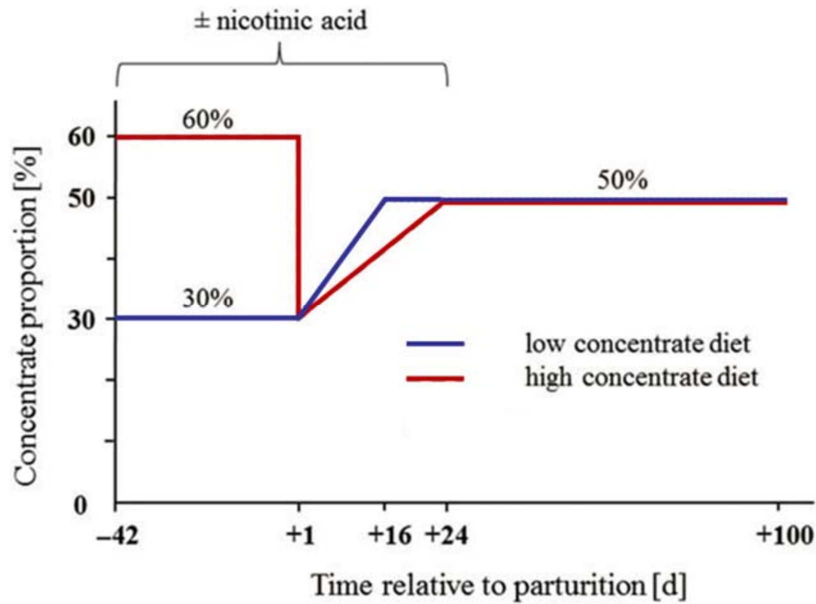


Figure 5: Feeding conditions during the animal experiment according to Tienken et al. (2015b).

Paper I addresses the influence of the feeding regimen on the apoptotic behaviour of blood leukocytes measured as functional flow cytometry assay and gene expression analysis of the selected apoptosis related genes BAX, BCL2, BCL-xL, CASP3 and RELA. With Spearman' rank correlation the relationship of these apoptotic parameters with each other and with blood and performance parameters was evaluated.

Paper II focusses on the feeding influences on anti-oxidative capacity in serum (ferric reducing ability (FRA), SOD and GPX enzyme activity), as well as on oxidative stress related gene expression of the genes GPX1, NRF2, PARP1, SOD2 and XDH in blood leukocytes. Spearman rank correlations also attempt to bring these examined data into context with metabolic and blood parameters.

Paper III outlines the examined data on phagocytosis and ROS production in the blood immune cells. In addition, it contains an in vitro evaluation of the effects of increasing amounts of NA or NAM on the ROS production of PMN.

An additional principal component analysis with the evaluated data on performance, metabolism, haematology, biochemical, and immunological parameters (Tienken et al., 2015a; Tienken et al., 2015b) (Paper I-III) was performed to examine overall associations and to find new approaches to interpret the data.

4. Paper I

S. Bühler, J. Frahm, R. Tienken, S. Kersten, U. Meyer, K. Huber, S. Dänicke (2016)

Influence of energy level and nicotinic acid supplementation on apoptosis of blood leukocytes of periparturient dairy cows.

Veterinary Immunology and Immunopathology 179, 36–45

Reprinted with permission of Elsevier.

Veterinary Immunology and Immunopathology 179 (2016) 36–45

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Influence of energy level and nicotinic acid supplementation on apoptosis of blood leukocytes of periparturient dairy cows

S. Bühler[a,*], J. Frahm[a], R. Tienken[a], S. Kersten[a], U. Meyer[a], K. Huber[b], S. Dänicke[a]

[a] Institute of Animal Nutrition, Friedrich-Loeffler-Insitute (FLI), Federal Research Institute for Animal Health, Braunschweig, Germany
[b] Institute of Animal Sciences, University of Hohenheim, Stuttgart, Germany

ARTICLE INFO

Article history:
Received 7 April 2016
Received in revised form 6 July 2016
Accepted 7 July 2016

Keywords:
Transition period
Niacin
Apoptosis
Blood leukocytes
qRT PCR
Flow cytometry

ABSTRACT

The periparturient period of dairy cows is accompanied by an immunosuppression that leaves the animal more susceptible to infections and metabolic disorders. Non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB) which peak shortly after parturition due to lipolysis are known to impair immune cell functions. Niacin with its well-known anti-lipolytic effect may have the ability to ameliorate this situation. Additionally, niacin shows also anti-inflammatory effects that may be beneficial to the immune status of the cow. To address this 29 multiparous and 18 primiparous German Holstein cows were subjected to four different feeding groups. They were fed either a ration with a high concentrate proportion of 60% (HC), or a low concentrate proportion of 30% (LC). After parturition both concentrate levels were reduced to 30% and increased again to 50% either within 16 days (LC-group) or within 24 days (HC-group). Half of the animals received either 24 g per day of nicotinic acid from 42 days prepartum until 24 days postpartum (LC-NA, HC-NA) or no supplement (LC-CON, HC-CON). Apoptosis in polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC) was examined with an Annexin V and propidium iodide (PI) based fluorescence flow cytometry assay and distinguished into early apoptotic (Annexin V positive and PI negative) and late apoptotic (Annexin V and PI positive) cells. Additionally, the pro-apoptotic gene BAX, the effector caspase CASP3, and the anti-apoptotic genes BCL2 and BCL-xL, as well as the NFκB subunit RELA were quantified by real-time PCR in blood leukocytes. All variables showed time dependencies that were mainly related to parturition ($p < 0.01$). Early apoptotic PBMC were significantly affected by concentrate level showing higher numbers of apoptotic cells in the HC groups ($p = 0.029$). PBMC were characterized by a more pronounced apoptosis than PMN and seemed to be more susceptible to the changes that occur around parturition. The genes BAX and CASP3 were positively correlated (0.631) and their peak preceded the apoptotic peak around parturition in the blood leukocytes. The LC animals showed a decrease in BCL2 expression before parturition, whereas the HC animals showed a continuous increase in BCL2 mRNA abundance ($p = 0.059$). RELA correlated stronger with the pro-apoptotic genes (0.715 and 0.650 with BAX and CASP3 respectively) and its expression was higher in primiparous than in multiparous cows ($p = 0.011$). Nicotinic acid supplementation did show some influence in increasing numbers of early apoptotic PMN and late apoptotic PBMC between 42 and 100 DIM.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

The periparturient period of dairy cows is accompanied by severe nutritional, metabolic, hormonal, and immunological changes that influence the incidence of infectious and metabolic diseases (Goff and Horst, 1997). With the onset of lactation the need for energy exceeds the dietary energy intake and leaves the cow in a temporary state of negative energy balance, which is additionally triggered by a decreasing dry matter intake (DMI) in the days before parturition. As a result, plasma glucose decreases and the cow, in an attempt to meet energy requirements, mobilizes body tissues (Bell, 1995). The forced lipolysis in adipose tissues increases the non-esterified fatty acid (NEFA) concentration in the blood. However, the liver fails to utilize the increased NEFA flux adequately leading to accumulation of triglycerides (TG) in the liver, impairing its function, and to an increase of ketone bod-

* Corresponding author at: Institute of Animal Nutrition, Friedrich-Loeffler-Insitute (FLI), Federal Research Institute for Animal Health, Bundesallee 50, 38116 Braunschweig, Germany.
E-mail address: Susanne.Buehler@fli.bund.de (S. Bühler).

http://dx.doi.org/10.1016/j.vetimm.2016.07.007
0165-2427/© 2016 Elsevier B.V. All rights reserved.

ies like beta-hydroxybutyrate (BHB) in the blood (Adewuyi et al., 2005; Drackley, 1999). By feeding cows different concentrate levels prepartum and increasing the concentrate level postpartum in different rates, the degree of lipolysis can be varied which influences NEFA and BHB generation (Schulz et al., 2014). The state of immunosuppression in periparturient dairy cows is well recognized in the literature (Burvenich et al., 2007; Ingvartsen and Moyes, 2013; Ingvartsen and Moyes, 2015). For example, parturition in dairy cattle is associated with a decreased chemotactic ability and an impaired phagocytic and oxidative burst activity in polymorphonuclear leukocytes (PMN) and therefore a decrease in the ability to fight bacterial infections (Hoeben et al., 2000; Kehrli et al., 1989). Blood lymphocytes show a decreased responsiveness to stimulation with mitogenic agents and B cells exhibit a decreased immunoglobulin production (Nonnecke et al., 2003). Increasing levels of NEFA and BHB have been shown to adversely affect functionality of PMN (Hammon et al., 2006; Scalia et al., 2006; Suriyasathaporn et al., 2000), and peripheral blood mononuclear cells (PBMC) (Brassard et al., 2007; Lacetera et al., 2004; Renner et al., 2012; Schulz et al., 2015; Ster et al., 2012). In addition, NEFA have been shown to affect the inflammatory response of periparturient dairy cows through Toll-like receptors and their subsequent signaling pathways (Sordillo et al., 2009). Gene expression analysis in spleen of postpartum dairy cows revealed that negative energy balance is associated with oxidative stress and increased apoptosis in spleen tissue with potential negative impact on innate and adaptive immune function (Morris et al., 2009). Furthermore, gene expression analysis in blood neutrophils of periparturient cows showed that the expression of apoptotic genes like BAX and RELA is influenced by parturition (Madsen et al., 2004).

Niacin, also known as vitamin B3, is found in two forms in the body, namely as nicotinic acid and as nicotinamide. Nicotinic acid is hereby readily transformed into nicotinamide that serves as precursor of β-nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$), cofactors for many enzymes in the vertebrate's body. Niacin is well known for its anti-lipolytic effect (Carlson, 2005) and nicotinic acid was shown to downregulate lipolysis *in vitro* (Kenez et al., 2014) and *in vivo* (Morey et al., 2011; Yuan et al., 2012) via its receptor GPR109A (Kenez et al., 2014). The nicotinic acid receptor GPR109A is expressed in different bovine tissues like adipose tissue, muscle and brain (Titgemeyer et al., 2011), and is also expressed in mature human neutrophils (Kostylina et al., 2008) as well as in monocytes and lymphocytes (Liu et al., 2014). Apart from the lipid altering effects, niacin influences the immune system via anti-inflammatory and immune-modulating properties (Yu and Zhao, 2007). Nicotinamide participates in the cellular energy metabolism, it influences oxidative stress, and modulates pathways that can either influence cell survival or death. It acts as cytoprotectant by blocking inflammatory cell activation, early apoptotic phosphatidylserine exposure, and late DNA degradation (Maiese et al., 2009). Based on the potential of niacin to influence immune-related parameters, we hypothesized that supplementing presumably not limited dairy cows with niacin during the critical transition period might affect blood immune cells. For testing this hypothesis, we investigated the apoptotic behavior of blood leukocytes by using a functional flow cytometry apoptosis assay and by expression analysis of genes known to play key roles in the apoptotic pathway of immune cells. The pro-apoptotic gene BAX (BCL2-associated X protein), the effector caspase CASP3, the anti-apoptotic genes BCL2 (B-cell lymphoma 2) and BCL-xL (B-cell lymphoma extra large), and the p65 subunit of the transcription factor NFκB (RELA) were examined on their transcriptional level with quantitative real time PCR.

2. Materials and methods

2.1. Experimental design and blood collection

The experiment was conducted at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Braunschweig, Germany in compliance to the German Animal Welfare Act, concerning the protection of experimental animals, and was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany. The experimental conditions are described in detail by Tienken et al. (2015b). Briefly, 47 pregnant and healthy German Holstein dairy cows (29 multiparous and 18 primiparous) were distributed homogeneously to one of four dietary treatment groups considering mean body weight (BW), body condition score (BCS), number of lactations, and milk yield of previous lactations. The experiment started at 42 days before expected parturition and ended at 100 days in milk (DIM). Six weeks prepartum half the animals received a low concentrate diet (30% concentrate and 70% roughage on dry matter (DM) basis; LC group) and the other half received a high concentrate diet (60% concentrate and 40% roughage on DM basis; HC group). Additionally, half of the cows of each concentrate level were supplemented with 24 g per day and cow of powdered and non-rumen protected nicotinic acid (NA) (Mianyang Vanetta Pharmaceutical Technology Co., Ltd, Sichuan, China) mixed in the pelleted concentrate resulting in feeding groups LC-NA (4 primiparous and 9 multiparous) and HC-NA (5 primiparous and 7 multiparous), whereas the not supplemented animals received a control concentrate yielding feeding groups LC-CON (5 primiparous and 7 multiparous) and HC-CON (4 primiparous and 6 multiparous), respectively. Supplementation was applied from day 42 prepartum until 24 DIM. After parturition the concentrate level was reduced to 30% concentrate and 70% roughage for all animals. The concentrate allowance increased for LC animals up to 50% of the diet in 16 days and for HC animals in 24 days, respectively. The different concentrate feeding strategies aimed at triggering cow groups differing in postpartum predisposition for developing lipolysis which should facilitate the investigation of niacin effects. Roughage mixture was composed of 50% corn silage and 50% grass silage on DM basis, and was fed *ad libitum* via self-feeding stations (type RIC, Insentec B.V., Marknesse, The Netherlands). All diets were formulated to meet the nutritional requirements of dairy cows stated by the Society of Nutrition Physiology GfE (2001). The mean daily intakes of nicotinic acid for the control diets during the time NA supplementation was applied were 714 ± 230 mg and 921 ± 202 mg for LC-CON and HC-CON, respectively (data not shown). The supplementation with 24 g NA, therefore, represented an approximately 30 fold higher level than the control dietary NA intake. Blood from the *Vena jugularis externa* was collected into EDTA containing tubes at day −42 (−40 ± 6d), −14 (−13 ± 2d), −7 (−7 ± 1d), −3 (−3 ± 1d), 3, 7, 14, 28, 42, 100 relative to parturition. The blood samples were processed within 2 h of collection.

2.2. Hematology

Granulocyte and lymphocyte counts were measured in EDTA blood with an automatic analyzer (Celltac αbMEK-6450, Nihon Kohden, Tokyo, Japan). Results were expressed as ratio between granulocytes and lymphocytes to facilitate the interpretation of gene expression measured at the level of mixed leukocyte RNA.

2.3. FACS analysis

For the detection of apoptotic cells in bovine blood samples, the FITC Annexin V Apoptosis Detection Kit II from BD Pharmingen™ (BD Biosciences, San Diego, CA, USA) was used, according to the

manufacturer's protocol. In brief, erythrocytes of 100 ml EDTA blood were lysed with 1 ml H_2O for a maximum of 15 s and the osmolarity was reestablished with 108 μl of 8.8% NaCl. After 5 min spin at 250 × g blood leukocytes were washed with 1 ml PBS and centrifuged again. Pellets were resuspended in 600 μl binding buffer (10 mM Hepes/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$). 100 μl cell suspension was incubated with 5 μl FITC Annexin V and 5 μl propidium iodide (PI) and incubated for 15 min at RT in the dark. 400 μl binding buffer was added and the samples were analyzed in duplicate by flow cytometry (FACSCanto™ II, BD Biosciences, San Jose, CA, USA). Excitation and emission wavelengths were 488 nm and 519 nm, respectively. A non-stained control of each sample was included. Polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC) were gated according to their size and granularity based on measurements of forward and side scatter. At least 10,000 cells were evaluated with FACSDiva software 6.1.3 (BD Biosciences, San Jose, CA, USA). Results were expressed as percentages of FITC Annexin V positive and PI negative (early apoptotic) or FITC Annexin V and PI positive (late apoptotic) cells of total PMN, PBMC or complete leukocyte fraction, respectively. To achieve normal distribution the data was log transformed for statistical analysis. For better interpretation the data was log-back transformed for charts.

2.4. RNA isolation

Total RNA of blood leukocytes was isolated at day −42 (−40 ± 6d), −14 (−13 ± 2d), −7 (−7 ± 1d), −3 (−3 ± 1d), 7, 14, 42, 100 relative to parturition. 2 ml EDTA blood was incubated with 10 ml H_2O on ice for a maximum of 15 s. After addition of 1.08 ml 8.8% NaCl, the samples were centrifuged at 4 °C and 400 x g for 12 min. Pellets were resuspended in 1 ml H_2O and 108 μl 8.8% NaCl was added followed by a 6 min spin at 4 °C and 450 × g. This step was repeated once leading to a cell pellet with no visible erythrocyte contamination. The final cell pellet was subjected to a total RNA isolation procedure with a chaotropic ions buffer system and a silica membrane (NucleoSpin® RNA II, Macherey Nagel, Düren, Germany), according to the manufacturer's protocol. Contaminating DNA was digested on-column and the RNA was eluted with 50 μl H_2O. Concentration and quality of the purified RNA were assessed spectrophotometrically with a NanoDrop® ND-1000 (NanoDrop, Wilmington, DE, USA). The RNA integrity was verified using 1.1% agarose gel electrophoresis. All samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits. RNA was stored at −80 °C until further processing.

2.5. cDNA synthesis and qRT PCR

750 ng total RNA was transcribed into cDNA with the qScript™ cDNA Synthesis Kit (Quanta Biosciences™, Inc, Gaithersburg, MD, USA) in a reaction volume of 20 μl at 42 °C for 30 min with a subsequent inactivation step at 85 °C for 10 min in a thermal cycler (Biometra, Göttingen, Germany), according to the manufacturer's protocol. cDNA was diluted 1:10 with H_2O and stored in aliquots at −20 °C for subsequent analysis. Gene-specific primer pairs were generated using Primer3 and BLAST, selecting for annealing temperatures of 60 °C, location on different exons, and, if possible, intron spanning. All used primers were obtained from Eurofins MWG Operon (Ebersberg, Germany) and are listed in Table 1. The primer efficiencies were calculated using a six point dilution series of a cDNA sample mixture of the experiment. The generation of a single PCR product was confirmed with a melting curve analysis from 60 °C to 95 °C in 0.5 °C increments for 5 s and, additionally, the product length was confirmed on 2% agarose gel electrophoresis. qPCR runs were conducted on a CFX96™ Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA) using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The primers were added in the concentrations listed in Table 1 and 5 μl cDNA was included. The samples were done in duplicate. An initial denaturation step for 30 s at 95 °C was followed by annealing and elongation at 60 °C for 30 s preceded by a 5 s denaturation step at 95 °C. After 40 cycles the generation of a single PCR product was confirmed with a melting curve from 60 °C to 95 °C in 0,5 °C increments for 5 s each. Every run was controlled by a no template control (NTC) in triplicate and, since the measurements were distributed over multiple plates, an inter-run calibrator (IRC), also in triplicate, was included. C_q-values of target and reference genes were obtained using CFX Manager™ Software 2.0. The evaluation was done using qbase+ (Version 2.5, Biogazelle, Zwijnaarde, Belgium). The optimal reference genes were identified with a geNorm analysis out of 6 reference targets (Vandesompele et al., 2002). B2M, UCHL5 and RPLP0, exhibiting a mean reference target stability M value of 0.43, were defined as best reference genes. The normalized, relative expression levels were calculated using the geometric mean of the above mentioned reference genes as normalization factor, relating the data to the geometric mean of all samples at time point −42 ($\Delta\Delta C_q$) and taking in consideration the primer pair specific amplification efficiencies (Hellemans et al., 2007). Statistical analysis was performed on the log data and for better interpretation the data was log-back transformed for charts.

2.6. Statistical analysis

For the FACS analysis at time point −42 only 21 samples could be measured. At this time point the animals have not received any dietary treatment, thus the mean values were considered to be representative for all animals. Therefore, the missing data was filled up with the averaged measured values for this time point. Statistical analysis were performed using the PROC MIXED procedure of SAS software package (Version 9.4, SAS Institute Inc., Cary, NC, USA) with the restricted maximum likelihood method. The model included time point (**T**; experimental day relative to parturition), dietary concentrate proportion (**C**; LC or HC diet), supplementation (**S**; CON or NA), parity (**P**; primiparous or multiparous cows), the triple interaction between T*C*S, and the quadruple interaction between T*C*S*P as fixed effects. The frequent measurements during the experiment for each individual cow were considered as repeated measurement. The covariance structures compound symmetry (CS), unstructured (UN), autoregressive of the first order (AR1), and variance component (VC) were tested and the one showing the lowest Akaike information criterion (AICC) was used. Degrees of freedom were calculated using the Kenward-Roger adjustment. Differences were considered to be significant at $p < 0.05$ and a tendency was noted if $0.10 > p > 0.05$. For the parameters that showed a significant parity effect a separate graphical representation for heifers and cows was used. All results are presented as LS-means and pooled standard errors (PSE) are stated. For correlation studies, Spearman rank correlation was calculated using Statistica (Version 12, Statsoft Inc., 2014).

3. Results

3.1. FACS analysis

Early apoptotic blood leukocytes were detected as Annexin V positive and PI negative cells. PMN (Fig. 1A) showed a lower percentage of early apoptotic cells than PBMC (Fig. 1B). The proportions of early apoptotic cells fluctuated significantly in the course of the experiment. They decreased prepartum and showed a slight peak just before calving. Until 42 DIM the percentage of apoptotic cells decreased further and with 100 DIM another increase in apoptotic

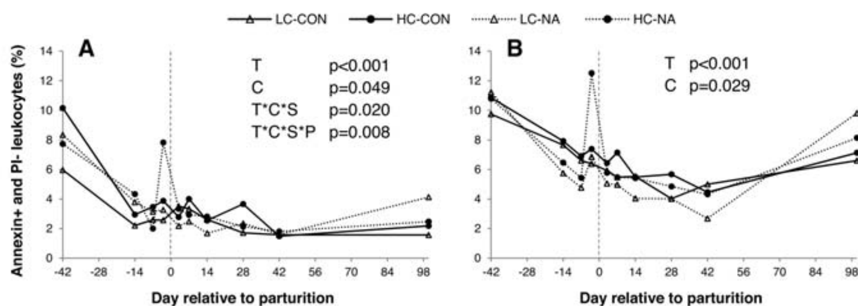


Fig. 1. Early apoptotic blood leukocytes. Percentage of Annexin V positive and PI negative PMN (A) and PBMC (B) of periparturient dairy cows. Cows were fed prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition cows were initially fed with a diet consisting of 30% concentrate. For LC animals this was increased continuously to 50% in the first 16 days wheras for HC animals this increase was achieved in 24 days. Animals were either fed a diet with (NA, 24 g NA/d from d 42 prepartum until 24 DIM) or without (CON) nicotinic acid. Symbols denote LSMEANS; pooled standard error (PSE) for PMN (A) PSE = 0.66 and for PBMC (B) PSE = 0.92. (n_{-42} = 47, n_{-14} = 31, n_{-7} = 23, n_{-3} = 18, n_{+3} = 39, n_{+7} = 47, n_{+14} = 43, n_{+28} = 44, n_{+42} = 47, n_{+100} = 47). T = time relative to parturition, C = concentrate level, S = supplementation, P = parity. Only p-values for significant ($p < 0.05$) fixed main and interaction effects are indicated.

Table 1
Characteristics of gene specific primers used for qRTPCR.

Gene	NCBI GenBank accession no.	Sequences (forward/reverse) (5'-3')	Amplicon size (bp)	Amount (nmol) for/rev	R^2	Efficiency (%)	M-value	origin
Reference genes								
ACTB	NM_173979.3	F-AGATCAAGATCATCGCGCCC R-GCCTAGAAGCATTTGCGGTG	154	250/250	0.999	106	0.71	this manuscript
B2M	NM_173893.3	F-AGCAGCACCATCGAGATTTGA R-TGGACATGTAGCACCCAAGG	172	250/250	0.996	105	0.57	this manuscript
RPLP0	NM_001012682.1	F-AACTCTGCATTCCCGCTTCC R-GCCTTGACCTTTTCAGCAAGTG	174	250/250	0.996	99	0.56	this manuscript
SDHA	NM_174178.2	F-TCTACGACACCGTGAAAGGC R-TGCCGTAATTCTCCAGCTCC	105	333/333	0.996	102	0.61	this manuscript
UCHL5	NM_174481.3	F-CAAAGACAACTTGCTGAGGAACC R-ACTGCTTGTGTTCTGCTAAAGTC	208	333/333	0.990	96	0.52	this manuscript
YWHAZ	NM_174814.2	F-CGGACACAGAACATCCAGTC R-TCCAAGATGACCTACGGGCT	198	333/333	0.997	103	0.58	this manuscript
Target genes								
BAX	NM_173894.1	F-GCTGCAGAGGATGATCGCAGCTGTG R-ATCAACTCGGGCACCTTGGTGCA	174	500/500	0.996	99		this manuscript
BCL2	NM_001166486.1	F-ACGGAGGCTGGGACGCCTTT R-AGGGTGATGCAAGCGCCCAC	121	250/333	0.993	102		this manuscript
BCL-xL	NM_001077486.2	F-CACTGTGCGTGGAAAGCGTA R-AAAGTGTCCCAGCCGCC	127	333/333	0.996	93		Valdez et al. 2005
CASP3	NM_001077840.1	F-CAGCGTCGTAGCTGAACGTA R-AGGCCATGCCAGTATTTTCG	221	500/500	0.992	101		this manuscript
RELA	NM_001080242.2	F-AACAACCCCTTCCAAGTTCCC R-CCCAGAGTTCCGATTCACCC	201	333/333	0.992	99		this manuscript

Abbreviations: ACTB actin, beta; B2 M beta-2-microglobulin; BAX BCL2-associated X protein; BCL2 B-cell CLL/lymphoma 2; BCL-xL B-cell lymphoma extra large; CASP3 caspase 3, apoptosis-related cysteine peptidase; RELA v-rel avian reticuloendotheliosis viral oncogene homolog A; RPLP0 ribosomal protein, large, P0; UCHL5 ubiquitin carboxyl-terminal hydrolase L5; SDHA succinate dehydrogenase complex, subunit A, flavoprotein (Fp); YWHAZ tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

cells was detected. This behavior was more pronounced for PBMC than for PMN. In PMN interactions of T*C*S*P were detected which arose from the differences at time point −42, the peak at day 3 before parturition in the HC-NA group (originating from three animals, one being a primiparous cow with the highest signal), and the increased apoptosis in NA supplemented animals at 100 DIM. In case of the early apoptotic PBMC a significant concentrate effect was detected. Late apoptotic blood leukocytes were defined as Annexin V and PI positive cells. Also the percentage of late apoptotic cells was higher in PBMC (Fig. 2B) than in PMN (Fig. 2A). Both cell types varied significantly over time. PMN displayed a slow decrease in late apoptotic cells until 14 DIM and a small peak at 42 DIM. However, for PBMC the proportion of late apoptotic cells increased distinctly around parturition in all groups. Additionally, a time-shift was noticed in the peaks between treatment groups. The LC animals seemed to reach this peak earlier (at day −3 and 3 DIM for LC-CON and LC-NA respectively) than the HC animals (at 7 DIM and 14 DIM for HC-CON and HC-NA respectively). Until 42 DIM the proportion of late apoptotic cells in PBMC decreased to exhibit another increase at 100 DIM. This increase was more pronounced for NA supplemented animals than for CON animals. In order to reflect the situation of the gene expression analysis, where no distinction between PMN and PBMC was made since the RNA was prepared of the whole blood leukocytes, the FACS data was also evaluated over a cell fraction containing both PMN and PBMC (Fig. 3). In contrast to the data shown for PMN and PBMC alone, the percentage of late apoptotic cells (Fig. 3B) was higher than for early apoptotic cells (Fig. 3A). This was due to the difference in gating the cells. The gate

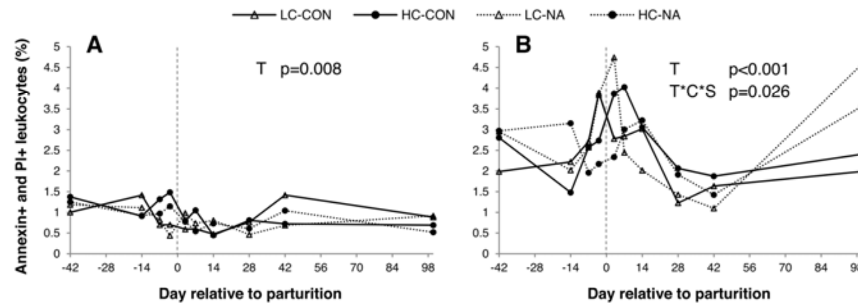


Fig. 2. Late apoptotic blood leukocytes. Percentage of Annexin V and PI positive PMN (A) and PBMC (B) of periparturient dairy cows. Cows were fed prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition cows were initially fed with a diet consisting of 30% concentrate. For LC animals this was increased continuously to 50% in the first 16 days wheras for HC animals this increase was achieved in 24 days. Animals were either fed a diet with (NA, 24 g NA/d from d 42 prepartum until 24 DIM) or without (CON) nicotinic acid. Symbols denote LSMEANS; pooled standard error (PSE) for PMN (A) PSE = 0.23 and for PBMC (B) PSE = 0.50. (n_{-42} = 47, n_{-14} = 31, n_{-7} = 23, n_{-3} = 18, n_{+3} = 39, n_{+7} = 47, n_{+14} = 43, n_{+28} = 44, n_{+42} = 47, n_{+100} = 47). T = time relative to parturition, C = concentrate level, S = supplementation. Only p-values for significant ($p < 0.05$) fixed main and interaction effects are indicated.

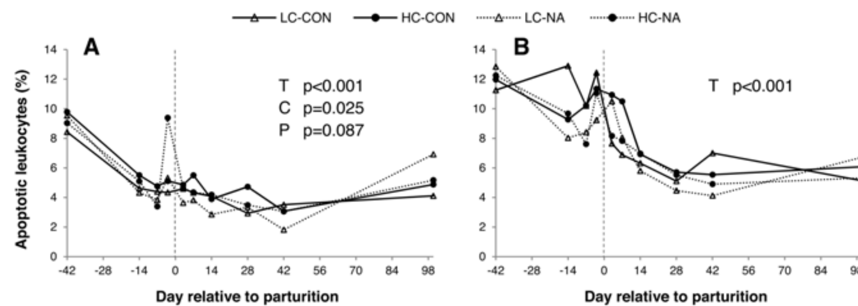


Fig. 3. Apoptotic blood leukocytes. Percentage of early apoptotic (Annexin V+ and PI−) leukocytes (A) and late apoptotic (Annexin V+ and PI+) leukocytes (B) of periparturient dairy cows. Cows were fed prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition cows were initially fed with a diet consisting of 30% concentrate. For LC animals this was increased continuously to 50% in the first 16 days wheras for HC animals this increase was achieved in 24 days. Animals were either fed a diet with (NA, 24 g NA/d from d 42 prepartum until 24 DIM) or without (CON) nicotinic acid. Symbols denote LSMEANS; pooled standard error (PSE) for early apoptotic leukocytes (A) PSE = 0.72 and for late apoptotic leukocytes (B) PSE = 1.21. (n_{-42} = 47, n_{-14} = 31, n_{-7} = 23, n_{-3} = 18, n_{+3} = 39, n_{+7} = 47, n_{+14} = 43, n_{+28} = 44, n_{+42} = 47, n_{+100} = 47). T = time relative to parturition, C = concentrate level, P = parity. Only p-values for significant ($p < 0.05$) and tendency ($0.10 > p > 0.05$) fixed main and interaction effects are indicated.

for the whole blood leukocytes included a cell fraction smaller in size and granularity which was neither included in the measurements for PMN nor PBMC alone. Apoptotic kinetics of cells changed significantly over time. In case of the early apoptotic leukocytes they decreased until time point −7 to show a peak around parturition. Until 42 DIM the percentage of early apoptotic leukocytes decreased further to exhibit another increase at 100 DIM. The late apoptotic leukocytes decreased also prepartum to exhibit a peak around parturition. Until 28 DIM the percentage of late apoptotic leukocytes decreased further and stayed at that level until 100 DIM. Early apoptotic leukocytes were additionally affected by the concentrate proportion. The HC animals showed higher percentages than the LC animals, except for time points −7 and +100. In addition, a tendency in parity for early apoptotic leukocytes was seen. Heifers displayed a tendency to have higher percentages of early apoptotic cells except at day 42 before parturition and 42 DIM (data not shown).

3.2. Gene expression analysis

Gene expression analysis also revealed significant changes over time for all tested genes (Figs. 4 and 5). The pro-apoptotic gene BAX showed hereby a quite similar development as the effector caspase CASP3. The mRNA amount increased to show a peak before calving. After calving it decreased again until 42 DIM. For BAX the mRNA abundance increased again slightly at 100 DIM whereas for CASP3 the level of mRNA remained unchanged after 42 DIM. In contrast, the curves for the anti-apoptotic genes BCL2 and BCL-xL showed a different progression over time. For BCL2 a decrease in mRNA amount until day 3 prepartum in LC animals was seen with a subsequent increase in mRNA level until 14 DIM and an additional increase at 100 DIM, whereas, for HC animals the expression level increased continuously until 100 DIM. In this case a tendency was noted for concentrate effect. The mRNA abundance of BCL-xL displayed a steady increase until 7 DIM with a subsequent decrease until 42 DIM and a slight increase again at 100 DIM. The mRNA abundance of RELA as transcription factor NFκB subunit changed also significantly over time (Fig. 5). Just before parturition a first peak was measured followed by a second peak at 14 DIM. In addition, parity significantly affected RELA mRNA abundance. Primiparous animals (Fig. 5A) showed higher RELA mRNA expression levels than multiparous animals (Fig. 5B). The amplitude of changes, especially around calving, was also higher in heifers than in multiparous cows.

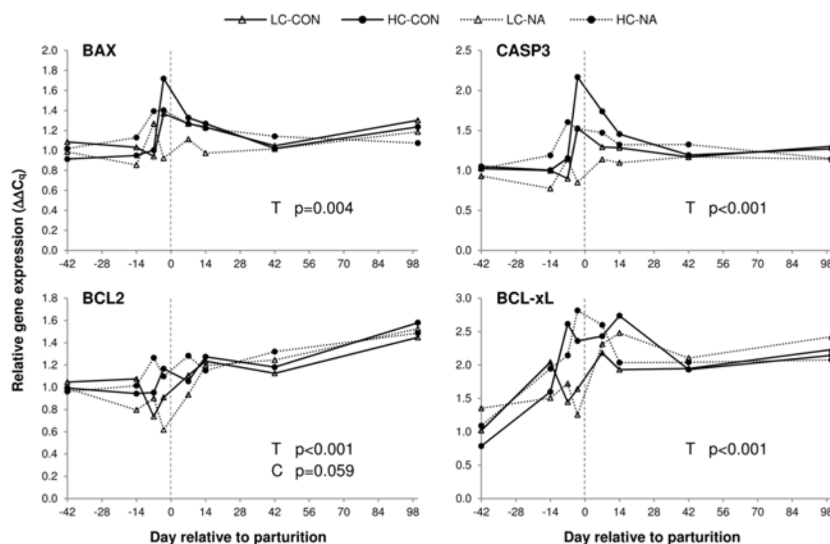


Fig. 4. Gene expression analysis of apoptotic genes. Relative expression levels of the pro-apoptotic genes BAX and CASP3 and the anti-apoptotic genes BCL2 and BCL-xL in blood leukocytes of periparturient dairy cows. Cows were fed prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition cows were initially fed with a diet consisting of 30% concentrate. For LC animals this was increased continuously to 50% in the first 16 days wheras for HC animals this increase was achieved in 24 days. Animals were either fed a diet with (NA, 24 g NA/d from d 42 prepartum until 24 DIM) or without (CON) nicotinic acid. Symbols denote LSMEANS; pooled standard error (PSE) BAX PSE = 0.13; CASP3 PSE = 0.20; BCL2 PSE = 0.12 and BCL-xL PSE = 0.35. (n_{-14} = 34, n_{-7} = 25, n_{-3} = 19, all other time points n = 47). T = time relative to parturition, C = concentrate level. Only p-values for significant (p < 0.05) and tendency (0.10 > p > 0.05) fixed main and interaction effects are indicated.

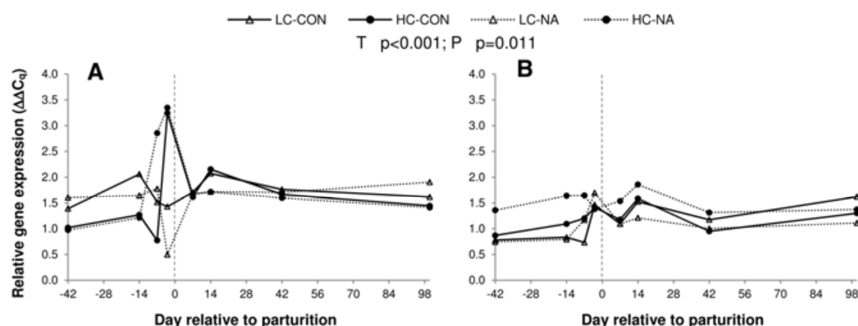


Fig. 5. Gene expression analysis of the NFκB transcription factor subunit RELA in blood leukocytes of primiparous (A) and multiparous (B) periparturient dairy cows. Cows were fed prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition cows were initially fed with a diet consisting of 30% concentrate. For LC animals this was increased continuously to 50% in the first 16 days wheras for HC animals this increase was achieved in 24 days. Animals were either fed a diet with (NA, 24 g NA/d from d 42 prepartum until 24 DIM) or without (CON) nicotinic acid. Symbols denote LSMEANS; pooled standard error (PSE) for primiparous (A) PSE = 0.12 and for multiparous (B) PSE = 0.09 cows. (n_{-14} = 34, n_{-7} = 25, n_{-3} = 19, all other time points n = 47). T = time relative to parturition, P = parity. Only p-values for significant (p < 0.05) fixed main and interaction effects are indicated.

3.3. Hematology

Since no distinction between granulocytes and lymphocytes was made for gene expression analysis, the ratio in granulocyte over lymphocyte counts was included in the examination. This ratio changed also strongly over time (Fig. 6). A peak just before parturition was noticeable and a second one at 14 DIM. A strong parity effect was also detectable. Multiparous cows (Fig. 6B) showed higher ratios than primiparous animals (Fig. 6A). Additionally, the curve progression looked more pronounced in terms of the two peaks for all feeding groups in multiparous animals. A tendency in the interaction T*C*S*P was observed, mostly driven by the increased ratios in the supplemented primiparous animals at 42 and 100 DIM.

3.4. Spearman rank correlations

Spearmen rank correlations were calculated to examine interesting co-progressions. The pro-apoptotic genes BAX and CASP3 showed stronger positive correlations with each other than with

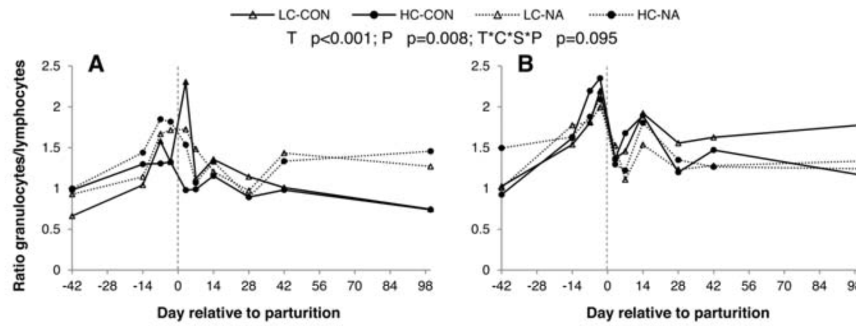


Fig. 6. Ratio of granulocytes/lymphocytes in periparturient primiparous (A) and multiparous (B) dairy cows. Cows were fed prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition cows were initially fed with a diet consisting of 30% concentrate. For LC animals this was increased continuously to 50% in the first 16 days wheras for HC animals this increase was achieved in 24 days. Animals were either fed a diet with (NA, 24 g NA/d from d 42 prepartum until 24 DIM) or without (CON) nicotinic acid. Symbols denote LSMEANS; pooled standard error (PSE) for primiparous (A) PSE = 0.29 and for multiparous (B) PSE = 0.23 cows. (n_{-14} = 44, n_{-7} = 36, n_{-3} = 40, all other time points n = 46). T = time relative to parturition, P = parity, C = concentrate level, S = supplementation. Only p-values for significant ($p < 0.05$) and tendency ($0.10 > p > 0.05$) fixed main and interaction effects are indicated.

Table 2
Spearman rank correlation coefficients between mRNA abundance of the measured apototic genes and late apoptotic leukocytes ($p < 0.001$).

Parameter	BAX	BCL2	BCL-xL	CASP3	RELA
BAX	–				
BCL2	0.540	–			
BCL-xL	0.514	0.582	–		
CASP3	0.631	0.382	0.460	–	
RELA	0.715	0.559	0.529	0.650	–
Leukocytes la	n.s.	−0.309	n.s.	n.s.	n.s.

Abbreviations: BAX BCL2-associated X protein; BCL2 B-cell CLL/lymphoma 2; BCL-xL B-cell lymphoma extra large; CASP3 caspase 3, apoptosis-related cysteine peptidase; RELA v-rel avian reticuloendotheliosis viral oncogene homolog A; leukocytes la late apoptotic (Annexin V+ and PI+) blood leukocytes; n.s. not significant.

Table 3
Spearman rank correlation coefficients between apoptotic parameters and metabolic and blood parameters ($p < 0.001$).

Parameter	EB	DMI	glucose	TG	cholesterol	GLDH	Hb
BCL2	n.s.	0.293	−0.219	−0.294	0.252	0.352	n.s.
BCL-xL	n.s.	n.s.	−0.213	−0.281	n.s.	0.198	n.s.
Leukocytes ea	0.389	n.s.	0.248	0.279	−0.189	−0.227	0.388
Leukocytes la	0.402	−0.314	0.198	0.374	−0.367	−0.349	0.419
PMN ea	0.297	−0.195	0.248	0.280	−0.256	−0.261	0.306
PMN la	0.276	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
PBMC ea	0.432	n.s.	0.256	0.308	n.s.	−0.178	0.401
PBMC la	n.s.	n.s.	n.s.	n.s.	−0.194	n.s.	n.s.

Abbreviations: EB energy balance; DMI dry matter intake; TG triglyceride; GLDH gutamate dehydrogenase; Hb hemoglobin; BCL2 B-cell CLL/lymphoma 2; BCL-xL B-cell lymphoma extra large; PMN polymorphonuclear leukocyte; PBMC peripheral blood mononuclear cell; ea early apoptotic (Annexin V+ and PI-); la late apoptotic (Annexin V+ and PI+); n.s. not significant.

the anti-apoptotic genes BCL2 and BCL-xL (Table 2). The same was true for the anti-apoptotic genes. RELA correlated stronger with the pro-apoptotic genes than with the anti-apoptotic genes. Correlating the genes with the functional FACS assay results, a small negative correlation of the BCL2 gene with the late apoptotic blood leukocytes was found. Correlations for metabolic and blood parameters (published in Tienken et al. (2015a,b)) to the measured apoptotic parameters were also calculated (Table 3). Although the shown correlation coefficients are rather weak they were proven to be significantly different from zero ($p < 0.001$). Especially the energy balance correlated positive with the measured FACS apoptosis values. Glucose and TG correlated positive with apoptotic leukocytes and with early apoptotic PMN and PBMC. In addition they displayed negative correlations with the mRNA abundance of the anti-apoptotic genes BCL2 and BCL-xL. Negative correlations were found between apoptotic leukocytes and DMI, cholesterol, and glutamate dehydrogenase (GLDH). They were accompanied by positive correlations with the BCL2 expression. Additionally, hemoglobin correlated positive with the apoptotic leukocytes and early apoptotic PMN and PBMC.

4. Discussion

The aim of this study was to induce differences in the energy metabolism of periparturient cows by feeding different concentrate levels prepartum, different concentrate increases postpartum, and a nicotinic acid supplementation peripartum and to examine the effects of these feeding strategies on the immune system, more precisely on the apoptosis of blood leukocytes. This study belongs to a larger trial and the feeding effects on production, metabolic, hematological, and biochemical variables are published elsewhere (Tienken et al., 2015a,b). In brief, the concentrate feeding strategies and the supplementation with 24 g NA did not reveal any effect on the metabolic parameters NEFA and BHB.

The periparturient dairy cow undergoes dramatic metabolic and physiological changes that are accompanied with an inflammatory and immuno-suppressed state in the transition period. Nutrient metabolism, oxidative stress and inflammation in transition cows are hereby interlinked as reviewed in Sordillo and Mavangira (2014). Since inflammation and oxidative stress can lead to apoptotic processes, we examined apoptosis in blood leukocytes. All measured apoptotic variables were significantly influenced by time related to parturition. The PMN, consisting mainly of neutrophilic granulocytes, exhibited lower percentages of apoptotic cells than the PBMC that is also seen by Tharwat et al. (2012). PMN are known to have a short half-life in circulation and spontaneously undergo apoptosis. However, they usually are recruited to sites of infection and apoptosis is influenced by inflammatory mediators, as well as adhesion and migration through epithelial cells (Paape et al., 2003). The decrease in PMN apoptosis visible in this study could contribute to the increase in granulocyte numbers around parturition (Schulz et al., 2015; Tienken et al., 2015a) that is also reflected by the granulocyte/lymphocyte ratio in this study. This neutrophilia is usually attributed to the downregulation of cell adhesion molecules through glucocorticoids (Weber et al., 2001). Additionally, glucocorticoids have been shown to increase neutrophil longevity *in vitro* (Chang et al., 2004). In this study, however, no decrease in PMN apoptosis that could be attributed to a glucocorticoid surge, induced by parturition, was detected. The decrease in apoptotic PMN was rather continuous from time point −42 until 14 DIM in case of the late apoptotic fraction, and continued from time point −42 until 42 DIM in the early apoptotic fraction. For early apoptotic PMN a slight increase just before parturition was recognized that could be explained with hormonal influences and a stressed situation since this signal originated from 3 animals one being a heifer exhibiting the highest value. Whereas the early apoptotic PBMC showed a quite similar behavior like the early apoptotic PMN, the late apoptotic PBMC exhibited a different progression. Here, after an initial decrease in apoptotic cells a pronounced increase in apoptosis around parturition was seen for all animals. This could be explained by hormonal changes around this time since glucocorticoids as well as other steroids can induce apoptosis in lymphocytes (Planey and Litwack, 2000). However, since hormonal changes are rather short-lived, there must be other factors influencing this increase of late apoptotic PBMC. The inflammation triggering effects of metabolites such as NEFA and the increase of oxidative stress in the transition period (Sordillo and Aitken, 2009) could be an explanation for increased apoptosis around parturition. In addition, macrophages are described to be affected by lipotoxicity and react by increasing inflammatory processes and subsequent apoptosis (Prieur et al., 2010). Taking the time effects together, PBMC seemed to be more susceptible to the changes around parturition than PMN, since their increase in apoptosis was higher.

For the early apoptotic PBMC a significant influence of the concentrate proportion was seen that was expressed in higher apoptotic cell numbers in the HC group. Zhou et al. (2015) showed that a prepartal higher energy level increases inflammatory and oxidative stress related gene expression in bovine neutrophils. Therefore, the higher percentages of early apoptotic cells in the HC group could derive from modulated inflammatory processes in this animal group. For the late apoptotic PBMC another interesting time by concentrate by supplementation interaction was seen. Here, the HC animals peaked considerably later than the LC animals and the supplemented groups did later than the unsupplemented controls. However, this could not be explained with above mentioned inflammatory processes in the HC group. Since NEFA and BHB concentrations show no comparable behavior (Tienken et al., 2015b) their increases do not explain this observation. Further research is needed to elucidate this effect and the factors inducing it.

Apoptosis was also evaluated over the complete blood leukocyte fraction to represent the situation of the gene expression analysis by setting a gate for FACS analysis that included both populations, PMN and PBMC. For the early apoptotic cells a similar picture than for the early apoptotic PMN and PBMC alone was shown with the same influences of time and concentrate proportion. Additionally, a tendency for parity was seen, meaning heifers showed higher percentages of early apoptotic cells than cows. This could either be explained by chronic stress that primiparous cows experience around parturition (González et al., 2003) or by growth hormones that are also known to influence apoptosis in blood leukocytes (Vangroenweghe et al., 2005). The late apoptotic blood leukocytes displayed also a time course similar to the late apoptotic PMN and PBMC alone. However, the absolute numbers were higher than the summation of the individual populations would have suggested. This is due to an additional fraction of smaller particles that was analyzed in the gate setting and included in the evaluation. It can be assumed, that this fraction was also included in the gene expression analysis. If this signal, however, arose of already fragmented and necrotic cells, maybe even produced during sample handling, or if it represented the event of apoptotic blebbing may not be differentiated.

The results of the gene expression analysis supported the functional FACS apoptosis assay. For the pro-apoptotic gene BAX and the effector caspase CASP3 an increase in mRNA abundance was seen before parturition that peaked at day 3 prepartum. This progression was in accordance to the apoptotic peaks seen around parturition. In addition, these two genes correlated stronger with each other than with the anti-apoptotic genes suggesting a common regulation. The expression of the anti-apoptotic gene BCL-xL increased strongly between day −42 and −14 prepartum and this could explain the simultaneous decrease in apoptotic leukocytes. mRNA abundance for BCL-xL showed also a peak at day 3 prepartum but decreased more slowly than the pro-apoptotic genes and stayed at a higher level compared to −42 prepartum. This is in support to the disappearance of the apoptotic peak after parturition and to the further decrease of apoptotic leukocytes postpartum. In LC animals, BCL2 expression slightly decreased before parturition that was expressed in a tendency for concentrate level. However this decrease did not implicate an absolute higher increase in apoptotic blood leukocytes in the LC group. But maybe it contributed to the shift seen between the LC and HC group in late apoptotic PBMC. Postpartum BCL2 expression increased leading presumably to the downregulation of the apoptotic peak and also to the further downregulation of apoptotic leukocytes. Also for the anti-apoptotic genes a stronger correlation with each other than with the pro-apoptotic genes was seen, implying common regulatory mechanisms. RELA, being a subunit of the transcription factor NFκB, is involved in many cellular processes including cellular metabolism, inflammation, and apoptosis. RELA mRNA abundance displayed 2 peaks: one 3 days prepartum and another one at 14 DIM. The first peak could be attributed to inflammatory processes accompanied with oxidative stress around parturition. The second peak at 14 DIM was accompanied by a higher granulocyte/lymphocyte ratio at this time point. Dairy cows resume their ovarian cycle after parturition and ovulate again about 15 days postpartum (Crowe, 2008). Therefore the increasing granulocyte/lymphocyte ratio and the peak in RELA mRNA abundance at 14 DIM could be attributed to changes in estrous hormones. Primiparous cows showed higher values of RELA mRNA abundance throughout the whole trial period. Since primiparous cows have not yet reached maturity this could reflect a higher transcription factor level in these animals. However, it could also reflect that RELA is more expressed in lymphocytes than in granulocytes, since the granulocyte/lymphocyte ratio in primiparous cows was lower than for multiparous cows. Grossmann et al. (2000) showed, for example, that RELA is important in B-cell

maturation partly by upregulating BCL2 expression to promote the survival of these cells. In addition, RELA correlated stronger with the pro-apoptotic genes than with the anti-apoptotic genes indicating its role in initiating anti-apoptotic gene expression.

To assess different expression levels in the different cell types, the granulocyte/lymphocyte ratio was included in the evaluation. This ratio varied over time, which is caused by the increase of granulocyte numbers during the periparturient period (Tienken et al., 2015a). Spearman rank correlations displayed only for the BCL2 mRNA abundance a significant ($p<0.001$) negative correlation with the granulocyte/lymphocyte ratio (−0.193) (data not shown). Including the granulocyte/lymphocyte ratio as covariate in the statistical analysis of the gene expression data, only for the BCL2 calculation this covariate became statistically significant ($p<0.05$) (data not shown). Therefore, it is evident that the change of expression in BCL2 is considerably influenced by blood leukocyte type. Similarly, Iwai et al. (1994) showed, that the Bcl-2 protein is strongly expressed in human lymphocytes but is essentially absent in human neutrophils.

Nicotinic acid supplementation effects were only detected as interactions. Some influence was seen in the increasing number of apoptotic cells in PBMC and in early apoptotic PMN. The supplemented animals displayed a considerably higher increase between 42 and 100 DIM than the control animals. However the NA supplementation ended at 24 DIM and this enhanced increase in apoptotic cells might be interpreted as a priming effect and requires further elucidation.

Spearman rank correlations were calculated to examine interesting co-progressions between apoptotic cells and metabolic and blood parameters. Apoptosis seems to be correlated to the energy status. This is reflected by positive correlations of apoptotic parameters to the energy balance, glucose, and TG. Apoptosis is an energy consuming process that is necessary for a normal functioning immune system especially in getting rid of cells that have been activated in inflammation and perform their defense mechanisms against pathogens. However, the proportion of apoptosis as an energy consuming process of the extent of the postpartum negative energy balance cannot be quantified so far. The positive correlation of above mentioned parameters to the apoptotic cells is paralleled by a negative correlation between glucose or TG and BCL2 expression. With more energy available the BCL2 mRNA abundance and, therefore, anti-apoptotic processes are reduced. This is also supported by the negative correlation of late apoptotic leukocytes and the positive correlation of BCL2 mRNA abundance with the DMI. For cholesterol a positive correlation to BCL2 mRNA abundance and a subsequent negative correlation to apoptotic cells are seen. Although, cholesterol causes a concentration dependent increase in apoptosis *in vitro* in human monocytes (Rauch et al., 2011), high density lipoprotein (HDL) is described to reduce inflammatory responses in human monocytes and neutrophils (Murphy et al., 2008; Murphy et al., 2011), therefore, offering an explanation for the negative correlation of cholesterol and apoptotic cells in this study. GLDH marks liver damage that takes place at a later stage in the periparturient period. For GLDH also a rather strong positive correlation with BCL2 mRNA abundance and negative correlations with apoptotic cells are seen. In what way, however, the liver damage is interlinked with the reduced apoptosis in peripheral blood cells needs to be clarified. Hemoglobin displays next to the EB the strongest positive correlations with the apoptotic cells. Free hemoglobin is known for its cytotoxicity in human neutrophils (Lee et al., 2015). More hemoglobin, however, could also indicate higher oxygen transport and subsequently a higher cell energy metabolism. This may result in more reactive oxygen species (ROS) in the cells and consequentially leading to enhanced apoptotic processes. The correlation studies revealed also that early apoptotic PMN or PBMC are more influenced by the considered parameters than the respective late apoptotic cells.

5. Conclusion

This is the first study to compare a functional apoptosis assay in PMN and PBMC to gene expression analysis in blood leukocytes throughout the periparturient period under the influence of different dietary energy levels and a nicotinic acid supplementation. The apoptotic behavior in PMN and PBMC differs and PBMC seem to be more susceptible to changes around parturition. Additionally early apoptotic cells are influenced by different concentrate levels and apoptosis is correlated to energy parameters. However to what extent increased apoptosis reflects a well-functioning immune system in its work against inflammatory processes and if a decrease in apoptosis leads to a compromized immune function needs further research.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors thank the Deutsche Forschungsgemeinschaft (DFG, Bonn, Germany) for financial support (DA 558/6-1). All co-workers of the Institute of Animal Nutrition and the Experimental Station of the Friedrich-Loeffler-Institute in Braunschweig who supported this work are gratefully acknowledged.

References

Adewuyi, A.A., Gruys, E., van Eerdenburg, F.J., 2005. Non esterified fatty acids (NEFA) in dairy cattle. A review. Vet. Q. 27, 117–126.
Bell, A.W., 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J. Anim. Sci. 73, 2804–2819.
Brassard, P., Larbi, A., Grenier, A., Frisch, F., Fortin, C., Carpentier, A.C., Fulop, T., 2007. Modulation of T-cell signalling by non-esterified fatty acids. Prostaglandins Leukot. Essent. Fatty Acids 77, 337–343.
Burvenich, C., Bannerman, D.D., Lippolis, J.D., Peelman, L., Nonnecke, B.J., Kehrli Jr., M.E., Paape, M.J., 2007. Cumulative physiological events influence the inflammatory response of the bovine udder to Escherichia coli infections during the transition period. J. Dairy Sci. 90 (Suppl. 1), E39–54.
Carlson, L.A., 2005. Nicotinic acid: the broad-spectrum lipid drug. A 50th anniversary review. J. Intern. Med. 258, 94–114.
Chang, L.C., Madsen, S.A., Toelboell, T., Weber, P.S., Burton, J.L., 2004. Effects of glucocorticoids on Fas gene expression in bovine blood neutrophils. J. Endocrinol. 183, 569–583.
Crowe, M.A., 2008. Resumption of ovarian cyclicity in post-partum beef and dairy cows. Reprod. Domest. Anim. 43 (Suppl. 5), 20–28.
Drackley, J.K., 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J. Dairy Sci. 82, 2259–2273.
GfE (Society of Nutrition Physiology), 2001. Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchtrinder (Recommendations of energy and nutrient supply for dairy cows and breeding cattle). DLG-Verlag, Frankfurt am Main, Germany.
Goff, J.P., Horst, R.L., 1997. Physiological changes at parturition and their relationship to metabolic disorders. J. Dairy Sci. 80, 1260–1268.
González, M., Yabuta, A.K., Galindo, F., 2003. Behaviour and adrenal activity of first parturition and multiparous cows under a competitive situation. Appl. Anim. Behav. Sci. 83, 259–266.
Grossmann, M., O'Reilly, L.A., Gugasyan, R., Strasser, A., Adams, J.M., Gerondakis, S., 2000. The anti-apoptotic activities of Rel and RelA required during B-cell maturation involve the regulation of Bcl-2 expression. EMBO J. 19, 6351–6360.
Hammon, D.S., Evjen, I.M., Dhiman, T.R., Goff, J.P., Walters, J.L., 2006. Neutrophil function and energy status in Holstein cows with uterine health disorders. Vet. Immunol. Immunopathol. 113, 21–29.
Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19.
Hoeben, D., Monfardini, E., Opsomer, G., Burvenich, C., Dosogne, H., De Kruif, A., Beckers, J.F., 2000. Chemiluminescence of bovine polymorphonuclear leucocytes during the periparturient period and relation with metabolic markers and bovine pregnancy-associated glycoprotein. J. Dairy Res. 67, 249–259.

Ingvartsen, K.L., Moyes, K., 2013. Nutrition, immune function and health of dairy cattle. Animal 7 (Suppl. 1), 112–122.

Ingvartsen, K.L., Moyes, K.M., 2015. Factors contributing to immunosuppression in the dairy cow during the periparturient period. Jpn. J. Vet. Res. 63 (Suppl. 1), S15–24.

Iwai, K., Miyawaki, T., Takizawa, T., Konno, A., Ohta, K., Yachie, A., Seki, H., Taniguchi, N., 1994. Differential expression of bcl-2 and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes, and neutrophils. Blood 84, 1201–1208.

Kehrli Jr., M.E., Nonnecke, B.J., Roth, J.A., 1989. Alterations in bovine neutrophil function during the periparturient period. Am. J. Vet. Res. 50, 207–214.

Kenez, A., Locher, L., Rehage, J., Danicke, S., Huber, K., 2014. Agonists of the G protein-coupled receptor 109A-mediated pathway promote antilipolysis by reducing serine residue 563 phosphorylation of hormone-sensitive lipase in bovine adipose tissue explants. J. Dairy Sci. 97, 3626–3634.

Kostylina, G., Simon, D., Fey, M.F., Yousefi, S., Simon, H.U., 2008. Neutrophil apoptosis mediated by nicotinic acid receptors (GPR109A). Cell Death Differ. 15, 134–142.

Lacetera, N., Scalia, D., Franci, O., Bernabucci, U., Ronchi, B., Nardone, A., 2004. Short communication: effects of nonesterified fatty acids on lymphocyte function in dairy heifers. J. Dairy Sci. 87, 1012–1014.

Lee, S.K., Goh, S.Y., Wong, Y.Q., Ding, J.L., 2015. Response of neutrophils to extracellular haemoglobin and LTA in human blood system. EBioMedicine 2, 225–233.

Liu, F., Fu, Y., Wei, C., Chen, Y., Ma, S., Xu, W., 2014. The expression of GPR109A, NF-kB and IL-1beta in peripheral blood leukocytes from patients with type 2 diabetes. Ann. Clin. Lab. Sci. 44, 443–448.

Madsen, S.A., Chang, L.C., Hickey, M.C., Rosa, G.J., Coussens, P.M., Burton, J.L., 2004. Microarray analysis of gene expression in blood neutrophils of parturient cows. Physiol. Genomics 16, 212–221.

Maiese, K., Chong, Z.Z., Hou, J., Shang, Y.C., 2009. The vitamin nicotinamide: translating nutrition into clinical care. Molecules 14, 3446–3485.

Morey, S.D., Mamedova, L.K., Anderson, D.E., Armendariz, C.K., Titgemeyer, E.C., Bradford, B.J., 2011. Effects of encapsulated niacin on metabolism and production of periparturient dairy cows. J. Dairy Sci. 94, 5090–5104.

Morris, D.G., Waters, S.M., McCarthy, S.D., Patton, J., Earley, B., Fitzpatrick, R., Murphy, J.J., Diskin, M.G., Kenny, D.A., Brass, A., Wathes, D.C., 2009. Pleiotropic effects of negative energy balance in the postpartum dairy cow on splenic gene expression: repercussions for innate and adaptive immunity. Physiol. Genomics 39, 28–37.

Murphy, A.J., Woollard, K.J., Hoang, A., Mukhamedova, N., Stirzaker, R.A., McCormick, S.P., Remaley, A.T., Sviridov, D., Chin-Dusting, J., 2008. High-density lipoprotein reduces the human monocyte inflammatory response. Arterioscler. Thromb. Vasc. Biol. 28, 2071–2077.

Murphy, A.J., Woollard, K.J., Suhartoyo, A., Stirzaker, R.A., Shaw, J., Sviridov, D., Chin-Dusting, J.P., 2011. Neutrophil activation is attenuated by high-density lipoprotein and apolipoprotein A-I in in vitro and in vivo models of inflammation. Arterioscler. Thromb. Vasc. Biol. 31, 1333–1341.

Nonnecke, B.J., Kimura, K., Goff, J.P., Kehrli Jr., M.E., 2003. Effects of the mammary gland on functional capacities of blood mononuclear leukocyte populations from periparturient cows. J. Dairy Sci. 86, 2359–2368.

Paape, M.J., Bannerman, D.D., Zhao, X., Lee, J.W., 2003. The bovine neutrophil: structure and function in blood and milk. Vet. Res. 34, 597–627.

Planey, S.L., Litwack, G., 2000. Glucocorticoid-induced apoptosis in lymphocytes. Biochem. Biophys. Res. Commun. 279, 307–312.

Prieur, X., Roszer, T., Ricote, M., 2010. Lipotoxicity in macrophages: evidence from diseases associated with the metabolic syndrome. Biochim. Biophys. Acta 1801, 327–337.

Rauch, S.J., Rosenkranz, A.C., Bohm, A., Meyer-Kirchrath, J., Hohlfeld, T., Schror, K., Rauch, B.H., 2011. Cholesterol induces apoptosis-associated loss of the activated leukocyte cell adhesion molecule (ALCAM) in human monocytes. Vascul. Pharmacol. 54, 93–99.Renner, L., von Soosten, D., Sipka, A., Doll, S., Beineke, A., Schuberth, H.J., Danicke, S., 2012. Effect of conjugated linoleic acid on proliferation and cytokine expression of bovine peripheral blood mononuclear cells and splenocytes ex vivo. Arch. Anim. Nutr. 66, 73–85.

Scalia, D., Lacetera, N., Bernabucci, U., Demeyere, K., Duchateau, L., Burvenich, C., 2006. In vitro effects of nonesterified fatty acids on bovine neutrophils oxidative burst and viability. J. Dairy Sci. 89, 147–154.

Schulz, K., Frahm, J., Meyer, U., Kersten, S., Reiche, D., Rehage, J., Danicke, S., 2014. Effects of prepartal body condition score and peripartal energy supply of dairy cows on postpartal lipolysis, energy balance and ketogenesis: an animal model to investigate subclinical ketosis. J. Dairy Res. 81, 257–266.

Schulz, K., Frahm, J., Kersten, S., Meyer, U., Reiche, D., Sauerwein, H., Danicke, S., 2015. Effects of elevated parameters of subclinical ketosis on the immune system of dairy cows: in vivo and in vitro results. Arch. Anim. Nutr. 69, 113–127.

Sordillo, L.M., Aitken, S.L., 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Vet. Immunol. Immunopathol. 128, 104–109.

Sordillo, L.M., Mavangira, V., 2014. The nexus between nutrient metabolism, oxidative stress and inflammation in transition cows. Anim. Prod. Sci. 54, 1204–1214.

Sordillo, L.M., Contreras, G.A., Aitken, S.L., 2009. Metabolic factors affecting the inflammatory response of periparturient dairy cows. Anim. Health Res. Rev./Conf. Res. Work. Anim. Dis. 10, 53–63.

Ster, C., Loiselle, M.C., Lacasse, P., 2012. Effect of postcalving serum nonesterified fatty acids concentration on the functionality of bovine immune cells. J. Dairy Sci. 95, 708–717.

Suriyasathaporn, W., Heuer, C., Noordhuizen-Stassen, E.N., Schukken, Y.H., 2000. Hyperketonemia and the impairment of udder defense: a review. Vet. Res. 31, 397–412.

Tharwat, M., Endoh, D., Oikawa, S., 2012. DNA damage in peripheral blood mononuclear cells and neutrophils of dairy cows during the transition period. Open Vet. J. 2, 65–68.

Tienken, R., Kersten, S., Frahm, J., Huther, L., Meyer, U., Huber, K., Rehage, J., Danicke, S., 2015a. Effects of prepartum dietary energy level and nicotinic acid supplementation on immunological: hematological and biochemical parameters of periparturient dairy cows differing in parity. Animals 5, 910–933.

Tienken, R., Kersten, S., Frahm, J., Meyer, U., Locher, L., Rehage, J., Huber, K., Kenez, A., Sauerwein, H., Mielenz, M., Danicke, S., 2015b. Effects of an energy-dense diet and nicotinic acid supplementation on production and metabolic variables of primiparous or multiparous cows in periparturient period. Arch. Anim. Nutr. 69, 319–339.

Titgemeyer, E.C., Mamedova, L.K., Spivey, K.S., Farney, J.K., Bradford, B.J., 2011. An unusual distribution of the niacin receptor in cattle. J. Dairy Sci. 94, 4962–4967.

Valdez, K.E., Cuneo, S.P., Turzillo, A.M., 2005. Regulation of apoptosis in the atresia of dominant bovine follicles of the first follicular wave following ovulation. Reproduction 130, 71–81.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 (Research0034).

Vangroenweghe, F., Lamote, I., Burvenich, C., 2005. Physiology of the periparturient period and its relation to severity of clinical mastitis. Domest. Anim. Endocrinol. 29, 283–293.

Weber, P.S., Madsen, S.A., Smith, G.W., Ireland, J.J., Burton, J.L., 2001. Pre-translational regulation of neutrophil L-selectin in glucocorticoid-challenged cattle. Vet. Immunol. Immunopathol. 83, 213–240.

Yu, B.L., Zhao, S.P., 2007. Anti-inflammatory effect is an important property of niacin on atherosclerosis beyond its lipid-altering effects. Med. Hypotheses 69, 90–94.

Yuan, K., Shaver, R.D., Bertics, S.J., Espineira, M., Grummer, R.R., 2012. Effect of rumen-protected niacin on lipid metabolism, oxidative stress, and performance of transition dairy cows. J. Dairy Sci. 95, 2673–2679.

Zhou, Z., Bu, D.P., Vailati Riboni, M., Khan, M.J., Graugnard, D.E., Luo, J., Cardoso, F.C., Loor, J.J., 2015. Prepartal dietary energy level affects peripartal bovine blood neutrophil metabolic, antioxidant, and inflammatory gene expression. J. Dairy Sci. 98, 5492–5505.

5. Paper II

S. Bühler, J. Frahm, R. Tienken, S. Kersten, U. Meyer, K. Huber, S. Dänicke (2018)

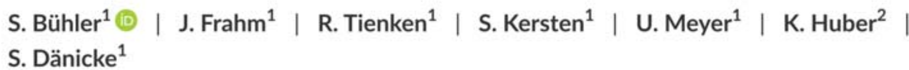
Effects of energy supply and nicotinic acid supplementation on serum anti-oxidative capacity and on expression of oxidative stress-related genes in blood leucocytes of periparturient primi-and pluriparous dairy cows.

Journal of Animal Physiology and Animal Nutrition 102, e87–e98.

Reprinted with permission of John Wiley and Sons

Received: 22 December 2016 | Accepted: 2 February 2017
DOI: 10.1111/jpn.12705

ORIGINAL ARTICLE



Effects of energy supply and nicotinic acid supplementation on serum anti-oxidative capacity and on expression of oxidative stress-related genes in blood leucocytes of periparturient primi- and pluriparous dairy cows

S. Bühler[1] | J. Frahm[1] | R. Tienken[1] | S. Kersten[1] | U. Meyer[1] | K. Huber[2] | S. Dänicke[1]

[1]Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Braunschweig, Germany

[2]Institute of Animal Sciences, University of Hohenheim, Stuttgart, Germany

Correspondence
S. Bühler, Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Braunschweig, Germany.
Email: Susanne.Buehler@fli.de

Summary

The periparturient period is accompanied by metabolic and oxidative stress. Niacin is known to decrease lipolysis but is also reported to have anti-oxidative effects. Therefore, we examined the effects of energy supply and a nicotinic acid (NA) supplementation on anti-oxidative serum parameters and on the expression of oxidative stress-related genes in blood leucocytes of periparturient dairy cows, differing in parity. Twenty-nine pluriparous and 18 primiparous cows were allocated to four different feeding groups 42 days before expected parturition until 100 days postpartum and fed a ration with either a low concentrate proportion of 30% (LC) or a high concentrate proportion of 60% (HC). After parturition, all animals received 30% concentrate which was increased to 50% either within 16 (LC group) or 24 days (HC group). Half of the animals per group were supplemented with 24 g NA per day from 42 days prepartum until 24 days postpartum. All investigated parameters varied significantly over time compared to parturition ($p < .05$). Ferric reducing ability (FRA) exhibited a nadir before parturition, and the antioxidant enzymes glutathione peroxidase (GPX) and superoxide dismutase (SOD) showed peak activities around parturition. Expression levels of GPX1, SOD2, xanthine dehydrogenase (XDH) and nuclear factor (erythroid-derived 2)-like 2 (NRF2) peaked before calving. The concentrate level influenced GPX activity and mRNA abundance of SOD2, XDH and poly (ADP-ribose) polymerase 1 (PARP1). Pluriparous animals exhibited higher serum GPX activities, a more distinct nadir for FRA and higher expression levels for GPX1, SOD2 and XDH. Primiparous cows displayed higher serum SOD activities. NA supplementation increased serum SOD activity antepartum in LC animals. Parturition was characterised by an increased need for antioxidants and an increased expression of oxidative stress-related genes that clearly differed with parity and was influenced by energy supply while NA exerted only minor effects on the investigated parameters.

KEYWORDS
ferric reducing ability, glutathione peroxidase, niacin, superoxide dismutase, transition period

 © 2017 Blackwell Verlag GmbH

1 | INTRODUCTION

The periparturient dairy cow is challenged with major nutritional, metabolic, hormonal and immunological changes during the transition from late gestation to early lactation. The transition period is furthermore accompanied by an increased susceptibility to and severity of metabolic and infectious diseases (Goff & Horst, 1997). With the onset of lactation, the energy demands cannot be met by dietary intake and the cow enters a state of negative energy balance that is further aggravated by a reduced dry matter intake (DMI) in the days before parturition (Hayirli, Grummer, Nordheim, & Crump, 2002). To meet energy requirements, the animals mobilise adipose tissues with a subsequent increase in non-esterified fatty acids (NEFA) in the blood (Bell, 1995). However, with massive lipolysis, the liver fails to oxidise NEFA completely for energy via the Kreb's cycle and converts them also into triglycerides (TG) and ketone bodies like beta-hydroxybutyrate (BHB) (Adewuyi, Gruys, & van Eerdenburg, 2005; Drackley, 1999). Both NEFA and BHB have been shown to adversely affect the functional capacity of polymorphonuclear leucocytes (PMN) (Hammon, Evjen, Dhiman, Goff, & Walters, 2006; Scalia et al., 2006; Suriyasathaporn et al., 1999) and peripheral blood mononuclear cells (PBMC) (Brassard et al., 2007; Lacetera et al., 2004; Renner et al., 2012; Schulz et al., 2015; Ster, Loiselle, & Lacasse, 2012). In addition, it was reported that NEFA induce inflammatory responses via toll-like receptor 4 (TLR4) pathways (Lee, Sohn, Rhee, & Hwang, 2001; Lee et al., 2003). Taken together, NEFA and BHB are thought to play a major role in the immunosuppression during the transition period (Ingvartsen & Moyes, 2013, 2015). The increased energy requirement at the onset of lactation, however, is also accompanied by increased oxygen consumption through cellular respiration. This increased metabolic activity results in enhanced accumulation of reactive oxygen species (ROS) that are produced as normal by-products in the respiratory chain in mitochondria (Valko et al., 2007). ROS, however, are also important signalling molecules. They activate nuclear factor kappa B (NFκB) and E2-related factor 2 (Nrf2), and consequentially promote inflammation and initiate antioxidant defences respectively. Among the most efficient antioxidants are enzymes that can directly catalyse the reduction in ROS such as glutathione peroxidase (GPX) and superoxide dismutase (SOD) (Sordillo & Aitken, 2009). However, when the production of ROS exceeds the neutralising capacity of antioxidants, oxidative stress develops (Sies, 1997). This condition occurs in cattle during the transition period (Bernabucci, Ronchi, Lacetera, & Nardone, 2002, 2005; Castillo et al., 2005; Miller, Brzezinska-Slebodzinska, & Madsen, 1993), and is a significant underlying factor leading to dysfunctional host immune and inflammatory responses and consequently increases cows' susceptibility to health disorders (Sordillo & Aitken, 2009). Increasing evidence suggests that nutrient metabolism, oxidative stress and inflammation are linked (Sordillo & Mavangira, 2014). For example, it was shown that the energy level prepartum influences oxidative stress and inflammation in dairy cows (Graugnard et al., 2012; Khan et al., 2015; Zhou et al., 2015). Niacin, the vitamin B3, is found as nicotinic acid and as nicotinamide in the body. Nicotinic acid is hereby readily transformed into nicotinamide that is a precursor of the coenzymes β-nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$), which participate in anabolic and catabolic pathways. Niacin is well known for its anti-lipolytic effect (Carlson, 2005), and nicotinic acid was shown in the bovine to downregulate lipolysis in vitro (Kenez, Locher, Rehage, Danicke, & Huber, 2014) while in vivo results are inconsistent showing either an anti-lipolytic effect (Morey et al., 2011; Pires & Grummer, 2007; Yuan, Shaver, Bertics, Espineira, & Grummer, 2012) or no protective effects (Kenez et al., 2015; Tienken, Kersten, Frahm, Meyer, et al., 2015). Nicotinamide participates in the cellular energy metabolism and influences oxidative stress. It acts as cytoprotectant in blocking inflammatory cell activation and has immune-modulating properties (Maiese, Chong, Hou, & Shang, 2009; Yu & Zhao, 2007).

Therefore, the objective of this study was to examine whether a nicotinic acid supplementation influences the anti-oxidative capacity of serum and the expression of oxidative stress-related genes in blood leucocytes of periparturient dairy cows that were fed different energy levels prepartum and different energy escalation strategies postpartum. Different energy feeding strategies were applied to induce variation in the degree of lipolysis and oxidative status.

2 | MATERIALS AND METHODS

2.1 | Experimental design and blood collection

The experiment was performed at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Braunschweig, Germany, in accordance with the German Animal Welfare Act concerning the protection of experimental animals and was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany. The experimental conditions are described in detail by Tienken, Kersten, Frahm and Meyer et al. (2015). Briefly, 47 pregnant and healthy German Holstein dairy cows (29 pluriparous and 18 primiparous) were allocated homogeneously to one of four dietary treatment groups considering mean body weight (BW), body condition score (BCS), number of lactations and milk yield of previous lactations (pluriparous cows). The experiment started at 42 days before expected parturition and ended at 100 days in milk (DIM). Before parturition, the animals received either a low concentrate diet (30% concentrate and 70% roughage on dry matter (DM) basis; LC group) or a high concentrate diet (60% concentrate and 40% roughage on DM basis; HC group) with or without a supplementation of 24 g per day and cow of powdered and non-rumen protected nicotinic acid (NA) (Mianyang Vanetta Pharmaceutical Technology, Sichuan, China) resulting in the feeding groups LC-NA (4 primiparous and 6 pluriparous) and HC-NA (5 primiparous and 7 pluriparous) as well as LC-CON (5 primiparous and 7 pluriparous) and HC-CON (4 primiparous and 9 pluriparous). Supplementation was applied from day 42 prepartum until 24 DIM. After parturition, all animals received initially a diet composed of 30% concentrate and 70% roughage. The concentrate proportion was increased up to 50% of the diet in the first 16 days for the LC animals and in the first 24 days for the HC animals and was maintained at 50%

until the end of the trial. The different concentrate feeding strategies aimed at triggering cow groups differing in the degree of postpartum lipolysis and oxidative status to possibly facilitate the investigation of niacin effects. Roughage (50% corn silage and 50% grass silage on DM basis) was fed ad libitum via self-feeding stations (type RIC, Insentec B.V., Marknesse, The Netherlands), and the animals had free access to water. All diets were formulated to meet the nutritional requirements of dairy cows stated by the Society of Nutrition Physiology GfE (2001).

Blood from a *Vena jugularis externa* was collected into EDTA-containing tubes and serum tubes at day −42 (−40 ± 6 [mean ± *SD*]), −14 (−13 ± 2), −7 (−7 ± 1), −3 (−3 ± 1), 3, 7, 14, 42 and 100 relative to parturition. After centrifugation at 2,000 *g* at 15°C for 15 min, serum was stored in aliquots at −80°C until further analysis. The blood samples were processed within 2 hrs after collection.

2.2 | Serum antioxidant enzyme activities

Glutathione peroxidase (GPX) activity was measured in serum with Ransel glutathione peroxidase assay reagents (Randox Laboratories, Crumlin, UK) based on the method of Paglia and Valentine (1967). In brief, GPX catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidised glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. Measurements were taken in duplicate according to the manufacturers' protocol, however, with adjusted volumes to fit a 96-well format: after pipetting 10 μl serum in a clear 96-well plate, 245 μl reagent R1 and 10 μl cumene hydroperoxide solution (R2) were added and the samples mixed well by shaking the plate. The decrease in NADPH was analysed by measuring the absorption at 340 nm and 37°C at two time points in the reaction linear range on a TECAN infinite M200 plate reader. The amount of NADPH that was decreased was calculated using the NADPH extinction coefficient of 0.00435/μM. The actual extinction coefficient for NADPH at 340 nm of 0.00622/μM cm was adjusted for the path length of the solution in the well (0.7 cm).

Superoxide dismutase (SOD) activity was measured in serum with Ransod superoxide dismutase assay reagents (Randox Laboratories, Crumlin, UK) in duplicate. Superoxide radicals are generated by the xanthine oxidase reaction and convert 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to a formazan dye. Conversion of superoxide radicals to hydrogen peroxide by superoxide dismutase inhibits dye formation, and the degree of inhibition serves as measure of the SOD activity. The assay was conducted according to the manufacturer's protocol; however, the volumes were adjusted correspondingly to amount to a final volume of 236 μl to fit a 96-well format.

2.3 | Ferric reducing ability

The ferric reducing ability (FRA) of serum was determined by the analytical procedure of Benzie and Strain (1996). It is a measure of the anti-oxidative capacity in serum that represents the non-enzymatic anti-oxidative systems. The method is based on the conversion of Fe^{3+}-tripyridyltriazine to Fe^{2+}-tripyridyltriazine by antioxidants present in the serum and the development of blue colour. The absorbance at 593 nm and 37°C was followed kinetically, and the end-point was taken at 15 min. With a calibration curve of Fe^{2+}, FRA values were calculated and expressed in nmol Fe^{2+} formed per ml serum.

2.4 | RNA isolation

Total RNA of blood leucocytes was isolated as described in detail by Bühler et al. (2016). Briefly, leucocytes of 2 ml EDTA blood were isolated by repeated lysing of erythrocytes with hypotonic shock (H_2O) and subsequent salt addition to restore isotonicity. The final cell pellet was subjected to a total RNA isolation procedure with a chaotropic ions buffer system and a silica membrane (NucleoSpin® RNA II; Macherey Nagel, Düren, Germany), according to the manufacturer's protocol. Contaminating DNA was digested on-column, and the RNA was eluted with 50 μl H_2O. Concentration and quality of the purified RNA were assessed spectrophotometrically with a NanoDrop® ND-1000 (NanoDrop, Wilmington, DE, USA). The RNA integrity was verified using 1.1% agarose gel electrophoresis. All samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits. RNA was stored at -80°C until further processing.

2.5 | cDNA synthesis and qRT PCR

Synthesis of cDNA and qRT PCR conditions are described in detail by Bühler et al. (2016). Briefly, 750 ng total RNA was transcribed into cDNA with the qScript™ cDNA Synthesis Kit (Quanta Biosciences™, Gaithersburg, MD, USA), according to the manufacturer's protocol. cDNA was diluted 1:10 with H_2O and stored at −20°C for subsequent analysis. Gene-specific primer pairs were generated using Primer3 and BLAST, selecting for annealing temperatures of 60°C, location on different exons, and, if possible, intron spanning. All used primers were obtained from Eurofins MWG Operon (Ebersberg, Germany) and are listed in Table 1. Primer efficiencies were calculated using a six-point dilution series of a cDNA sample mixture of the experiment. The generation of a single PCR product was confirmed with a melting curve analysis from 60 to 95°C in 0.5°C increments for 5 s, and the product length was confirmed on 2% agarose gel electrophoresis. qPCR runs were conducted on a CFX96™ Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA) using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) in a final volume of 15 μl. The primers were added in the concentrations listed in Table 1 and 5 μl cDNA was included. Samples were done in duplicate. After an initial denaturation step for 30 s at 95°C, 40 cycles of 5-s denaturation at 95°C and 30-s annealing and elongation at 60°C followed. The generation of a single PCR product was confirmed with a melting curve analysis. Every run was controlled by a no template control (NTC) in triplicate, and as the measurements were distributed over multiple plates, two inter-run calibrators (IRC), also in triplicate, were included. C_q values of target and reference genes were obtained using CFX Manager™ Software 2.0. The evaluation was done using qbase+ (version 2.5; Biogazelle, Zwijnaarde, Belgium). The normalised, relative expression levels ($\Delta\Delta C_q$) were calculated using the geometric mean of the

TABLE 1 Characteristics of gene-specific primers used for qRTPCR

Gene	NCBI GenBank accession no.	Sequences (forward/reverse) (5′–3′)	Amplicon size (bp)	Amount (nmol) for/rev	R^2	Efficiency (%)
Reference genes						
B2M	NM_173893.3	F-AGCAGCACCATCGAGATTTGA R-TGGACATGTAGCACCCAAGG	172	250/250	.996	105
RPLP0	NM_001012682.1	F-AACTCTGCATTCCCGCTTCC R-GCCTTGACCTTTTCAGCAAGTG	174	250/250	.996	99
UCHL5	NM_174481.3	F-CAAAGACAACTTGCTGAGGAACC R-ACTGCTTGTGTTCTGCTAAAGTC	208	333/333	.990	96
Target genes						
GPX1	NM_174076.3	F-GAGCCCTTCAACCTGTCCTC R-GCGTTTTCCTGATGCCCAAAC	179	250/250	.999	93
NRF2	NM_001011678.2	F-AGCTCAGCATGATGGACTTGGA R-CAGCTCATGCTCCTTCTGTCG	152	333/333	.999	92
PARP1	NM_174751.2	F-CGGACAGATGTTTCAGGCAAAG R-TGGGGCTTATCGGGGTACA	179	250/250	.983	93
SOD2	NM_201527.2	F-CGTGACTTTGGTTCCTTTGCC R-GCGTCCCTGCTCCTTATTGA	108	250/250	.998	100
XDH	NM_173972.2	F-AAGTCACGGCTCTCAGTGTG R-CCACAGCATCCACCATTCTTG	192	250/250	.991	94

B2M, beta 2-microglobulin; RPLP0, ribosomal protein, large, P0; UCHL5, ubiquitin carboxyl-terminal hydrolase L5; GPX1, glutathione peroxidase 1; NRF2, nuclear factor (erythroid-derived 2)-like 2; PARP1, poly (ADP-ribose) polymerase 1; SOD2, superoxide dismutase 2, mitochondrial; XDH, xanthine dehydrogenase.

reference genes B2M, UCHL5 and RPLP0 (mean reference target stability *M* value = 0.43) as normalisation factor, relating the data to the geometric mean of all samples at time point −42 and considering the primer pair specific amplification efficiencies (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007). Statistical analysis was performed on the log data, and for better interpretation, the data were log back transformed for charts.

2.6 | Statistical analysis

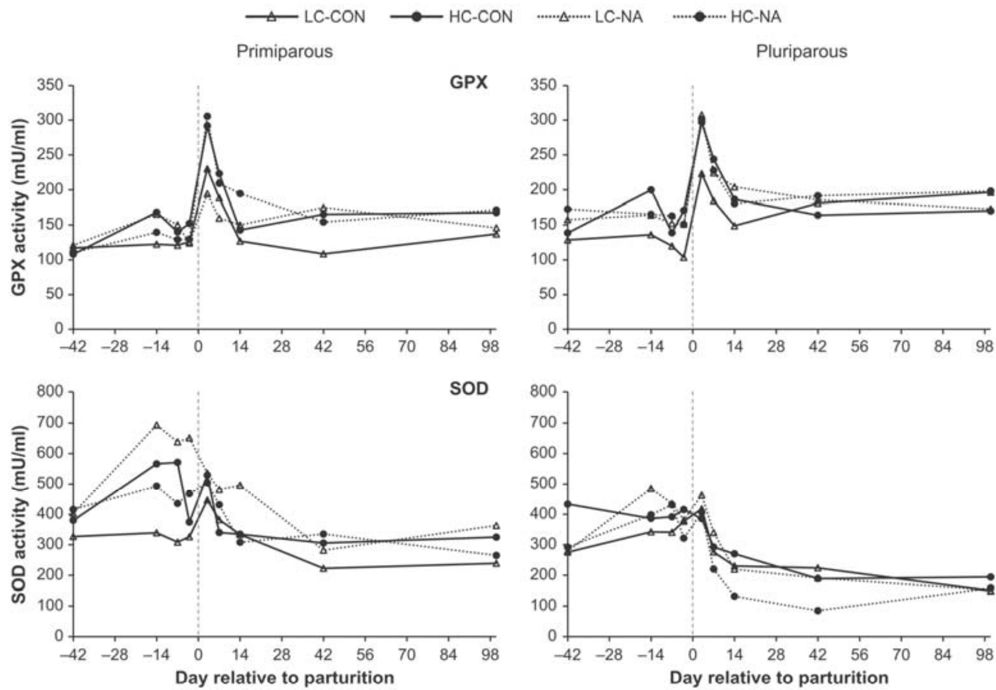
Statistical analyses were performed using the PROC MIXED procedure of SAS software package (version 9.4; SAS Institute, Cary, NC, USA) with the restricted maximum likelihood method. The model included time point (*T*; experimental day relative to parturition), dietary concentrate proportion (C; LC or HC diet), supplementation (S; CON or NA), parity (P; primiparous or pluriparous cows) and the quadruple interaction between $T \times C \times S \times P$ as fixed effects. The frequent measurements during experiment for each individual cow were considered as repeated measurement. The covariance structure compound symmetry (CS) was used showing the lowest Akaike information criterion (AICC). Degrees of freedom were calculated using the Kenward–Roger adjustment. Statistical differences were declared significant at $p \le .05$ and tendencies at $0.10 \ge p > .05$. All results are presented as LS means and pooled standard errors (PSE) are stated. For correlation studies, Spearman's rank correlation was calculated using Statistica (version 12, Statistica for Windows. Version 12; Statsoft Inc, Tulsa, OK, USA).

3 | RESULTS

Data on feed intake, milk yield, NA and NAM serum concentrations have been previously published (Tienken, Kersten, Frahm, Huther, et al., 2015; Tienken, Kersten, Frahm, Meyer, et al., 2015). Briefly summarised, prepartum DMI was 13.2 ± 0.3 kg/day for LC animals and 15.1 ± 0.3 kg/day for HC animals, whereas it did not differ significantly in the lactation period (17.0 ± 0.4 kg/day, LC; 16.7 ± 0.3 kg/day, HC) (Tienken, Kersten, Frahm, Meyer, et al., 2015). DMI for pluriparous cows was prepartum 2.7 kg/day and in the lactation period 3.1 kg/day higher than for primiparous cows. NA supplementation had no effect on DMI. Milk yield was not influenced by feeding strategies, but it was 11.8 kg/day higher for pluriparous (39.1 ± 1.0 kg/day) than for primiparous (27.3 ± 1.2 kg/day) cows. NA concentrations were not detectable in serum samples; however, NAM concentrations increased with NA supplementation to mean values of 3.35 ± 1.65 µg/ml compared to 2.01 ± 1.10 µg/ml in CON animals during the supplementation period (Tienken, Kersten, Frahm, Huther, et al., 2015).

3.1 | Antioxidant enzyme activity

Glutathione peroxidase (GPX) and superoxide dismutase (SOD) serum activities fluctuated significantly in the course of the experiment (Figure 1). GPX activity showed a pronounced peak at 3 DIM for all animals. Postpartum activities were higher than the initial values at



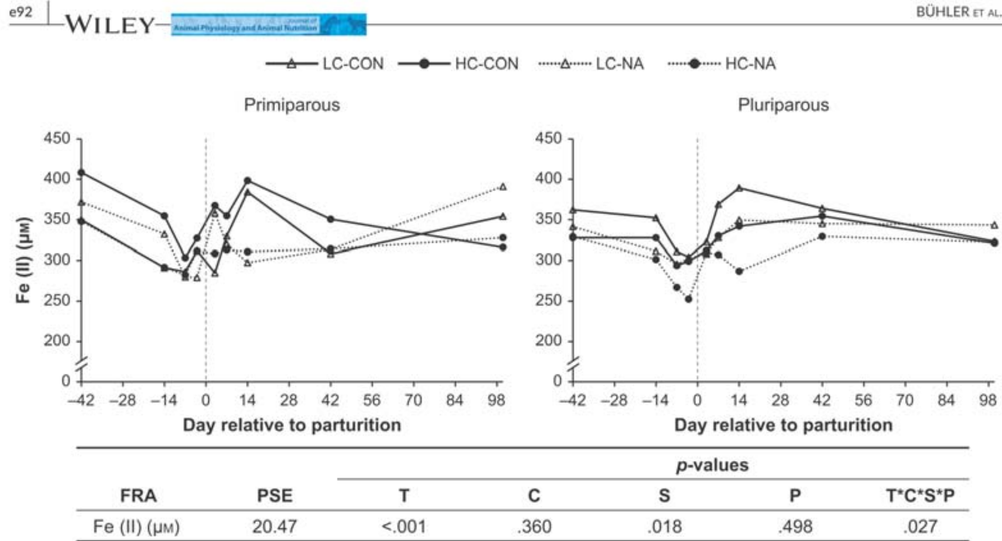
Enzyme	PSE	*p*-values T	C	S	P	T*C*S* P
GPX	26.15	<.001	.026	.193	.014	.947
SOD	62.94	<.001	.809	.226	.001	.094

FIGURE 1 Antioxidant enzyme activities of glutathione peroxidase (GPX, upper panel) and superoxide dismutase (SOD, lower panel) in serum of primiparous (left column) and pluriparous (right column) periparturient dairy cows. Animals received prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition, cows were initially fed with a diet consisting of 30% concentrate. The concentrate proportion was continuously increased up to 50% either within 16 (LC) or 24 days (HC). Animals were fed a diet either with (NA, 24 g NA/day from day 42 prepartum until 24 DIM) or without (CON) nicotinic acid. The dashed line indicates parturition. Symbols denote LS means (*n* − 14 = 44, *n* − 7 = 36, *n* − 3 = 38, all other time points *n* = 47). PSE, pooled standard error; *T*, time relative to parturition; C, concentrate level; S, supplementation; P, parity

day −42 for all groups with the exception of LC-CON in primiparous animals at 42 DIM. Additionally, a significant concentrate effect was detected that arose from the higher GPX activities in the HC groups around parturition, seen more pronounced in primiparous cows. Mostly driven was the concentrate effect, however, by the low GPX activities in the LC-CON group in pluriparous animals from time point −42 until +14 and in primiparous animals at 42 DIM. The parity effect for GPX activity resulted from the higher activities in pluriparous animals at time points −42, 42 and 100 DIM. SOD activity in serum showed a different course over time. A clear peak was not visible at a particular time point for all animals, but signals increased in the course of the experiment before or around parturition. Postpartal activities were lower than prepartal ones starting from 14 DIM for cows and 42 DIM for heifers. Levels of SOD activity in primiparous cows were higher compared to pluriparous cows clearly seen postpartum starting from 7 DIM. The LC-NA group showed the highest SOD activities from −14 until 14 DIM in primiparous cows, whereas the HC-NA group exhibited lowest activities in pluriparous animals postpartum at 7, 14 and 42 DIM. These preceding effects gave rise to a tendency in the interaction $T \times C \times S \times P$ for SOD activity.

3.2 | Ferric reducing ability

Ferric reducing ability in serum varied significantly over time compared to parturition (Figure 2). The nadir seen before parturition was more distinct in pluriparous cows, although the decrease in FRA was



FRA	PSE	T	C	S	P	T*C*S*P
		p-values				
Fe (II) (μM)	20.47	<.001	.360	.018	.498	.027

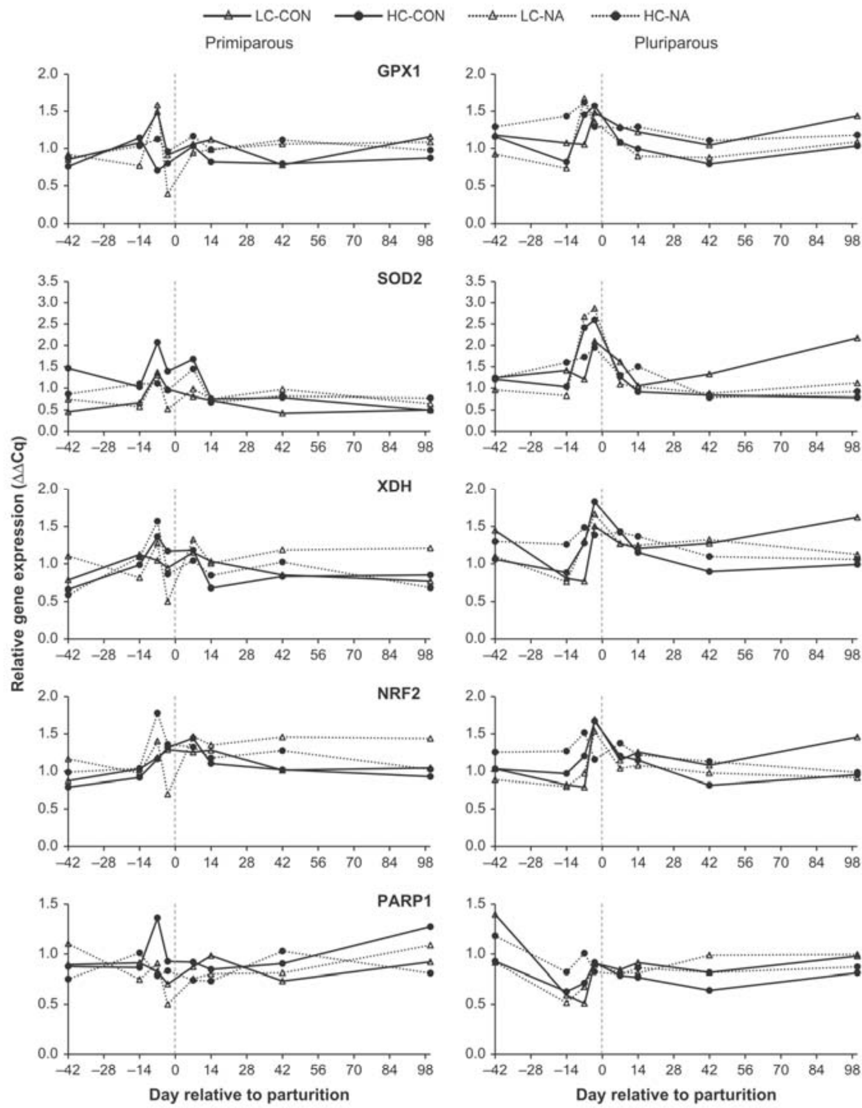
FIGURE 2 Ferric reducing ability (FRA) of serum of primiparous (left panel) and pluriparous (right panel) periparturient dairy cows measured as Fe^{2+} equivalents. Animals received prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition, cows were initially fed with a diet consisting of 30% concentrate. The concentrate proportion was continuously increased up to 50% either within 16 (LC) or 24 days (HC). Animals were fed a diet either with (NA, 24 g NA/day from day 42 prepartum until 24 DIM) or without (CON) nicotinic acid. The dashed line indicates parturition. Symbols denote LS means ($n - 14 = 44$, $n - 7 = 36$, $n - 3 = 38$, all other time points $n = 47$). PSE, pooled standard error; *T*, time relative to parturition; C, concentrate level; S, supplementation; P, parity

higher in primiparous animals as the −42 values were higher than in pluriparous animals. At 14 DIM, the niacin-supplemented animals showed a reduced FRA in primiparous animals that was seen in pluriparous animals only for the HC-NA group. However, this group showed the lowest FRA at time points −7 and −3 in pluriparous cows. These observations lead to a significant interaction for *T* × C × S × P.

3.3 | Gene expression analysis

All oxidative stress-related genes measured in this study changed significantly over time compared to parturition (Figure 3). Except for PARP1, all genes showed peak expression levels around parturition that were more pronounced in pluriparous cows. For GPX1 mRNA abundance, a significant parity effect was detected additionally. The pluriparous animals exhibited higher mRNA levels than the primiparous ones, especially at days 42 and 3 before parturition. In case of SOD2 mRNA abundance, the interaction of *T* × C × S × P became significant mainly driven by the higher gene expression levels in pluriparous animals at time point −3 as well as 14 and 100 DIM. The higher SOD2 mRNA abundance in primiparous HC animals at the time points −42 and +7 and the increased SOD2 gene expression level at 42 and 100 DIM in the LC-CON group of pluriparous animals also contributed to the significant interaction of *T* × C × S × P. For XDH, a tendency in the interaction *T* × C × S × P was observed that originated from the higher mRNA abundances in pluriparous cows at time points −42, −3, +14 and +100. Moreover, the XDH gene expression levels of supplemented animals at 42 DIM were higher than in not supplemented cows. This effect was more pronounced in primiparous animals. The pluriparous cows, however, showed at 42 and 100 DIM lower mRNA abundances in the HC groups compared to the LC groups. Regarding PARP1, the significant interaction of *T* × C × S × P was mostly driven by the pronounced decrease in mRNA abundance in pluriparous animals between time points −42 and −14. Additionally, in the two weeks before parturition, the HC animals showed a higher PARP1 mRNA level than the LC animals that was more pronounced in pluriparous cows. Furthermore, at 42 DIM, the supplemented animals

FIGURE 3 Relative expression levels of oxidative stress-related genes in blood leucocytes of primiparous (left column) and pluriparous (right column) periparturient dairy cows. Animals received prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition, cows were initially fed with a diet consisting of 30% concentrate. The concentrate proportion was continuously increased up to 50% either within 16 (LC) or 24 days (HC). Animals were fed a diet either with (NA, 24 g NA/day from day 42 prepartum until 24 DIM) or without (CON) nicotinic acid. The dashed line indicates parturition. Symbols denote LS means ($n - 14 = 34$, $n - 7 = 25$, $n - 3 = 19$, all other time points $n = 47$). GPX1, glutathione peroxidase 1; SOD2, superoxide dismutase 2; XDH, xanthine dehydrogenase; NRF2, nuclear factor (erythroid-derived 2)-like 2; PARP1, poly (ADP-ribose) polymerase 1; PSE, pooled standard error; *T*, time relative to parturition; C, concentrate level; S, supplementation; P, parity



Gene	PSE	*p*-values				
		T	C	S	P	T*C*S*P
GPX1	.165	.016	.877	.706	.001	.109
SOD2	.282	<.001	.223	.782	<.001	.030
XDH	.197	.008	.795	.569	.001	.065
NRF2	.198	<.001	.675	.528	.697	.267
PARP1	.127	.001	.436	.674	.332	.038

TABLE 2 Spearman's rank correlation coefficients between anti-oxidative parameters in serum and metabolic and blood parameters ($p < .001$)

Parameter	FRA	GPX	SOD
DMI	n.s.	n.s.	−0.309
EB	n.s.	−0.248	0.334
BCS	−0.308	n.s.	0.239
glucose	−0.167	−0.264	0.236
TG	n.s.	−0.309	0.207
NEFA	n.s.	0.270	n.s.
BHB	n.s.	0.214	n.s.
Cholesterol	0.217	n.s.	−0.482
AST	n.s.	0.407	n.s.
γ-GT	n.s.	0.307	−0.242
GLDH	n.s.	n.s.	−0.225
Hb	n.s.	n.s.	0.280

FRA, ferric reducing ability; GPX, glutathione peroxidase activity; SOD, superoxide dismutase activity; DMI, dry matter intake; EB, energy balance; BCS, body condition score; TG, triglyceride; NEFA, non-esterified fatty acid; BHB, beta-hydroxybutyrate; AST, aspartate aminotransferase; γ-GT, gamma glutamyl transferase; GLDH, glutamate dehydrogenase; Hb, haemoglobin; n.s., not significant.

showed a higher gene expression level for PARP1 than the not supplemented animals.

3.4 | Spearman's rank correlations

Spearman's rank correlations were calculated to examine interesting co-progressions of the anti-oxidative parameters in serum with metabolic and blood parameters (Table 2). FRA was negatively correlated with BCS and glucose and positively correlated with cholesterol. GPX activity was inversely correlated with energy balance (EB), glucose and triglyceride (TG) and positively correlated with NEFA and BHB as well as with the enzymes aspartate aminotransferase (AST) and gamma glutamyl transferase (γ-GT). Positive correlations with SOD activity were detected for EB, BCS, glucose, TG and haemoglobin (Hb), whereas dry matter intake (DMI), cholesterol as well as enzymes γ-GT and glutamate dehydrogenase (GLDH) showed negative correlations.

4 | DISCUSSION

During the transition from late gestation to early lactation, dairy cows are confronted with dramatic metabolic and physiological changes that are accompanied by increased inflammation (Sordillo, Contreras, & Aitken, 2009; Trevisi, Amadori, Cogrossi, Razzuoli, & Bertoni, 2012), oxidative stress (Sordillo & Aitken, 2009) and a dysfunctional immune response (Ingvartsen & Moyes, 2013). Nutrient metabolism, oxidative stress and inflammation are hereby interlinked as reviewed by Sordillo and Mavangira (2014).

The experiment of the present study aimed at inducing differences in energy metabolism and oxidative status of periparturient dairy heifers and cows by feeding different energy dense diets prepartum, different rates in dietary energy concentration increase postpartum, and a nicotinic acid supplementation from 42 days prepartum until 24 DIM. The effects of these feeding strategies on anti-oxidative parameters in serum and on oxidative stress-related gene expression in blood leucocytes were investigated. This study was part of a larger trial from which feeding effects on production, metabolic, haematological and biochemical parameters are previously published (Tienken, Kersten, Frahm, Huther, et al., 2015; Tienken, Kersten, Frahm, Meyer, et al., 2015). Briefly summarised, the feeding strategies had no effects on the metabolic parameters NEFA and BHB.

4.1 | Effects of time

All measured parameters showed significant changes over time mainly due to parturition. The increased antioxidant activities of GPX and SOD as well as the decrease in FRA around parturition are in accordance with existing literature (Bernabucci et al., 2005; Didara et al., 2015; Wullepit et al., 2012). Together they confirm the increased need for antioxidants around parturition. However, antioxidants are not only used for eliminating ROS. They are also transferred into colostrum (Goff & Stabel, 1990). This partly explains the reduced FRA and therefore antioxidants availability at parturition, additionally enhanced by a decreased intake of dry matter and hence dietary antioxidants during this time. Accordingly, it was shown that non-enzymatic antioxidants like retinol and α-tocopherol that comprise part of the FRA capacity are strongly decreased around parturition (Gessner et al., 2015; Goff & Stabel, 1990). The well-established oxidative stress during the transition period originates from multiple factors: stress of parturition, stress of metabolic adjustments to the negative energy balance with its increased lipolysis and stressors thereof, the increased energy need with enhanced cell metabolism and cellular respiration and increased ROS production because of inflammatory processes. All this explains the increased activities of SOD and GPX and the reduced FRA around parturition. The increased metabolic activity that is necessary for milk production and the subsequent increased ROS formation as a by-product of the respiratory chain, however, should take place in a more extended period, as milk production continues and reaches peak levels approximately 42 DIM (Tienken, Kersten, Frahm, Meyer, et al., 2015). This may explain the higher levels of GPX activity postpartum compared to activities at 42 days before parturition seen also by Bernabucci et al. (2005) and Didara et al. (2015). However, as the GPX serum activity correlated with the enzymes AST and γ-GT, which are measured to assess liver function, the higher GPX activities postpartum could also result from liver cell damage and subsequent leakage of cytosolic GPX into serum. Postpartum SOD activities decreased considerably even under the values of 42 days before parturition. Reduction in zinc and copper availability in the early postpartum period of dairy cows might explain the reduction in SOD activity, especially as a supplementation with trace minerals including Zn and Cu was demonstrated to increase postpartum oxygen radical absorbance capacity

(ORAC) (Osorio et al., 2016). Extracellular SOD, usually bound to the cell surface, can get glycosylated depending on blood glucose level and released into serum (Fattman, Schaefer, & Oury, 2003). For example, it is reported that in diabetic patients higher concentrations of glycated extracellular SOD are found in the serum (Adachi, Ohta, Hirano, Hayashi, & Marklund, 1991). As in this trial the glucose concentration decreased postpartum (Tienken, Kersten, Frahm, Meyer, et al., 2015), this could explain a reduced SOD serum activity. Supported is this view additionally by the correlation of serum SOD activity and glucose concentration. The fact that the nadir for FRA precedes the peak activities for antioxidant enzyme activities allows the assumption that the enzymatic activities are an adaptation mechanism to increased ROS production before parturition. This is furthermore supported by the peak expression levels of oxidative stress-related genes before parturition. NRF2, a redox-sensitive transcription factor which regulates the transcription of many genes with anti-oxidative and cytoprotective functions (Ma, 2013) as well as the anti-oxidative genes GPX1 and SOD2, showed peak expression levels just before parturition also indicating an oxidative tensed situation at this time point. Increased expression levels around parturition for GPX and SOD were also found in spleen, liver and neutrophils (Loor et al., 2005; Morris et al., 2009; Zhou et al., 2015). Gessner et al. (2013) showed a strong upregulation of Nrf2 target genes in the liver of transition dairy cows and concluded a protective mechanism of the liver against damage by inflammation processes and increased ROS generation. The increase in NRF2, GPX1 and SOD2 gene expression seen in this study could hint at a similar conclusion for blood leucocytes. The calculation of Spearman's rank correlations revealed clear positive correlations ($p < .001$) with the granulocyte/lymphocyte ratio for GPX1 (0.216) and SOD2 (0.506) mRNA abundance (data not shown). This ratio exhibited peak values just before parturition (Bühler et al., 2016), and the peaks in mRNA abundance may therefore represent the increased granulocyte compared to lymphocyte numbers. As granulocytes are the first line of defence of the innate immune system via killing invading bacteria with ROS production, the increased expression levels of anti-oxidative genes may protect the cells against ROS damage during the time of parturition that goes in hand with invading organisms. XDH can be converted to xanthine oxidase (XO) by protein modification and as such generates ROS that are involved in cell signalling and anti-microbial defence (Harrison, 2004). An increase in the expression of this gene therefore implies also a possible increase in ROS production around parturition. A most interesting progression in mRNA abundance was displayed by PARP1. Its gene expression level strongly decreased between 42 and 14 days before parturition. PARP1 activity gets induced by DNA strand breaks caused among other things by ROS and takes considerable part in the regulation of oxidative stress-induced cell death (Hegedus & Virag, 2014). Investigations into the apoptotic behaviour of blood leucocytes (Bühler et al., 2016) revealed a strong decrease in the percentage of apoptotic cells in the same time frame from −42 to −14. Therefore, a reduced gene expression level of PARP1 may explain a reduced apoptotic behaviour of the blood leucocytes. PARP1 is, however, also influenced by metabolic factors. It consumes NAD+ for its activity and it was shown in mice that PARP1 activity decreases with fasting (Bai et al., 2011). Furthermore, PARP1 inhibition in cells increases the NAD+ content and enhances oxidative metabolism (Bai et al., 2011). Hence, the decreased PARP1 mRNA abundance could be a consequence of decreased energy availability. However, to further elucidate these apparently opposing effects more research is needed especially with regard to regulation on gene expression and protein level.

4.2 | Effects of energy supply

Prepartal energy supply is known to affect oxidative parameters (Graugnard et al., 2012; Khan et al., 2015; Zhou et al., 2015). In this study, a clear concentrate effect was detected for GPX activity. HC animals exhibited higher GPX activity than LC animals, especially in the transition period. It was shown in liver as well as in neutrophils that with a higher prepartal energy level the expression of NFκB and inflammatory genes is increased (Khan et al., 2015; Zhou et al., 2015). As a consequence, NRF2 expression could also be elevated to exert its anti-inflammatory and cytoprotective functions (Kim, Cha, & Surh, 2010) and thus GPX gene expression could be increased. This study revealed a concentrate effect neither on NRF2 nor on GPX1 gene expression in blood leucocytes, suggesting that the higher GPX enzyme activity may result from increased expression in other tissues. However, to elucidate this point further, also the expression of the extracellular GPX isoform GPX3 should be evaluated. The LC-CON group showed the lowest GPX activities throughout the experiment. This suggests that with a lower energy level a lower metabolic rate with lower cellular respiration and a lower burden of produced ROS develops, and therefore, less antioxidant activity is necessary. However, this was not paralleled to the same extent by FRA and SOD activity. In the two weeks before parturition, higher gene expression levels were detected in the HC groups for the genes SOD2, XDH and PARP1. SOD2 as being the mitochondrial superoxide dismutase directly involved with the respiratory chain may reflect the higher cell metabolism with higher energy availability. XDH involved in ROS production is also higher with more energy around, and as mentioned above, PARP1 may decrease in its mRNA abundance more slowly when more energy is available. Postpartal mRNA abundances of SOD2 and GPX1 seemed to be lower in the HC group. The LC-CON group showed a strong increase in mRNA abundance of these genes at 100 DIM in pluriparous cows. This is paralleled by an increased granulocyte-to-lymphocyte ratio (Bühler et al., 2016) and therefore possibly reflecting a change in blood cell type.

4.3 | Effects of nicotinic acid supplementation

Niacin being a precursor for NAD+ and NADP+ is involved in metabolic processes, oxidative stress and inflammatory processes. Thus, and additionally because of its potential to exert anti-lipolytic effects, it is a most interesting supplementation to examine in the transition period of dairy cows, where all these processes are in disarray because of the metabolic adaptations and the confrontation with inflammation. However, we could not demonstrate clear-cut supplementation

effects on the measured parameters. This may be due to the fact that the experiment was not able to provoke differences in the metabolic parameters NEFA and BHB (Tienken, Kersten, Frahm, Meyer, et al., 2015). Nevertheless, LC-NA animals exhibited highest values for SOD activity before parturition until 7 DIM, whereas LC-CON animals showed lowest SOD activities. This could be evidence for an increased energy status in the LC-NA animals due to higher NAD+ and NADP+ levels, leading to an increase in cell respiration and consequently SOD activity. However, this was not paralleled in the HC animals. At 14 DIM, FRA values in the supplemented animals were clearly lower than in the not supplemented ones suggesting an oxidative more stressed situation with a concomitant reduced anti-oxidative capacity in the serum. But no similar effect was seen in antioxidant enzyme activity. NA is known to decrease plasma cholesterol in humans as a consequence of its anti-lipolytic effect (Carlson, 2005). As the fat-soluble antioxidant vitamin E is transported in blood as a component of plasma lipoproteins (Herdt & Smith, 1996), NA could have decreased the vitamin E concentration and therefore FRA values. Cholesterol values were decreased around parturition (Tienken, Kersten, Frahm, Huther, et al., 2015) and the correlation of FRA with cholesterol (0.217, Table 2) supports the association; however, NA had no significant effect on cholesterol in this trial (Tienken, Kersten, Frahm, Huther, et al., 2015). Therefore, the question why FRA values decreased in NA-supplemented animals at 14 DIM needs further research.

4.4 | Effects of parity

Heifers exhibit higher variability in measured parameters during the transition period than pluriparous cows (Jonsson et al., 2013; Tienken, Kersten, Frahm, Huther, et al., 2015) which is also confirmed in this study.

Only few investigations, however, compare anti-oxidative and oxidative parameters in heifers vs. cows (Pilarczyk et al., 2012). The results of the present study made it quite obvious that cows differing in age have different levels of antioxidants and oxidative stress-related genes. Future research on oxidative status in periparturient animals should take this more into account when assigning animals to treatment groups. Pluriparous animals exhibited higher GPX activities in serum as well as higher GPX1, SOD2 and XDH mRNA abundances in blood leucocytes. Serum SOD activities were higher in primiparous animals, and the nadir in FRA just before parturition was more distinct in pluriparous cows. All this suggests that pluriparous cows are confronted with a greater oxidative burden than primiparous cows. Pluriparous cows show a greater DMI throughout the experiment, lower glucose levels prepartum and a higher milk yield postpartum (Tienken, Kersten, Frahm, Meyer, et al., 2015), thus explaining the need for increased anti-oxidative capacity. Furthermore, the decrease in PARP1 mRNA abundance antepartum was more pronounced in pluriparous cows. As PARP1 gene expression may be associated with energy availability (Bai et al., 2011), this is also indicative for a decreased energy availability in blood leucocytes because of increased metabolic activity in pluriparous cows.

4.5 | Spearman's rank correlations

Also the Spearman rank correlations confirm the connection between energy level, metabolism and anti-oxidative capacity. FRA is inversely correlated with BCS and glucose. Bernabucci et al. (2005) reported that the animals experience more oxidative stress with higher BCS, which implicates a decreased anti-oxidative capacity and thus FRA. With more available glucose and thus more metabolic activity, FRA also depleted explaining the negative correlation. The inverse correlation of GPX serum activity with the parameters EB, glucose and TG suggests that extracellular GPX activity originates from other factors than increased metabolic activity and cell respiration. The positive correlations of GPX serum activity with the parameters NEFA and BHB that are associated with metabolic stress in liver and the additional correlations of GPX with AST and γ-GT may point towards liver cell damage and accompanied leakage of cytosolic GPX activity. In contrast, increasing energy means increasing serum SOD activity seen with the positive correlations of SOD activity and EB, glucose and TG. Additionally, SOD activity correlates with Hg suggesting an increased cell respiration with available energy and the associated necessary SOD activity. DMI and cholesterol are correlated (0.672, data not shown) and SOD activity is inversely correlated with both. Higher cholesterol means higher liver turnover with its implications to liver damage. Therefore, SOD activity also correlates negatively to liver enzymes γ-GT and GLDH. The correlations suggest that the antioxidant serum enzyme activities of GPX and SOD originate from different cellular sources.

5 | CONCLUSION

This study examined the influences of different dietary energy levels and a nicotinic acid supplementation on the anti-oxidative capacity of serum and on the expression of oxidative stress-related genes in blood leucocytes of periparturient heifers and cows. It confirmed the increased need for antioxidants at parturition. Together with the peak expressions of oxidative stress-related genes in blood leucocytes before calving, it supports findings pointing at oxidative stress around parturition. Higher dietary energy level induced higher GPX activity and higher expression levels for some of the evaluated genes and anti-oxidative parameters correlate with energy and metabolic parameters. Heifers and cows clearly exhibited different levels in anti-oxidative capacity and oxidative stress-related gene expression. Nicotinic acid supplementation increased prepartal serum SOD activity in animals fed with a lower concentrate level maybe indicating a higher metabolic activity in these animals.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

The authors thank the Deutsche Forschungsgemeinschaft (DFG, Bonn, Germany) for financial support (DA 558/6-1). The help of all

co-workers of the Institute of Animal Nutrition and the Experimental Station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute in Braunschweig, Germany, who supported this work is gratefully acknowledged.

REFERENCES

Adachi, T., Ohta, H., Hirano, K., Hayashi, K., & Marklund, S. L. (1991). Non-enzymic glycation of human extracellular superoxide dismutase. *Biochemical Journal*, *279*, 263–267.

Adewuyi, A. A., Gruys, E., & van Eerdenburg, F. J. (2005). Non esterified fatty acids (NEFA) in dairy cattle. A review. *Veterinary Quarterly*, *27*, 117–126.

Bai, P., Cantó, C., Oudart, H., Brunyánszki, A., Cen, Y., Thomas, C., ... Auwerx, J. (2011). PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation. *Cell Metabolism*, *13*, 461–468.

Bell, A. W. (1995). Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. *Journal of Animal Science*, *73*, 2804–2819.

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry*, *239*, 70–76.

Bernabucci, U., Ronchi, B., Lacetera, N., & Nardone, A. (2002). Markers of oxidative status in plasma and erythrocytes of transition dairy cows during hot season. *Journal of Dairy Science*, *85*, 2173–2179.

Bernabucci, U., Ronchi, B., Lacetera, N., & Nardone, A. (2005). Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. *Journal of Dairy Science*, *88*, 2017–2026.

Brassard, P., Larbi, A., Grenier, A., Frisch, F., Fortin, C., Carpentier, A. C., & Fulop, T. (2007). Modulation of T-cell signalling by non-esterified fatty acids. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *77*, 337–343.

Bühler, S., Frahm, J., Tienken, R., Kersten, S., Meyer, U., Huber, K., & Dänicke, S. (2016). Influence of energy level and nicotinic acid supplementation on apoptosis of blood leukocytes of periparturient dairy cows. *Veterinary Immunology and Immunopathology*, *179*, 36–45.

Carlson, L. A. (2005). Nicotinic acid: The broad-spectrum lipid drug. A 50th anniversary review. *Journal of Internal Medicine*, *258*, 94–114.

Castillo, C., Hernandez, J., Bravo, A., Lopez-Alonso, M., Pereira, V., & Benedito, J. L. (2005). Oxidative status during late pregnancy and early lactation in dairy cows. *Veterinary Journal*, *169*, 286–292.

Didara, M., Poljicak-Milas, N., Milinkovic-Tur, S., Masek, T., Suran, J., Pavic, M., ... Speranda, M. (2015). Immune and oxidative response to linseed in the diet of periparturient Holstein cows. *Animal*, *9*, 1349–1354.

Drackley, J. K. (1999). ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: The final frontier? *Journal of Dairy Science*, *82*, 2259–2273.

Fattman, C. L., Schaefer, L. M., & Oury, T. D. (2003). Extracellular superoxide dismutase in biology and medicine. *Free Radical Biology & Medicine*, *35*, 236–256.

Gessner, D. K., Most, E., Schlegel, G., Kupczyk, K., Schwarz, F. J., & Eder, K. (2015). Concentrations of retinol and tocopherols in the milk of cows supplemented with conjugated linoleic acid. *Journal of Animal Physiology and Animal Nutrition*, *99*, 1039–1046.

Gessner, D. K., Schlegel, G., Keller, J., Schwarz, F. J., Ringseis, R., & Eder, K. (2013). Expression of target genes of nuclear factor E2-related factor 2 in the liver of dairy cows in the transition period and at different stages of lactation. *Journal of Dairy Science*, *96*, 1038–1043.

GfE (2001). *Empfehlungen zur Energie- und Nährstoffversorgung von Milchkühen und Aufzuchtrindern (Recommendations of energy and nutrient supply for dairy cows and breeding cattle)*. Frankfurt am Main, Germany: DLG-Verlag.

Goff, J. P., & Horst, R. L. (1997). Physiological changes at parturition and their relationship to metabolic disorders. *Journal of Dairy Science*, *80*, 1260–1268.

Goff, J. P., & Stabel, J. R. (1990). Decreased plasma retinol, alpha-tocopherol, and zinc concentration during the periparturient period: Effect of milk fever. *Journal of Dairy Science*, *73*, 3195–3199.

Graugnard, D. E., Bionaz, M., Trevisi, E., Moyes, K. M., Salak-Johnson, J. L., Wallace, R. L., ... Loor, J. J. (2012). Blood immunometabolic indices and polymorphonuclear neutrophil function in peripartum dairy cows are altered by level of dietary energy prepartum. *Journal of Dairy Science*, *95*, 1749–1758.

Hammon, D. S., Evjen, I. M., Dhiman, T. R., Goff, J. P., & Walters, J. L. (2006). Neutrophil function and energy status in Holstein cows with uterine health disorders. *Veterinary Immunology and Immunopathology*, *113*, 21–29.

Harrison, R. (2004). Physiological roles of xanthine oxidoreductase. *Drug Metabolism Reviews*, *36*, 363–375.

Hayirli, A., Grummer, R. R., Nordheim, E. V., & Crump, P. M. (2002). Animal and dietary factors affecting feed intake during the prefresh transition period in Holsteins. *Journal of Dairy Science*, *85*, 3430–3443.

Hegedus, C., & Virag, L. (2014). Inputs and outputs of poly(ADP-ribosyl) ation: Relevance to oxidative stress. *Redox Biology*, *2*, 978–982.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., & Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology*, *8*, R19.

Herdt, T. H., & Smith, J. C. (1996). Blood-lipid and lactation-stage factors affecting serum vitamin E concentrations and vitamin E cholesterol ratios in dairy cattle. *Journal of Veterinary Diagnostic Investigation*, *8*, 228–232.

Ingvartsen, K. L., & Moyes, K. (2013). Nutrition, immune function and health of dairy cattle. *Animal*, *7*(Suppl 1), 112–122.

Ingvartsen, K. L., & Moyes, K. M. (2015). Factors contributing to immunosuppression in the dairy cow during the periparturient period. *Japanese Journal of Veterinary Research*, *63*(Suppl 1), S15–S24.

Jonsson, N. N., Fortes, M. R., Piper, E. K., Vankan, D. M., de Cisneros, J. P., & Wittek, T. (2013). Comparison of metabolic, hematological, and peripheral blood leukocyte cytokine profiles of dairy cows and heifers during the periparturient period. *Journal of Dairy Science*, *96*, 2283–2292.

Kenez, A., Locher, L., Rehage, J., Danicke, S., & Huber, K. (2014). Agonists of the G protein-coupled receptor 109A-mediated pathway promote antilipolysis by reducing serine residue 563 phosphorylation of hormone-sensitive lipase in bovine adipose tissue explants. *Journal of Dairy Science*, *97*, 3626–3634.

Kenez, A., Tienken, R., Locher, L., Meyer, U., Rizk, A., Rehage, J., ... Huber, K. (2015). Changes in lipid metabolism and beta-adrenergic response of adipose tissues of periparturient dairy cows affected by an energy-dense diet and nicotinic acid supplementation. *Journal of Animal Science*, *93*, 4012–4022.

Khan, M. J., Jacometo, C. B., Riboni, M. V., Trevisi, E., Graugnard, D. E., Correa, M. N., & Loor, J. J. (2015). Stress and inflammatory gene networks in bovine liver are altered by plane of dietary energy during late pregnancy. *Functional & Integrative Genomics*, *15*, 563–576.

Kim, J., Cha, Y. N., & Surh, Y. J. (2010). A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. *Mutation Research*, *690*, 12–23.

Lacetera, N., Scalia, D., Franci, O., Bernabucci, U., Ronchi, B., & Nardone, A. (2004). Short communication: Effects of nonesterified fatty acids on lymphocyte function in dairy heifers. *Journal of Dairy Science*, *87*, 1012–1014.

Lee, J. Y., Plakidas, A., Lee, W. H., Heikkinen, A., Chanmugam, P., Bray, G., & Hwang, D. H. (2003). Differential modulation of Toll-like receptors by fatty acids: Preferential inhibition by n-3 polyunsaturated fatty acids. *Journal of Lipid Research*, *44*, 479–486.

Lee, J. Y., Sohn, K. H., Rhee, S. H., & Hwang, D. (2001). Saturated fatty acids, but not unsaturated fatty acids, induce the expression of

cyclooxygenase-2 mediated through Toll-like receptor 4. *Journal of Biological Chemistry*, *276*, 16683–16689.

Loor, J. J., Dann, H. M., Everts, R. E., Oliveira, R., Green, C. A., Guretzky, N. A., … Drackley, J. K. (2005). Temporal gene expression profiling of liver from periparturient dairy cows reveals complex adaptive mechanisms in hepatic function. *Physiological Genomics*, *23*, 217–226.

Ma, Q. (2013). Role of nrf2 in oxidative stress and toxicity. *Annual Review of Pharmacology and Toxicology*, *53*, 401–426.

Maiese, K., Chong, Z. Z., Hou, J., & Shang, Y. C. (2009). The vitamin nicotinamide: Translating nutrition into clinical care. *Molecules*, *14*, 3446–3485.

Miller, J. K., Brzezinska-Slebodzinska, E., & Madsen, F. C. (1993). Oxidative stress, antioxidants, and animal function. *Journal of Dairy Science*, *76*, 2812–2823.

Morey, S. D., Mamedova, L. K., Anderson, D. E., Armendariz, C. K., Titgemeyer, E. C., & Bradford, B. J. (2011). Effects of encapsulated niacin on metabolism and production of periparturient dairy cows. *Journal of Dairy Science*, *94*, 5090–5104.

Morris, D. G., Waters, S. M., McCarthy, S. D., Patton, J., Earley, B., Fitzpatrick, R., … Wathes, D. C. (2009). Pleiotropic effects of negative energy balance in the postpartum dairy cow on splenic gene expression: Repercussions for innate and adaptive immunity. *Physiological Genomics*, *39*, 28–37.

Osorio, J. S., Trevisi, E., Li, C., Drackley, J. K., Socha, M. T., & Loor, J. J. (2016). Supplementing Zn, Mn, and Cu from amino acid complexes and Co from cobalt glucoheptonate during the peripartal period benefits postpartal cow performance and blood neutrophil function. *Journal of Dairy Science*, *99*, 1868–1883.

Paglia, D. E., & Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory and Clinical Medicine*, *70*, 158–169.

Pilarczyk, B., Jankowiak, D., Tomza-Marciniak, A., Pilarczyk, R., Sablik, P., Drozd, R., … Skolmowska, M. (2012). Selenium concentration and glutathione peroxidase (GSH-Px) activity in serum of cows at different stages of lactation. *Biological Trace Element Research*, *147*, 91–96.

Pires, J. A., & Grummer, R. R. (2007). The use of nicotinic acid to induce sustained low plasma nonesterified fatty acids in feed-restricted Holstein cows. *Journal of Dairy Science*, *90*, 3725–3732.

Renner, L., von Soosten, D., Sipka, A., Doll, S., Beineke, A., Schuberth, H. J., & Danicke, S. (2012). Effect of conjugated linoleic acid on proliferation and cytokine expression of bovine peripheral blood mononuclear cells and splenocytes ex vivo. *Archives of Animal Nutrition*, *66*, 73–85.

Scalia, D., Lacetera, N., Bernabucci, U., Demeyere, K., Duchateau, L., & Burvenich, C. (2006). In vitro effects of nonesterified fatty acids on bovine neutrophils oxidative burst and viability. *Journal of Dairy Science*, *89*, 147–154.

Schulz, K., Frahm, J., Kersten, S., Meyer, U., Reiche, D., Sauerwein, H., & Danicke, S. (2015). Effects of elevated parameters of subclinical ketosis on the immune system of dairy cows: In vivo and in vitro results. *Archives of Animal Nutrition*, *69*, 113–127.

Sies, H. (1997). Oxidative stress: Oxidants and antioxidants. *Experimental Physiology*, *82*, 291–295.

Sordillo, L. M., & Aitken, S. L. (2009). Impact of oxidative stress on the health and immune function of dairy cattle. *Veterinary Immunology and Immunopathology*, *128*, 104–109.

Sordillo, L. M., Contreras, G. A., & Aitken, S. L. (2009). Metabolic factors affecting the inflammatory response of periparturient dairy cows. *Animal Health Research Reviews*, *10*, 53–63.

Sordillo, L. M., & Mavangira, V. (2014). The nexus between nutrient metabolism, oxidative stress and inflammation in transition cows. *Animal Production Science*, *54*, 1204–1214.

Ster, C., Loiselle, M. C., & Lacasse, P. (2012). Effect of postcalving serum nonesterified fatty acids concentration on the functionality of bovine immune cells. *Journal of Dairy Science*, *95*, 708–717.

Suriyasathaporn, W., Daemen, A. J., Noordhuizen-Stassen, E. N., Dieleman, S. J., Nielen, M., & Schukken, Y. H. (1999). Beta-hydroxybutyrate levels in peripheral blood and ketone bodies supplemented in culture media affect the in vitro chemotaxis of bovine leukocytes. *Veterinary Immunology and Immunopathology*, *68*, 177–186.

Tienken, R., Kersten, S., Frahm, J., Huther, L., Meyer, U., Huber, K., … Dänicke, S. (2015). Effects of prepartum dietary energy level and nicotinic acid supplementation on immunological, hematological and biochemical parameters of periparturient dairy cows differing in parity. *Animals*, *5*, 910–933.

Tienken, R., Kersten, S., Frahm, J., Meyer, U., Locher, L., Rehage, J., … Danicke, S. (2015). Effects of an energy-dense diet and nicotinic acid supplementation on production and metabolic variables of primiparous or multiparous cows in periparturient period. *Archives of Animal Nutrition*, *69*, 319–339.

Trevisi, E., Amadori, M., Cogrossi, S., Razzuoli, E., & Bertoni, G. (2012). Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows. *Research in Veterinary Science*, *93*, 695–704.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry & Cell Biology*, *39*, 44–84.

Wullepit, N., Hostens, M., Ginneberge, C., Fievez, V., Opsomer, G., Fremaut, D., & De Smet, S. (2012). Influence of a marine algae supplementation on the oxidative status of plasma in dairy cows during the periparturient period. *Preventive Veterinary Medicine*, *103*, 298–303.

Yu, B. L., & Zhao, S. P. (2007). Anti-inflammatory effect is an important property of niacin on atherosclerosis beyond its lipid-altering effects. *Medical Hypotheses*, *69*, 90–94.

Yuan, K., Shaver, R. D., Bertics, S. J., Espineira, M., & Grummer, R. R. (2012). Effect of rumen-protected niacin on lipid metabolism, oxidative stress, and performance of transition dairy cows. *Journal of Dairy Science*, *95*, 2673–2679.

Zhou, Z., Bu, D. P., Vailati Riboni, M., Khan, M. J., Graugnard, D. E., Luo, J., … Loor, J. J. (2015). Prepartal dietary energy level affects peripartal bovine blood neutrophil metabolic, antioxidant, and inflammatory gene expression. *Journal of Dairy Science*, *98*, 5492–5505.

How to cite this article: Bühler S, Frahm J, Tienken R, et al. Effects of energy supply and nicotinic acid supplementation on serum anti-oxidative capacity and on expression of oxidative stress-related genes in blood leucocytes of periparturient primi- and pluriparous dairy cows. *J Anim Physiol Anim Nutr*. 2018;102:e87–e98. https://doi.org/10.1111/jpn.12705

6. Paper III

S. Bühler, J. Frahm, W. Liermann, R. Tienken, S. Kersten, U. Meyer, K. Huber, S. Dänicke (2018)

Effects of energy supply and nicotinic acid supplementation on phagocytosis and ROS production of blood immune cells of periparturient primi- and pluriparous dairy cows.

Research in Veterinary Science 116, 62–71.

Reprinted with permission of Elsevier

Research in Veterinary Science 116 (2018) 62–71

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Effects of energy supply and nicotinic acid supplementation on phagocytosis and ROS production of blood immune cells of periparturient primi- and pluriparous dairy cows

S. Bühler[a,*], J. Frahm[a], W. Liermann[b], R. Tienken[a], S. Kersten[a], U. Meyer[a], K. Huber[c], S. Dänicke[a]

[a] Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Braunschweig, Germany
[b] Institute of Nutritional Physiology 'Oskar Kellner', Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany
[c] Institute of Animal Sciences, University of Hohenheim, Stuttgart, Germany

ARTICLE INFO

Keywords:
Transition period
Niacin
Polymorphonuclear leukocytes (PMN)
Peripheral blood mononuclear cells (PBMC)
Oxidative burst
Phagocytosis
Flow cytometry

ABSTRACT

Effects of energy supply and nicotinic acid (NA) supplementation on the phagocytic activity of polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC), and on ROS production in PMN of periparturient cows differing in parity were examined. 29 pluriparous and 18 primiparous cows were allocated to four different feeding groups from 42 days prepartum until 100 days postpartum. They were fed either a ration with a low concentrate proportion of 30% (LC) or a high concentrate proportion of 60% (HC). After parturition all animals received 30% concentrate which was increased to 50% either within 16 (LC) or within 24 days (HC). The different concentrate feeding strategies aimed at triggering differences in postpartum lipolysis. Half of the animals per group were supplemented with 24 g per day of NA from 42 days prepartum until 24 days postpartum. All investigated parameters varied significantly over time compared to parturition ($p < 0.05$). Numbers of phagocytosing PMN and PBMC increased in the course of the experiment, whereas the amount of engulfed bacteria per cell decreased between 42 and 11 days prepartum. Percentage of basal ROS producing PMN decreased strongly before parturition and reached initial values only at 28 days in milk again. Mean fluorescence intensity (MFI) in these ROS producing cells, however, increased before parturition. Oxidative burst stimulation in PMN was reduced around parturition but the amount of ROS produced in the stimulated cells was increased. Pluriparous cows exhibited higher numbers of basal ROS producing PMN and phagocytic PBMC. NA supplementation influenced phagocytosis in blood leukocytes.

1. Introduction

The transition from late gestation to early lactation challenges the periparturient dairy cow with severe nutritional, metabolic, hormonal and immunological changes. Additionally, this period is accompanied with an increased susceptibility to and severity of metabolic and infectious diseases (Goff and Horst, 1997; Mallard et al., 1998). Research has, furthermore, shown that this phase goes along with increased inflammation (Sordillo et al., 2009; Trevisi et al., 2012), oxidative stress (Sordillo and Aitken, 2009) and a dysfunctional immune response (Ingvartsen and Moyes, 2015). In the literature, it is widely accepted that cows experience around parturition a transient period of immunosuppression (Aleri et al., 2016; Ingvartsen and Moyes, 2015). Neutrophil function, lymphocyte responsiveness to mitogen stimulation, antibody responses, and cytokine production by immune cells are components of the cow's host defense that are reported to be altered during the transition period (Mallard et al., 1998). Impaired neutrophil function around parturition has additionally been linked to the occurrence of diseases like mastitis, metritis, and retained placenta (Cai et al., 1994; Hammon et al., 2006; Kimura et al., 2002).

Causes for altered immune cell function and consequently immunosuppression are manifold. Glucocorticoids like the stress hormone cortisol, which exhibits increased blood concentrations at parturition, is reported to impair neutrophil migration as well as lymphocyte proliferation and development (Aleri et al., 2016; Burton et al., 1995).

Non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB) have also been shown to adversely affect the functional capacity of polymorphonuclear leukocytes (PMN) (Hammon et al., 2006; Hoeben et al., 1997; Sartorelli et al., 1999; Scalia et al., 2006; Suriyasathaporn et al., 1999) and peripheral blood mononuclear cells (PBMC) (Brassard

* Corresponding author at: Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Bundesallee 50, 38116 Braunschweig, Germany.
E-mail address: Susanne.Buehler@fli.de (S. Bühler).

http://dx.doi.org/10.1016/j.rvsc.2017.09.012
Received 21 June 2017; Received in revised form 31 August 2017; Accepted 9 September 2017
0034-5288/ © 2017 Elsevier Ltd. All rights reserved.

et al., 2007; Lacetera et al., 2004; Renner et al., 2012; Schulz et al., 2015; Ster et al., 2012). Particularly NEFA is also reported to induce inflammatory responses *via* toll-like receptor 4 (TLR4) pathways (Lee et al., 2001). The blood concentrations of NEFA and BHB are increased in the periparturient period, in consequence of increased lipolysis to meet the energy requirements of the animal (Bell, 1995; Drackley, 1999). The oxidative stress the animals experience in the transition phase (Bernabucci et al., 2005; Castillo et al., 2005; Miller et al., 1993) is also a significant factor leading to dysfunctional host immune and inflammatory responses and consequently increasing cows' susceptibility to health disorders (Sordillo and Aitken, 2009). This oxidative stress is caused, among other things, by the enhanced metabolic activity in consequence of the increased energy demands, by a reduced anti-oxidant capacity, and by increased reactive oxygen species (ROS) production in the fight against invading organisms.

Niacin, the vitamin B3, is found as nicotinic acid (NA) and as nicotinamide (NAM) in the vertebrates' body. NA is hereby readily transformed into NAM that is a precursor of the coenzymes nicotinamide adenosine dinucleotide (NAD +) and nicotinamide adenosine dinucleotide phosphate (NADP +). As such, it participates in many redox reactions including anabolic and catabolic pathways. The anti-lipolytic effect of niacin is well documented (Carlson, 2005). In the bovine niacin was shown to downregulate lipolysis *in vitro* (Kenez et al., 2014) while *in vivo* results varied showing either an anti-lipolytic (Morey et al., 2011; Pires and Grummer, 2007; Yuan et al., 2012) or no effect (Kenez et al., 2015; Tienken et al., 2015b). Niacin, however, is also known to have anti-oxidative and anti-inflammatory effects. It acts as cytoprotectant in blocking inflammatory cell activation and has immune modulating properties (Maiese et al., 2009; Yu and Zhao, 2007). Hence, niacin is an interesting supplementation substance to test in the periparturient cow, where metabolic and oxidative stress as well as increased inflammation occur and are associated with a dysfunctional immune response.

The aim of this study was to investigate the effects of niacin on the functional capacity of blood leukocytes of dairy cows. This was addressed firstly by an *in vitro* experiment of incubating whole blood with either NA or NAM and examining the capability of PMN to produce ROS. And secondly, by feeding a nicotinic acid supplementation to periparturient primi- and pluriparous dairy cows that were fed with differing energy supplies antepartum and differing energy escalation strategies after parturition to induce differences in the degree of postpartum lipolysis and ketogenesis (Schulz et al., 2014). The effect of these feeding strategies on the phagocytic activity of PMN and PBMC as well as on the capability of ROS production in PMN was then examined by flow cytometry. Phagocytosis and ROS production being, hereby, two major functions of the innate immune system in the defense against invading organisms.

2. Materials and methods

2.1. In vitro niacin incubation

For the *in vitro* niacin incubation studies blood from four cows in their second lactation at 84 days in milk (DIM) was incubated in triplicates either 30 or 180 min at 37 °C with increasing concentrations (0 to 4 µg/mL) of either nicotinic acid (NA) or nicotinamide (NAM). The ability to generate ROS after this incubation was then measured as described below.

2.2. Animal experiment, design and blood collection

The experiment was performed at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Braunschweig, Germany in accordance with the German Animal Welfare Act concerning the protection of experimental animals and was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany. A detailed description of the experimental conditions is found in Tienken et al. (2015b). Briefly, 29 pluriparous and 18 primiparous pregnant and healthy German Holstein dairy cows were allocated homogeneously to one of four dietary treatment groups considering mean body weight (BW), body condition score (BCS), and in case of the pluriparous cows number of lactations, and milk yield of previous lactations. The experiment started at 42 days before expected parturition and ended at 100 DIM. Before parturition, the animals received either a low concentrate diet (30% concentrate and 70% roughage on dry matter (DM) basis; LC) or a high concentrate diet (60% concentrate and 40% roughage on DM basis; HC) with or without a supplementation of 24 g per day and cow of powdered and non-rumen protected nicotinic acid (NA) (Mianyang Vanetta Pharmaceutical Technology Co., Ltd., Sichuan, China) resulting in the feeding groups LC-NA (4 primiparous and 6 pluriparous) and HC-NA (5 primiparous and 7 pluriparous) as well as LC-CON (5 primiparous and 7 pluriparous) and HC-CON (4 primiparous and 9 pluriparous). Supplementation was applied from day 42 prepartum until 24 DIM. After parturition, all animals received initially a diet of 30% concentrate and 70% roughage. This concentrate proportion was increased up to 50% of the diet either, within 16 days (LC animals) or 24 days (HC animals) and was maintained at 50% until the end of the trial. The different concentrate feeding strategies aimed at triggering cow groups differing in degree of postpartum lipolysis (Schulz et al., 2014) which should facilitate the investigation of niacin effects. Roughage (50% corn silage and 50% grass silage on DM basis) was fed *ad libitum via* self-feeding stations (type RIC, Insentec B.V., Marknesse, The Netherlands) and the animals had free access to water. All diets were formulated to meet the nutritional requirements of dairy cows stated by the Society of Nutrition Physiology GfE (2001).

Blood from a *Vena jugularis externa* was collected into EDTA containing tubes at day −42 (−40 ± 6 (mean ± SD)), −21 (−20 ± 2), −14 (−13 ± 2), −7 (−7 ± 1), −3 (−3 ± 1), 1, 3, 7, 14, 21, 28, 35, 42, 63, 84, and 100 relative to parturition. For the phagocytosis assay blood was collected into heparin containing tubes at day −42 (−41 ± 6), −14 (−13 ± 3), −7 (−6 ± 3), 7, 14, 28, 42, and 100 relative to parturition. The blood samples were processed within 2 h of collection.

2.3. Phagocytosis

Phagocytic activity of polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC) was investigated using the PHAGOTEST™ reagent kit (Glycotope Biotechnology, Heidelberg, Germany) according to the manufacturer's protocol. Phagocytosis was hereby determined in duplicate by the ingestion of fluorescein (FITC)-labeled opsonized E.coli bacteria. In brief, 100 µL heparinized blood was incubated for 10 min on ice. 20 µL E.coli bacteria solution (4×10^7 bacteria) was added and samples incubated for 10 min at 37 °C. A negative control sample remained on ice. Phagocytosis was stopped by placing the samples on ice and 100 µL ice cold quenching solution was added to quench the FITC fluorescence of surface bound bacteria in order to finally measure only internalized bacteria. After two washing steps with 3 mL wash buffer and a subsequent spin at 250 × g and 4 °C for 5 min, the cell pellets were resuspended in 1 mL lysing solution and incubated for 20 min at room temperature for simultaneously lysing erythrocytes and fixing leukocytes. After an additional wash step DNA was stained by resuspending the cells in 500 µL Hepes buffered saline (HBS, 14 mM Hepes, 0.9% NaCl) and 2.5 µL propidium iodide (PI) solution (1 mg/mL) and incubating them on ice for 10 min. This step is necessary for the cytometric discrimination of bacterial and cellular aggregation artifacts during leukocyte analysis. FITC labelling was measured by flow cytometry (FACSCanto™ II, BD Biosciences, San Jose, CA, USA) and excitation and emission wavelengths were 488 nm and 519 nm, respectively. PMN and PBMC were gated according to their size and granularity based on measurements of forward and side

Table 1
Reactive oxygen species formation of non stimulated (basal) and TPA stimulated polymorphonuclear leukocytes (PMN) after *in vitro* incubation with different concentrations of nicotinic acid (NA) or nicotinamide (NAM) for either 30 or 180 min. Proportions (%) and mean fluorescence intensities (MFI) of rhodamin positive cells (R123 +) are presented. Blood samples were taken from cows at 84 days in milk of their second lactation.

	NA (μg/mL)					NAM (μg/mL)					PSE	*p*-Values			
	0	0.25	0.40	1.00	4.00	0	0.25	0.40	1.00	4.00		Sub	Conc	IT	Sub * Conc * IT
Basal R123 + (%)															
30 min	10.6	10.5	10.7	11.0	10.7	10.4	9.8	10.5	10.3	10.1	2.0	0.474	0.816	0.197	0.028
180 min	11.3	10.3	10.4	9.6	8.9	9.8	10.3	9.9	10.4	11.0					
Stimulated R123 + (%)															
30 min	99.0	99.3	99.2	99.2	99.2	99.1	98.6	98.9	99.0	98.7	0.5	0.558	0.307	0.847	0.800
180 min	99.0	98.4	99.0	98.8	99.8	99.1	98.4	99.3	99.3	99.4					
Basal R123 + (MFI)															
30 min	7686	8742	9172	9577	9878	7780	8367	8881	9794	10,234	602	0.573	0.000	0.398	0.999
180 min	7689	7980	8629	9357	9892	7865	8491	8737	9533	10,233					
Stimulated R123 + (MFI)															
30 min	50,971	51,433	51,399	49,763	49,509	51,018	51,622	51,673	51,541	52,005	2233	0.400	0.259	0.006	0.802
180 min	50,401	53,193	52,348	54,036	56,552	50,938	53,049	54,009	54,090	56,856					

Notes: Values are least squares means (LSMeans), n = 4, TPA = 12-O-tetradecanoylphorbol-13-acetate, PSE = pooled standard error, Sub = Substance (NA or NAM), Conc = NA or NAM concentration, IT = 30 or 180 minutes incubation time.

scatter. At least 10,000 cells were evaluated with FACSDiva™ software 6.1.3 (BD Biosciences, San Jose, CA, USA). Results were expressed as percentage of FITC labeled PMN or PBMC, as mean fluorescence intensity (MFI) of FITC labeled PMN or PBMC describing the amount of internalized *E.coli* per cell, and as phagocytic total PMN or total PBMC. The latter corresponds to the product of PMN or PBMC blood concentrations (Tienken et al., 2015a) multiplied with the proportion of phagocytosing PMN or PBMC, respectively. The statistical analysis of the phagocytosis data made it necessary to pool time points − 14 and − 7 into time point − 10 (− 10 ± 3.5) to achieve the necessary statistical power.

2.4. ROS production

The capacity of PMN to produce reactive oxygen species (ROS) was analyzed by flow cytometry based on the intracellular oxidation of the non-fluorescent dye dihydrorhodamine (DHR) 123 to the fluorescent rhodamine (R) 123 by hydrogen peroxide. As a positive control the protein kinase C activation agent 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was used that induces a maximum NADPH-oxidase activity and, therefore, a respiratory burst. Diphenylene iodonium (DPI) as NADPH-oxidase inhibitor was used as a negative control. Briefly, 50 μL EDTA blood was incubated with 100 μL DHR solution (40 μM in Hepes buffered saline (HBS) (0.9% NaCl, 14 mM Hepes, pH: 7.3–7.4)) with or without TPA (30 μM) and/or DPI (5 μM) for 15 min at 37 °C. Erythrocytes were lysed for 10 min in the dark by addition of 500 μL lysis buffer (BD Pharm Lyse™, BD Bioscience San Jose, CA, USA). After a 5 min spin at 250 x g and 4 °C the cells were resuspended in HBS and analyzed by flow cytometry (FACSCanto™ II, BD Biosciences, San Jose, CA, USA) in duplicate. Excitation and emission wavelengths were 488 nm and 519 nm, respectively. PMN were gated according to their size and granularity based on measurements of forward and side scatter. At least 10,000 cells were evaluated with FACSDiva™ software 6.1.3 (BD Biosciences, San Jose, CA, USA). Results of R123 labeled (R123 +) and, therefore, ROS producing PMN either non-stimulated (basal) or TPA-stimulated were expressed as percentage of PMN, as mean fluorescence intensity (MFI) (a measure of the amount of ROS per cell) and in case of the animal experimental data as concentration of peripheral blood granulocytes. The latter being the product of granulocyte blood count (Tienken et al., 2015a) and the percentage of basal or stimulated R123 + PMN.

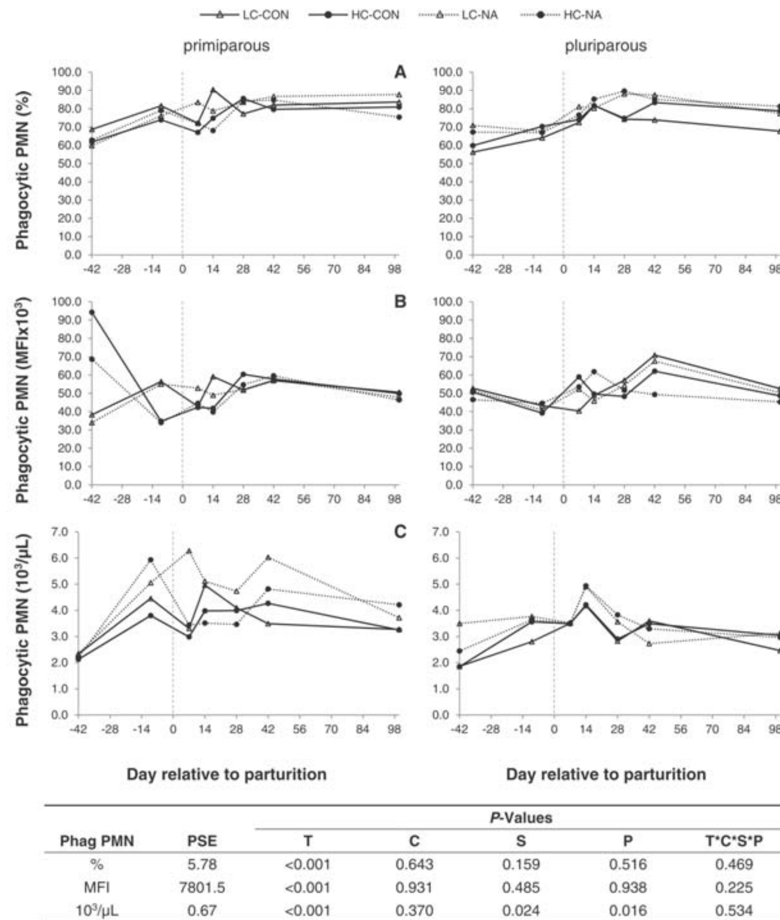
2.5. Statistical analysis

Statistical analysis were performed using the PROC MIXED procedure of SAS software package (Version 9.4, SAS Institute Inc., Cary, NC, USA) with the restricted maximum likelihood method. The model for the *in vitro* niacin incubation studies included substance (**Sub**; NA or nicotinamide (NAM)), concentration (**Conc**; niacin concentration), incubation time (**IT**, 30 or 180 min) and the triple interaction between Sub * Conc * IT as fixed effects. The cow was considered as random effect and the control values without NA or NAM (0 μg/mL) were considered as co-variates. Variance components (VC) was used as covariance structure, since it exhibited the lowest Akaike information criterion (AICC). The model for the phagocytosis and the ROS production data of the animal experiment included time point (**T**; experimental day relative to parturition), dietary concentrate proportion (**C**; LC or HC diet), supplementation (**S**; CON or NA), parity (**P**; primiparous or pluriparous cows), and the quadruple interaction between T * C * S * P as fixed effects. The frequent measurements during experiment for each individual cow were considered as repeated measurement. The covariance structure compound symmetry (CS) showing the lowest AICC was used. Degrees of freedom were calculated using the Kenward-Roger adjustment. Differences were considered to be significant at $p < 0.05$ and a tendency was noted if $0.10 > p \geq 0.05$. All results are presented as LS-means and pooled standard errors (PSE) are stated.

3. Results

3.1. ROS production in PMN after in vitro incubation with NA and NAM

The percentage of basal ROS producing PMN was significantly influenced by the NA concentration after 180 min incubation time with the highest concentration of 4 μg/mL exhibiting the lowest percentage leading to the significant interaction for Sub * Conc * IT (Table 1). The MFI, a measure of the amount of ROS produced per cell, of basal R123 + PMN was significantly influenced by the niacin concentration at both incubation times showing increasing numbers with increasing NA or NAM concentration. The MFI of R123 + PMN after TPA stimulation was significantly affected by incubation time exhibiting higher values after 180 min incubation than after 30 min incubation.



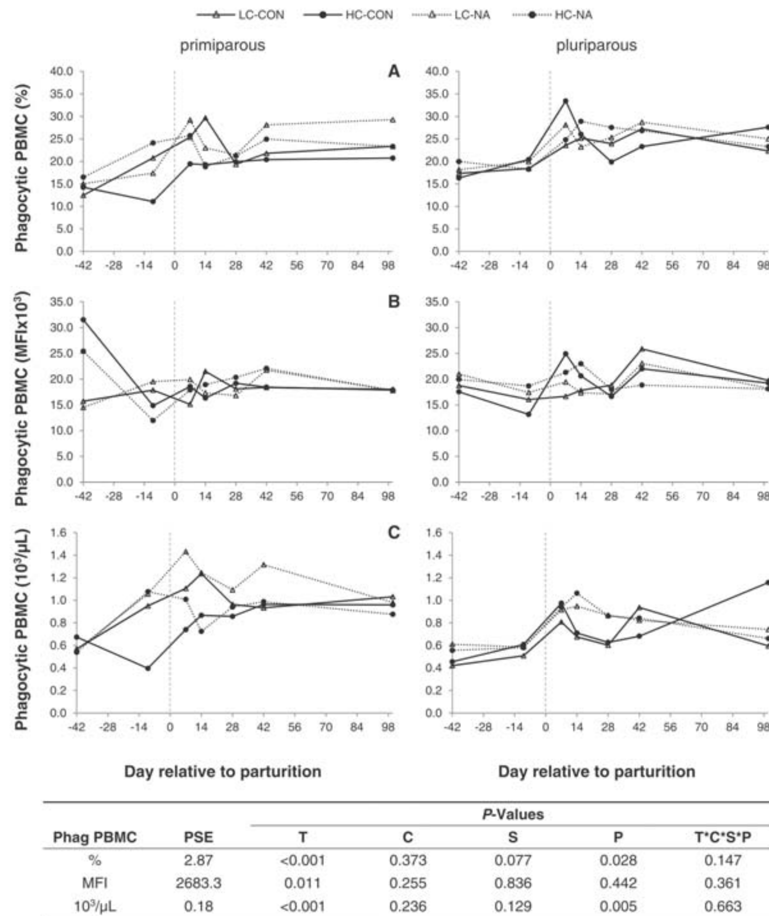
Phag PMN	PSE	T	C	S	P	T*C*S*P
		P-Values				
%	5.78	<0.001	0.643	0.159	0.516	0.469
MFI	7801.5	<0.001	0.931	0.485	0.938	0.225
10^3/µL	0.67	<0.001	0.370	0.024	0.016	0.534

Fig. 1. Phagocytic polymorphonuclear leukocytes (PMN) of primiparous (left column) and pluriparous (right column) periparturient dairy cows. Shown are the percentage (A), the mean fluorescence intensity (MFI) (B) and the total blood concentration (C) of phagocytosing PMN. Animals received prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition cows were initially fed with a diet consisting of 30% concentrate. The concentrate proportion was continously increased up to 50% either within 16 (LC) or 24 days (HC). Animals were either fed a diet with (NA, 24 g NA/d from d 42 prepartum until 24 DIM) or without (CON) nicotinic acid. Symbols denote LSMEANS. (Primiparous: LC-CON, *n* = 5; HC-CON, *n* = 4; LC-NA, n = 4; HC-NA, *n* = 5; pluriparous: LC-CON, *n* = 7; HC-CON, *n* = 9; LC-NA, *n* = 6; HC-NA, *n* = 7). PSE = pooled standard error, T = time relative to parturition, C = concentrate level, S = supplementation, P = parity.

3.2. Phagocytosis in PMN and PBMC

Phagocytic PMN changed significantly over time in relation to parturition (Fig. 1). Percentage of phagocytosing PMN steadily increased to reach maximum signals at 42 DIM and decreased afterwards slightly until 100 DIM not reaching initial −42 values but rather staying on a 25% higher level (Fig. 1A). The MFI, which is a measure of the internalized bacteria per cell, decreased before parturition and exhibited afterwards a steady increase until 42 DIM (Fig. 1B). The signal decreased again until 100 DIM and reached initial −42 values. The total number of phagocytosing PMN in blood increased before parturition and exhibited a slight decrease in the week after parturition to increase again afterwards (Fig. 1C). In pluriparous cows peak signals were reached at 14 DIM. Then the signals decreased again but showed slightly higher values at 100 DIM compared to 42 days antepartum. For the primiparous animals the peak at 14 DIM was less pronounced, however, the values at 100 DIM were 60% higher than initial values at time point −42. The parity effect resulted of the higher signals in primiparous than in pluriparous animals especially 10 days before parturition and at time points 28, 42 and 100 DIM. The total number of phagocytosing PMN in blood additionally exhibited a supplementation effect. Supplemented animals showed higher numbers than the control animals. This was especially obvious in primiparous cows at time points −10, 42 and 100 and in pluriparous cows at 14 and 28 DIM.

Phagocytic PBMC also fluctuated significantly in the course of the experiment (Fig. 2). Whereas > 60% of the PMN exhibited phagocytosis only 15–20% of the PBMC phagocytized E coli (Fig. 2A). The percentage of phagocytosing PBMC increased before parturition to



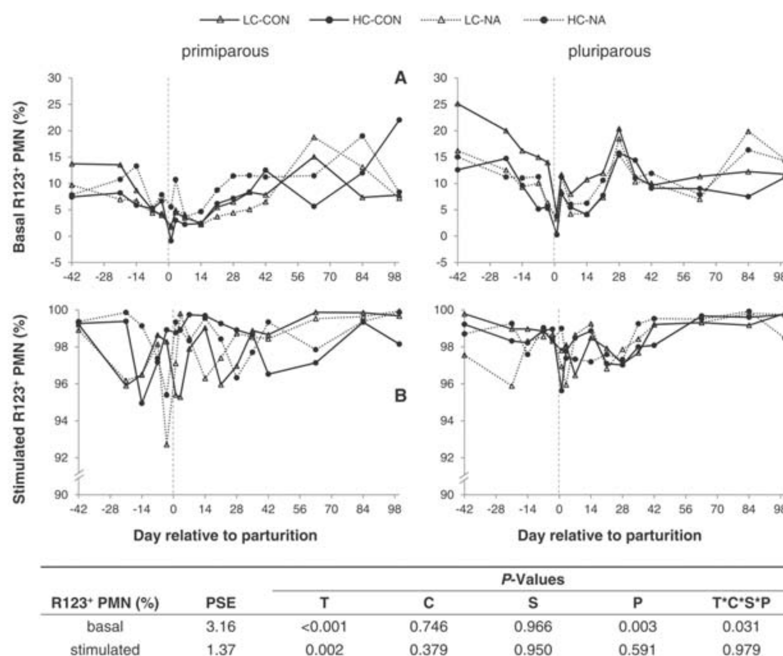
Phag PBMC	PSE	*P*-Values				
		T	C	S	P	T*C*S*P
%	2.87	<0.001	0.373	0.077	0.028	0.147
MFI	2683.3	0.011	0.255	0.836	0.442	0.361
10³/µL	0.18	<0.001	0.236	0.129	0.005	0.663

Fig. 2. Phagocytic peripheral blood mononuclear cells (PBMC) of primiparous (left column) and pluriparous (right column) periparturient dairy cows. Shown are the percentage (A), the mean fluorescence intensity (MFI) (B) and the total blood concentration (C) of phagocytosing PBMC. Animals received prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition cows were initially fed with a diet consisting of 30% concentrate. The concentrate proportion was continously increased up to 50% either within 16 (LC) or 24 days (HC). Animals were either fed a diet with (NA, 24 g NA/d from d 42 prepartum until 24 DIM) or without (CON) nicotinic acid. Symbols denote LSMEANS. (Primiparous: LC-CON, $n = 5$; HC-CON, $n = 4$; LC-NA, $n = 4$; HC-NA, $n = 5$; pluriparous: LC-CON, $n = 7$; HC-CON, $n = 9$; LC-NA, $n = 6$; HC-NA, $n = 7$). PSE = pooled standard error, T = time relative to parturition, C = concentrate level, S = supplementation, P = parity.

exhibit a first peak in the two weeks after parturition. Until 28 DIM the proportion of phagocytic PBMC decreased and showed a second peak at 42 DIM. Afterwards the signals decreased again until 100 DIM however exhibiting 67% higher values in primiparous and 38% higher values in pluriparous cows compared to initial values at time point − 42. The significant parity influence resulted from the higher phagocytic proportion of PBMC in cows than in heifers mostly obvious at time points − 42, 14, 28 and 42. The tendency in supplementation influence originated from the higher percentages of phagocytic PBMC in supplemented animals at 28 and 42 DIM. The MFI of phagocytic PBMC showed an overall decrease before parturition (Fig. 2B). It exhibited an increase in the two weeks after parturition followed by a nadir at 28 DIM and another peak at 42 DIM. This curve progression was more pronounced in pluriparous cows. The total number of phagocytic PBMC in blood increased before parturition and showed a first peak in the two weeks after parturition (Fig. 2C). Another peak at 42 DIM followed a slight nadir at 28 DIM. Numbers of phagocytosing PBMC at 100 DIM were 65% higher in primiparous and 59% higher in pluriparous cows than at 42 days antepartum. Primiparous cows exhibited significantly higher numbers of phagocytic PBMC in blood than pluriparous cows, most pronounced at time points − 10, 7, 28 and 42.

3.3. ROS production in PMN

The percentage of ROS producing PMN fluctuated significantly in the course of the experiment (Fig. 3). The basal ROS production decreased prepartum to show a distinct nadir at the first day after parturition (Fig. 3A). In primiparous cows this decrease averaged 8 and in



R123⁺ PMN (%)	PSE	T	C	S	P	T*C*S*P
		P-Values				
basal	3.16	<0.001	0.746	0.966	0.003	0.031
stimulated	1.37	0.002	0.379	0.950	0.591	0.979

Fig. 3. Percentage of basal (A) and TPA stimulated (B) R123^{+} polymorphonuclear leukocytes (PMN) of primiparous (left column) and pluriparous (right column) periparturient dairy cows. Animals received prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition cows were initially fed with a diet consisting of 30% concentrate. The concentrate proportion was continously increased up to 50% either within 16 (LC) or 24 days (HC). Animals were either fed a diet with (NA, 24 g NA/d from d 42 prepartum until 24 DIM) or without (CON) nicotinic acid. Symbols denote LSMEANS. (Primiparous: LC-CON, n = 5; HC-CON, n = 4; LC-NA, n = 4; HC-NA, n = 5; pluriparous: LC-CON, n = 7; HC-CON, n = 9; LC-NA, n = 6; HC-NA, n = 7). PSE = pooled standard error, T = time relative to parturition, C = concentrate level, S = supplementation, P = parity.

pluriparous cows 14 percentage points to reach a mean of 2 and 3% ROS producing PMN, respectively. After a slight increase at 3 DIM another nadir formed between 7 and 14 DIM. Afterwards the primiparous animals showed a continuous increase in the basal ROS production that was at 63 and 84 DIM even higher than at the starting point at − 42. At 100 DIM, the values reached however this initial level again. In pluriparous cows, another peak of basal ROS production formed at 28 DIM and decreased again until 63 DIM. At 100 DIM, it also reached initial values again. The significant interaction for T ∗ C ∗ S ∗ P was additionally driven by the higher levels in basal ROS production of pluriparous cows and the higher basal ROS production in supplemented animals at 84 DIM. The percentage of TPA stimulated ROS producing cells changed also significantly in relation to parturition (Fig. 3B). It decreased before parturition and showed lowest values around parturition and at 21 and 28 DIM. Values reached again − 42 levels at 63 DIM for pluriparous and at 84 DIM for primiparous cows.

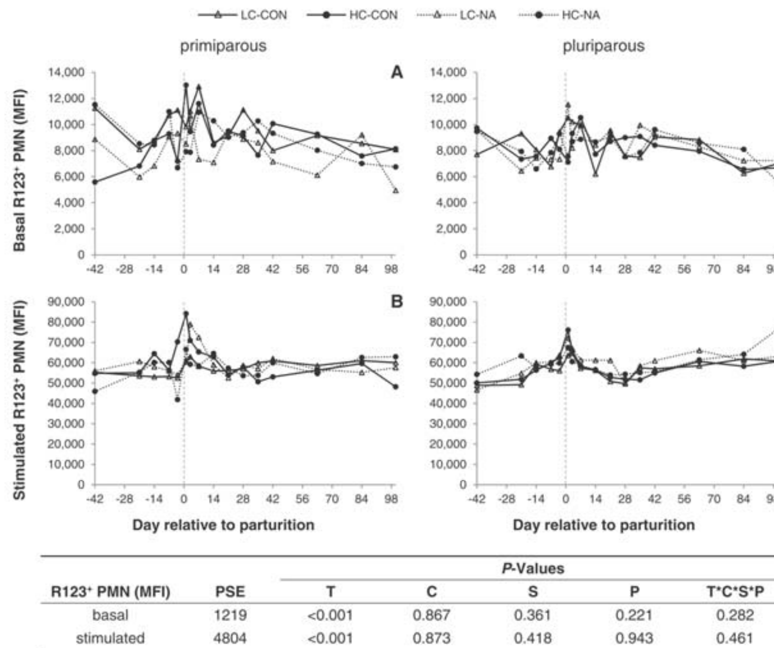
The MFI of ROS producing PMN, that is a measure of the amount of ROS produced per cell, changed also significantly in the course of the experiment (Fig. 4). The basal MFI decreased from time point − 42 until − 21 and continuously increased afterwards until 7 DIM (Fig. 4A). At 14 DIM a small nadir showed followed by a slightly higher signal at 21 DIM. Afterwards the signal decreased and exhibited at 100 DIM clearly lower values than at − 42. The MFI of TPA stimulated ROS producing PMN increased to express a peak at 1 day after parturition (Fig. 4B). Afterwards it decreased to initial values until 28 DIM and ascended again until 100 DIM. The values at 100 DIM were mostly higher than the values at 42 days antepartum. However, this was more pronounced in pluriparous cows.

The blood concentration of ROS producing PMN varied also significant in the course of the experiment (Fig. 5). The concentration of basal ROS producing cells decreased antepartum and showed lowest values at the day after parturition. At 3 DIM the signals increased slightly but decreased again at 7 and 14 DIM. Afterwards the signals increased continuously until 42 DIM in heifers whereas the pluriparous cows exhibited another clear peak at 28 DIM. In pluriparous cows, the signals decreased again until 63 DIM and reached, thereafter, initial values. The primiparous animals, however, showed for the experimental days > 42 DIM heterogenous results. The higher concentration of ROS producing PMN in pluriparous cows prepartum and in the 4 weeks after parturition resulted in a tendency for parity. The concentration of TPA stimulated R123 + PMN increased prepartum to show peak signals in the 3 days after parturition and decreased rapidly to stay on a slightly higher level than the initial values.

4. Discussion

The objective of the present study was to examine the influence of niacin on the functional capacity of blood leukocytes of dairy cows *in vitro* and *in vivo*.

The incubation of PMN *in vitro* with increasing concentrations of either nicotinic acid or nicotinamide revealed influences on the ROS production and oxidative burst capability of these cells. Whereas the percentage of basal ROS producing cells decreased with increasing NA concentration after 180 min incubation, the MFI, and therefore the



R123⁺ PMN (MFI)	PSE	*P*-Values T	C	S	P	T*C*S*P
basal	1219	<0.001	0.867	0.361	0.221	0.282
stimulated	4804	<0.001	0.873	0.418	0.943	0.461

Fig. 4. Mean fluorescence intensity (MFI) of basal (A) and TPA stimulated (B) R123^{+} polymorphonuclear leukocytes (PMN) of primiparous (left column) and pluriparous (right column) periparturient dairy cows. Animals received prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition cows were initially fed with a diet consisting of 30% concentrate. The concentrate proportion was continously increased up to 50% either within 16 (LC) or 24 days (HC). Animals were either fed a diet with (NA, 24 g NA/d from d 42 prepartum until 24 DIM) or without (CON) nicotinic acid. Symbols denote LSMEANS. (Primiparous: LC-CON, n = 5; HC-CON, n = 4; LC-NA, n = 4; HC-NA, n = 5; pluriparous: LC-CON, n = 7; HC-CON, n = 9; LC-NA, n = 6; HC-NA, n = 7). PSE = pooled standard error, T = time relative to parturition, C = concentrate level, S = supplementation, P = parity.

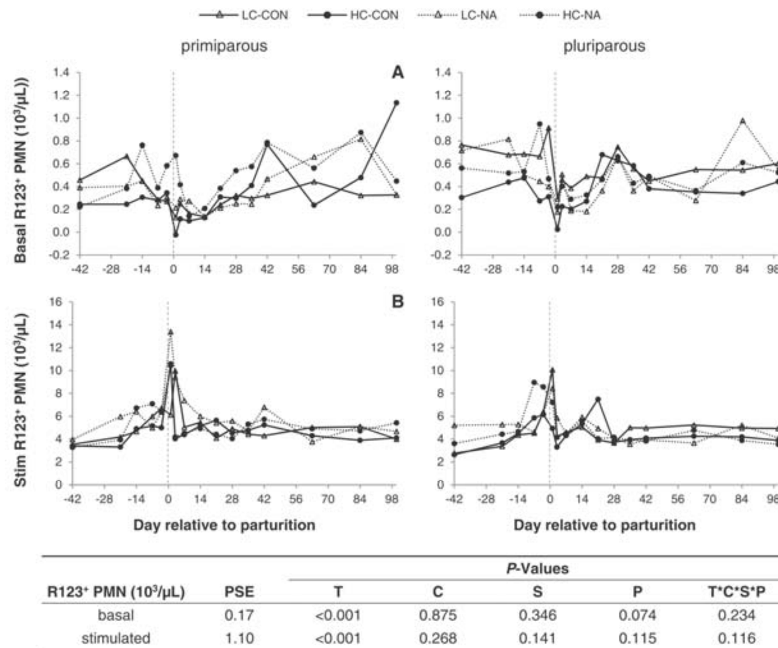
amount of ROS per cell, of basal ROS producing cells increased with either increasing NA or NAM concentrations at the different incubation times. Since niacin is a precursor for the coenzymes NAD + and NADP + this could be explained with higher NAD +/NADP + concentrations in those cells. Although, there is evidence that niacin reduces ROS and oxidative stress in blood leukocytes (Choi et al., 2015; Ganji et al., 2014; Montserrat-de la Paz et al., 2017), Crowley et al. (2000) also showed an increase in ROS production in Jurkat cells after incubation with niacin. Since ROS are important signaling molecules for the clearance of oxidative stress (Fialkow et al., 2007), the authors discussed the increase of ROS with an early oxidative stress signal to induce anti-oxidative processes. This study measured the increased ROS production with niacin incubation after 30 and 180 min, whereas, studies that revealed a decrease in the ROS production measured the signal after far longer incubation times with niacin (Choi et al., 2015; Ganji et al., 2014; Montserrat-de la Paz et al., 2017). Therefore, our results may support a niacin effect of the nature of an early oxidative stress signal. However, another difference between the data of this study and the aforementioned research was the use of whole blood in the niacin incubations instead of isolated cells.

The significant effect of incubation time for both substances on the MFI of TPA stimulated ROS production may be explained by the higher affinity of NADPH oxidase for NADPH than that of glutathione reductase (GR) (Blacker and Duchen, 2016). With the TPA stimulation, NADPH oxidase is highly activated, therefore, using up the NADPH that is no longer available for the GR and, therefore, the clearance of ROS through the glutathione system is impaired. Hence, the higher ROS signals measured after 180 min niacin incubation than after 30 min incubation.

The reduced percentage of basal ROS producing cells with the highest NA concentration at 180 min incubation supports the anti-oxidative capacity of niacin (Maiese et al., 2009). However, this effect was not paralleled for NAM. A pH effect could be excluded, since the final blood solutions of the different NA concentrations were tested on pH and showed no difference (data not shown).

With the potential of niacin to influence immune cells, the examination of a nicotinic acid supplementation during the periparturient period of dairy cows was of special interest, since this period goes along with impaired immune cell functions.

The animal experiment of the present study aimed, hereby, at inducing differences in energy metabolism and lipolysis by feeding different energy dense diets prepartum and different rates in dietary energy concentration increase postpartum that should facilitate the investigation of niacin effects that was applied as a nicotinic acid supplementation from 42 days prepartum until 24 DIM. This study was part of a larger trial from which feeding effects on production, metabolic, hematological and biochemical parameters are published elsewhere (Tienken et al., 2015a; Tienken et al., 2015b). Briefly summarized, the feeding strategies did not influence the metabolic parameters NEFA and BHB. Schulz et al. (2014) could show differences in postpartum lipolysis and ketogenesis by using different energy dense diets prepartum and different concentrate escalation strategies postpartum on cows that were preselected for BCS. In contrast, this study used homogenously distributed animals concerning their BCS and, therefore,



R123+ PMN (10³/µL)	PSE	*P*-Values T	C	S	P	T*C*S*P
basal	0.17	<0.001	0.875	0.346	0.074	0.234
stimulated	1.10	<0.001	0.268	0.141	0.115	0.116

Fig. 5. Blood concentration of basal (A) and TPA stimulated (B) R123$^+$ polymorphonuclear leukocytes (PMN) of primiparous (left column) and pluriparous (right column) periparturient dairy cows. Animals received prepartal either a low concentrate diet LC (30% concentrate) or a high concentrate diet HC (60% concentrate). After parturition cows were initially fed with a diet consisting of 30% concentrate. The concentrate proportion was continously increased up to 50% either within 16 (LC) or 24 days (HC). Animals were either fed a diet with (NA, 24 g NA/d from d 42 prepartum until 24 DIM) or without (CON) nicotinic acid. Symbols denote LSMEANS. (Primiparous: LC-CON, n = 5; HC-CON, n = 4; LC-NA, n = 4; HC-NA, n = 5; pluriparous: LC-CON, n = 7; HC-CON, n = 9; LC-NA, n = 6; HC-NA, n = 7). PSE = pooled standard error, T = time relative to parturition, C = concentrate level, S = supplementation, P = parity.

aimed at not preselecting the animals genetically in that respect (Tienken et al., 2015b).

In accordance to Meglia et al. (2005), this study revealed no effect of energy supply on the measured functional parameters of blood leukocytes that can be explained by the also non-existing effects on NEFA and BHB (Tienken et al., 2015b).

The supplementation of 24 g rumen protected NA per day increased the serum NAM concentration from a mean NAM concentration of 1.9 ± 0.4 µg/mL in control animals to a mean NAM concentration of 3.2 ± 0.6 µg/mL in NA supplemented cows (Tienken et al., 2015a). The results of this study revealed a significant influence of the nicotinic acid supplementation on the concentration of phagocytosing PMN, being higher for supplemented animals. However, the results were not congruent between primi- and pluriparous cows. Primiparous cows showed the highest signals in NA supplemented animals at time points − 10, 7 and 42 whereas the highest signals in supplemented pluriparous cows were detected at time points 14, 28 and − 42. These differences may be explained with differing hormonal signals at the different time points in the two age groups. However, also genetic characteristics are known to influence blood leukocyte function (Paape et al., 2002). Phagocytosis is a process that involves also cell signaling with ROS participation (Fialkow et al., 2007). Therefore, the increased ROS production with niacin *in vitro* could explain an increased phagocytic activity of PMN in NA supplemented animals. However, this was not paralleled in the percentage of phagocytosing PMN and only to some degree in the percentage of phagocytosing PBMC. In case of the percentage of basal ROS producing PMN another interesting supplementation effect as T ∗ C ∗ S ∗ P interaction was observed. At 84 DIM, the NA supplemented animals exhibited an increased basal ROS production. However, the supplementation with NA ended at 24 DIM and this effect may be of a priming nature and needs further elucidation.

Research suggests that the functional capacity of PMN and PBMC is impaired around parturition. Since the transition period is accompanied by an increased susceptibility to health disorders (Goff and Horst, 1997; Mallard et al., 1998) this led to the terminology of immunosuppression in the periparturient cow (Aleri et al., 2016). PMN and PBMC, especially the neutrophils and monocytes (the blood progenitor cells of tissue resident macrophages) belong to the innate immune system and fight against invading pathogens by engulfing and killing them with ROS. Therefore, the phagocytic activity and the capability to exert an oxidative burst are key qualities of these cells. However, reports of changes in both phagocytosis and oxidative burst during the periparturient period vary. Kehrli and Goff (1989) reported higher phagocytosis around parturition, whereas other researchers reported a decrease (Tan et al., 2012) or no change (Graugnard et al., 2012; Sander et al., 2011; Vailati-Riboni et al., 2017) in phagocytic activity in the periparturient dairy cow. Meglia et al. (2005) and Vailati-Riboni et al. (2017) reported no change in oxidative burst, whereas others (Kehrli and Goff, 1989; Piccinini et al., 2004) reported a decrease in oxidative burst in the periparturient period. These differences may be due to varying experimental designs and assays used. A key reason seems to be the difference between used blood leukocytes. Isolated blood cells seem to exhibit changes in their functional capacity whereas cells in whole

blood assays mostly do not (Vailati-Riboni et al., 2017).

This study used whole blood assays, and all measured parameters on the functional capacity of PMN and PBMC varied significantly over time in relation to parturition. Different aspects of the data on phagocytosis and ROS production were examined. The percentage of positively labeled cell type and its mean fluorescence intensity (being a measure of the amount of signal per cell) depicted, hereby, a qualitative approach. Relating the percentage of positively labeled cells to the absolute cell count of the animal, on the other hand, aimed at a quantification of the signal. The measured data on phagocytosis does not imply an overall impairment on the phagocytic activity of blood leukocytes in periparturient dairy cows depending on the considered aspect. The percentage of phagocytosing PMN as well as PBMC increased in the peripartal period and was higher postpartum compared to initial values at 42 days antepartum. The MFI, a measure of the number of phagocytosed bacteria per cell, decreased slightly between 42 and 10 days antepartum, which could reflect some kind of impairment during this period, but afterwards it increased as well. Some time points repeatedly stand out in the curve progressions of the measured parameters. They may be primarily related to hormone changes of either glucocorticoids or sex steroids. Those hormones reportedly influence the functional capacity of blood leukocytes (Chaveiro and Moreira da Silva, 2009; Lamote et al., 2004; Vangroenweghe et al., 2005) as well as the numbers of circulating PMN (Burton et al., 1995). Time point 7 DIM could be associated with the cortisol rise at parturition (Mallard et al., 1997; Preisler et al., 2000a, 2000b), whereas 28 and 42 DIM are accompanied with changes in the oestrus cycle (Crowe, 2008).

Percentage of basal ROS producing PMN exhibited a pronounced decrease before parturition and showed a distinct nadir at the day after parturition. Most likely this is associated with an increase in the stress hormone cortisol at parturition. The TPA stimulated fraction of ROS producing PMN was also slightly decreased around parturition. However, the MFI of either basal or TPA stimulated ROS producing PMN increased at parturition. That means, although there are less ROS producing cells, the ones that do produce ROS do this in a greater amount. ROS are increasingly recognized as important signaling molecules in a well-functioning immune system (Fialkow et al., 2007) and the described effects may be a consequence of that.

Primi- and pluriparous cows have different blood concentrations of PMN and PBMC and this is also evident during the periparturient period were it was shown that primiparous animals do have higher numbers for PMN and PBMC (Sander et al., 2011; Schulz et al., 2015; Tienken et al., 2015a). Additionally, it was shown that the viability of PMN is higher in primiparous cows and that these animals exhibit higher numbers of circulating immature PMN (Mehrzad et al., 2002). Primiparous cows with ongoing growth during pregnancy and first lactation have other levels of, for example, growth hormones. These are known to influence blood cell maturation in the bone marrow and may be an explanation for the different numbers in circulating blood cells (Vangroenweghe et al., 2005). Our results revealed parity effects for the calculated blood concentrations of phagocytosing PMN and PBMC, both being higher in primiparous cows. This possibly results of the above mentioned higher cell numbers for PMN and PBMC in primiparous animals (Mehrzad et al., 2002; Tienken et al., 2015a). However, the percentage of phagocytosing PBMC was higher in pluriparous cows. This may be due to more mature blood leukocytes in pluriparous animals (Mehrzad et al., 2002) that are able to better phagocytize.

The percentage of basal ROS producing PMN during the periparturient period also varied significantly between primi- and pluriparous animals. Pluriparous cows exhibited a higher basal ROS production from 42 days antepartum until 42 DIM with a distinct peak at 28 DIM. The higher basal ROS production may result from aging issues since it is known that older individuals experience more oxidative stress. Furthermore, pluriparous cows exhibit an increased need for anti-oxidative capacity during the periparturient period compared to primiparous animals (Bühler et al., 2017) accompanied with higher dry mater intake and milk yield that allow the assumption of higher cell respiration. Therefore, the higher basal ROS production in pluriparous cows might be an output of this increased respiratory rate. The peak at 28 DIM coincides with the time point of the first oestrus after calving. Estradiol was shown *in vitro* to increase oxidative burst activity (Chaveiro and Moreira da Silva, 2010) and therefore increased estradiol concentrations could influence basal ROS production in these cells as well.

5. Conclusion

Nicotinic acid affected phagocytic capacity and ROS production in blood leukocytes of dairy cows. To understand, however, the underlying mechanisms on the cellular level, further research is necessary. Although, the investigated parameters on the functional capacity of blood leukocytes varied in the course of the experiment, the results could not entirely be interpreted as expressing impaired functionalities. Depending on the aspect of the flow cytometry data (percentage of positively labeled cells or their MFI), increasing as well as decreasing signals have been measured illustrating the importance of considering both when interpreting the results. Between primi- and pluriparous animals there exist clearly differences that could be explained by different cellular equipment and hormonal influences.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors thank the Deutsche Forschungsgemeinschaft (DFG, Bonn, Germany) for financial support (DA 558/6-1). All co-workers of the Institute of Animal Nutrition and the Experimental Station of the Friedrich-Loeffler-Institute in Braunschweig who supported this work are gratefully acknowledged.

References

Aleri, J.W., Hine, B.C., Pyman, M.F., Mansell, P.D., Wales, W.J., Mallard, B., Fisher, A.D., 2016. Periparturient immunosuppression and strategies to improve dairy cow health during the periparturient period. Res. Vet. Sci. 108, 8–17.

Bell, A.W., 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J. Anim. Sci. 73, 2804–2819.

Bernabucci, U., Ronchi, B., Lacetera, N., Nardone, A., 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J. Dairy Sci. 88, 2017–2026.

Blacker, T.S., Duchen, M.R., 2016. Investigating mitochondrial redox state using NADH and NADPH autofluorescence. Free Radic. Biol. Med. 100, 53–65.

Brassard, P., Larbi, A., Grenier, A., Frisch, F., Fortin, C., Carpentier, A.C., Fulop, T., 2007. Modulation of T-cell signalling by non-esterified fatty acids. Prostaglandins Leukot. Essent. Fat. Acids 77, 337–343.

Bühler, S., Frahm, J., Tienken, R., Kersten, S., Meyer, U., Huber, K., Dänicke, S., 2017. Effects of energy supply and nicotinic acid supplementation on serum anti-oxidative capacity and on expression of oxidative stress-related genes in blood leucocytes of periparturient primi- and pluriparous dairy cows. J. Anim. Physiol. Anim. Nutr. 1–12. http://dx.doi.org/10.1111/jpn.12705.

Burton, J.L., Kehrli Jr., M.E., Kapil, S., Horst, R.L., 1995. Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone. J. Leukoc. Biol. 57, 317–325.

Cai, T.Q., Weston, P.G., Lund, L.A., Brodie, B., McKenna, D.J., Wagner, W.C., 1994. Association between neutrophil functions and periparturient disorders in cows. Am. J. Vet. Res. 55, 934–943.

Carlson, L.A., 2005. Nicotinic acid: the broad-spectrum lipid drug. A 50th anniversary review. J. Intern. Med. 258, 94–114.

Castillo, C., Hernandez, J., Bravo, A., Lopez-Alonso, M., Pereira, V., Benedito, J.L., 2005. Oxidative status during late pregnancy and early lactation in dairy cows. Vet. J. (Lond. Engl. 1997) 169, 286–292.

Chaveiro, A., Moreira da Silva, F., 2009. Effect of oestrous cycle on the oxidative burst activity of blood polymorphonuclear leucocytes in cows. Reprod. Domest. Anim. 44, 900–906.

Chaveiro, A., Moreira da Silva, F., 2010. In vitro effect of the reproductive hormones on the oxidative burst activity of polymorphonuclear leucocytes from cows: a flow cytometric study. Reprod. Domest. Anim. 45, e40–45.

Choi, H.J., Jang, S.Y., Hwang, E.S., 2015. High-dose nicotinamide suppresses ROS

generation and augments population expansion during CD8(+) T cell activation. Mol. Cell 38, 918–924.

Crowe, M.A., 2008. Resumption of ovarian cyclicity in post-partum beef and dairy cows. Reprod. Domest. Anim. 43 (Suppl. 5), 20–28.

Crowley, C.L., Payne, C.M., Bernstein, H., Bernstein, C., Roe, D., 2000. The NAD+ precursors, nicotinic acid and nicotinamide protect cells against apoptosis induced by a multiple stress inducer, deoxycholate. Cell Death Differ. 7, 314–326.

Drackley, J.K., 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J. Dairy Sci. 82, 2259–2273.

Fialkow, L., Wang, Y., Downey, G.P., 2007. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. Free Radic. Biol. Med. 42, 153–164.

Ganji, S.H., Kamanna, V.S., Kashyap, M.L., 2014. Niacin decreases leukocyte myeloperoxidase: mechanistic role of redox agents and Src/p38MAP kinase. Atherosclerosis 235, 554–561.

GfE, 2001. Empfehlungen zur Energie- und Nährstoffversorgung von Milchkühen und Aufzuchtrindern (Recommendations of energy and nutrient supply for dairy cows and breeding cattle). DLG-Verlag, Frankfurt am Main, Germany.

Goff, J.P., Horst, R.L., 1997. Physiological changes at parturition and their relationship to metabolic disorders. J. Dairy Sci. 80, 1260–1268.

Graugnard, D.E., Bionaz, M., Trevisi, E., Moyes, K.M., Salak-Johnson, J.L., Wallace, R.L., Drackley, J.K., Bertoni, G., Loor, J.J., 2012. Blood immunometabolic indices and polymorphonuclear neutrophil function in peripartum dairy cows are altered by level of dietary energy prepartum. J. Dairy Sci. 95, 1749–1758.

Hammon, D.S., Evjen, I.M., Dhiman, T.R., Goff, J.P., Walters, J.L., 2006. Neutrophil function and energy status in Holstein cows with uterine health disorders. Vet. Immunol. Immunopathol. 113, 21–29.

Hoeben, D., Heyneman, R., Burvenich, C., 1997. Elevated levels of beta-hydroxybutyric acid in periparturient cows and in vitro effect on respiratory burst activity of bovine neutrophils. Vet. Immunol. Immunopathol. 58, 165–170.

Ingvartsen, K.L., Moyes, K.M., 2015. Factors contributing to immunosuppression in the dairy cow during the periparturient period. Jpn. J. Vet. Res. 63 (Suppl. 1), S15–24.

Kehrli Jr., M.E., Goff, J.P., 1989. Periparturient hypocalcemia in cows: effects on peripheral blood neutrophil and lymphocyte function. J. Dairy Sci. 72, 1188–1196.

Kenez, A., Locher, L., Rehage, J., Danicke, S., Huber, K., 2014. Agonists of the G protein-coupled receptor 109A-mediated pathway promote antilipolysis by reducing serine residue 563 phosphorylation of hormone-sensitive lipase in bovine adipose tissue explants. J. Dairy Sci. 97, 3626–3634.

Kenez, A., Tienken, R., Locher, L., Meyer, U., Rizk, A., Rehage, J., Danicke, S., Huber, K., 2015. Changes in lipid metabolism and beta-adrenergic response of adipose tissues of periparturient dairy cows affected by an energy-dense diet and nicotinic acid supplementation. J. Anim. Sci. 93, 4012–4022.

Kimura, K., Goff, J.P., Kehrli Jr., M.E., Reinhardt, T.A., 2002. Decreased neutrophil function as a cause of retained placenta in dairy cattle. J. Dairy Sci. 85, 544–550.

Lacetera, N., Scalia, D., Franci, O., Bernabucci, U., Ronchi, B., Nardone, A., 2004. Short communication: effects of nonesterified fatty acids on lymphocyte function in dairy heifers. J. Dairy Sci. 87, 1012–1014.

Lamote, I., Meyer, E., Duchateau, L., Burvenich, C., 2004. Influence of 17beta-estradiol, progesterone, and dexamethasone on diapedesis and viability of bovine blood polymorphonuclear leukocytes. J. Dairy Sci. 87, 3340–3349.

Lee, J.Y., Sohn, K.H., Rhee, S.H., Hwang, D., 2001. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. J. Biol. Chem. 276, 16683–16689.

Maiese, K., Chong, Z.Z., Hou, J., Shang, Y.C., 2009. The vitamin nicotinamide: translating nutrition into clinical care. Molecules (Basel, Switzerland) 14, 3446–3485.

Mallard, B.A., Wagter, L.C., Ireland, M.J., Dekkers, J.C., 1997. Effects of growth hormone, insulin-like growth factor-I, and cortisol on periparturient antibody response profiles of dairy cattle. Vet. Immunol. Immunopathol. 60, 61–76.

Mallard, B.A., Dekkers, J.C., Ireland, M.J., Leslie, K.E., Sharif, S., Vankampen, C.L., Wagter, L., Wilkie, B.N., 1998. Alteration in immune responsiveness during the peripartum period and its ramification on dairy cow and calf health. J. Dairy Sci. 81, 585–595.

Meglia, G.E., Johannisson, A., Agenas, S., Holtenius, K., Waller, K.P., 2005. Effects of feeding intensity during the dry period on leukocyte and lymphocyte sub-populations, neutrophil function and health in periparturient dairy cows. Vet. J. (Lond. Engl. 1997) 169, 376–384.

Mehrzad, J., Duchateau, L., Pyorala, S., Burvenich, C., 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. J. Dairy Sci. 85, 3268–3276.

Miller, J.K., Brzezinska-Slebodzinska, E., Madsen, F.C., 1993. Oxidative stress, antioxidants, and animal function. J. Dairy Sci. 76, 2812–2823.

Montserrat-de la Paz, S., Naranjo, M.C., Lopez, S., Abia, R., Muriana, F.J., Bermudez, B., 2017. Niacin and its metabolites as master regulators of macrophage activation. J. Nutr. Biochem. 39, 40–47.

Morey, S.D., Mamedova, L.K., Anderson, D.E., Armendariz, C.K., Titgemeyer, E.C., Bradford, B.J., 2011. Effects of encapsulated niacin on metabolism and production of periparturient dairy cows. J. Dairy Sci. 94, 5090–5104.

Paape, M., Mehrzad, J., Zhao, X., Detilleux, J., Burvenich, C., 2002. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J. Mammary Gland Biol. Neoplasia 7, 109–121.

Piccinini, R., Binda, E., Belotti, M., Casirani, G., Zecconi, A., 2004. The evaluation of non-specific immune status of heifers in field conditions during the periparturient period. Vet. Res. 35, 539–550.

Pires, J.A., Grummer, R.R., 2007. The use of nicotinic acid to induce sustained low plasma nonesterified fatty acids in feed-restricted Holstein cows. J. Dairy Sci. 90, 3725–3732.

Preisler, M.T., Weber, P.S., Tempelman, R.J., Erskine, R.J., Hunt, H., Burton, J.L., 2000a. Glucocorticoid receptor down-regulation in neutrophils of periparturient cows. Am. J. Vet. Res. 61, 14–19.

Preisler, M.T., Weber, P.S., Tempelman, R.J., Erskine, R.J., Hunt, H., Burton, J.L., 2000b. Glucocorticoid receptor expression profiles in mononuclear leukocytes of periparturient Holstein cows. J. Dairy Sci. 83, 38–47.

Renner, L., von Soosten, D., Sipka, A., Doll, S., Beineke, A., Schuberth, H.J., Danicke, S., 2012. Effect of conjugated linoleic acid on proliferation and cytokine expression of bovine peripheral blood mononuclear cells and splenocytes ex vivo. Arch. Anim. Nutr. 66, 73–85.

Sander, A.K., Piechotta, M., Schlamberger, G., Bollwein, H., Kaske, M., Sipka, A., Schuberth, H.J., 2011. Ex vivo phagocytic overall performance of neutrophilic granulocytes and the relation to plasma insulin-like growth factor-I concentrations in dairy cows during the transition period. J. Dairy Sci. 94, 1762–1771.

Sartorelli, P., Paltrinieri, S., Agnes, F., 1999. Non-specific immunity and ketone bodies. I: in vitro studies on chemotaxis and phagocytosis in ovine neutrophils. Zentralblatt fur Veterinarmedizin. Reihe A 46, 613–619.

Scalia, D., Lacetera, N., Bernabucci, U., Demeyere, K., Duchateau, L., Burvenich, C., 2006. In vitro effects of nonesterified fatty acids on bovine neutrophils oxidative burst and viability. J. Dairy Sci. 89, 147–154.

Schulz, K., Frahm, J., Meyer, U., Kersten, S., Reiche, D., Rehage, J., Danicke, S., 2014. Effects of prepartal body condition score and peripartal energy supply of dairy cows on postpartal lipolysis, energy balance and ketogenesis: an animal model to investigate subclinical ketosis. J. Dairy Res. 81, 257–266.

Schulz, K., Frahm, J., Kersten, S., Meyer, U., Reiche, D., Sauerwein, H., Danicke, S., 2015. Effects of elevated parameters of subclinical ketosis on the immune system of dairy cows: in vivo and in vitro results. Arch. Anim. Nutr. 69, 113–127.

Sordillo, L.M., Aitken, S.L., 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Vet. Immunol. Immunopathol. 128, 104–109.

Sordillo, L.M., Contreras, G.A., Aitken, S.L., 2009. Metabolic factors affecting the inflammatory response of periparturient dairy cows. Anim. Health Res. Rev. 10, 53–63.

Ster, C., Loiselle, M.C., Lacasse, P., 2012. Effect of postcalving serum nonesterified fatty acids concentration on the functionality of bovine immune cells. J. Dairy Sci. 95, 708–717.

Suriyasathaporn, W., Daemen, A.J., Noordhuizen-Stassen, E.N., Dieleman, S.J., Nielen, M., Schukken, Y.H., 1999. Beta-hydroxybutyrate levels in peripheral blood and ketone bodies supplemented in culture media affect the in vitro chemotaxis of bovine leukocytes. Vet. Immunol. Immunopathol. 68, 177–186.

Tan, X., Li, W.W., Guo, J., Zhou, J.Y., 2012. Down-regulation of NOD1 in neutrophils of periparturient dairy cows. Vet. Immunol. Immunopathol. 150, 133–139.

Tienken, R., Kersten, S., Frahm, J., Huther, L., Meyer, U., Huber, K., Rehage, J., Danicke, S., 2015a. Effects of prepartum dietary energy level and nicotinic acid supplementation on immunological, hematological and biochemical parameters of periparturient dairy cows differing in parity. Animals 5, 910–933.

Tienken, R., Kersten, S., Frahm, J., Meyer, U., Locher, L., Rehage, J., Huber, K., Kenez, A., Sauerwein, H., Mielenz, M., Danicke, S., 2015b. Effects of an energy-dense diet and nicotinic acid supplementation on production and metabolic variables of primiparous or multiparous cows in periparturient period. Arch. Anim. Nutr. 69, 319–339.

Trevisi, E., Amadori, M., Cogrossi, S., Razzuoli, E., Bertoni, G., 2012. Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows. Res. Vet. Sci. 93, 695–704.

Vailati-Riboni, M., Zhou, Z., Jacometo, C.B., Minuti, A., Trevisi, E., Luchini, D.N., Loor, J.J., 2017. Supplementation with rumen-protected methionine or choline during the transition period influences whole-blood immune response in periparturient dairy cows. J. Dairy Sci. 100, 3958–3968.

Vangroenweghe, F., Lamote, I., Burvenich, C., 2005. Physiology of the periparturient period and its relation to severity of clinical mastitis. Domest. Anim. Endocrinol. 29, 283–293.

Yu, B.L., Zhao, S.P., 2007. Anti-inflammatory effect is an important property of niacin on atherosclerosis beyond its lipid-altering effects. Med. Hypotheses 69, 90–94.

Yuan, K., Shaver, R.D., Bertics, S.J., Espineira, M., Grummer, R.R., 2012. Effect of rumen-protected niacin on lipid metabolism, oxidative stress, and performance of transition dairy cows. J. Dairy Sci. 95, 2673–2679.

7. General Discussion

The aim of the present animal trial was to induce different degrees of lipolysis in periparturient dairy cows by feeding different concentrate proportions before parturition, different concentrate escalation strategies after parturition and additionally a nicotinic acid supplementation from 42 days before parturition until 24 DIM. The effect of these feeding strategies on the functional capacity of blood immune cells and on anti-oxidative parameters in the serum throughout the periparturient period, that is among other things characterized by a dysfunctional immune response (Ingvartsen and Moyes, 2013; Mallard et al., 1998) and by oxidative stress (Sordillo and Aitken, 2009), was analysed in the present study. With a comparable concentrate feeding strategy, Schulz et al. (2014) were able to induce differences in lipolysis in periparturient dairy cows as seen by increased NEFA, BHB and liver total lipid concentrations postpartum in the animals fed with a higher concentrate proportion prepartum and a prolonged escalation of the concentrate proportion after parturition. These animals experienced additionally a more severe and extended negative energy balance. However, in the present study no significant differences in the NEFA and BHB concentrations as indicators of different degrees of lipolysis could be established by the feeding regimen (Tienken et al., 2015b). The lower gap in concentrate proportion prepartum, 30% and 60% compared to 20% and 60% in the study by Schulz et al., could have contributed to this outcome. More likely, however, is that by dividing the animals by their BCS and allocating the high BCS cows to the higher concentrate group and the low BCS animals to the lower concentrate group Schulz et al. may have genetically preselected the cows for lipolytic capacity. In the current trial, the animals were allocated homogeneously to the feeding groups regarding their BCS to avoid a possible genetic pre-selection for the degree of lipolysis in the groups and, therefore, examining only feeding induced effects.

7.1. The influences of the feeding regimen

7.1.1. NA supplementation effects

The supplementation with 24 g non-rumen-protected NA per day and cow increased the serum NAM concentration to a mean value of 3.35 + 1.65 µg/ml in NA supplemented cows compared to a mean NAM concentration of 2.01 + 1.10 µg/ml in control animals during the supplementation period, however, NA was not detectable in the serum (Tienken et al., 2015a). Dietary NA tends to be converted to NAD^+ in the intestine or liver and released into the systemic circulation as NAM and, additionally, hepatocytes are very capable of removing NA from the portal circulation (Kirkland, 2009) explaining the not detectable NA

concentrations in serum. However, NA is able to decrease lipolysis via its receptor GPR109A in vitro, whereas, NAM exerts no anti-lipolytic effect on the lipolytic response of bovine adipose tissue explants (Kenez et al., 2014). Since NA seems to be the substance that is responsible for the anti-lipolytic effect and NA concentrations were under the detection limit in serum in the present study this explains the non-existing effects on NEFA and BHB values in the outcome of the present trial (Tienken et al., 2015b).

With the NA supplementation some interesting influences were recognized. GPR109A, the receptor for NA, is expressed in bovine PMN (Agrawal et al., 2017) as well as in human monocytes (Digby et al., 2012). Influences on early apoptotic PMN as well as on apoptotic PBMC became evident in a clear increase of apoptotic cells in the NA supplemented animals at 100 DIM (Paper I). In addition, the percentage of basal ROS producing PMN of NA supplemented animals showed higher values at 84 DIM than the control animals (Paper III). If ROS are interpreted as signal transducers for apoptotic processes (Redza-Dutordoir and Averill-Bates, 2016), this result could explain a higher apoptotic signal in the blood leukocytes at 100 DIM although NA supplementation lasted only until 24 DIM. NAM and NA concentrations were assessed in serum until 35 DIM and no statement about later niacin concentrations can be made. The serum NAM concentrations already declined at 35 DIM to the initial values before supplementation. Therefore, the described niacin effects must be interpreted as a priming effect that needs further elucidation. The ferric reducing ability (FRA) of serum, a measure of the non-enzymatic antioxidant capacity, was decreased in NA supplemented animals most pronounced in the 2 weeks after parturition (Paper II). An explanation for this effect could be the plasma cholesterol lowering effect of NA in humans (Carlson, 2005). With reduced cholesterol, smaller amounts of the lipid-soluble antioxidant vitamin E could be transported in the serum of NA supplemented animals, therefore being responsible for reduced FRA values. In favour of this explanation is the significant positive correlation of FRA and cholesterol (Paper II), however, the NA supplementation was not able to induce significant differences in the cholesterol concentrations (Tienken et al., 2015a). A further explanation for the NA effect on FRA values could arise from increased NAD^+ concentrations with NA supplementation that shift the redox pair ratio of $NADP^+$/NADPH to an unfavourable direction. Consequentially, reduced antioxidants, like glutathione, may not be supplied in oxidized form to the same extent than in control animals, hence, leading to lower FRA values in NA supplemented animals. Additionally, with a higher NAD^+ supply through NA supplementation, the overall metabolic activity in these animals could be higher leading to more ROS accumulation in these animals, that needs to be scavenged through antioxidants, hence the lower FRA values. This explanation is also supported by the higher serum SOD enzyme activity antepartum in LC-NA animals (Paper II) that also indicates a higher metabolic rate with related higher ROS production and the

need to scavenge this in the supplemented animals. The greater ROS production in cells with niacin and subsequent NAD^+ supply is furthermore affirmed by the higher basal ROS production (MFI) in PMN seen after incubation with increasing concentrations of NA and NAM (Paper III). The blood concentration of phagocytosing PMN was higher in NA supplemented animals than in control animals (Paper III). Additionally, the percentage of phagocytosing PBMC tended to be higher in NA supplemented animals (Paper III). Phagocytosis is a process that involves cell signalling with ROS participation (Fialkow et al., 2007; Vernon and Tang, 2013). The higher ROS production in PMN with increased niacin incubation (Paper III) could therefore already explain a higher phagocytic activity of PMN and PBMC in NA supplemented animals.

7.1.2. Effects of energy supply

The feeding of the higher concentrate proportion in the HC animals influenced anti-oxidative and apoptotic parameters. The serum GPX activity was higher in HC cows indicating a higher metabolic rate in these animals and a subsequent higher oxidative burden (Paper II) that needs to be cleared off. Supported is this view also by antepartum higher mRNA abundances of the genes SOD2, XDH and PARP1 in the HC animals (Paper II) indicating energy effects on mitochondrial respiratory chain, ROS formation and regulation of oxidative stress induced cell death. Since ROS can induce apoptotic processes (Sinha et al., 2013), a higher metabolic rate with increased ROS production as a side product of the respiratory chain could already explain the higher proportion in early apoptotic PMN and PBMC that was seen in HC cows (Paper I). Additionally, the mRNA abundance of the anti-apoptotic gene BCL2 tended to be lower in LC animals in the week before parturition until 7 DIM, also indicating at lower oxidative stress in these animals.

Zhou et al. (2015) and Khan et al. (2015) also observed higher expression levels of oxidative stress related and inflammatory genes in PMN and liver cells with cows that were overfed antepartum. This indicates a higher inflammatory state of HC cows that also entails an increase in ROS and oxidative stress.

7.2. Influences over the course of time

All examined parameters on immune cell functional activity and gene expression as well as the antioxidant parameters were significantly influenced by time in relation to parturition. This supports that the transition dairy cow undergoes dramatic changes in immune response (Ingvartsen and Moyes, 2015) and oxidative stress (Sordillo and Aitken, 2009) during the periparturient period.

Apoptosis expressed an overall reduction during the periparturient period from day – 14 until 42 DIM seen for the early apoptotic as well as for the late apoptotic leukocytes (Paper I). The values at 100 DIM did not reach the original values from 6 weeks before parturition. PMN expressed hereby lower percentages of apoptotic cells than PBMC (Paper I) that is also seen by Tharwat et al. (2012). The reduced percentage in apoptotic cells could be causative for the higher neutrophil cell counts in the periparturient cow (Schulz et al., 2015; Tienken et al., 2015a). Crookenden et al. (2016) detected an increased expression in anti-apoptotic genes and a decreased expression of pro-apoptotic genes in bovine neutrophils postpartum compared to the pre-calving state. According to the authors, this decreased apoptosis and the presumably longer life span of the cells may be advantageous to the immune system, since the onset of apoptosis leads to functional impairment in neutrophils (Whyte et al., 1993). However, the PBMC showed, especially for the late apoptotic cells, a strong increase of the apoptotic proportion around parturition. Since glucocorticoids and other steroid hormones induce apoptosis in lymphocytes (Planey and Litwack, 2000), this increase could be partly explained by hormonal changes at parturition. Albeit, hormonal changes are rather short-lived and the increased numbers of apoptotic PBMC could additionally be explained by the inflammation inducing effects of NEFA and the oxidative stress around parturition (Sordillo and Aitken, 2009). In particular, macrophages are described to be affected by lipotoxicity and react by increasing inflammatory processes and subsequent apoptosis (Prieur et al., 2010). Taken together, the PBMC seemed to be more affected by the changes around parturition in their apoptotic behaviour than the PMN (Paper I). Consistent with Crookenden et al. (2016) the present study also revealed increased gene expression levels for the anti-apoptotic genes BCL2 and BCL-xL after parturition supporting the decreased apoptotic behaviour of the blood leukocytes post-partum (Paper I). For the pro-apoptotic genes BAX and CASP3, however, a pronounced expression peak just before parturition became evident that preceded the apoptotic peak in late apoptotic PBMC (Paper I). Similar expression peaks for the oxidative stress related genes (Paper II) accompanied the expression of the pro-apoptotic genes indicating at the relationship between oxidative stress and apoptosis (Sinha et al., 2013). With the onset of lactation, the increased energy requirement is accompanied by increased oxygen consumption through cellular respiration. The emerging ROS as by-products of the respiratory chain (Valko et al., 2007) are also important signalling molecules that activate transcription factors like NFκB and NRF2 and consequentially promote inflammation and initiate antioxidant defences (Franchina et al., 2018). The expression peaks for the genes NRF2, GPX1, SOD2 and XDH just before parturition (Paper II) support the occurrence of oxidative stress in the transition dairy cow (Bernabucci et al., 2002, 2005; Castillo et al., 2005; Miller et al., 1993). FRA values exhibited a pronounced nadir just before parturition (Paper II) also suggesting an oxidative stressed

situation. Since FRA depicts the non-enzymatic antioxidant activity, in this case in the serum, the pronounced nadir just before parturition may mostly be deduced from the transfer of antioxidants into colostrum (Goff and Stabel, 1990) and this depletion being further aggravated by the reduced DMI before parturition (Tienken et al., 2015b). The serum activity of the antioxidant enzyme GPX peaked just after parturition (Paper II) therefore following the gene expression peak and possibly being an adaption to the increased need for antioxidants. Postpartum GPX activities stayed on a higher level than prepartum activities (Paper II) also seen by other researchers (Bernabucci et al., 2005; Didara et al., 2015) supporting the higher metabolic rate that is necessary with ongoing lactation and the subsequent need for antioxidants. However, the strong correlations of the GPX serum activity with liver enzymes aspartate aminotransferase (AST) and gamma glutamyl transferase (γGT) as well as with NEFA and BHB (Paper II) led to the assumption that an increased GPX serum activity may also be a consequence of liver cell damage. Serum enzyme activities for SOD decreased in the lactation period and exhibited lower activities than before parturition showing only a slight peak right after parturition (Paper II). Therefore, it displayed quite a different time course than GPX and may not directly be connected with the strong expression peak of SOD2 before parturition (Paper II). SOD2 is the mitochondrial SOD and its' expression was measured in blood leukocytes, whereas the serum SOD enzyme activity rather origins from an extracellular form. Extracellular SOD is usually bound to the cell surface but depending on the blood glucose level it gets glycosylated and released into the serum (Fattman et al., 2003). For example in diabetic patients higher concentrations of glycated extracellular SOD are found in the serum (Adachi et al., 1991). The postpartum decrease of the glucose concentration (Tienken et al., 2015b) therefore explains the decrease in SOD serum enzyme activity supported by the significant correlation of SOD activity and glucose concentration (Paper II). The negative correlations of SOD activity with the liver enzymes γ-GT and glutamate dehydrogenase (GLDH) (Paper II) strengthening the different cellular origin of GPX and SOD serum enzyme activities.

Research suggests that the functional capacity of PMN and PBMC is impaired around parturition and since the transition period is accompanied by an increased susceptibility to health disorders (Goff and Horst, 1997; Mallard et al., 1997), the terminology of immunosuppression in the periparturient cow was established (Aleri et al., 2016; Ingvartsen and Moyes, 2015). However, the results on phagocytic activity and the capability to perform an oxidative burst, key qualities of neutrophils and monocytes, are inconsistent in the literature depending on the assays used, time points evaluated, and the treated cells, e.g. isolated cells or whole blood assays (Vailati-Riboni et al., 2017). In the present study, the proportion of phagocytic PMN and PBMC exhibited an increase throughout the periparturient period, whereas the MFI, a measure of the internalized bacteria per cell,

decreased prepartum and increased again after parturition (Paper III). The decrease in MFI could be interpreted as an impaired function since individual cells incorporated less bacteria. However, the higher proportion of phagocytosing cells may compensate for that. The percentage of PMN displaying a basal ROS production strongly decreased before parturition and exhibited a pronounced nadir the day after parturition (Paper III). That points at a decreased cell respiration in the PMN, maybe caused by decreased energy availability. However, it disagrees with the increase in oxidative stress related gene expression of blood leukocytes just before parturition (Paper II). Presumably, hormonal influences especially the rise in cortisol before parturition is of more significance at this point (Preisler et al., 2000). The MFI of basal ROS producing PMN, in this case a measure for the amount of ROS produced per cell, decreased between days -42 and -14 to increase afterwards and exhibited a peak at 14 DIM (Paper III). The TPA stimulated PMN showed comparable curve fits, with a decreased percentage of ROS producing cells and a nadir around parturition and an increase in MFI with a peak just after parturition (Paper III). Several time points repeatedly stood out in the curve progressions of the phagocytosis and ROS data that could be related to hormonal changes of either glucocorticoids or sex steroids during the periparturient period. Those hormones reportedly influence the functional capacity of blood leukocytes (Chaveiro and Moreira da Silva, 2009; Lamote et al., 2004; Vangroenweghe et al., 2005). In this context the cortisol rise at parturition (Preisler et al., 2000) and the changes in the oestrus cycle at 28 and 42 DIM (Crowe, 2008) may influence the examined parameters.

The present study examined healthy animals that passed the complete experimental period without metabolic and infectious diseases. Therefore, the changes in the functional activities and capacities of the blood leukocytes represent the adaptations of the immune cells to the challenges and changes in the periparturient period.

7.3. The differences between primiparous and pluriparous animals

Primiparous cows exhibit a greater variability in measured parameters during the transition period compared to pluriparous cows (Jonsson et al., 2013; Tienken et al., 2015a). During the evaluation of the present study, it became furthermore evident that primi- and pluriparous animals differ greatly in many of the measured parameters on the functional capacity and gene expression of blood leukocytes and antioxidant serum capacity (Figure 6). Primi- and pluriparous cows have different blood concentrations of PMN and PBMC and this is also demonstrated in the periparturient period where primiparous animals have higher numbers for PMN and PBMC (Sander et al., 2011; Schulz et al., 2015; Tienken et al., 2015a). Consequentially, the total blood concentrations of phagocytosing PMN and PBMC

were higher in primiparous cows (Paper III). Mehrzad et al. (2002) revealed that PMN viability and the number of circulating immature PMN is higher in primiparous cows. With ongoing growth during the first pregnancy and lactation, these animals exhibit for example different levels of growth hormones than mature cows. These hormones are known to influence blood cell maturation in the bone marrow, therefore, explaining the different numbers in circulating blood cells (Vangroenweghe et al., 2005). The mRNA abundance in blood leukocytes of the NFκB transcription factor subunit RELA was also higher in primiparous cows throughout the periparturient period (Paper I) supporting the view of the immature primiparous animal. In addition, Grossmann et al. (2000) revealed that RELA is important in B-cell maturation and survival. Hence, the higher gene expression levels for RELA could also result from the higher lymphocyte numbers in primiparous cows. The percentage of phagocytosing PBMC was higher in pluriparous cows (Paper III). This could also hint at a higher level of mature blood leukocytes in pluriparous animals (Mehrzad et al., 2002) that are able to better phagocytize. Serum activity of the antioxidant enzyme GPX as well as the gene expression levels for GPX1, SOD2 and XDH were higher in pluriparous cows through most of the periparturient period (Paper II). In addition to the higher percentage of basal ROS producing PMN (Paper III), this supports the theory of a greater oxidative burden in pluriparous cows, that may result from a higher metabolic rate that is linked to the greater milk production in pluriparous animals (Tienken et al., 2015b). Considering the higher percentage of phagocytosing PBMC, however, this may also indicate an elevated inflammatory state in the pluriparous cows. Percentage in early apoptotic leukocytes tended to be lower in pluriparous cows most pronounced between days -14 and 28 DIM (Paper I) indicating that the antioxidant mechanisms (Paper II) were able to support the survival of the cells albeit the presence of higher percentages of basal ROS producing PMN (Paper III). The increased serum SOD enzyme activity in primiparous cows (Paper II), however, is not in support of the abovementioned aspects. The glucose concentrations were significant higher in primiparous animals in the dry period and tended to be higher in primiparous cows in the lactation period (Tienken et al., 2015b). Since SOD is usually bound to the cell surface and depending on the glucose level it gets glycosylated and released into the serum (Fattman et al., 2003) the higher glucose concentrations in primiparous cows explain the higher serum SOD enzyme activities in these animals.

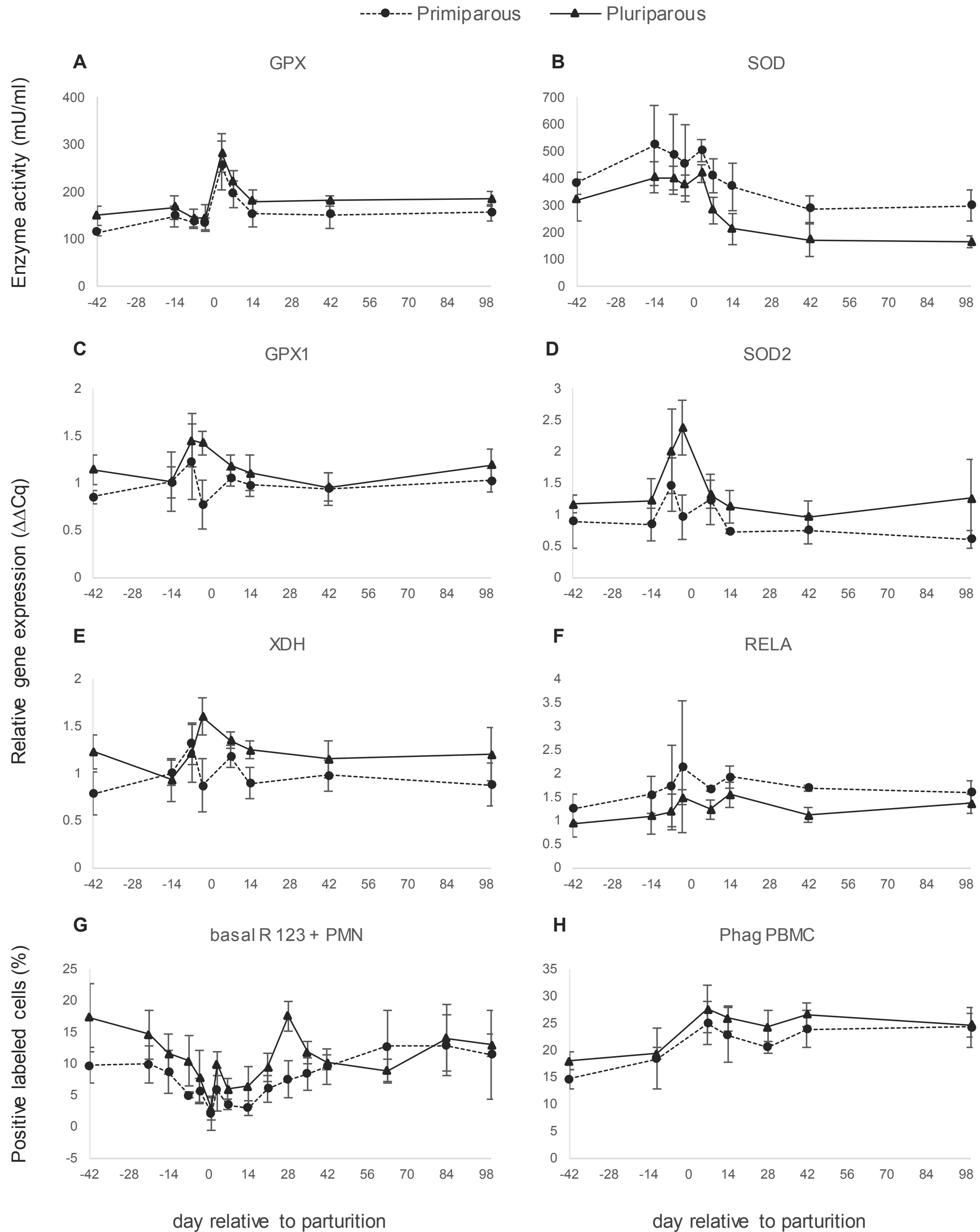


Figure 6: Parameters that differ significantly between primiparous and pluriparous dairy cows during the periparturient period: Serum antioxidant enzyme activities of glutathione peroxidase (GPX; A) and superoxide dismutase (SOD; B); gene expression of the oxidative stress related genes GPX1 (C), SOD2 (D) and xanthine dehydrogenase (XDH; E) as well as the anti-apoptotic and inflammation associated transcription factor NF-κB subunit v-rel avian reticuloendotheliosis viral oncogene homolog A (RELA; F); the proportion of basal reactive oxygen species producing polymorphonuclear leukocytes (PMN; G), and the proportion of phagocytic peripheral blood mononuclear cells (PBMC; H).

7.4. Principal component analysis

To connect the present data on the functional activity and gene expression of blood leukocytes and antioxidant serum parameters with other established data on production, metabolic, biochemical, haematological as well as immunological parameters (Tienken et al., 2015a; Tienken et al., 2015b) (Paper I-III) and to visualize the relationship between this large number of variables, a principal component analysis (PCA) was conducted based on correlations. On total 63 parameters of each individual cow during the periparturient period were included in the PCA to detect interesting interactions. The results revealed that the first two principal components (PC1 and PC2) extracted about 20% of the total variance. The scree plot as a visualization between the step-by-step extracted components and the corresponding eigenvalue did not reveal a distinct break point that would depict the separation between the most important components and the ones not significantly contributing to the total explained variance. Nineteen extracted components with an eigenvalue of >1.0 (the mean value of all 63 eigenvalues) explained approximately 74% of the total variance. Figure 7 illustrates the 63 variables plotted in the space between PC1 and PC2 to visualize their relationship to each other and to both components. Since the PCA was based on correlations, a localization of the variable close to the cross at the centre means only poor or no correlation of this particular variable to both components. The closer the localization of a variable to the outer circle, the closer the correlation to 1.0 or -1.0 of that variable to PC1 or PC2 or to both and the better its variance can be explained by that PC. Variables that are in close proximity to each other can be interpreted as being positively correlated to each other, whereas variables that have opposing localizations in the plot rather display negative correlations.

The apoptotic parameters late apoptotic (la) and early apoptotic (ea) leukocytes (Leukos la, Leukos ea), PMN ea and PBMC ea are positively correlated to PC1 and negatively correlated to PC2. They cluster with the energy parameters glucose, energy balance (EB) and TG as well as haemoglobin and haemoglobin associated parameters mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). Apoptosis is an energy-consuming process that is necessary for a well-functioning immune system, e.g. in getting safely rid of phagocytes after oxidative burst. Since blood leukocytes prefer glucose as energy source, the reduced apoptotic activity of blood leukocytes after parturition may be ascribed to the reduced blood glucose concentration postpartum (Paper I) (Tienken et al., 2015b). However, an increased glucose concentration could also entail higher cell metabolism with higher ROS production in the cell and subsequent apoptotic processes. The proximity of the proportion of basal ROS producing PMN may support this view. Increased haemoglobin could imply increased oxygen transport and subsequent higher

cellular respiration in the cells with the impact on ROS production and apoptotic processes (Paper I). The close proximity of apoptotic parameters to TG can be attributed to lipotoxic effects of fatty acids on macrophages and the initiation of inflammatory and subsequent apoptotic processes (Prieur et al., 2010). The early apoptotic cells are more influenced by the above mentioned parameters than the late apoptotic ones (Paper I).

The proportions of phagocytic PMN and PBMC are negatively correlated to PC 1 and PC2. Spearman rank correlations showed that they are negatively correlated to the apoptotic parameters of blood leukocytes. Onset of apoptosis leads to functional impairment in neutrophils (Whyte et al., 1993) hence the negative correlation. Percentage of phagocytic PMN and PBMC are also negatively correlated to glucose, EB and TG, therefore the opposing location to the apoptotic cluster. Although this is not consistent, since phagocytosis should also require energy to be performed. The phagocytic proportion of blood leukocytes is associated with liver enzymes AST, γGT and GLDH. These enzymes are connected with liver damage and maybe the associated inflammatory processes lead to increased phagocytosis in PMN and PBMC, whereby the latter are more affected.

With the PCA the differences in serum GPX and SOD enzyme activity also become apparent. Whereas SOD enzyme activity is positively correlated to PC1 and PC2, GPX enzyme activity is negatively correlated to both. Again, the association of GPX activity to liver enzymes becomes evident (Paper II) while SOD activity is associated to blood cells strengthening the conclusion that the antioxidant enzyme activities originate from different cellular sources (Paper II).

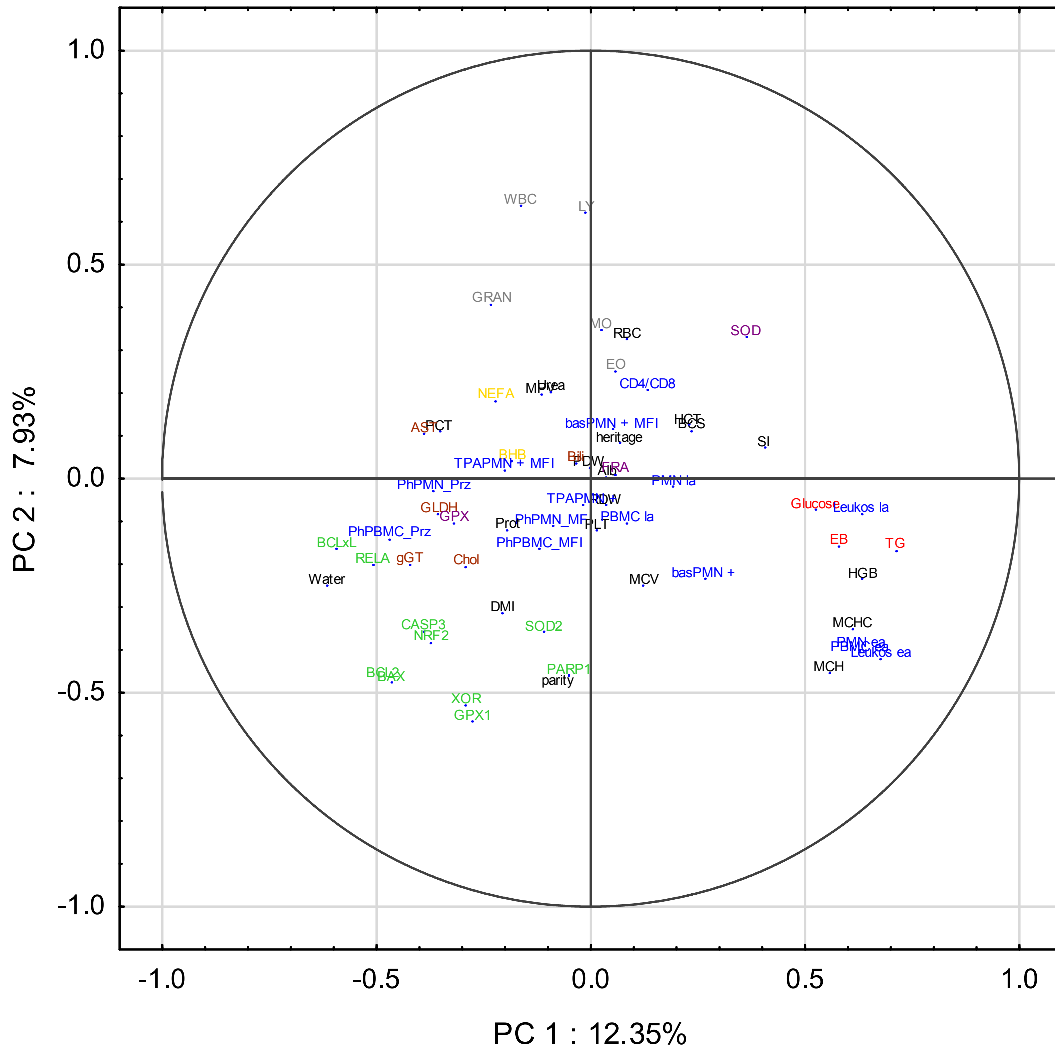


Figure 7: Principal component analysis for the two-dimensional visualization of the relationships between 63 variables collected from the experiment. Colour code and abbreviations of the depicted variables: leukogram: WBC white blood cell count, LY lymphocyte concentration, MO monocyte concentration, GRAN neutrophil granulocyte concentration, EO eosinophil granulocyte concentration; erythrogram: RBC red blood cell count, HGB haemoglobin, HCT haematocrit, MCV mean corpuscular volume, MCH mean corpuscular haemoglobin, MCHC mean corpuscular haemoglobin concentration, PLT platelet count, PCT plateletcrit, MPV mean platelet volume, PDW platelet distribution width, RDW red cell distribution width; gene expression data of the in Table 1 listed genes of interest; serum anti-oxidative parameters: GPX glutathione peroxidase activity, SOD superoxide dismutase activity, FRA ferric reducing ability; flow cytometry parameters: CD4/CD8 ratio between CD4+ and CD8+ cells, PMN ea proportion of early apoptotic polymorphonuclear leukocytes (PMN), PMN la proportion of late apoptotic PMN, PBMC ea proportion of early apoptotic peripheral blood mononuclear cells (PBMC), PBMC la proportion of late apoptotic PBMC, Leukos ea proportion of early apoptotic blood leukocytes, Leukos la proportion of late apoptotic blood leukocytes, PhPMN_Prz proportion of phagocytosing PMN, PhPMN_MFI mean fluorescence intensity (MFI) of phagocytosing PMN, PhPBMC_Prz proportion of phagocytosing PBMC, PhPBMC_MFI MFI of phagocytosing PBMC, basPMN + proportion of basal reactive oxygen species (ROS) positive PMN,

TPAPMN + proportion of ROS positive PMN after 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulation, basPMN + MFI MFI of basal ROS positive PMN, TPAPMN + MFI MFI of ROS positive PMN after TPA stimulation; SI stimulation index relation between concanavalin A stimulated PBMC and unstimulated PBMC, Liver associated parameters: Chol cholesterol, AST aspartate-aminotransferase, gGT gamma-glutamyl transferase, GLDH glutamate dehydrogenase, Bili bilirubin; Alb albumin, Prot protein, urea, BHB 3-β-hydroxybutyrate, NEFA non-esterified fatty acids; Glucose, TG triglycerides, EB energy balance, BCS body condition score, DMI dry mater intake, water water intake, parity primi-or pluriparous, heritage according to bull.

The PCA did not reveal any differences between the feeding groups (Figure 8). The projection of cases (individual cows) are shown here between the principal components PC 1 and PC 2 at the different time points labelled according to their feeding groups. However, the changing parameters according to time can be depicted in this figure. The marks clearly change during time around the axes coordinates. At 42 days before expected parturition, they start out widely spread, mostly in the lower right panel of the coordinate axes, confirming the high animal individual variability. Later on, the marks seem to perform a counter clockwise rotation until 100 DIM. The representations for the time points 7, 14 and 42 DIM look rather similar and more condensed which can be interpreted as a closer relation of the variables at these times. At 100 DIM the marks are again broader distributed and indicate a higher variability between the animals.

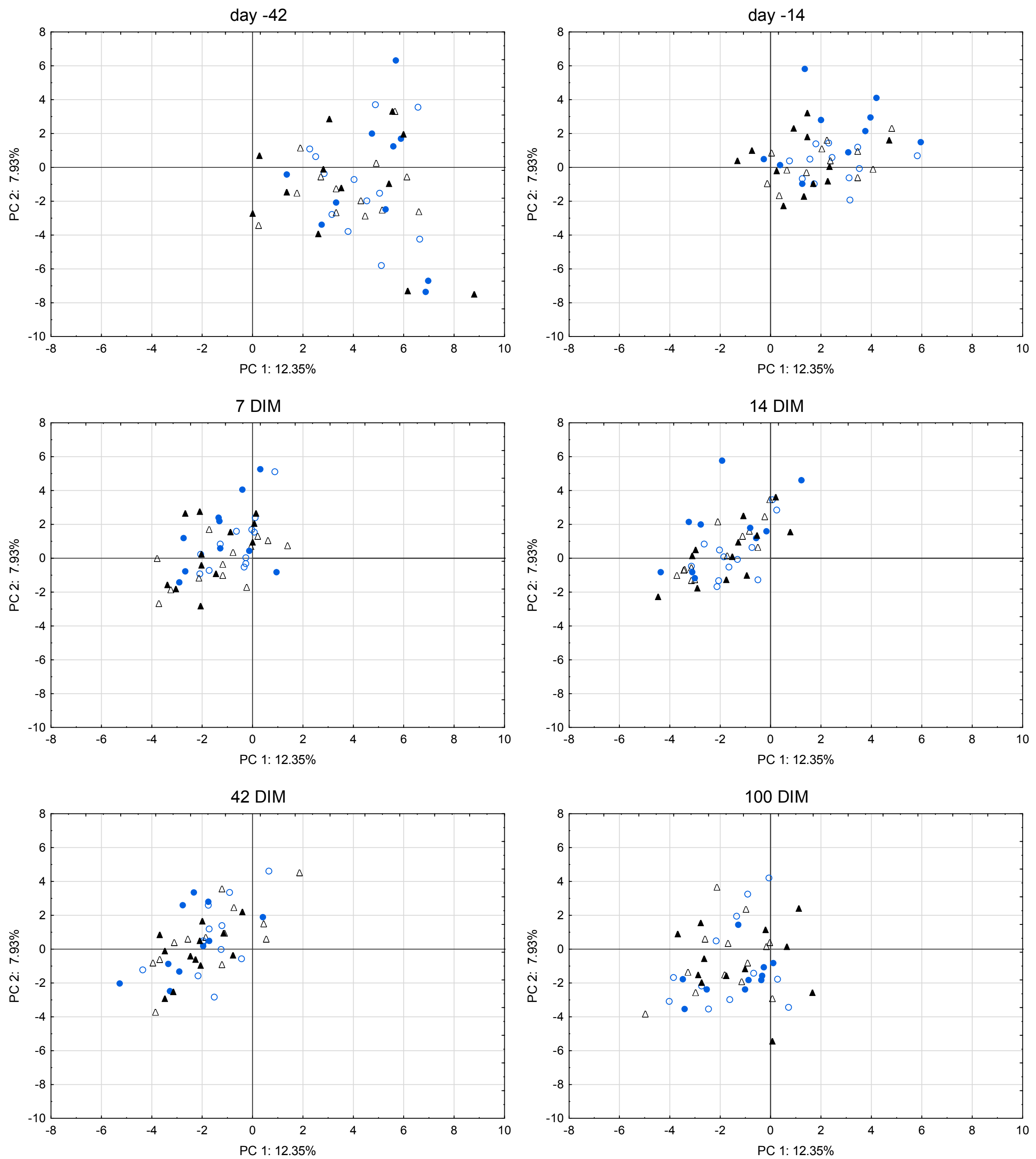


Figure 8: Principal component analysis projection of the individual cows of the four feeding groups at the different time points of the experiment. ○ LC-CON (n=12), ● LC-NA (n=13), △ HC-CON (n=10), ▲ HC-NA (n=12)

In addition, the differences between primi- and pluriparous animals became further evident with the PCA. To emphasize this, the analysis was done for each time point individually and Figure 9 shows the projections of the cases (individual cows) into the two dimensional space between each PC 1 and PC 2 pair calculated for the different time points. The primi- and pluriparous animals displayed clearly clusters supporting the differences between these two animal groups throughout the periparturient period.

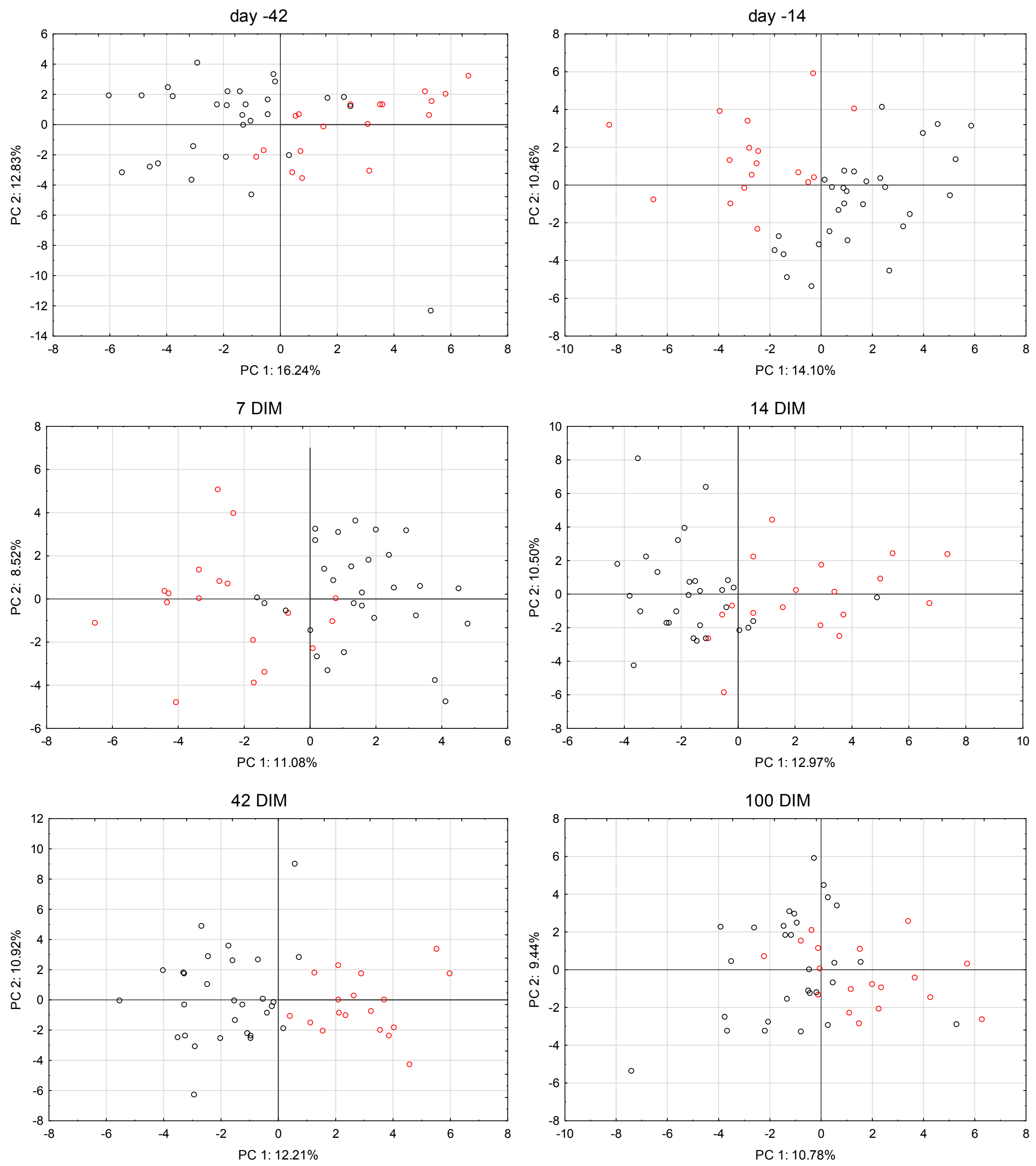


Figure 9: Principal component analysis projection of the individual cows for the individual time points of the experiment. Red circles depict primiparous cows (n=18) and black circles pluriparous animals (n=29).

8. Conclusion

Although the feeding experiment did not induce differences in the degree of lipolysis and the blood concentrations of NEFA and BHB, niacin effects on the functional capacity of blood leukocytes as well as on antioxidant serum parameters were detected that may mostly be explained by higher NAD^+ availability in the NA supplemented cows. Prepartum energy supply affected the antioxidant capacity of the serum and gene expression in blood leukocytes. Hereby, the higher energy level may be responsible for a higher metabolic rate in these animals with a subsequent higher oxidative stress level as produced by ROS, generated from the respiratory chain. However, the need for higher antioxidant capacity could also result from increased inflammatory processes in the cows fed with higher energy supply. Additionally, apoptosis was associated to energy parameters. Parturition clearly is accompanied by changes in functional activity and capacity of blood leukocytes as well as in antioxidant capacity of the serum. This is in accordance to in the literature reported changes in immune response and oxidative stress in the transition period. However, a clear dysfunction of blood leukocytes was not detected. Since the present study was performed on healthy animals with no signs of metabolic and infectious diseases, the results rather demonstrate the adaptations of the immune cells to the diverse changes in the transition period. Primi- and pluriparous cows clearly differ in their equipment in blood immune cells and their functional capacity of blood leukocytes and antioxidant parameters during the periparturient period. Primiparous cows are furthermore not fully-grown animals and presumably differ in hormonal environment that shows subsequent influences on the immune blood cells and their functional capacity emphasizing the need to treat these cows differently in the periparturient period from pluriparous animals.

9. Summary

Periparturient dairy cows experience vast physiological, metabolic, hormonal and immunological changes during the transition from late gestation to early lactation. This period is furthermore associated with oxidative stress, inflammation and immunosuppression and a high incidence in metabolic and infectious diseases. Since the energy intake does not meet the energy requirement that accompanies the needs for foetus growth and starting milk production, the animals experience a negative energy balance that makes it necessary for the cow to mobilise energy reserves from body tissues. The increased lipolysis in adipose tissue leads to increased blood concentrations of NEFA and subsequently, since the liver is not able to use up all these fatty acids in energy conversion through the Krebs cycle, of increased BHB blood concentrations. Both, NEFA and BHB are adversely affecting blood immune cell functions and are thought to play a major part in the immunosuppression during the transition period that leaves the animals more susceptible to infectious diseases like mastitis and metritis. In particular, NEFA also induces inflammatory processes through the TLR4 pathway. Niacin is known to have anti-lipolytic effects and has the potential to decrease postpartum NEFA and BHB concentrations but it is also described to exert anti-inflammatory and anti-oxidative influences on immune cells. Therefore, it seems to be an ideal supplementation to test in the periparturient period. To study possible influences of niacin on the functions of blood immune cells and anti-oxidative serum parameters in the periparturient dairy cow a feeding experiment was undertaken that aimed at inducing different degrees of lipolysis.

To address this, 29 pluri- and 18 primiparous healthy and pregnant German Holstein-Friesian dairy cows were allocated homogenously to four different feeding groups considering their body weight, BCS as well as number of lactations and milk yield of previous lactations. The experiment started 42 days before expected parturition and ended at 100 DIM. Before parturition, the cows received either a low concentrate diet (30% concentrate and 70% roughage on dry matter (DM) basis; LC) or a high concentrate diet (60% concentrate and 40% roughage on DM basis; HC) with or without a supplementation of 24 g per day and cow of powdered non-rumen protected NA resulting in the feeding groups LC-NA and HC-NA as well as LC-CON and HC-CON. Supplementation was applied from 42 days antepartum until 24 DIM. After parturition, all animals received initially a diet of 30% concentrate and 70% roughage. This concentrate proportion was increased up to 50% either within 16 days (LC animals) or 24 days (HC animals) and was maintained at 50% until the end of the trial. Blood samples were collected from the *Vena jugularis externa.*

Polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC) were examined on a functional level with flow cytometry. Apoptosis was investigated with an Annexin V and propidium iodide (PI) based assay and distinguished into early apoptotic (annexin V + and PI -) and late apoptotic (annexin V + and PI +) cells. Phagocytosis was determined by the ingestion of FITC-labeled opsonized *E.coli* and the capability of PMN to produce reactive oxygen species (ROS) was examined by the intracellular oxidation of the non-fluorescent dye dihydrorhodamine 123 (DHR) to the fluorescent rhodamine 123. In addition, the expression of apoptotic (BAX, BCL2, BCL-xL, CASP3 and RELA) and oxidative stress-related (GPX1, NRF2, PARP1, SOD2 and XDH) genes was quantified with real-time PCR in blood leukocytes. Furthermore, serum enzyme activities of glutathione peroxidase (GPX) and superoxide dismutase (SOD) were analyzed spectrophotometrically as well as serum ferric reducing ability (FRA) by the conversion of $Fe3^{+}$-tripyridyltriazine to $Fe2^{+}$-tripyridyltriazine.

Although the feeding experiment achieved no differences in lipolysis as seen by uninfluenced NEFA and BHB values, all measured variables exhibited a time dependency which was mainly related to parturition. PBMC were characterized by a more pronounced apoptosis than PMN and early apoptosis in PBMC was significantly influenced by concentrate level. The HC groups showed higher numbers of apoptotic cells than the LC groups. LC animals exhibited a decrease in the expression of the anti-apoptotic gene BCL2 before parturition, whereas the HC animals showed a continuous increase. Additionally, apoptosis in blood leukocytes correlated to energy variables. Serum GPX activity was higher in HC animals. Oxidation variables were strongly influenced by parity. Pluriparous cows exhibited higher expression levels for the oxidative stress related genes GPX1, SOD2 and XDH, higher serum GPX activity, increased percentages of phagocytizing PBMC and a greater proportion of basal ROS production in PMN. Whereas, primiparous animals showed higher RELA gene expression and SOD serum activity. Nicotinic acid supplementation did exhibit some influence in increasing numbers of early apoptotic PMN and late apoptotic PBMC between 42 and 100 DIM and increased the percentage of basal ROS producing PMN at 84 DIM. It furthermore tended to increase PBMC phagocytosis and decreased FRA values in the 2 weeks after parturition.

The present study confirmed that parturition is a period of multifold changes with considerable impact on blood immune cell functional activity and gene expression as well as on antioxidant serum parameters. Parity in this context plays an important role, since animals differing in age had different prerequisites and functional abilities in blood leukocytes to respond to the stressful period of parturition. However, the changes in functional activity and capability of blood leukocytes can not be interpreted as dysfunctional

responses. They rather represent the adaptation to the challenges during the transition phase. Energy availability however seems to play a major role in this respect.

10. Zusammenfassung

Peripartale Milchkühe durchleben große physiologische, metabolische, hormonelle und immunologische Veränderungen während des Übergangs von der späten Trächtigkeit in die frühe Laktation. Zudem ist diese Periode mit oxidativem Stress, Entzündungen, Immunsuppression und einer hohen Inzidenz an Stoffwechsel- und Infektionskrankheiten verbunden. Da die Energieaufnahme den Energiebedarf nicht deckt, der mit dem Wachstum des Fötus und der Milchproduktion einhergeht, erleben die Tiere eine negative Energiebilanz, welche es notwendig macht, dass die Kuh Energiereserven aus dem Körpergewebe mobilisiert. Die erhöhte Lipolyse des Fettgewebes führt zu erhöhten Blutkonzentrationen von NEFA und, da die Leber nicht in der Lage ist, all diese Fettsäuren bei der Energieumwandlung durch den Krebs-Zyklus zu verbrauchen, folglich auch zu erhöhten BHB Konzentrationen im Blut. NEFA als auch BHB beeinträchtigen Funktionen von Immunzellen des Blutes und spielen eine wichtige Rolle bei der Immunsuppression während der Übergangszeit, welche die Tiere anfälliger für Infektionskrankheiten wie Mastitis und Metritis macht. Im Besonderen induziert NEFA auch Entzündungsprozesse über den TLR4-Weg. Es ist bekannt, dass Niacin anti-lipolytische Wirkung und das Potential besitzt, postnatale NEFA- und BHB-Konzentrationen zu verringern, aber es wird auch beschrieben, dass Niacin entzündungshemmende und anti-oxidative Einflüsse auf Immunzellen ausübt. Niacin schein daher ein ideales Futterergänzungsmittel zu sein, um es in der peripartalen Periode zu testen. Um mögliche Einflüsse von Niacin auf die Funktion von Blutimmunzellen und anti-oxidative Serumparameter in der peripartalen Milchkuh zu untersuchen, wurde ein Fütterungsversuch unternommen, der verschiedene Ausprägungen von Lipolyse induzieren sollte.

Hierzu wurden 29 pluri- und 18 primipare gesunde und trächtige Milchkühe der Rasse Deutsche Holstein gleichmäßig in vier verschiedene Fütterungsgruppen hinsichtlich ihres Körpergewichts, BCS, der Laktationszahl sowie der Milchleistung vorangegangener Laktationen eingeteilt. Das Experiment begann 42 Tage vor der erwarteten Kalbung und endete 100 Tage nach dem Abkalben. Vor der Kalbung erhielten die Kühe entweder eine Diät mit niedrigerem Kraftfutteranteil (30% Kraftfutter und 70% Raufutter auf Trockenmassebasis; LC) oder eine Diät mit höherem Kraftfutteranteil (60% Kraftfutter und 40% Raufutter auf Trockenmassebasis; HC) mit oder ohne eine Supplementierung von 24 g pulverförmiger nicht Pansen-geschützter NA pro Tag und Kuh, was zu den Fütterungsgruppen LC-NA und HC-NA sowie LC-CON und HC-CON führte. Die Supplementierung wurde von 42 Tagen antepartum bis zum 24. Laktationstag appliziert. Nach dem Abkalben erhielten alle Tiere zunächst eine Diät mit 30% Kraftfutter und 70% Raufutter. Dieser Kraftfutteranteil wurde bei den LC-Tieren innerhalb von 16 Tagen und bei

den HC-Tieren innerhalb von 24 Tagen auf 50% erhöht und bis zum Ende des Versuchs beibehalten. Blutproben wurden von der *Vena jugularis externa* gesammelt.

Polymorphkernige Leukozyten (PMN) und periphere mononukleäre Blutzellen (PBMC) wurden auf funktioneller Ebene mittels Durchflusszytometrie untersucht. Die Apoptose wurde mit einem auf Annexin V und Propidiumiodid (PI) basierenden Test untersucht und in früh-apoptotische (Annexin V + und PI -) und spät-apoptotische (Annexin V + und PI +) Zellen unterschieden. Die Phagozytose wurde durch die Aufnahme von FITC-markierten opsonisierten *E.coli* Bakterien bestimmt und die Fähigkeit von PMN, reaktive Sauerstoffspezies (ROS) zu produzieren, wurde durch die intrazelluläre Oxidation des nicht-fluoreszierenden Farbstoffs Dihydrorhodamin 123 (DHR) zum fluoreszierenden Rhodamin 123 untersucht. Zusätzlich wurde die Expression von apoptotischen (BAX, BCL2, BCL-xL, CASP3 und RELA) und oxidativem Stress assoziierten (GPX1, NRF2, PARP1, SOD2 und XDH) Genen in Blutleukozyten mittels Real-Time PCR quantifiziert. Darüber hinaus wurden die Serum-Enzym-Aktivitäten von Glutathionperoxidase (GPX) und Superoxiddismutase (SOD) spektrophotometrisch analysiert sowie die Eisen-Reduktionsfähigkeit (FRA) des Serums durch die Umwandlung von $Fe3^{+}$ -Tripyridyltriazin zu $Fe2^{+}$ -Tripyridyltriazin bestimmt.

Obwohl das Fütterungsexperiment keine Unterschiede in der Lipolyse hervorrief, wie die unbeeinflussten NEFA- und BHB-Werte zeigten, wiesen alle gemessenen Variablen eine Zeitabhängigkeit auf, die hauptsächlich auf die Abkalbung zurückzuführen war. PBMC waren durch eine ausgeprägtere Apoptose als PMN gekennzeichnet, und die frühe Apoptose in PBMC wurde signifikant durch den Kraftfutteranteil der Diät beeinflusst. Die HC-Gruppen zeigten eine höhere Anzahl an apoptotischen Zellen als die LC-Gruppen. LC-Tiere zeigten vor der Geburt eine Abnahme der Expression des anti-apoptotischen Gens BCL2, während die HC-Tiere einen kontinuierlichen Anstieg zeigten. Zusätzlich korrelierte Apoptose in Blutleukozyten mit Energievariablen. Die Serum-GPX-Aktivität war bei HC-Tieren höher. Die Oxidationsvariablen wurden stark von der Parität beeinflusst. Pluripare Kühe zeigten höhere Expressionsniveaus für die mit oxidativem Stress in Zusammenhang stehenden Gene GPX1, SOD2 und XDH, höhere Serum-GPX-Aktivität, erhöhte Prozentsätze an phagozytierenden PBMC und einen größeren Anteil an basaler ROS-Produktion in PMN. Primipare Tiere zeigten eine höhere RELA-Genexpression und SOD-Serumaktivität. Die Supplementierung mit Nicotinsäure zeigte einen gewissen Einfluss auf die Zunahme der früh-apoptotischen PMN und der spät-apoptotischen PBMC zwischen 42 und 100 Tage nach der Abkalbung und erhöhte den Anteil an basal ROS produzierenden PMN am 84. Laktationstag. Die NA Supplementierung tendierte außerdem dazu, die

PBMC-Phagozytose zu erhöhen und die Eisen-Reduktionsfähigkeit des Serums (FRA) in den 2 Wochen nach der Abkalbung zu verringern.

Die Studie bestätigte, dass die Kalbung ein Zeitraum von vielfachen Veränderungen mit beträchtlichen Auswirkungen auf die funktionelle Aktivität und Genexpression von Immunzellen des Blutes sowie auf anti-oxidative Parameter des Serums ist. Parität spielt in diesem Zusammenhang eine wichtige Rolle, da Tiere mit unterschiedlichem Alter unterschiedliche Voraussetzungen und Funktionsfähigkeiten in Blutleukozyten haben, um auf die stressreiche Zeit des Gebärens zu reagieren. Die Veränderungen in der funktionellen Aktivität und Fähigkeit von Blutleukozyten können jedoch nicht als dysfunktionale Reaktionen interpretiert werden. Sie repräsentieren vielmehr die Anpassung an die Herausforderungen in der Übergangsphase. Die Verfügbarkeit von Energie scheint jedoch in dieser Hinsicht eine wichtige Rolle zu spielen.

11. References

(Used in Introduction, Background and General Discussion)

Adachi, T., Ohta, H., Hirano, K., Hayashi, K., Marklund, S.L., 1991. Non-enzymic glycation of human extracellular superoxide dismutase. The Biochemical journal 279 (Pt 1), 263-267.

Agrawal, A., Alharthi, A., Vailati-Riboni, M., Zhou, Z., Loor, J.J., 2017. Expression of fatty acid sensing G-protein coupled receptors in peripartal Holstein cows. Journal of animal science and biotechnology 8, 20.

Aleri, J.W., Hine, B.C., Pyman, M.F., Mansell, P.D., Wales, W.J., Mallard, B., Fisher, A.D., 2016. Periparturient immunosuppression and strategies to improve dairy cow health during the periparturient period. Research in veterinary science 108, 8-17.

Barghouthi, S., Everett, K.D., Speert, D.P., 1995. Nonopsonic phagocytosis of Pseudomonas aeruginosa requires facilitated transport of D-glucose by macrophages. Journal of immunology (Baltimore, Md. : 1950) 154, 3420-3428.

Bell, A.W., 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. Journal of animal science 73, 2804-2819.

Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical biochemistry 239, 70-76.

Bernabucci, U., Ronchi, B., Lacetera, N., Nardone, A., 2002. Markers of oxidative status in plasma and erythrocytes of transition dairy cows during hot season. J Dairy Sci 85, 2173-2179.

Bernabucci, U., Ronchi, B., Lacetera, N., Nardone, A., 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J Dairy Sci 88, 2017-2026.

Bionaz, M., Trevisi, E., Calamari, L., Librandi, F., Ferrari, A., Bertoni, G., 2007. Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. J Dairy Sci 90, 1740-1750.

Brassard, P., Larbi, A., Grenier, A., Frisch, F., Fortin, C., Carpentier, A.C., Fulop, T., 2007. Modulation of T-cell signalling by non-esterified fatty acids. Prostaglandins, leukotrienes, and essential fatty acids 77, 337-343.

Burton, J.L., Kehrli, M.E., Jr., Kapil, S., Horst, R.L., 1995. Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone. Journal of leukocyte biology 57, 317-325.

Burvenich, C., Paape, M.J., Hoeben, D., Dosogne, H., Massart-Leen, A.M., Blum, J., 1999. Modulation of the inflammatory reaction and neutrophil defense of the bovine lactating mammary gland by growth hormone. Domestic animal endocrinology 17, 149-159.

Cai, T.Q., Weston, P.G., Lund, L.A., Brodie, B., McKenna, D.J., Wagner, W.C., 1994. Association between neutrophil functions and periparturient disorders in cows. American journal of veterinary research 55, 934-943.

Carlson, L.A., 2005. Nicotinic acid: the broad-spectrum lipid drug. A 50th anniversary review. J Intern Med 258, 94-114.

Castillo, C., Hernandez, J., Bravo, A., Lopez-Alonso, M., Pereira, V., Benedito, J.L., 2005. Oxidative status during late pregnancy and early lactation in dairy cows. Veterinary journal (London, England : 1997) 169, 286-292.

Chaveiro, A., Moreira da Silva, F., 2009. Effect of oestrous cycle on the oxidative burst activity of blood polymorphonuclear leucocytes in cows. Reprod Domest Anim 44, 900-906.

Crookenden, M.A., Heiser, A., Murray, A., Dukkipati, V.S.R., Kay, J.K., Loor, J.J., Meier, S., Mitchell, M.D., Moyes, K.M., Walker, C.G., Roche, J.R., 2016. Parturition in dairy cows temporarily alters the expression of genes in circulating neutrophils. J Dairy Sci 99, 6470-6483.

Crowe, M.A., 2008. Resumption of ovarian cyclicity in post-partum beef and dairy cows. Reprod Domest Anim 43 Suppl 5, 20-28.

Daha, M.R., 2011. Grand challenges in molecular innate immunity. Frontiers in immunology 2, 16.

Detilleux, J.C., Kehrli, M.E., Jr., Stabel, J.R., Freeman, A.E., Kelley, D.H., 1995. Study of immunological dysfunction in periparturient Holstein cattle selected for high and average milk production. Vet Immunol Immunopathol 44, 251-267.

Didara, M., Poljicak-Milas, N., Milinkovic-Tur, S., Masek, T., Suran, J., Pavic, M., Kardum, M., Speranda, M., 2015. Immune and oxidative response to linseed in the diet of periparturient Holstein cows. Animal 9, 1349-1354.

Digby, J.E., Martinez, F., Jefferson, A., Ruparelia, N., Chai, J., Wamil, M., Greaves, D.R., Choudhury, R.P., 2012. Anti-inflammatory effects of nicotinic acid in human monocytes are mediated by GPR109A dependent mechanisms. Arteriosclerosis, thrombosis, and vascular biology 32, 669-676.

do Amaral, B.C., Connor, E.E., Tao, S., Hayen, M.J., Bubolz, J.W., Dahl, G.E., 2011. Heat stress abatement during the dry period influences metabolic gene expression and improves immune status in the transition period of dairy cows. J Dairy Sci 94, 86-96.

Drackley, J.K., 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J Dairy Sci 82, 2259-2273.

Fattman, C.L., Schaefer, L.M., Oury, T.D., 2003. Extracellular superoxide dismutase in biology and medicine. Free radical biology & medicine 35, 236-256.

Fialkow, L., Wang, Y., Downey, G.P., 2007. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. Free radical biology & medicine 42, 153-164.

Franchina, D.G., Dostert, C., Brenner, D., 2018. Reactive Oxygen Species: Involvement in T Cell Signaling and Metabolism. Trends in immunology.

Goff, J.P., Horst, R.L., 1997. Physiological changes at parturition and their relationship to metabolic disorders. J Dairy Sci 80, 1260-1268.

Goff, J.P., Stabel, J.R., 1990. Decreased plasma retinol, alpha-tocopherol, and zinc concentration during the periparturient period: effect of milk fever. J Dairy Sci 73, 3195-3199.

Grossmann, M., O'Reilly, L.A., Gugasyan, R., Strasser, A., Adams, J.M., Gerondakis, S., 2000. The anti-apoptotic activities of Rel and RelA required during B-cell maturation involve the regulation of Bcl-2 expression. The EMBO journal 19, 6351-6360.

Grummer, R.R., 1995. Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. Journal of animal science 73, 2820-2833.

Hammon, D.S., Evjen, I.M., Dhiman, T.R., Goff, J.P., Walters, J.L., 2006. Neutrophil function and energy status in Holstein cows with uterine health disorders. Vet Immunol Immunopathol 113, 21-29.

Hoeben, D., Heyneman, R., Burvenich, C., 1997. Elevated levels of beta-hydroxybutyric acid in periparturient cows and in vitro effect on respiratory burst activity of bovine neutrophils. Vet Immunol Immunopathol 58, 165-170.

Hotchkiss, R.S., Strasser, A., McDunn, J.E., Swanson, P.E., 2009. Cell death. The New England journal of medicine 361, 1570-1583.

Ingvartsen, K.L., Moyes, K., 2013. Nutrition, immune function and health of dairy cattle. Animal 7 Suppl 1, 112-122.

Ingvartsen, K.L., Moyes, K.M., 2015. Factors contributing to immunosuppression in the dairy cow during the periparturient period. The Japanese journal of veterinary research 63 Suppl 1, S15-24.

Janeway, C.A.T., P.; Walport, M.; Shlomchik, M.J. et al. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; 20, 2001. Immunobiology. New York and London: Garland Science 5th ed.

Jonsson, N.N., Fortes, M.R., Piper, E.K., Vankan, D.M., de Cisneros, J.P., Wittek, T., 2013. Comparison of metabolic, hematological, and peripheral blood leukocyte cytokine profiles of dairy cows and heifers during the periparturient period. J Dairy Sci 96, 2283-2292.

Kehrli, M.E., Jr., Nonnecke, B.J., Roth, J.A., 1989. Alterations in bovine neutrophil function during the periparturient period. American journal of veterinary research 50, 207-214.

Kelley, K.W., Weigent, D.A., Kooijman, R., 2007. Protein hormones and immunity. Brain, behavior, and immunity 21, 384-392.

Kenez, A., Locher, L., Rehage, J., Danicke, S., Huber, K., 2014. Agonists of the G protein-coupled receptor 109A-mediated pathway promote antilipolysis by reducing serine residue 563 phosphorylation of hormone-sensitive lipase in bovine adipose tissue explants. J Dairy Sci 97, 3626-3634.

Khan, M.J., Jacometo, C.B., Riboni, M.V., Trevisi, E., Graugnard, D.E., Correa, M.N., Loor, J.J., 2015. Stress and inflammatory gene networks in bovine liver are altered by plane of dietary energy during late pregnancy. Functional & integrative genomics 15, 563-576.

Kimura, K., Goff, J.P., Kehrli, M.E., Jr., 1999. Effects of the presence of the mammary gland on expression of neutrophil adhesion molecules and myeloperoxidase activity in periparturient dairy cows. J Dairy Sci 82, 2385-2392.

Kimura, K., Goff, J.P., Kehrli, M.E., Jr., Harp, J.A., Nonnecke, B.J., 2002. Effects of mastectomy on composition of peripheral blood mononuclear cell populations in periparturient dairy cows. J Dairy Sci 85, 1437-1444.

Kimura, K., Reinhardt, T.A., Goff, J.P., 2006. Parturition and hypocalcemia blunts calcium signals in immune cells of dairy cattle. J Dairy Sci 89, 2588-2595.

Kirkland, J.B., 2009. Niacin status, NAD distribution and ADP-ribose metabolism. Current pharmaceutical design 15, 3-11.

Lacetera, N., Scalia, D., Bernabucci, U., Ronchi, B., Pirazzi, D., Nardone, A., 2005. Lymphocyte functions in overconditioned cows around parturition. J Dairy Sci 88, 2010-2016.

Lacetera, N., Scalia, D., Franci, O., Bernabucci, U., Ronchi, B., Nardone, A., 2004. Short communication: effects of nonesterified fatty acids on lymphocyte function in dairy heifers. J Dairy Sci 87, 1012-1014.

Lamote, I., Meyer, E., De Ketelaere, A., Duchateau, L., Burvenich, C., 2006. Influence of sex steroids on the viability and CD11b, CD18 and CD47 expression of blood neutrophils from dairy cows in the last month of gestation. Veterinary research 37, 61-74.

Lamote, I., Meyer, E., Duchateau, L., Burvenich, C., 2004. Influence of 17beta-estradiol, progesterone, and dexamethasone on diapedesis and viability of bovine blood polymorphonuclear leukocytes. J Dairy Sci 87, 3340-3349.

LeBlanc, S., 2010. Monitoring metabolic health of dairy cattle in the transition period. The Journal of reproduction and development 56 Suppl, S29-35.

LeBlanc, S.J., Lissemore, K.D., Kelton, D.F., Duffield, T.F., Leslie, K.E., 2006. Major advances in disease prevention in dairy cattle. J Dairy Sci 89, 1267-1279.

Lee, J.Y., Sohn, K.H., Rhee, S.H., Hwang, D., 2001. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. The Journal of biological chemistry 276, 16683-16689.

Lippolis, J.D., 2008. Immunological signaling networks: integrating the body's immune response. Journal of animal science 86, E53-63.

Maiese, K., Chong, Z.Z., Hou, J., Shang, Y.C., 2009. The vitamin nicotinamide: translating nutrition into clinical care. Molecules (Basel, Switzerland) 14, 3446-3485.

Mallard, B.A., Dekkers, J.C., Ireland, M.J., Leslie, K.E., Sharif, S., Vankampen, C.L., Wagter, L., Wilkie, B.N., 1998. Alteration in immune responsiveness during the peripartum period and its ramification on dairy cow and calf health. J Dairy Sci 81, 585-595.

Mallard, B.A., Wagter, L.C., Ireland, M.J., Dekkers, J.C., 1997. Effects of growth hormone, insulin-like growth factor-I, and cortisol on periparturient antibody response profiles of dairy cattle. Vet Immunol Immunopathol 60, 61-76.

Martinez, N., Sinedino, L.D., Bisinotto, R.S., Ribeiro, E.S., Gomes, G.C., Lima, F.S., Greco, L.F., Risco, C.A., Galvao, K.N., Taylor-Rodriguez, D., Driver, J.P., Thatcher, W.W., Santos, J.E., 2014. Effect of induced subclinical hypocalcemia on physiological responses and neutrophil function in dairy cows. J Dairy Sci 97, 874-887.

McGrath, J., Duval, S.M., Tamassia, L.F.M., Kindermann, M., Stemmler, R.T., de Gouvea, V.N., Acedo, T.S., Immig, I., Williams, S.N., Celi, P., 2017. Nutritional strategies in ruminants: A lifetime approach. Research in veterinary science.

Meglia, G.E., Johannisson, A., Petersson, L., Waller, K.P., 2001. Changes in some blood micronutrients, leukocytes and neutrophil expression of adhesion molecules in periparturient dairy cows. Acta veterinaria Scandinavica 42, 139-150.

Mehrzad, J., Dosogne, H., Meyer, E., Heyneman, R., Burvenich, C., 2001. Respiratory burst activity of blood and milk neutrophils in dairy cows during different stages of lactation. The Journal of dairy research 68, 399-415.

Mehrzad, J., Duchateau, L., Pyorala, S., Burvenich, C., 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. J Dairy Sci 85, 3268-3276.

Miller, G.Y., Bartlett, P.C., Erskine, R.J., Smith, K.L., 1995. Factors affecting serum selenium and vitamin E concentrations in dairy cows. Journal of the American Veterinary Medical Association 206, 1369-1373.

Miller, J.K., Brzezinska-Slebodzinska, E., Madsen, F.C., 1993. Oxidative stress, antioxidants, and animal function. J Dairy Sci 76, 2812-2823.

Mordak, R., Stewart, P.A., 2015. Periparturient stress and immune suppression as a potential cause of retained placenta in highly productive dairy cows: examples of prevention. Acta veterinaria Scandinavica 57, 84.

Morey, S.D., Mamedova, L.K., Anderson, D.E., Armendariz, C.K., Titgemeyer, E.C., Bradford, B.J., 2011. Effects of encapsulated niacin on metabolism and production of periparturient dairy cows. J Dairy Sci 94, 5090-5104.

Moyes, K.M., Drackley, J.K., Salak-Johnson, J.L., Morin, D.E., Hope, J.C., Loor, J.J., 2009. Dietary-induced negative energy balance has minimal effects on innate immunity during a Streptococcus uberis mastitis challenge in dairy cows during midlactation. J Dairy Sci 92, 4301-4316.

Niehoff, I.D., Huther, L., Lebzien, P., 2009. Niacin for dairy cattle: a review. The British journal of nutrition 101, 5-19.

Nonnecke, B.J., Kimura, K., Goff, J.P., Kehrli, M.E., Jr., 2003. Effects of the mammary gland on functional capacities of blood mononuclear leukocyte populations from periparturient cows. J Dairy Sci 86, 2359-2368.

Oliveira, L.J., Barreto, R.S., Perecin, F., Mansouri-Attia, N., Pereira, F.T., Meirelles, F.V., 2012. Modulation of maternal immune system during pregnancy in the cow. Reprod Domest Anim 47 Suppl 4, 384-393.

Paape, M.J., Bannerman, D.D., Zhao, X., Lee, J.W., 2003. The bovine neutrophil: Structure and function in blood and milk. Veterinary research 34, 597-627.

Pires, J.A., Grummer, R.R., 2007. The use of nicotinic acid to induce sustained low plasma nonesterified fatty acids in feed-restricted Holstein cows. J Dairy Sci 90, 3725-3732.

Planey, S.L., Litwack, G., 2000. Glucocorticoid-induced apoptosis in lymphocytes. Biochemical and biophysical research communications 279, 307-312.

Preisler, M.T., Weber, P.S., Tempelman, R.J., Erskine, R.J., Hunt, H., Burton, J.L., 2000. Glucocorticoid receptor down-regulation in neutrophils of periparturient cows. American journal of veterinary research 61, 14-19.

Prieur, X., Roszer, T., Ricote, M., 2010. Lipotoxicity in macrophages: evidence from diseases associated with the metabolic syndrome. Biochimica et biophysica acta 1801, 327-337.

Raphael, W., Sordillo, L.M., 2013. Dietary polyunsaturated fatty acids and inflammation: the role of phospholipid biosynthesis. International journal of molecular sciences 14, 21167-21188.

Redza-Dutordoir, M., Averill-Bates, D.A., 2016. Activation of apoptosis signalling pathways by reactive oxygen species. Biochimica et biophysica acta 1863, 2977-2992.

Renner, L., von Soosten, D., Sipka, A., Doll, S., Beineke, A., Schuberth, H.J., Danicke, S., 2012. Effect of conjugated linoleic acid on proliferation and cytokine expression of bovine peripheral blood mononuclear cells and splenocytes ex vivo. Archives of animal nutrition 66, 73-85.

Roth, J.A., Kaeberle, M.L., Hsu, W.H., 1982. Effect of estradiol and progesterone on lymphocyte and neutrophil functions in steers. Infection and immunity 35, 997-1002.

Sander, A.K., Piechotta, M., Schlamberger, G., Bollwein, H., Kaske, M., Sipka, A., Schuberth, H.J., 2011. Ex vivo phagocytic overall performance of neutrophilic granulocytes and the relation to plasma insulin-like growth factor-I concentrations in dairy cows during the transition period. J Dairy Sci 94, 1762-1771.

Sartorelli, P., Paltrinieri, S., Agnes, F., 1999. Non-specific immunity and ketone bodies. I: In vitro studies on chemotaxis and phagocytosis in ovine neutrophils. Zentralblatt fur Veterinarmedizin. Reihe A 46, 613-619.

Scalia, D., Lacetera, N., Bernabucci, U., Demeyere, K., Duchateau, L., Burvenich, C., 2006. In vitro effects of nonesterified fatty acids on bovine neutrophils oxidative burst and viability. J Dairy Sci 89, 147-154.

Schulz, K., Frahm, J., Kersten, S., Meyer, U., Reiche, D., Sauerwein, H., Danicke, S., 2015. Effects of elevated parameters of subclinical ketosis on the immune system of dairy cows: in vivo and in vitro results. Archives of animal nutrition 69, 113-127.

Schulz, K., Frahm, J., Meyer, U., Kersten, S., Reiche, D., Rehage, J., Danicke, S., 2014. Effects of prepartal body condition score and peripartal energy supply of dairy cows on postpartal lipolysis, energy balance and ketogenesis: an animal model to investigate subclinical ketosis. The Journal of dairy research 81, 257-266.

Schwab, E.C., Caraviello, D.Z., Shaver, R.D., 2005. Review: A Meta-Analysis of Lactation Responses to Supplemental Dietary Niacin in Dairy Cows. The Professional Animal Scientist 21, 239-247.

Sinha, K., Das, J., Pal, P.B., Sil, P.C., 2013. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. Archives of toxicology 87, 1157-1180.

Sladek, Z., Rysanek, D., 2001. Neutrophil apoptosis during the resolution of bovine mammary gland injury. Research in veterinary science 70, 41-46.

Sordillo, L.M., 2016. Nutritional strategies to optimize dairy cattle immunity. J Dairy Sci 99, 4967-4982.

Sordillo, L.M., Aitken, S.L., 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Vet Immunol Immunopathol 128, 104-109.

Sordillo, L.M., Contreras, G.A., Aitken, S.L., 2009. Metabolic factors affecting the inflammatory response of periparturient dairy cows. Animal health research reviews / Conference of Research Workers in Animal Diseases 10, 53-63.

Sordillo, L.M., Mavangira, V., 2014. The nexus between nutrient metabolism, oxidative stress and inflammation in transition cows. Anim Prod Sci 54, 1204-1214.

Spears, J.W., Weiss, W.P., 2008. Role of antioxidants and trace elements in health and immunity of transition dairy cows. Veterinary journal (London, England : 1997) 176, 70-76.

Ster, C., Loiselle, M.C., Lacasse, P., 2012. Effect of postcalving serum nonesterified fatty acids concentration on the functionality of bovine immune cells. J Dairy Sci 95, 708-717.

Suriyasathaporn, W., Daemen, A.J., Noordhuizen-Stassen, E.N., Dieleman, S.J., Nielen, M., Schukken, Y.H., 1999. Beta-hydroxybutyrate levels in peripheral blood and ketone bodies supplemented in culture media affect the in vitro chemotaxis of bovine leukocytes. Vet Immunol Immunopathol 68, 177-186.

Tharwat, M., Endoh, D., Oikawa, S., 2012. DNA damage in peripheral blood mononuclear cells and neutrophils of dairy cows during the transition period. Open veterinary journal 2, 65-68.

Tienken, R., Kersten, S., Frahm, J., Huther, L., Meyer, U., Huber, K., Rehage, J., Danicke, S., 2015a. Effects of Prepartum Dietary Energy Level and Nicotinic Acid Supplementation on Immunological, Hematological and Biochemical Parameters of Periparturient Dairy Cows Differing in Parity. Animals : an open access journal from MDPI 5, 910-933.

Tienken, R., Kersten, S., Frahm, J., Meyer, U., Locher, L., Rehage, J., Huber, K., Kenez, A., Sauerwein, H., Mielenz, M., Danicke, S., 2015b. Effects of an energy-dense diet and nicotinic acid supplementation on production and metabolic variables of primiparous or multiparous cows in periparturient period. Archives of animal nutrition 69, 319-339.

Trevisi, E., Amadori, M., Bakudila, A.M., Bertoni, G., 2009. Metabolic changes in dairy cows induced by oral, low-dose interferon-alpha treatment. Journal of animal science 87, 3020-3029.

Trevisi, E., Amadori, M., Cogrossi, S., Razzuoli, E., Bertoni, G., 2012. Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows. Research in veterinary science 93, 695-704.

Trevisi, E., Minuti, A., 2018. Assessment of the innate immune response in the periparturient cow. Research in veterinary science 116, 47-54.

Vailati-Riboni, M., Zhou, Z., Jacometo, C.B., Minuti, A., Trevisi, E., Luchini, D.N., Loor, J.J., 2017. Supplementation with rumen-protected methionine or choline during the transition period influences whole-blood immune response in periparturient dairy cows. J Dairy Sci.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39, 44-84.

Van Oostveldt, K., Vangroenweghe, F., Dosogne, H., Burvenich, C., 2001. Apoptosis and necrosis of blood and milk polymorphonuclear leukocytes in early and midlactating healthy cows. Veterinary research 32, 617-622.

Vangroenweghe, F., Lamote, I., Burvenich, C., 2005. Physiology of the periparturient period and its relation to severity of clinical mastitis. Domestic animal endocrinology 29, 283-293.

Vernon, P.J., Tang, D., 2013. Eat-me: autophagy, phagocytosis, and reactive oxygen species signaling. Antioxidants & redox signaling 18, 677-691.

Whyte, M.K., Meagher, L.C., MacDermot, J., Haslett, C., 1993. Impairment of function in aging neutrophils is associated with apoptosis. Journal of immunology (Baltimore, Md. : 1950) 150, 5124-5134.

Yu, B.L., Zhao, S.P., 2007. Anti-inflammatory effect is an important property of niacin on atherosclerosis beyond its lipid-altering effects. Medical hypotheses 69, 90-94.

Yuan, K., Shaver, R.D., Bertics, S.J., Espineira, M., Grummer, R.R., 2012. Effect of rumen-protected niacin on lipid metabolism, oxidative stress, and performance of transition dairy cows. J Dairy Sci 95, 2673-2679.

Zhou, Z., Bu, D.P., Vailati Riboni, M., Khan, M.J., Graugnard, D.E., Luo, J., Cardoso, F.C., Loor, J.J., 2015. Prepartal dietary energy level affects peripartal bovine blood neutrophil metabolic, antioxidant, and inflammatory gene expression. J Dairy Sci 98, 5492-5505.

Curriculum vitae

Susanne Bühler

Born on 4th of November 1970
in Schwäbisch Hall, Germany

08/1981 - 07/1990	Ganerben-Gymnasium Künzelsau, Germany Degree: Allgemeine Hochschulreife (Abitur)
10/1991 - 10/1997	Study of Biology at the University of Konstanz, Germany Degree: Diplom
11/1997 - 03/1998	Research assistant at the University of Konstanz, Germany, in the group of Prof. Dirk Pette
04/1998 - 11/1998	Pharmaceutical representative with 3M Medica, Borken, Germany
02/1999-03/2003	Research assistant at the Friedrich-Miescher-Laboratory of the Max-Planck-Society, Tübingen, Germany, in the group of Dr. Andreas Mayer
08/2003-02/2005	Research assistant at the University of Colorado, Boulder, USA, in the group of Prof. Joseph J. Falke
03/2005-05/2012	Family leave
Since 06/2012	Research associate at the Institute of Animal Nutrition of the Friedrich-Loeffler-Institute, Braunschweig, Germany

Braunschweig August 2018

Eidesstattliche Erklärung / Declaration under Oath

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

………………………………… ……………………………….

Ort, Datum */ Place, Date* Susanne Bühler

Danksagung

An dieser Stelle möchte ich mich bei all den Menschen bedanken, die diese Arbeit möglich gemacht haben.

Zunächst möchte ich mich bei Herrn Prof. Dr. Dr. Sven Dänicke bedanken für die Überlassung des Themas dieser Doktorarbeit und die Möglichkeit, sie am Institut für Tierernährung in Braunschweig anfertigen zu können. Vielen Dank für das Vertrauen in meine Fähigkeiten sowie die Geduld und die Unterstützung beim Erstellen der Arbeit.

Frau Prof. Dr. Annette Zeyner danke ich für die Übernahme der Betreuung dieser Promotionsarbeit an der Martin-Luther-Universität Halle-Wittenberg.

Für die Durchführung der Durchflusszytometrie danke ich Frau Dr. Jana Frahm ebenso wie für die vielen anregenden Diskussionen zum Verständnis der Zellen des Immunsystems der Milchkuh.

Frau Dr. Susanne Kersten danke ich für die Unterstützung bei der Erstellung der Manuskripte und die stets offene Tür für meine Fragen. Zudem weis ich es sehr zu schätzen, dass sie mir die Freiräume für die Erstellung der Doktorarbeit gewährt hat. Vielen Dank dafür!

Ein riesiges Dankeschön geht an Frau Dr. Reka Tienken und Frau Dr. Wendy Liermann ohne deren Einsatz und zuverlässige Probenahme während des Tierexperiments diese Arbeit gar nicht möglich gewesen wäre. Reka Tienken möchte ich zudem für eine wunderschöne gemeinsame Bürozeit danken.

Mein besonderer Dank gilt allen Mitarbeitern des Instituts für Tierernährung auf der Versuchsstation und in den Laboren da sich solch ein großer Milchkuhversuch nur mit Hilfe vieler durchführen lässt. Ein besonderes Dankeschön geht jedoch an Frau Nicola Mickenautsch, die durch ihre freundliche und kompetente Art das Labor der Immuno-Nutrition so gut am Laufen hält und Frau Lara Lindner, mit der es eine Freude war, die Enzym-Assays zu etablieren.

Zum Gelingen einer solchen Arbeit ist es jedoch auch wichtig, dass man ein angenehmes und freundliches Arbeitsklima vorfindet. Deshalb ein herzliches Dankeschön an alle jetzigen und ehemaligen Mitarbeiter des Instituts für Tierernährung für das herzliche und offene Miteinander während des Erstellens dieser Doktorarbeit. Meiner Bürokollegin Julia

Hartwiger gilt zudem ein besonderer Dank für die Unterstützung in allen Computerfragen und den gemeinsamen Spaß bei der Arbeit.

Mein größter Dank gilt jedoch meiner Familie für die Unterstützung, das Mut machen und den Glauben an mich und meine Fähigkeiten. Meinem Mann Piet möchte ich für die Freiräume danken, welche er mir trotz eines immer vollen Terminkalenders zugestanden hat und meinen Kindern Jule und Ben danke ich für das Groß- und Selbständig-werden in der Zeit der Anfertigung dieser Arbeit, mit dem sie mich auf Ihre Weise unterstützt haben.