Effects of grape pomace and a polyphenolic plant product during the transition period of high-yielding dairy cows

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1.1 Introduction

The transition period, spanning the period of 3 weeks pre-partum to 3 weeks post-partum, is widely recognised as the most metabolically challenging time in the production cycle of high-yielding dairy cows due to metabolic and inflammatory stimuli, which are associated with the development of stress of the endoplasmic reticulum (ER) in the liver, which contributes to the development of liver-associated diseases (Gessner et al. 2014, Ringseis et al. 2014, van Knegsel et al. 2014). During the transition period, ER stress-induced stimuli in the liver are mainly non-esterified fatty acids (NEFA), pro-inflammatory cytokines, reactive oxygen species (ROS) and microbial components, such as lipopolysaccharides (LPS). The consequently disturbed ER homeostasis activates an adaptive response known as unfolded protein response (UPR) which leads to a variety of symptoms in the liver examined in rat models of obesity or diabetes, which are similar to those observed in periparturient dairy cows. This variety of symptoms including the development of fatty liver (Pagliassotti 2012, Gentile et al. 2011), an induction of fibroblast growth factor (FGF) 21 (Schaap et al. 2013), an enhancement of the antioxidant and cytoprotective capacity by activation of nuclear factor E2-related factor 2 (Nrf2) (Cullinan et al. 2003, Cullinan and Diehl 2006) and an induction of inflammation by activation of nuclear factor- kappa B (NF-κB, Zhang and Kaufmann 2008, Rath and Haller 2011). Polyphenols as a dietary supplement have antioxidant as well as anti-inflammatory properties which might be useful for dairy cows during the transition period based on the prevailing inflammatory conditions, whereas various studies have revealed that polyphenols fed to non-ruminant animals are able to reduce oxidative stress and inflammation.

The present thesis focuses on the effects of polyphenols, especially those from grape pomace (GP), green tea and curcuma extract, on the liver of periparturient dairy cows associated with the impact on the prevailing ER stress and inflammation during this period (**studies 1, 2 and 3**). Additionally, little is known about the feeding value and the polyphenol content of GP of different grape varieties in Rhineland-Palatinate (Germany). Therefore, **study 4** investigated the feeding value of dried white, dried red and ensiled white GP associated with the contents of metabolisable energy (ME) and net energy lactation (NEL). In addition, **study 4** is concerned with the determination of crude

nutrient contents and polyphenol contents of different GP varieties in Rhineland-Palatinate (Germany). In the following chapter, inflammation, the transcription factor NF-κB as a master regulator of inflammatory responses, ER stress and ER stress-induced stimuli (NEFA, pro-inflammatory cytokines, ROS, microbial components) associated with the UPR-induced stimulation of antioxidant and cytoprotective capacity of the cell by activation of Nrf2 and the UPR-triggered stimulation of ketogenesis by the induction of FGF21 will be explained. Furthermore, GP as dietary supplement and their antioxidant and anti-inflammatory effects are considered and the hypothesis underlying the three studies will be presented.

1.2 Fat mobilization in periparturient dairy cows

During the last 3 weeks of pregnancy, the nutrient demands by the foetal calf and the placenta reach their maximum (Bell 1995). After parturition, within the onset of lactation, the milk yield, milk proteins, fat and lactose increase rapidly associated with a decreased feed dry matter intake by up to 40% (Hayirli and Grummer 2004) which leads to a negative energy balance (NEB) in early lactation (Janovick et al. 2011). The high nutrient demands for galactopoiesis are compensated by the mobilisation of body fat reserves stored in adipose tissue (Weber et al. 2013) associated with a marked influx of NEFA released into the blood (Xu et al. 2008) and partially taken up by the liver. The greatest part of NEFA in the liver is oxidised to carbon dioxide to provide energy or is re-esterified into triglycerides (TG; Brickner et al. 2009) which mostly exceeds the liver capacity to secrete TG (Drackley et al. 2001). This process is associated with the formation of ketone bodies such as ß-Hydroxybutyrate (BHBA), which reflects the complete oxidation of fat in the liver. Ketone bodies (acetone and acetoacetate) are the intermediate metabolites of the oxidation of fatty acids, which is caused by the incomplete oxidation of fatty acids to acetyl CoA by ß-oxidation (Herdt 2000). The overproduced TGs during early lactation are stored as triacylglycerol (TAG) in the liver which leads to a decrease of metabolic functions (Bionaz et al. 2007) of the liver and causes the development of fatty liver (Gruffat et al. 1996). Thus, the synthesis and accumulation of TAG in the liver are related to the NEFA concentration in the blood (Rukkwamsuk et al. 2000). At normal levels of NEFA uptake into the liver, the TAGs formed in the liver are secreted as very low-density lipoproteins (VLDL) and incorporated into the blood, which prevents the development of fatty liver. Additionally, the development of fatty liver impairs the gluconeogenic activity of liver tissue, which is metabolically reflected by a reduction in blood insulin, glucose and insulin-like growth factor-1 (IGF-1) concentrations (Butler et al. 2003). The overproduced ketones accumulate in the bloodstream, and at strong NEB, ketones can appear in the blood, milk and urine, which is referred to as ketosis (Goff and Horst 1997). Besides this metabolic stress, the transition period of high-yielding dairy cows is characterised by inflammatory-like conditions (Trevisi et al. 2010, Trevisi et al. 2012) due to the release of pro-inflammatory mediators such as cytokines [tumour necrosis factor α (TNFα), interleukins 1 and 6 (IL-1 and IL-6)], lipopolysaccharides (LPS) and reactive oxygen species (ROS).

1.3 Inflammation

The inflammation is defined as a part of the complex biological response of animal tissue to harmful physical, chemical and biological stimuli. Thereby, inflammation helps the body adapt to and eliminate the harmful stimuli and avoid the expansion of injury associated with the restoration of tissue homeostasis. A distinction is made between acute and subacute inflammation. Meanwhile, acute inflammation is induced by infection and injury and thereafter resolves, subacute inflammation is affiliated with tissue malfunction. The body responds to acute inflammatory stimuli by enhancing the expression and the release of inflammatory mediators, such as adhesion molecules, cytokines, chemokines, eicosanoids and complement proteins (Newton and Dixit 2012). The following formation of these molecules to complex regulatory networks supports the increased blood flow to the infected tissue, immune cell activation and infiltration, and systemic responses comprising enhanced body temperature and heart rate, and decreased appetite (Dantzer and Kelly 2007). Cytokines play important roles in the inflammatory response.

A mild enhancement of inflammatory mediators is caused by the subacute inflammation resulting in a contribution to chronic and progressive changes in tissue function. For example, in obesity, subacute inflammation is triggered by an excess of nutrients in metabolic tissue which finally activates multiple types of immune cells (Gregor and Hotamisligil 2011). This activation leads to an unresolved tissue inflammatory response which frequently disturbs metabolism by mechanisms such as inhibiting insulin action (Gregor and Hotamisligil 2011). Although acute and subacute inflammation induce different responses, both share most signalling pathways.

The nuclear factor-kappa B (NF-κB) is considered the master regulator of inflammatory responses.

Nuclear factor-kappa B (NF-κB)

NF-κB is a eukaryotic transcription factor found in almost all animal cell types and tissues, belonging to the family of rapid acting transcription factors. This protein complex controls transcription of DNA, cytokine production and cell survival. NF-κB binds to DNA as a dimer consisting of the NF-κB proteins and the Rel proteins, whereby five mammalian NF-κB subunits have been characterised: two NF-κB proteins including p50 (also NF-κB1 or its precursor p105) and p52 (also NF-κB2 or its precursor p100), and three Rel proteins including RelA (also p65),

RelB and c-Rel (Ghosh and Karin 2002). All of those Rel proteins contain a conserved N-terminal region, called the Rel homologous domain (RHD). The RHD comprises the DNA-binding and dimerisation domains and the nuclear localisation signal, and is responsible for binding to inhibitor proteins and regulatory elements of target genes (Barnes 1997). The NF-κB1 and NF-κB2 proteins are synthesised as the large precursor proteins p105 and p100, which undergo processing mediated by the ubiquitin/proteasome pathway or sometimes by arrested translation generating the mature NF-κB subunits, p50 and p52, respectively (Karin and Ben-Neriah 2000). The dimerisation of subunits is required for DNA binding, whereby subunits forms a homo- or heterodimeric complex based on their RHD and selectively modulate target genes, with different potentials for transactivation leading to the activation of inhibitory effects. Different dimer combinations act as transcriptional activators or repressors.

In an inactivated state, the NF-κB dimer remains inactive and is located in the cytosol bound to the inhibitory protein IκBα. In response to a wide variety of extracellular inducers such as pathogens, cytokines, and growth factors, the enzyme IκB kinase (IKK) is activated and rapidly phosphorylates the IκBα protein (can be induced by ROS and blocked by antioxidants) resulting in bonding of ubiquitin residues with IκBα. The IKK complex controls the IκBα-NF-κB interaction and is composed of two catalytic subunits IKKα (=IKK1) and IKKβ (=IKK2) and the regulatory subunit IKKγ (=NEMO) (DiDonato et al. 1997, Rothwarf and Karin 1999). The ubiquitination leads to a dissociation of IκBα from the NF-κB and proteasomal degradation of IκBα by the ubiquitin-proteasome system. After the degradation of IκBα, the activated NF-κB is translocated into the nucleus and binds to specific sequences of DNA called response elements (RE) (Karin and Ben-Neriah 2000). NF-κB is intimately involved in the regulation of transcriptional activation of numerous target genes which mainly encode pro-inflammatory proteins, cell adhesion molecules, acute phase proteins, stress response genes, growth factors, and factors regulating cell proliferation and apoptosis (Barnes 1997, Sica et al. 1997). The activated NF-κB leads to the expression of the IκBα gene, which can enter the nucleus, removing NF-κB from the DNA and translocating the complex back to the cytoplasm; thereby, the transcriptional activity is terminated unless a persistent activation signal is present. Overall, NF-κB is involved in cellular responses to stimuli such as cytokines, lipopolysaccharides, reactive oxygen species, and bacterial or viral antigens (Figure 1).

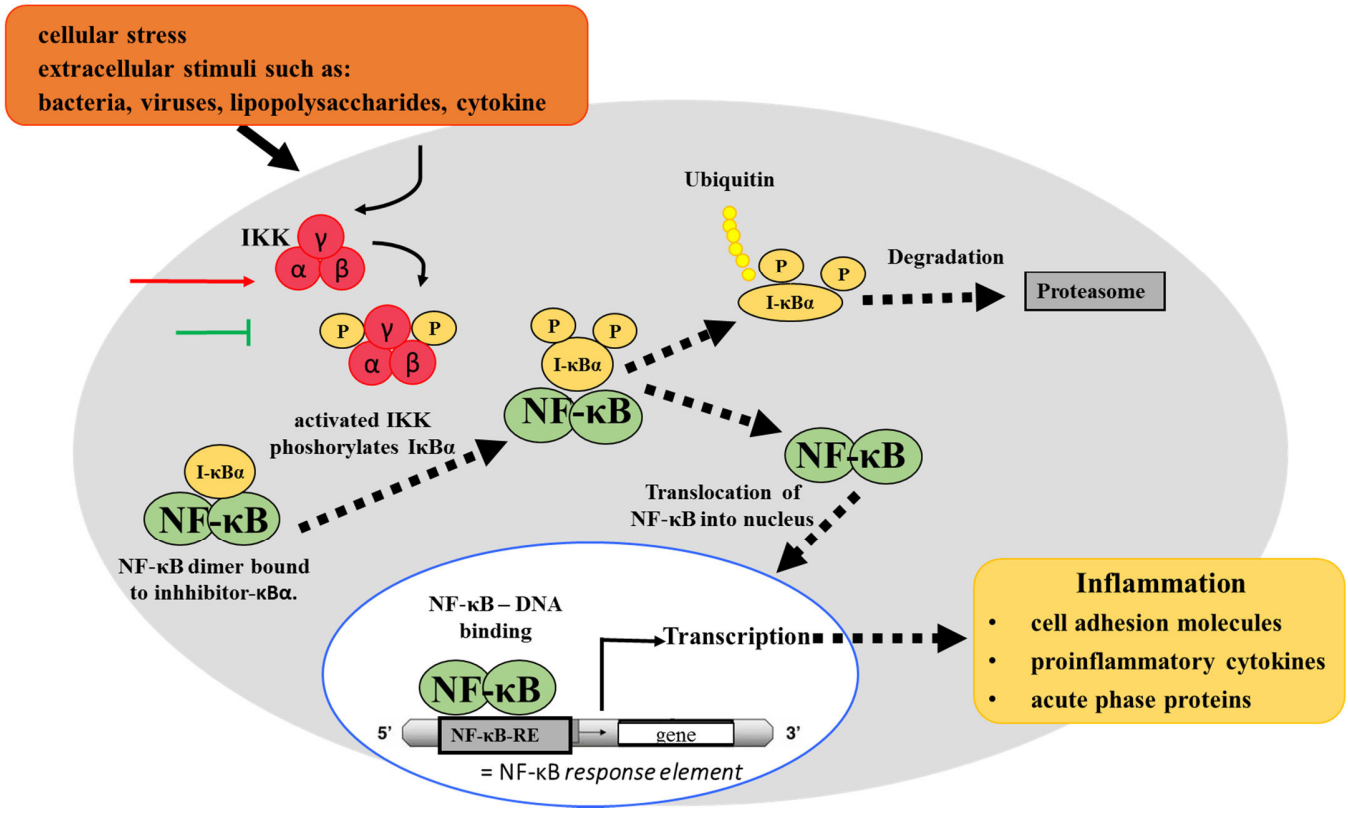

Figure 1: The activation of NF-κB (adapted from Ghosh and Karin 2002)

Pro-inflammatory cytokines

It has been suggested that the hepatic expression of pro-inflammatory cytokines, such as TNFα, is increased in dairy cows during early lactation in comparison with late pregnancy (Gessner et al. 2013a, Graugnard et al. 2013). Moreover, Ohtsuka et al. (2001) reported that the transition period induces an elevation of serum TNFα levels during early lactation, suggesting that TNFα might play a role in development of the fatty liver. These cytokines induce a systemic reaction known as acute phase response (APR; Fleck 1989) and stimulate the synthesis of positive acute phase proteins (+APP) in the liver such as serum amyloid A (SAA), haptoglobin (HP), or C-reactive protein (CRP), which are released by the hepatocytes after cytokine stimulation (Heinrich et al. 1990, 1998). The hepatic mRNA up-regulation of those APPs is associated with reduced hepatic synthesis of negative acute phase proteins (−APP), which constitutes essential liver proteins, such as albumins, enzymes, lipoproteins, transferrin or carriers of vitamins (e.g. retinol-binding proteins) and hormones (Carroll et al. 2009, Ceciliani et al. 2012). The main function of these APPs is protection against pathological damage and contribution to the restoration of homeostasis, as well

as to the regulation of different stages of inflammation (Petersen et al. 2004). The induction of immune stress by cytokines can lead to an increase of heat production (fever) and the synthesis of unusual proteins in liver and immune system tissues (Elsasser et al., 2000). Thereby, the most important APPs in ruminants are HP and SAA, whose concentrations increase particularly in response to acute inflammatory conditions (Eckersall and Bell 2010) and which are triggered by IL-1 and IL-6 (Yap et al. 1991). IL-1 and IL-6 are produced by Kupffer cells in response to endotoxin.

Haptoglobin is mainly synthesised by the liver and consists of four polypeptide chains, two α and two ß chains, which are connected by disulphide bridges (Morimatsu et al. 1991). The primary function of HP is binding free haemoglobin, which is released from erythrocytes, thus inhibiting its oxidative activity (Yang et al. 2003) associated with the prevention of iron uptake (essential for bacteria growth) from bacteria (Murata et al. 2004). This prevents the oxidative tissue damage which may be mediated by the free haemoglobin (Langlois and Delanghe 1996).

SAA proteins are a family of apolipoproteins which are synthesised predominantly by the liver. After secretion into the circulation, they associate with high-density lipoprotein (HDL) in the plasma (Uhlar et al. 1994). The main functions of SAA include the reserve transport of cholesterol from tissues to hepatocytes, thereby binding circulating toxins associated with an accelerated release of lipoproteins by the liver (Malle et al. 1993). Seven different isoforms of SAA have been reported in the blood of dairy cows, but the precise functions of these isoforms are not yet known (Takahashi 2009). During inflammation, SAA1 and SAA2 are mainly expressed in the liver in response to pro-inflammatory stimuli, whereas the third isoform SAA3 is expressed by adipose tissue, mammary glands, intestinal epithelial cells and macrophages, and is not transported by HDL (Meek et al. 1992, Chiba et al. 2009, Eckhardt et al. 2010). The fourth isoform, SAA4, does not respond to external stimuli.

Microbial components

The transition period is associated with an increased risk of infectious diseases, such as mastitis, because of the onset of lactation, physiological stress of calving and the risk of infection during parturition (Ringseis et al. 2014). The infectious diseases are caused by gram-negative bacteria such as *Escherichia coli*, which is consisted of LPS found in abundance in their outer membrane,

and contributes to the development of an inflammation process in the liver of dairy cows during early lactation (Zebeli and Ametaj 2009). LPS are relatively thermostable compounds from lipoid and sugar components (polysaccharides) which have a toxic effect via the degradation of bacteria. Thus, these parts are referred to as bacterial endotoxins. The potential source for LPS could be the mammary gland, causing pathological changes in the mammary tissue affiliated to the development of local inflammatory conditions (Wenz et al. 2001, Ametaj et al. 2012). However, the origin of LPS might be from other sources such as the gastrointestinal tract and uterus (Wenz et al. 2001). During an infection, LPS are released from the bacteria cell wall, and translocated to the liver via blood stream. During the transition period, the free ruminal LPS concentration increased, especially when cows are switched from the dry off period diets that are low in grain to high-grain diets during early postpartum. A sudden depression of ruminal pH, induced by high-grain diets, is associated with the onset of subacute ruminal acidosis and the contribution to the challenge of the liver with LPS (Ametaj et al. 2010, Dong et al. 2011). A low pH value in the rumen can disturb the rumen ecosystem, which results in the release of cell-free LPS in the rumen milieu. Furthermore, the permeability of the ruminal epithelial barrier enhanced, which is caused by the low pH-value in the rumen and certain strains of gram-negative bacteria and their products including LPS. Thereby, tight junctions and other cell adhesion proteins became disrupted. LPS, located in the rumen, are able to reach the systemic circulation via the lymphatic duct associated with the induction of systemic inflammation or enter the liver via the portal vein (Zebeli and Metzler- Zebeli 2012, Zebeli et al. 2012). Furthermore, it is evident that rumen LPS are released into the bloodstream and further translocated into the mammary gland where the function of the mammary epithelial cells is suppressed (Dong et al. 2011, Emmanuel et al. 2007). The recognition of LPS in the liver occurs via a family of pattern recognition receptors (PRRs), such as the toll-like receptors (TLRs), which are expressed by almost all types of liver cells (including hepatocytes, Kupffer, stellate and sinusoidal endothelial cells). In particular, TLR4 recognises gram-negative microorganisms, where LPS acts as an agonistic ligand (Pandey and Agrawal 2006), including saturated fatty acids (Mamedova et al. 2013), which are greatly elevated during the transition period of high yielding dairy cows. The activation of TLR4 triggers an intracellular signalling cascade which can result in a translocation of the NF-κB into the nucleus and up-regulation of pro-inflammatory genes (Bannerman and Goldblum 2003). Additionally, the detection of other pathogen-associated

molecular patterns such as nucleic acids, virus or fungi or damage-associated molecular patterns released from the damaged tissues are also implemented by the PRRs. Thus, the TLRs microbial detection induces a specific stress response called the microbial stress response (MSR) which involves the activation of transcriptional programs that induce an adaptive response to react to the infection, for example by the production of pro-inflammatory cytokines and antiviral factors, including IFNγ (Cláudio et al. 2013). It has been shown that some molecular determinants of MSR are in common with the UPR induced by the ER stress (Cláudio et al. 2013). Martinon et al. (2010) and Savic et al. (2014) have already shown that the activation of TLR2 and/or TLR4 activates the ER stress sensor IRE1α and its downstream target XBP1 in different cell types, with the latter being required for the optimal production of pro-inflammatory cytokines. According to this, microbial components can also induce ER stress in the liver of periparturient dairy cows.

Reactive oxygen species (ROS)

The energy metabolism during the transition period of dairy cows is associated with increased rates of ROS production and may result in oxidative stress (Gaal et al. 2006), which is caused by a strongly reduced plasma concentration of antioxidants in transition cows (Calderón et al. 2007). The aerobic cellular metabolism requires oxygen for efficient energy production and consequently results in the production of ROS including oxygen ions, free radicals and lipid hydroperoxides. During the transition period of dairy cows, increased oxygen metabolism elevates the rate of ROS production and the subsequent degradation of important antioxidant defences (Sordillo et al. 2007, Sordillo et al. 2009). Thus, the ROS production exceeds the capacity of antioxidant defences to neutralise the pro-oxidants triggering oxidative damage of lipids, DNA, proteins and other macromolecules (Brenneisen et al. 2005), which leads to oxidative stress in periparturient dairy cows. There are two main reasons for the occurrence of oxidative stress in transition cows. The first is the systemic inflammation during the transition period, a time of high metabolic demand, which induces oxidative stress by stimulating the production of ROS in the mitochondria and NADPH oxidase during the respiratory burst activity of phagocytic cells (Valko et al. 2007). The second is the increased load of the liver with NEFA released from adipose tissue associated with an increased production of energy from fatty acids resulting in an enhancement of the production of superoxide radicals in the electron chain and consequently leads to oxidative stress in the liver. Thus, a continuous oxidation of fatty acid induces ER stress (Huang et al. 2011, Huang et al. 2012),

whereas an inhibited oxidation of fatty acid protects hepatocytes from ER stress (Tyra et al. 2012). The ROS can participate in the activation of several pathways, whereby the activated redox-sensitive transcription factor NF-κB enhances the inflammation which can stimulate ROS production again. Several studies have demonstrated that oxidative stress primarily contributes to ER stress (Kaneto et al. 2005, Hotamisligil 2010) and the increased ROS levels act as local messengers between ER stress and the mitochondria (Csordás and Hajnóczky 2009). Furthermore, a recent study has shown that the induction of ER stress by palmitic acid causes mitochondrial DNA damage resulting in oxidative stress (Yuzefovych et al. 2013a). Thereby, the palmitic-induced mitochondrial DNA damage led to mitochondrial dysfunction, which results in a defective electron transfer, thus causing supplementary ROS production, oxidative stress and ER stress. Similar results were obtained with the ROS scavenger N-acetylcysteine (NAC), which reduced palmitate-induced mitochondrial dysfunction and ER stress in cultured L 6 myotubes (Yuzefovych et al. 2013a). Additionally, an *in vivo* experiment with obese mice fed a high-fat diet demonstrated that the induction of ER stress is associated with mitochondrial damage and dysfunction and oxidative stress (Yuzefovych et al. 2013b).

1.4 Stress of the endoplasmic reticulum

The metabolic load and inflammation-like conditions during the transition period of high-yielding dairy cows can frequently lead to a higher risk of diseases like ketosis (Katoh et al. 2002), abomasal displacement (Guzelbektes et al. 2010), subacute rumen acidose (Plaizier et al. 2008), mastitis (Contreras and Rodríguez 2011, Jørgensen et al. 2012), retained placenta (LeBlanc et al. 2004) and endometritis (Burke et al. 2010, Akbar et al. 2014).

Recent studies demonstrated that metabolic stress and inflammation (NEFA, pro-inflammatory cytokines, ROS, LPS) induce the development of stress of the endoplasmic reticulum (ER) in the liver of early lactating cows (Gessner et al. 2014, Ringseis et al. 2014). During the transition period of dairy cows, high concentrations of fatty acids, especially saturated fatty acids, can lead to a disruption of ER membranes and to disturbed ER homeostasis in the liver associated with the activation of an adaptive response (Wei et al. 2009, Fu et al. 2012). Disrupted ER function leads to several problems, including the release of stored calcium from organelles and an imbalance between the protein folding capacity of the ER and the protein load, which consequently leads to the accumulation of unfolded or misfolded proteins in the ER lumen (Cnop et al. 2012). Thereby, the accumulation of unfolded and misfolded proteins induces an adaptive response called the unfolded protein response (UPR) to decrease the unfolded protein load and restore the ER homeostasis and functions. Thereby, the UPR alleviates stress by three kinds of protective cellular responses: 1) up-regulation of ER chaperones, such as immunoglobulin heavy-chain binding protein (BiP), to support the process of protein refolding, 2) the alleviation of protein translation and 3) degraded misfolded proteins by the proteasome by a process called ER-associated degradation (ERAD; Marciniak and Ron 2006, Ron and Walter 2007). The activated UPR may lead either to cell survival of cells causing the synthesis of ER chaperone proteins associated with a decrease in general protein translation or to cell death by the induction of apoptosis (Breckenridge et al. 2003). The three ER-resident transmembrane protein sensors of ER stress, which become activated when unfolded or misfolded proteins accumulate in the ER lumen, are: 1) inositol requiring 1 (IRE1), 2) PKR-like ER kinase (PERK), and the activation of transcription factor 6 (ATF6; Ron and Walter 2007, Cnop et al. 2012, Figure 2). An accumulation of misfolded proteins in the ER lumen results in dissociation of BiP from the stress transducers to chaperone the misfolded proteins leading to activated ER stress transducers and initiation of the UPR (Bertolotti et al. 2000). The

activation of PERK results in a stimulation of the phosphorylation of eukaryotic translation initiation factor (eIF) 2α, which alleviates the translation of proteins (Harding et al. 1999). The activation of IRE1 results in site-specific splicing of X-box binding protein 1 (XBP1) mRNA which generates a translation into the transcription factor XBP1 (Ron and Walter 2007) regulating the expression of genes involved in ER biogenesis and ER-associated degradation. Additionally, the stimulation of IRE1α by ER stress binding to the adaptor protein TNF-receptor-associated factor 2 (TRAF2) leads to formation of the IRE1α-TRAF2 complex (Urano et al. 2000). This complex is capable of activating signalling cascades like NF-κB and c-Jun N-terminal kinase (JNK), which results in the production of pro-inflammatory cytokines. NF-κB is a transcription factor involved in inflammation, and the induction of pro-apoptotic genes (Momoi 2004, Cnop et al. 2012). XBP1 up-regulated ER chaperons (components of ERAD) stimulates phospholipid biosynthesis leading to an expanded ER membrane (Marciniak and Ron 2006, Cnop et al. 2012, Fu et al. 2012). ATF6 is an ER stress-regulated transmembrane transcription factor, which is activated by processing via translocation (site 1 and site 2 proteases) in the Golgi apparatus associated with an induced expression of genes which are involved in ERAD, lipid biosynthesis, ER expansion and protein folding (Cnop et al. 2012).

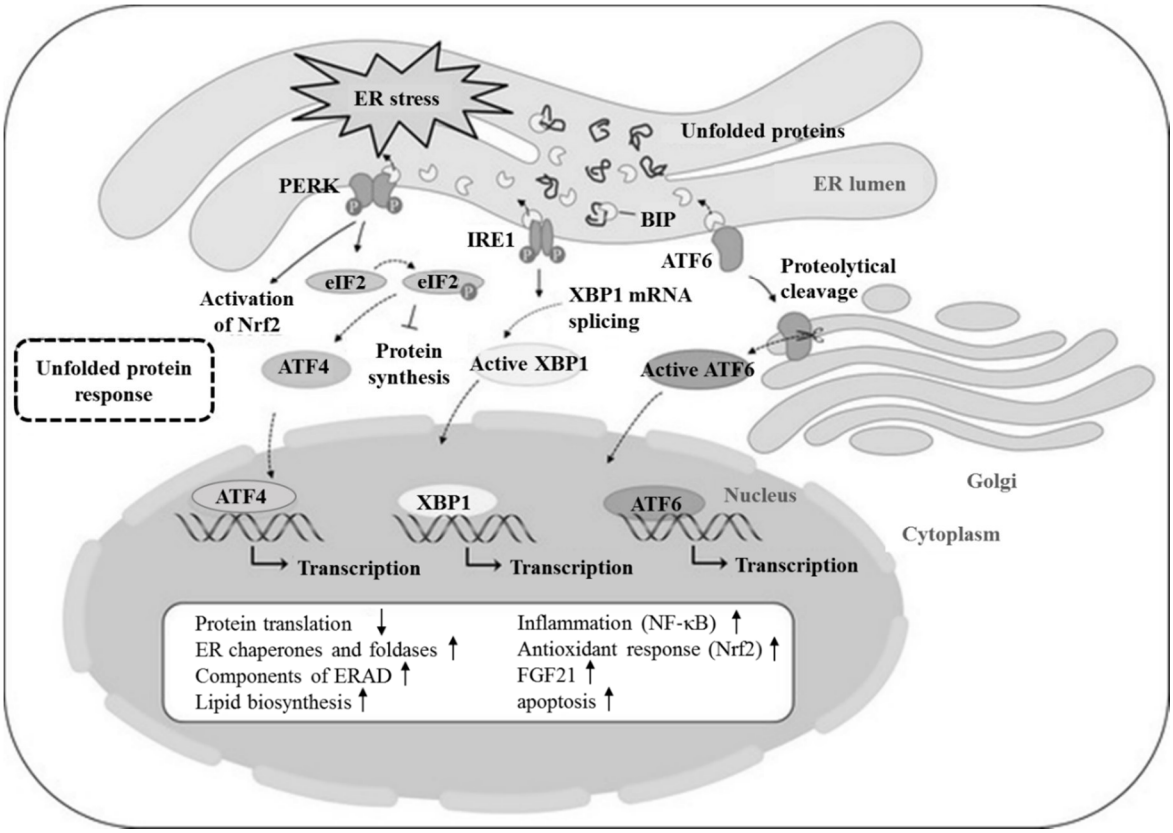

Figure 2: schematic model of endoplasmic reticulum (ER) stress-induced unfolded protein response UPR mediated by three ER stress transducers (PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) (Ringseis et al. 2014)

The ER stress-induced UPR are associated with a variety of symptoms in the liver, which were similarly observed in periparturient dairy cows, such as the development of fatty liver (Gentile et al. 2011, Pagliassotti 2012). Beside the activation of the UPR, which may play a key role for the development of fatty liver, further inductions are initiated (Figure 3). Therefore, ketosis and insulin resistance might also be important for cell survival under stressful conditions during early lactation. Furthermore, the activation of lipid biosynthesis, the reduction of fatty acid oxidation and the reduced secretion of lipids by VLDL which is caused by the UPR leading to the development of fatty liver. The reduced insulin sensitivity and the inhibition of glycolysis and gluconeogenesis induced by UPR can clearly explain the insulin resistance which is generally observed in post-parturient dairy cows. Additionally, the UPR-induced stimulation of inflammation by an activation of NF-κB, and the enhancement of the antioxidant and cytoprotective capacity of the cell by activation of nuclear factor-erythroid 2-related factor-2 (Nrf2, Cullinan et al. 2003, Cullinan and Diehl 2006) contribute to the maintenance of cell survival and cell function. Finally, the stimulation of ketogenesis might be generated by the

UPR-triggered induction of the fibroblast growth factor 21 (FGF21) which is the key regulator of ketogenesis (Schaap et al. 2013).

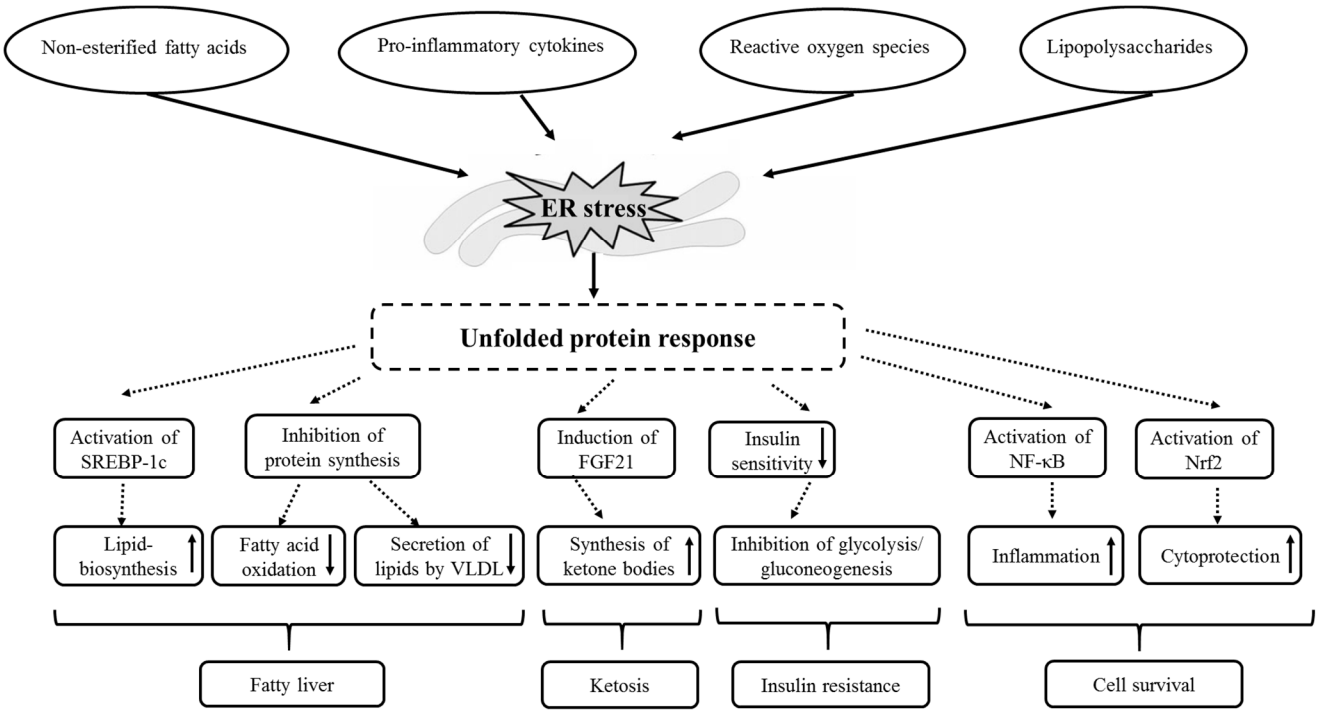

Figure 3: schematic summary of UPR-induced effects (Ringseis et al. 2014)

Fibroblast growth factor 21 (FGF21)

FGF21 has been detected and identified as an important hormonal factor regulating the stimulation of hepatic lipid oxidation, ketogenesis and gluconeogenesis during energy deprivation (Badman et al. 2007, Inagaki et al. 2007, Seo et al. 2010) and is expressed in the liver, pancreas, white adipose tissue and skeletal muscle (Nishimura et al. 2000, Wang et al. 2008, Izumiya et al. 2008). Additionally, FGF21 restores insulin sensitivity in animal models of obesity-related disorders and insulin resistance (Xu et al. 2009, Li et al. 2011, Hale et al. 2012).

In dairy cows, several recent studies have reported that the expression of FGF21 in the liver and plasma is markedly up-regulated during the transition from late pregnancy to early lactation (Carriquiry et al. 2009, Schoenberg et al. 2011, Schlegel et al. 2012). Recently, it has been found that FGF21 is immediately induced by ER stress mediated by an activation of the PERK cascade indicating that the up-regulation of FGF21 in the liver during the transition period is initiated by an ER stress-induced UPR (Schaap et al. 2013, Gessner et al. 2014). Schaap et al. (2013) reported that different ER stress inducers lead to an up-regulation of FGF21 expression via the PERK/eIF2α/ATF4 cascade in hepatocytes. Whereas FGF21 stimulates ketogenesis,

Gessner et al. (2014) indicate that ER stress is present in the liver of periparturient cows leading to an enhancement of ketosis development due to an up-regulation of FGF21. Therefore, it has been proposed that the induction of ER stress and the associated UPR contribute to pathophysiologic conditions during the transition period of high-yielding dairy cows, such as fatty liver, ketosis and hepatic inflammation (Gessner et al. 2014). The elevation of serum levels of FGF21 in animal models of fatty liver indicates that FGF21 is involved in the development of fatty liver, as well (Hale et al. 2012). In dairy cows at parturition, plasma FGF21 levels are markedly elevated until the level is stabilised at lower, chronically elevated concentrations during early lactation (Schoenberg et al. 2011, Khan et al. 2014). Recently, it has been demonstrated that the plasma FGF21 level or the hepatic FGF21 mRNA level was positively correlated with the plasma NEFA level and the estimated energy deficit (Carriquiry et al. 2009, Schoenberg et al. 2011), indicating that the blood FGF21 level is a useful biomarker of stress in the post-partum dairy cow.

Nuclear factor-erythroid 2-related factor-2 (Nrf2)

Additionally, ER stress induced pro-inflammatory cytokines and ROS activates cytoprotective pathways, such as the nuclear factor E2-related factor 2 (Nrf2) pathway. Nrf2 is an important redox-sensitive transcription factor which regulates the expression of various antioxidative and cytoprotective proteins. Under basal conditions, the Nrf2 remains inactive and is located in the cytosol bound to the cysteine-rich cytosolic inhibitory Kelch-like ECH-associated protein 1 (Keap1, Ohnuma et al. 2010, Wakabayashi et al. 2010). The Keap1 protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase (Cullinan et al. 2004). While Nrf2 is inactive, Nrf2 is constantly ubiquitinated, resulting in proteasomal degradation (Cullinan et al. 2004, McMahon et al. 2006). Upon exposure to stimuli such as oxidative stress, Nrf2 dissociates from Keap1, translocates into the nucleus, heterodimerises with small musculoaponeurotic fibrosarcoma (Maf) proteins activating the transcription of target genes encoding antioxidant, detoxifying, anti-inflammatory proteins by binding to the antioxidant response element (ARE, Niture et al. 2010, Kim et al. 2010). In response to ER-induced stimuli such as ROS or pro-inflammatory cytokines, the activated PERK pathway leads to a phosphorylation-dependent dissociation of Nrf2 from Keap1 and add-on translocation of Nrf2 into the nucleus binding to ARE (figure 4). The activated Nrf2 leads to a reduction of sensitivity of tissues to oxidative damage and cytotoxicity and contributes to the suppression of pro-inflammatory signalling, the attenuation of inflammatory damage and the neutralisation of ROS produced under pro-inflammatory

conditions (Köhle and Bock 2007, Baird and Dinkova-Kostova 2011). It has recently been observed that the activated Nrf2 leads to an up-regulation of Nrf2 target genes including catalase, glutathione peroxidase 3, microsomal glutathione S-transferase 3, haem oxygenase 2, metallothionein 2A, NAD(P)H dehydrogenase, quinone 1, superoxide dismutase 1 and UDP glucuronosyltransferase 1 family, polypeptide A1, in the liver of high-yielding dairy cows during early lactation (Gessner et al., 2013a). The ER- induced UPR and the observed following activation of Nrf2 in the liver of dairy cows at early lactation may be a compensatory method to protect the liver against inflammation- and ROS-induced damage (Gessner et al. 2013a).

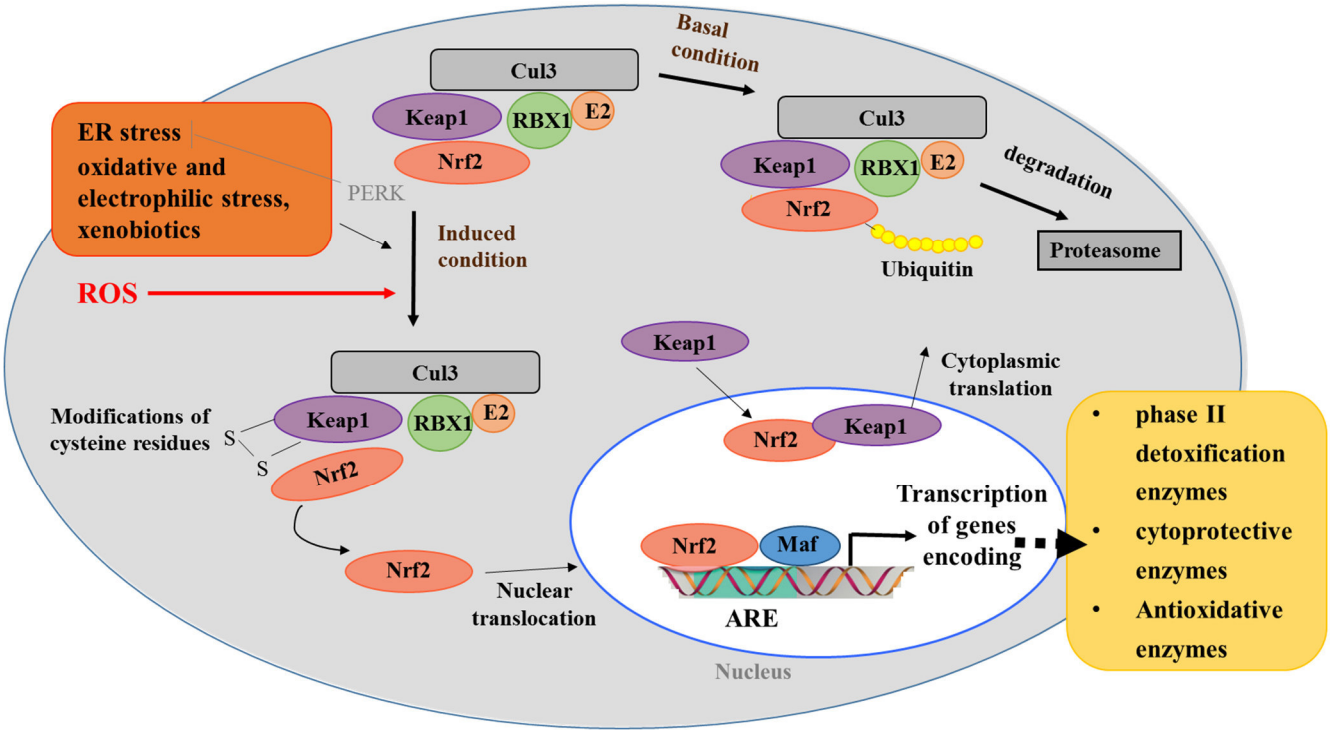

Figure 4: The activation of Nrf2 (adapted from Dinkova-Kostova et al. 2002)

1.5 Grape pomace (GP)

Grapes are the most widely cultivated fruit crop in the world. The vineyard cultivation globally represents 7,436,000 ha on which a total volume of 277 million hectolitres of wine was produced in 2013 and cultivated mainly as *Vitis vinifera* for wine production (German wine institution 2014). The winemaking process generates a residue left after juice extraction by pressing called grape pomace (GP) which constitutes about 13% of the total weight of grapes (Torres et al. 2002). This incidental and inexpensive press residue, consisting of skins, seed and stems, has been recognised as an important source of polyphenols (Kammerer et al. 2004, Toaldo et al. 2013). Thus, grape seeds and skins are increasingly used to obtain functional food ingredients, such as antioxidants and dietary supplements (Goni et al. 2005). The polyphenols of GP are mainly proanthocyanidins, anthocyanins, flavonols, flavanols, phenolic acids and stilbenes (resveratrol, Monagas et al. 2006, Novaka et al. 2008, Hernandez-Jimenez et al. 2009). Proanthocyanidins are the major phenolic compounds in grape skins and seeds (Hernandez-Jimenez et al. 2009). Anthocyanins, which constitute a subgroup of flavonoids (glycosidic-linked flavonoids), are mainly found in red grapes (Jackson 2008), a pigment which mainly exists in grape skins, whereas flavonoids are widely distributed in grapes, especially in seeds and stems. The latter basically contain (+)-catechins, (−)-epicatechin and procyanidin polymers and are more abundant in white varieties (Cantos et al. 2002, Chacona et al. 2009).

Concerning the general usage of GP, seeds are most often used for the extraction of oligomeric procyanidins (OPCs) and for the production of grape seed oil (Spranger et al. 2008, Deng et al. 2011), whereby the skins and stems are used for preparing grape resveratrol extracts (Sun et al. 2006). Further investigations focused on yeast production or extraction and recovery of phenolic compounds (Lo Curto and Tripodo 2001, Louli et al. 2004). Other previous studies reported that composted GP is a high-quality substitute to improve the plant growth (Baran et al. 2001, Bustamante et al. 2007, Bustamante et al. 2008). Additionally, GP can be recycled by applying these residues as organic fertilisers directly into the soil (Ferrer et al. 2001).

Polyphenolic compounds, especially flavonoids, have received increasing attention due to the various health-promoting effects, which are largely based on their anti-inflammatory activities by regulating the activities of key regulators of inflammation (Kim et al. 2008, Gessner et al. 2013b). Several recent studies reported antioxidant, anti-proliferative and anti-angiogenic effects of resveratrol, as well as anti-inflammatory impacts (Tipoe et al. 2007, Catalgol et al.

2012, Peluso et al. 2013, Bognar et al. 2013). Furthermore, Nishiumi et al. (2012) showed that red GP contains an abundance of effective compounds for anti-inflammatory action. Other previous numerous studies demonstrated the neuroprotective, cardioprotective, antioxidant and antimicrobial health benefit of GP (Hoensch and Oertel 2012, Oliveira et al. 2013, Tognon et al. 2014, Ky et al. 2014, Liang et al. 2014, de Sá et al. 2014).

Polyphenols and their antioxidant effects

As polyphenolic compounds, flavonoids have the ability to act as antioxidants. Regarding this, it has been reported that feeding diets rich in polyphenols could lead to an improvement of antioxidant status of plasma and tissues associated with an enhancement of tocopherol concentrations due to a vitamin E-sparing effect (Zhu et al. 1999). Some studies observed that feeding various types of flavonoids increases the plasma and tissue tocopherol concentrations in rats (Nanjo et al. 1993, Choi et al. 2003, Frank et al. 2003), whereas other studies reported that there are no effects (Frémont et al. 2000, Yamagishi et al. 2001, Ameho et al. 2008). Additionally, studies exist dealing with the effects of grape seed and GP on the antioxidant system in the liver, mostly in rats or rabbits (Chis et al. 2009, Choi et al. 2010, Choi et al. 2012). Thus, Chis et al. (2009) demonstrated that an orally administered grape seed extract (GSE) reduced the levels of lipid peroxides and carbonylated proteins and led to an improvement of antioxidant activity in plasma and hepatic tissue in rats suggesting that GSE enhanced the antioxidant defence against ROS produced under hyperglycaemic conditions. Similar results reported Choi et al. (2012) suggesting that adding grape seeds as dietary supplement are able to suppress lipid peroxidation in high fat-fed rats. Choi et al. (2010) investigated that GP can activate the antioxidant enzyme system and prevent damage with hypercholesterolemia in diet-induced hypercholesterolemic rabbits. Luehring et al. (2011) suggested that quercetin has a tocopherol-sparing effect under conditions of low dietary vitamin E concentration in pigs. In contrast, other studies demonstrated that adding dietary flavonoids to diets with nutritionally adequate vitamin E concentrations did not elevate the concentrations of tocopherol in plasma and tissue in pigs (Augustin et al. 2008, Wiegand et al. 2010). Wiegand et al. (2010) observed that the metabolism of vitamin E remained unchanged due to the fact that dietary flavonoids do not modify the expression of hepatic genes which are involved in the transfer of tocopherols into plasma lipoproteins, decomposition and excretion into the bile. Hogan et al. (2010) reported that the antioxidant activity of grape pomace extract (GPE) was elevated by oxygen radical absorbance, whereby a supplementation of dietary GPE did not improve oxidative stress in diet-

induced obese mice. A further recent study reported the prevention of oxidative processes in monogastric organisms associated with reduced lipid peroxidation by adding GP in diets for broilers (Vossen et al. 2011). Furthermore, Gessner et al. (2013b) shows that a supplementation of grape seeds and grape marc rich in polyphenols do not lead to an improvement of vitamin E status and antioxidant status of pigs associated with an adequate supply of dietary vitamin E. Concerning an alleviation of ER stress in the liver, Liu et al. (2013) have shown that quercetin leads to an attenuation of lead-induced ER stress in the liver of rats caused by their antioxidant activity, indicating that the reduced oxidative stress acts by inhibiting ER stress *in vivo*. In line with this, it was found that a grape seed proanthocyanidin extract high in antioxidant potential effected a reduction of oxidative stress in the skeletal muscle of diet-induced diabetic rats (Ding et al. 2013), which indicates that antioxidants as a dietary supplement have the potential to alleviate ER stress.

Polyphenols and their anti-inflammatory effects

Beside the antioxidant effects of flavonoids, it has been observed that polyphenols have an anti-inflammatory potential, as well (Garcia-Lafuente et al. 2009). Thus, recent studies have focused on the anti-inflammatory effects of plant compounds such as green tea or grape seeds (Rahman et al. 2006, Park et al. 2012, Recio et al. 2012). Under *in vitro* conditions, Gessner et al. (2011) already reported that an ethanolic grape seed and grape meal-based feed additive exerts anti-inflammatory effects in intestinal cells by a reduced TNFα-induced NF-κB transactivation and a decreased expression of NF-κB target genes. Recent studies have shown that polyphenols inhibit the NF-κB DNA binding by a suppression of phosphorylation and degradation of the inhibitory peptide IκB (Erlejman et al. 2008; Sahin et al. 2010), which prevents the translocation of NF-κB from the cytosol into the nucleus, which might be explained by the reduced formation of ROS (Lee et al., 2006). Certainly, Erlejman et al. (2008) suggested that the inhibited TNFα-induced NF-κB activation in Caco-2 cells might be caused by the reduced production of cellular oxidants. A review of effects of flavonoids (in vitro) conclude that flavonoids are almost anti-inflammatory on different cell types reducing the expression of a variety of inflammatory mediators including eicosanoids, adhesion molecules and cytokines (González et al. 2011). Another study shows the anti-inflammatory effects of flavonoids on immune cells focusing on their ability to modulate multiple redox-sensible pathways involved in inflammation (Izzi et al. 2012). Under *in vivo* conditions, Gessner et al. (2013b) investigated that adding grape seed and grape marc meal extract as a dietary supplement could lead to a suppression of the inflammatory

process by reducing the activity of NF-κB in the duodenal mucosa of pigs. This latter finding that polyphenols are able to suppress the process of inflammation is in line with several *in vitro* studies using intestinal epithelial cells and *in vivo* studies mainly carried out in rodent models of acute or chronic colitis using mainly green tea as dietary polyphenols (Romier et al. 2009). Fiesel et al. (2014) showed a lower expression of various pro-inflammatory genes within the duodenum, ileum and colon by supplementing grape seed and grape marc meal extracts to diets of weaning pigs. Other studies reported that anthocyanins from grapes or grape seeds have inhibitory effects on the inflammation process in human intestinal Caco-2 cells (Romier-Crouzet et al. 2009) or the intestine of rats with ulcerative colitis (Wang et al. 2011). Suggesting a potential anti-inflammatory effect by dietary grape pomace extract (GPE), the supplementation of GPE reduced the levels of plasma C-reactive protein by 15.5% in the high fat diet fed mice (Hogan et al. 2010). In rodents, it has been observed that the ingestion and systemic administration of four polyphenols (resveratrol, epigallocatechin gallate, curcumin and quercetin) lead to an inhibition of NF-κB-dependent gene expression associated with an induction of phase II antioxidant and detoxifying proteins (Shapiro et al. 2007).

Several studies have observed that polyphenols, especially those in green tea, are able to induce the inhibition NF-κB and activation of Nrf2 in the liver (Rahman et al. 2006, Park et al. 2012). Furthermore, various studies suggested that polyphenols can induce an activation of Nrf2 associated with an enhancement of the expression of several cytoprotective and antioxidative genes in the small intestine (Scapagnini et al. 2011, Cheng et al. 2013). In contrast, Gessner et al. (2013b) investigated that supplementing grape seed and grape marc meal extract as a dietary supplement lowered the transactivation of Nrf2 and their expression of various target genes in the duodenal mucosa in pigs due to the suppressed production of ROS and pro-inflammatory cytokines in the surrounding of intestinal cells by the applied polyphenols.

Formulation of the hypotheses underlying study 1, 2 and 3

Based on these findings (polyphenols and their antioxidant and anti-inflammatory effects) and likewise in regard to the fact that the potential effects of polyphenols on the antioxidant status, inflammation and ER stress in the liver of dairy cows have not yet been investigated so far, studies 1 and 2 of this thesis were carried out. Consequently, we investigated in **study 1** the hypothesis that adding grape seed and grape marc meal extract (GSGME) as a plant extract rich in flavonoids has the potential to reduce inflammation and ER stress in the liver of dairy cows during early lactation. Beyond that, the effect on the metabolic and antioxidant status of dairy

cows received the GSGME was considered as well. For this purpose, two groups of dairy cows received a total mixed ration, whereby the ration of the treatment group was supplemented with 1% of GSGME from week 3 prepartum to week 9 postpartum. As study 1 indeed showed that the supplementation of GSGME significantly reduced the mRNA abundancy of FGF21, a key marker of ER stress, in the liver in week 1 and 3 postpartum and that the milk yield was increased associated with a dry matter intake which was not different between both groups, study 2 was carried out. **Study 2** investigated the hypothesis that supplementing a plant product consisting of green tea (95%) and curcuma extract (5%) rich in polyphenols attenuates inflammation and ER stress in the liver of periparturient dairy cows. Therefore, metabolic and antioxidant parameters [NEFA, TAG, retinol, α-tocopherol, β-carotene, Trolox equivalent antioxidative capacity (TEAC)] as well as the relative mRNA concentrations of FGF21, inflammatory genes and genes of the UPR in the liver were determined. Whereas the mode of action of the improved milk yield in study 1 could not be explained by the collected parameters, **study 3** was carried out. As polyphenols exert a broad spectrum of metabolic effects, we hypothesized that feeding of GSGME influences metabolic pathways in the liver which could induce the positive effects of GSGME in dairy cows. In order to identify these pathways, we performed genome-wide transcript profiling in the liver and lipid profiling in plasma of dairy cows fed GSGME during the transition period at 1 week postpartum.

Grape pomace and their nutritional factors

In order to implement an energetic feed evaluation of GP for ruminants, determination of nutrient digestibility have to be carried out according to the guidelines of Gesellschaft für Ernährungsphysiologie (GfE) (1991). It has already been established that GP, either in fresh, dried or ensiled form, has a low energy content and can be included in feeding rations for ruminant animals, particularly when fed near to maintenance (Baumgärtel et al. 2007). However, little is known about the feeding value of fresh and dried GP from different grape varieties, especially in German regions. In addition, studies performed in Iran and Portugal suggested that ensiling of GP as an inexpensive conservation technique are able to increase the feeding value of GP (Alipour and Rouzbehan 2007, De Pina and Hogg 1999). Based on these non-German studies and in the light of the scarcity of informations about the effect of ensiling of GP originating from Germany on crude nutrient contents, polyphenol stability as well as nutrient digestibility, new data are required.

In the recent past, the determined contents of crude nutrients of GP underlying strong variations associated with great differences of the following generated values of nutrient digestibilities (Pirmohammadi et al. 2007, Goni et al. 2008, Bahrami et al. 2010, Mirzaei-Aghsaghali et al. 2011). Additionally, only one study investigating the feed value for sheep of fresh GP from two different grape varieties originating from Germany (Saale-Unstrut) is known (Baumgärtel et al. 2007). Other recent digestibility studies are frequently performed in non-local regions (De La Cerda-Carrasco et al. 2014, Basalan et al. 2011, Spanghero et al. 2009) suggesting that regional German GP varieties have to be determined for their crude nutrients to generate their digestibilities in ruminants.

For the purpose of inclusion GP in ruminant diets, the contents of nutrients, but also the anti-nutritive factors have to be observed and identified. The use of GP as animal feed have largely been restricted due to its low feeding value for ruminants (Baumgärtel et al. 2007). Thereby, the main factors limiting the usage of GP as a ruminant feed are the presence of grape seeds which are high in lignified fibre (D'Urso and Asmundo 1983) and the high condensed and hydrolysable tannins content (Abarghuei et al. 2010, Abarghuei et al. 2011) which can negatively affect the rumen microbial population at high levels (Leinmüller and Menke 1990, Wang et al. 1997). The anti-nutritive effects of tannins are associated with their ability to bind dietary proteins, polymers such as cellulose, hemicellulose and pectin and minerals which retarded their digestion (McSweeney et al. 2001a). Further studies have already shown that tannins from grapes and fruits inhibits digestive enzymes including α-amylase, α-glucosidase and lipase as well as nutrient digestibility (McDougall et al. 2008, Chamorro et al. 2013). The polyphenol contents of GP underlie strong variations as well, which might be caused by edaphic, geographic, weather-related factors, viticultural practice and winemaking techniques (Obreque-Slier et al. 2013, De la Cerda-Carrasco et al. 2014) suggesting that regional German GP varieties have to be determined for their polyphenol content.

Furthermore, differences between white and red GP should be made calculating energy contents, whereas in the feeding value tables of the DLG (1997) only one energy value of dried GP is provided. Considering that Rhineland-Palatinate produced just about a third of GP of whole Germany, new data of contents of crude nutrient and polyphenols of GP and their digestibilities are in great demand to give recommendations for use of GP in ruminants.

Determination of feeding value of GP - study 4

Based on these preceding findings, study 4 was performed. Thus, in **study 4** we determined the content of crude nutrients and polyphenols of GP from different grape varieties originating from an important winegrowing area in Rhineland-Palatinate (Germany) to calculate their energy contents. In order to investigate the effect of ensiling on the feeding value of GP, these different GP were moreover ensiled with as well as without an ensiling additive and determined for their contents of crude nutrient and polyphenols. Additionally, we determined the feeding value of dried white, dried red and ensiled white GP for sheep by calculating nutrient digestibility and the contents of metabolisable energy (ME) and net energy lactation (NEL).

1 PUBLICATIONS

The present cumulative thesis consists of three articles which have been published in peer reviewed, international journals.

Paper I

Gessner, D.K., Koch, C., Romberg, F.-J., Winkler, A., Dusel, G., Herzog, E., Most, E. & K. Eder (2015): The effect of grape seed and grape marc meal extract on milk performance and the expression of genes of endoplasmic reticulum stress and inflammation in the liver of dairy cows in early lactation. J. Dairy Sci. 98: 1-13.

Paper II

Winkler, A., Gessner, D.K., Koch, C., Romberg, F.-J., Dusel, G., Herzog, E., Most, E. & K. Eder (2015): Effects of a plant product consisting of green tea and curcuma extract on milk production and the expression of hepatic genes involved in endoplasmic stress response and inflammation in dairy cows. Arch. Anim. Nutr. 69(6): 425-441.

Paper III

Gessner, D.K., Winkler, A., Koch, C., Dusel, G., Liebisch, G., Ringseis, R. & K. Eder (2017): Analysis of hepatic transcript profile and plasma lipid profile in early lactating dairy cows fed grape seed and grape marc meal extract. BMC Genomics 18(253): 1-17.

Paper IV

Winkler, A., Weber, F., Ringseis, R., Eder, K. & G. Dusel (2015): Determination of polyphenol and crude nutrient content and nutrient digestibility of dried and ensiled white and red grape pomace cultivars. Arch. Anim. Nutr. 69(3): 1-14.

PAPER I

The effect of grape seed and grape marc meal extract on milk performance and the expression of genes of endoplasmic reticulum stress and inflammation in the liver of dairy cows in early lactation

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The effect of grape seed and grape marc meal extract on milk performance and the expression of genes of endoplasmic reticulum stress and inflammation in the liver of dairy cows in early lactation

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ABSTRACT

During the periparturient phase, cows are typically in an inflammation-like condition, and it has been suggested that inflammation associated with the development of stress of the endoplasmic reticulum (ER) in the liver contributes to the development of fatty liver syndrome and ketosis. In the present study, we investigated the hypothesis that feeding grape seed and grape marc meal extract (GSGME) as a plant extract rich in flavonoids attenuates inflammation and ER stress in the liver of dairy cows. Two groups of cows received either a total mixed ration as a control diet or the same total mixed ration supplemented with 1% of GSGME over the period from wk 3 prepartum to wk 9 postpartum. Dry matter intake during wk 3 to 9 postpartum was not different between the 2 groups. However, the cows fed the diet supplemented with GSGME had an increased milk yield and an increased daily milk protein yield. Cows supplemented with GSGME moreover had a significantly reduced mRNA abundancy of fibroblast growth factor (*FGF*) 21, a stress hormone induced by various stress conditions, in the liver in wk 1 and 3 postpartum. In contrast, mRNA abundances of a total of 3 genes involved in inflammation and 14 genes involved in ER stress response, as well as concentrations of triacylglycerols and cholesterol, in liver samples of wk 1 and 3 postpartum did not differ between the 2 groups. Overall, this study shows that supplementation of GSGME did not influence inflammation or ER stress in the liver but increased milk yield, an effect that could be due to effects on ruminal metabolism.
Key words: dairy cow, liver, inflammation, grape seed and grape marc meal extract

INTRODUCTION

The transition period spanning the time period between wk 3 prepartum and wk 3 postpartum represents the most critical period in the productive life of high-yielding dairy cows. During this period, the liver experiences pronounced metabolic stress due to a marked influx of NEFA, which are mobilized from adipose tissue triacylglycerol (**TAG**) stores due to a severe negative energy balance (Drackley, 1999). Approximately one-third of the whole-body NEFA flux is taken up into the liver and exceeds its oxidation capacity and, thus, promotes liver-associated diseases, such as fatty liver and ketosis (Drackley et al., 2001). Besides this metabolic stress, the liver of early lactating cows is exposed to diverse inflammatory challenges, such as microbial components, pro-inflammatory cytokines, and reactive oxygen species. These inflammatory challenges are resulting from infectious diseases, such as mastitis, endometritis, but also from subacute rumen acidosis and abomasal displacement (Plaizier et al., 2008; Vels et al., 2009; Zebeli and Metzler-Zebeli, 2012), which frequently occur during parturition, the onset of lactation, or both. As a consequence of this, transition dairy cows develop an inflammation-like condition in the liver (Bionaz et al., 2007; Vels et al., 2009), which is evident from the induction of an acute phase response. The acute phase response is characterized by the production of positive acute phase proteins, such as serum amyloid A, haptoglobin, or C-reactive protein, which compete with the production of essential liver proteins, also called negative acute phase proteins, such as albumins, enzymes, lipoproteins, transferrin, or carriers of vitamins and hormones (Carroll et al., 2009). The role of inflammation during transition to lactation for health and performance of dairy cows has been recently highlighted in a review in this journal (Bradford et al., 2015). It has been found that metabolic and inflammatory stress induces stress of the endoplasmic reticulum

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(**ER**) in the liver of early lactating cows (Gessner et al., 2014). Endoplasmic reticulum stress is defined as an imbalance between the folding capacity of the ER and the protein load, with the consequence that unfolded or misfolded proteins accumulate in the ER lumen, thereby disturbing ER homeostasis (Cnop et al., 2012). It is known from studies in humans and rodents that this causes activation of an adaptive response, termed unfolded protein response (**UPR**), which aims to decrease the unfolded protein load and restore ER function (Cnop et al., 2012). The activation of the UPR, such as observed in rat models of obesity or diabetes or induced by application of chemical ER stress inducers, leads to a variety of symptoms in the liver, which are similar to those observed in periparturient dairy cows. This includes the development of fatty liver (Gentile et al., 2011; Lee et al., 2012; Pagliassotti, 2012), an enhancement of the antioxidant and cytoprotective capacity by activation of Nrf2 (Cullinan et al., 2003; Cullinan and Diehl, 2006; Gessner et al., 2013b), an induction of inflammation (Zhang and Kaufman, 2008; Rath and Haller, 2011), and an induction of FGF21 (Schaap et al., 2013). Therefore, it has been proposed that ER stress-induced UPR contributes to the pathophysiologic conditions commonly observed in the liver of periparturient cows, such as the development of fatty liver, ketosis, or inflammation (Ringseis et al., 2015).

Polyphenols are members of a large family of plant-derived compounds classified as flavonoids and nonflavonoids. Polyphenols of the flavonoid class have been shown to exert several beneficial effects in humans and animals. Besides their antioxidative potential, flavonoids have strong antiinflammatory properties. Studies with various models of inflammation such as obese rats, rats fed a high-fat diet or rats challenged with endotoxins, dietary supplementation with various types of flavonoids reduced the level of inflammation in the liver and attenuated the development of hepatic steatosis, which is a typical feature in these animal models (Terra et al., 2009; Bharrhan et al., 2012; Heber et al., 2014). Recently, it has been found that flavonoids are moreover able to attenuate ER stress in liver and muscle cells (Giordano et al., 2014; Rodriguez et al., 2015).

Whereas a great number of studies dealing with antiinflammatory properties of flavonoids has been performed in humans and rodents, less information is available so far about potential beneficial effects of flavonoids in farm animals. In the present study, we investigated the hypothesis that supplementation of flavonoids could reduce inflammation and ER stress in the liver of dairy cows during early lactation. Cows were fed a diet supplemented with grape seed and grape marc meal extract (**GSGME**), an inexpensive byproduct of wine and grape juice processing, rich in flavonoids. The most abundant flavonoids in grape seeds (**GS**) are gallic acid, catechin, epigallocatechin-3-gallate, epigallocatechin, epicatechin-3-gallate, epicatechin, and proanthocyanidins (Auger et al., 2004). Grape marc (**GM**) contains fewer procyanidins (with the exception of the procyanidin dimer B1) but contains significant amounts of anthocyanins, such as cyanidine 3-glucoside, malividin 3-glucoside, cyanidin, and peonidin, which are absent in GS (Auger et al., 2004). Due to their antioxidant and free radical scavenging activities, GS meal and GM meal are used as feed additives for oxidative stabilization of feedstuff (Bonilla et al., 1999). In recent studies, grape products have been considered as supplements for ruminants, and it has been found that these products are able to reduce methane production, probably due to their high concentrations of tannins, which have inhibitory effects on methanogenic bacteria (Moate et al., 2014; Ishida et al., 2015; Khiaosa-Ard et al., 2015). In contrast, the potential effects of grape products on inflammation and ER stress in the liver of dairy cows have not yet been investigated so far.

MATERIALS AND METHODS

The study was conducted at the Educational and Research Centre for Animal Husbandry Hofgut Neumuehle in Rhineland-Palatinate (Münchweiler an der Alsenz, Germany) and were approved by the local department for animal welfare affairs (23 177–07/G 12–20–074).

Animals

Twenty-eight primiparous and multiparous Holstein cows with an average parity number of 2.8 were used as experimental animals for this study. The cows were allocated into 2 experimental groups, either a control group or a group supplemented with GSGME (GSGME group). Multiparous cows were allocated to the groups according to parity number and previous milk performance; heifers were allocated randomly. Each group was composed of 4 primiparous and 10 multiparous cows with an average parity number of 2.8 for the control group and 2.9 for the GSGME group. Body weights of the cows did not differ before parturition (wk 8 prepartum: control group, 692 ± 21 kg; treatment group, 664 ± 21 kg; mean ± SE) or after parturition (wk 2 postpartum: control group, 657 ± 28 kg; GSGME group, 632 ± 22 kg; mean ± SE) between the 2 groups. The cows were housed in a freestall barn with boxes bedded with short cut straw. Five days before the expected calving date until 5 d after calving, the cows were kept separately from the herd in calving pens bedded with short cut straw. During this time, feed intake of the individual cows could not be recorded. After cows were

transferred to the freestall barn, individual feed intake was recorded by using feed bins, which were equipped with an automatic weighing system (Roughage Intake Control; Insentec B.V., Marknesse, the Netherlands).

Diets

The experimental period consisted of wk 3 before the expected calving date of the cows to wk 9 postpartum. In the period between wk 3 prepartum and calving, a TMR was calculated to meet the demand of net energy and CP requirement of a dry cow with a BW of 650 kg and an assumed DMI of 12 kg of DM/d, according to German Society of Nutrition Physiology (GfE, 2001; Table 1). After calving, all animals were offered a basal TMR calculated to meet the demand of net energy and CP requirement for producing 34 kg of milk, with an assumed daily DMI of 22 kg (Table 1).

In the time period from 3 wk before the expected calving date until wk 9 postpartum, the basal TMR of the GSGME group were supplemented with 1% of GSGME (Antaox, Dr. Eckel, Niederzissen, Germany) based on DM content. The GSGME was mixed homogenously into the TMR using a feed mixer. The GSGME product used had a total flavonoid content of 52 mg gallic acid equivalents per gram [according to determination of total polyphenol content by the Folin-Ciocalteu method (Singleton and Rossi, 1965)]. The TMR of the control group was supplemented with 1% of wheat bran for an energetic adjustment. The TMR was fed once per day at 0730 h. Feed residuals were checked visually daily for sorting behavior of the cows. All cows had free access to drinking water.

Feed Samples and Analyses

Samples of all feedstuffs were collected every second week and stored at −20°C until analysis. Feed samples were analyzed for crude ash, CP, crude fat, and crude fiber according to the official methods of Verband der Deutschen Landwirtschaftlichen Untersuchungs- und Forschungsanstalten (VDLUFA, 2007). The NDF and ADF were analyzed according to Van Soest et al. (1991). The NE_L and CP of the diets were calculated according to the German Society of Nutrition Physiology (GfE, 2001).

Milk and Blood Samples and Liver Biopsies

Cows were milked twice daily at 0500 and 1530 h in a combined milking parlor offering space for 8 cows in the herringbone parlor and a side-by-side parlor for 10 cows, manufactured by GEA Farm Technologies (Boenen, Germany). Daily milk yield was recorded electronically via the herd management system Dairy Plan C21 from GEA and stored. From wk 2 to 9 postpartum, milk samples were collected weekly as aliquots from one evening and the next morning milking and pooled for further analysis. Bronopol (2-bromo-2-nitropropane-1,3-diol) preservative treated milk samples

Table 1. Ingredient composition and chemical composition (% of DM, unless otherwise noted)offered during dry period and lactation to the control group or the grape seed and grape marc meal extract (GSGME) group

	Dry period		Lactation	
	−21 to 0 DIM		1 to 63 DIM	
Item	Control	GSGME	Control	GSGME
Ingredient				
Grass silage	30.7	30.7	22.7	22.7
Corn silage	35.7	35.7	18.0	18.0
Pressed beet pulp silage	0	0	12.7	12.7
Wheat straw and hay	6.5	6.5	4.9	4.9
Barley	6.1	6.1	10.5	10.5
Corn grain, cracked	6.1	6.1	10.5	10.5
Soybean meal	0	0	5.6	5.6
Canola meal	12.6	12.6	13.1	13.1
Wheat bran	1.0	—	1.0	—
GSGME	—	1.0	—	1.0
Vitamin and mineral mix	0.8	0.8	0.8	0.8
Urea	0.5	0.5	0.2	0.2
Chemical composition				
NE_L[1] (MJ/kg of DM)	6.49	6.47	6.79	6.77
CP	14.0	14.0	16.5	16.6
NDF	38.4	38.2	35.6	35.5
ADF	31.6	31.8	19.7	19.8
Crude fiber	19.0	19.1	15.9	16.0

[1]Calculated values from the analyses of all feedstuffs according to GfE (2001).

were transported to the regional laboratory of the milk recording organization (Landeskontrollverband Rheinland-Pfalz-Saar e.V., Bad Kreuznach, Germany) for analyzing concentrations of fat, protein, lactose, and SCC via infrared analyzer (MilkoScan FT-6000, Foss Analytical A/S, Hillerød, Denmark; LKV Rheinland-Pfalz-Saar e.V., Bad Kreuznach, Germany). Energy-corrected milk was calculated according to GfE (2001), and was adjusted to 4% fat and 3.4% protein.

Blood samples were taken at wk 1 (d 7 ± 2), wk 3 (d 21 ± 2), and wk 5 (d 35 ± 2) postpartum before feeding between 0730 and 0830 h and were centrifuged immediately. For this purpose, cows were separated after the morning milking. Blood from the vena caudalis was collected into tubes containing EDTA as an anticoagulant (S-Monovette, Sarstedt, Nümbrecht, Germany) and centrifuged to obtain plasma which was stored at −20°C until analysis.

In addition, liver biopsies were taken after sampling of blood at wk 1 (d 7 ± 2) and wk 3 (d 21 ± 2) postpartum. Therefore, the relevant cow was separated after morning milking and its head was fixed in a locking yoke. After shaving and disinfecting of the liver biopsy site on the right side of the cow between the 11th and 12th rib on a line between the olecranon and the tuber coxae, a local subcutaneous anesthesia with 5 mL of Isocaine 2% (Procainhydrochloride/Epinephrin, Selectavet, Weyarn/Holzolling, Germany) was administered. A small incision was made with a scalpel (carbon steel scalpel blades #11, Aesculap AG, Tuttlingen, Germany), and biopsies were taken with a sterile 14-G biopsy needle (Dispomed Witt oHG, Gelnhausen, Germany). Approximately 50 mg of liver tissue was immediately snap-frozen in liquid nitrogen and stored at −80°C until further analysis. After the biopsy, the puncture site was treated with antiseptic spray (Oxytetracycline spray blue, Bayer Health Care AG, Leverkusen, Germany).

Analysis of Plasma and Liver Samples

Concentrations of retinol, tocopherols, and β-carotene in plasma samples were determined by HPLC (L-7100, LaChrom, Merck-Hitachi, Darmstadt, Germany) according to Balz et al. (1993). Samples of 0.2 mL of plasma were mixed with 2 mL of a 10 g/L of pyrogallol solution (in ethanol, absolute) and 300 μL of a saturated sodium hydroxide solution. After flushing with nitrogen, this mixture was heated for 30 min at 70°C in closed glass tubes. The vitamins were then extracted by addition of 2 mL of n-hexane and 2 mL of distilled water. After centrifugation, an aliquot of the hexane phase was evaporated to dryness under nitrogen and re-dissolved in methanol containing 0.05% of butylated hydroxytoluene. Retinol and tocopherols were separated isocratically by HPLC using a mixture of methanol and water (96:4, vol/vol) as the mobile phase and a LiChrospher 100 RP18 column (5 μm particle size, 125 mm length, 4.6 mm internal diameter, Merck-Hitachi) and detected by fluorescence (Fluorescence Detector L-7480, Merck-Hitachi; retinol: excitation wavelength, 325 nm; emission wavelength, 475 nm; tocopherols: excitation wavelength, 295 nm; emission wavelength, 325 nm). For measurement of β-carotene, an aliquot of the hexane phase was directly injected on a LiChrospher 100 Diol column (5 μm particle size, 125 mm length, 4.6 mm internal diameter, Merck-Hitachi) with hexane as the mobile phase and detected by absorption (UV-VIS-Detector L4250, Merck-Hitachi, absorption wavelength 455 nm). The temperature of the columns was set at 40°C for the determination of retinol and tocopherols and 30°C for β-carotene using a column oven (L-7360, Merck-Hitachi). The vitamins were calculated by an external calibration based on linear regression. Following analytical standards were used: retinol (Sigma-R7632, Sigma-Aldrich, Taufkirchen, Germany); DL-all-*rac*-α-tocopherol (Supelco-47783, Sigma-Aldrich), γ-tocopherol (Supelco-4778, Sigma-Aldrich), and β-carotene (Sigma-22040, Sigma-Aldrich). All the vitamins and β-carotene showed good linearity, with a correlation coefficient over 0.999. Instrumental precision and stability were determined by analyzing the same sample and standards a second time at the end, whereas the repeatability or precision of the method was obtained by processing one sample 5 times and calculating the variation. The stability for the standards and samples were between 95 and 102% per day and the sample pretreatment showed acceptable variations with 4 to 5% for all vitamins and β-carotene.

Thiobarbituric acid-reactive substances (**TBARS**) in plasma were measured using a modified version of the method of Sidwell et al. (1954). Sample aliquots were mixed with thiobarbituric acid reagent [thiobarbituric acid (8 g/L)/perchloric acid (70 g/L), 2:1 (vol/vol)] and heated for 60 min at 95°C. The TBARS were extracted with *n*-butanol and measured by fluorescence (excitation wavelength, 532 nm; emission wavelength, 553 nm; Fluorescence Spectrometer LS55, PerkinElmer, Rodgau, Germany). Concentrations were calculated via a standard curve with 1,1,3,3-tetraethoxypropane.

To determine TAG and cholesterol concentrations in the liver, lipids from liver biopsy samples were extracted with a mixture of n-hexane and isopropanol (3:2, vol/vol; Hara and Radin, 1978). Aliquots of the lipid extracts were concentrated under a nitrogen atmosphere at 37°C for 5 min (Techne sample concentrator, Bibby Scientific, Staffordshire, United Kingdom). The lipids were then dissolved using a 1:1-mixture of chloroform and Triton X-100 (De Hoff et al., 1978).

After vacuum drying at 42°C for 2 h, liver TAG and cholesterol content were measured using enzymatic reagent kits [Fluitest CHOL (Cat. No. 4241), Fluitest TG (Cat. No. 5741), Analyticon Biotechnologies AG, Lichtenfels, Germany], respectively.

The Trolox equivalent antioxidant capacity (**TEAC**) of plasma was determined following the protocol of Re et al. (1999). Oxidation of 2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; **ABTS**) with potassium persulfate generates the blue/green radical mono cation ($ABTS^{+\bullet}$), which is reduced in the presence of hydrogen-donating antioxidants including hydrophilic and lipophilic antioxidants. Antioxidants contained in the sample reduce $ABTS^{+\bullet}$ to ABTS and therefore cause decolorization proportional to their concentration. The absorbance was measured in a microplate-reader (Infinite M200, Tecan, Germany) at a wavelength of 600 nm, and antioxidant capacity of the plasma was calculated against Trolox as a standard. The TEAC values expressed the millimoles of Trolox having the antioxidant capacity corresponding to 1.0 mmol of the test substance (Betancor-Fernandez et al., 2002).

Concentrations of NEFA and BHBA in plasma samples were analyzed using enzymatic reagent kits (Code No. 436–396–91995 and 417–73501, Wako Chemicals GmbH, Neuss, Germany).

Quantitative PCR

Total RNA isolation from liver biopsies, cDNA synthesis, and quantitative(q)PCR were performed as described recently in detail (Gessner et al., 2013a). Expression values of the examined genes were normalized using the GeNorm normalization factor according to Vandesompele et al. (2002) with *ACTB* (forward 5′-ACTTGCGCAGAAAACGAGAT-3′ and reverse 5′-CACCTTCACCGTTCCAGTTT-3′), *ATP5B* (forward 5′-GGACTCAGCCCTTCAGCGCC-3′ and reverse 5′- GCCTGGTCTCCCTGCCTTGC-3′), and *SDHA* (forward 5′- GCAGAACCTGATGCTTTGTG-3′ and reverse 5′-CGTAGGAGAGCGTGTGCTT-3′), being the 3 most stable out of 6 tested potential reference genes in the liver. Details about gene-specific primer pairs for the target genes catalase (*CAT*), C-reactive protein (*CRP*), glutathione peroxidase 3 (*GPX3*), haptoglobin (*HP*), microsomal glutathione S-transferase 3 (*MGST3*), NAD(P)H dehydrogenase, quinone 1 (*NQO1*), superoxide dismutase 1 (*SOD1*), tumor necrosis factor α (*TNF*), UDP glucuronosyltransferase 1 family, polypeptide A1 (*UGT1A1*), and activating transcription factor 4 (*ATF4*), BCL2-antagonist/killer 1 (*BAK1*), BCL2-associated X protein (*BAX*), heat shock 70 kDa protein 5 (*HSPA5*), apoptosis-related cysteine peptidase 3 (*CASP3*), DNA-damage-inducible transcript 3 (*DDIT3*), ER degradation enhancer, mannosidase α-like (*EDEM1*), protein disulfide isomerase family A, member 4 (*PDIA4*), X-box binding protein 1 (*XBP1*) can be obtained from recent publications (Gessner et al., 2013a, 2014). The following primer pair was used for the detection of mRNA abundancy of fibroblast growth factor 21 (*FGF21*): forward 5′-GGCATCATCCGTGTAGAGGT-3′ and reverse 5′-TTCAAGCACTTGGGACTGTG-3′). The mean of wk 1 postpartum was set to 1, and relative expression ratios of wk 3 and the average of wk 1 and 3 are expressed as fold changes compared with wk 1 postpartum. The size of the PCR products were verified using 1.5% agarose gel electrophoresis, stained with GelRed nucleic acid gel stain (Biotium, Hayward, CA), and visualized under UV light with a digital camera (SynGene, Cambridge, United Kingdom).

Statistics

Data were analyzed with a linear mixed-effects model using packages lmerTest (Kuznetsova et al., 2014) and lsmeans (Lenth and Hervé, 2013) in R version 3.1.1 (R Core Team 2014). The linear mixed-effects model included treatment, week, lactation number (1, 2–3, or ≥4), and the treatment × week interaction as fixed factors and cow as a random factor to account for repeated measures over the weeks on the same animal. Pairwise comparisons between the overall treatment means and means at each sampling time point were performed using linear contrasts for least squares means. Differences at $P < 0.05$ were considered as statistically significant, and differences at $P < 0.1$ were considered as trends.

RESULTS

No significant difference in the deviation of actual from expected calving dates occurred between the control group and the GSGME group (1.1 ± 3.2 versus −1.7 ± 4.3, respectively). Actual calving dates ranged from 7 d before to 7 d after the expected calving date.

Feed Intake, Energy Balance, Milk Production, and Composition

No visual indications were present for sorting behavior of the cows. Thus, it can be assumed that the cows of the GSGME group consumed the proposed amount of GSGME. Feed intake, milk production, and composition were determined weekly in the period of wk 2 to 9 postpartum. As expected, feed and energy intake, energy balance, milk yield, and milk composition were

influenced by week of lactation ($P < 0.05$, Table 2). However, no interactions between treatment and time were observed (Table 2). Cows of the GSGME group did not differ in DMI, NE_L intake, and energy balance from cows of the control group in the time period of wk 2 to 9 postpartum (Table 2). However, milk yield and ECM were higher in the GSGME group than in the control group ($P < 0.05$, Table 2). Milk composition (concentrations of fat, protein, and lactose) was not different between both groups of cows (Table 2). The daily amount of milk protein was higher in the GSGME group than in the control ($P = 0.028$); the daily amount of fat did not differ between the 2 groups of cows (Table 2). The time course of DMI, milk yield, and ECM is shown in Figure 1. The DMI was not different between the 2 groups of cows at any time point (Figure 1A). Milk yield was significantly higher ($P < 0.05$) from the cows supplemented with GSGME at wk 4, 5, and 6 and tended to be increased ($P < 0.10$) in wk 3 and 7 in comparison with the control group (Figure 1B). The ECM was significantly increased in the cows supplemented with GSGME in wk 4, 5, 7, and 9 in comparison with the control cows ($P < 0.05$, Figure 1C).

Metabolic and Antioxidant Parameters in Plasma

Concentration of various metabolic and antioxidant parameters in plasma are shown in Table 3. All these parameters were determined in wk 1, 3, and 5 postpartum. Most of the parameters considered were influenced by week of lactation. The TAG, retinol, TBARS, TEAC, α-tocopherol, and β-carotene concentrations were lowest during wk 1 and were thereafter increasing (Table 2). Plasma NEFA concentration was highest during wk 1 and was thereafter decreasing (Table 2). Plasma BHBA concentration was not different in the time interval between wk 1 and 5 (Table 2). No interactions were found between treatment and time for all parameters considered, indicating that effects of the treatment (GSGME vs. control) were independent of week of lactation. Cows of the GSGME group had higher concentrations of BHBA and retinol in plasma than cows of the control group ($P < 0.05$, Table 3). In contrast, concentrations of NEFA, TAG, and all the parameters related to the antioxidant system (α-tocopherol, β-carotene, TEAC, and TBARS) determined in plasma samples did not differ between the 2 groups of cows (Table 3).

Liver TAG and Cholesterol Concentrations

Concentrations of TAG and cholesterol in the liver, determined in samples of wk 1 and 3, did not differ between GSGME group and the control group (Table 4).

mRNA Abundances of Genes of UPR, Nrf2 Pathway, and Inflammation in the Liver

The mRNA abundances of inflammatory genes, genes of the UPR, and genes of the Nrf2 pathway in liver biopsy samples were determined at 1 to 3 wk postpartum. The mRNA abundances of most of the genes considered were influenced by week of lactation (Table 4). The mRNA abundances of the majority of the genes considered (*ATF4*, *BAK1*, *EDEM1*, *PDIA4*, *CAT*, *NQO1*, *SOD1*, *UGT1A1*, *CRP*) were higher at wk 3 than at wk 1 ($P < 0.05$); mRNA abundances of fewer genes (*BAX*, *DDIT3*, *GPX3*) were lower at wk 3 than at wk 1 ($P < 0.05$). The mRNA abundances of *CASP3*, *FGF21*, *HSPA5*, *XBP1*, *MGST3*, *HP*, and *TNF* did not differ between wk 1 and 3 (Table 4). No interactions were found between time and treatment for mRNA abundances of all the genes considered (Table 4). Hepatic mRNA abundancy of *FGF21* was significantly reduced

Table 2. Feed intake, milk production, and milk composition of Holstein cows fed the control diet or the diet supplemented with 1% of grape seed and grape marc meal extract (GSGME) on average over wk 2 to 9 of lactation[1]

	Wk 2 to 9			*P*-value		
Variable	Control	GSGME	SEM	GSGME	Time	GSGME × time
DMI (kg/d)	16.6	17.2	0.63	0.515	<0.001	0.629
Net energy intake (MJ/d)	114.3	118.4	4.33	0.515	<0.001	0.629
Energy balance (MJ NE_L/d)	−29.9	−36.5	5.40	0.390	<0.001	0.631
Milk yield (kg/d)	35.4	39.0	1.06	0.029	<0.001	0.678
ECM[2] (kg/d)	33.9	37.0	1.04	0.045	0.076	0.312
Fat (%)	4.03	3.92	0.08	0.329	<0.001	0.711
Protein (%)	3.20	3.22	0.06	0.871	<0.001	0.954
Lactose (%)	4.80	4.83	0.03	0.470	<0.001	0.931
Fat (kg/d)	1.37	1.47	0.05	0.142	0.884	0.478
Protein (kg/d)	1.09	1.21	0.04	0.028	0.887	0.328

[1]Values are least squares means, n = 14 for each group.

[2]ECM, adjusted to 4% fat and 3.4% protein.

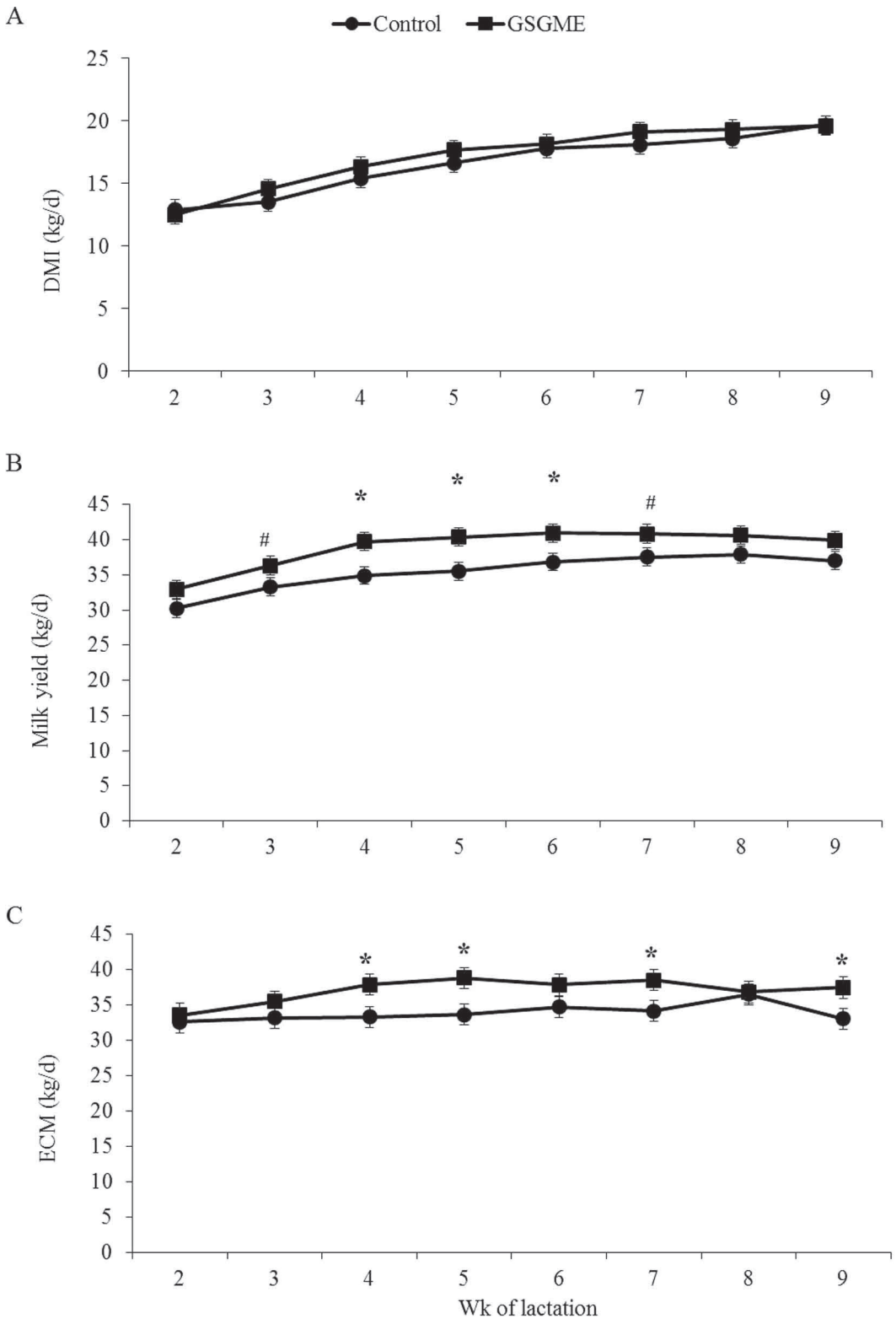

Figure 1. Dry matter intake (A), milk yield (B), and ECM (C) in control cows and cows supplemented with grape seed and grape marc meal extract (GSGME) in the period from wk 2 to 9 of lactation. Values are least squares means ± standard errors; control, n = 14 for each group. Symbols indicate difference from control group ($*P < 0.05$; $\#P < 0.10$).

Table 3. Metabolic parameters in plasma of Holstein cows fed the control diet or the diet supplemented with 1% of grape seed and grape marc meal extract (GSGME) at wk 1, 3, and 5 of lactation[1,2]

	Wk 1		Wk 3		Wk 5			*P*-value		
Variable	Control	GSGME	Control	GSGME	Control	GSGME	SEM	GSGME	Time	GSGME × time
BHBA (mmol/L)	0.41	0.55	0.38	0.55	0.50	0.55	0.03	0.003	0.273	0.299
NEFA (mmol/L)	0.57	0.45	0.26	0.27	0.27	0.27	0.02	0.289	<0.001	0.135
TAG (mmol/L)	0.14	0.12	0.16	0.17	0.17	0.18	0.01	0.886	<0.001	0.078
Retinol (µmol/L)	0.66	0.80	0.87	1.05	1.05	1.12	0.03	0.043	<0.001	0.719
TBARS (µmol/L)	6.49	6.17	6.63	6.13	7.35	6.76	0.28	0.259	0.026	0.919
TEAC (µmol/L)	290	283	321	320	334	333	8.80	0.829	0.003	0.974
α-Tocopherol (µmol/L)	3.71	4.08	6.57	6.29	8.49	8.61	0.37	0.683	<0.001	0.960
β-Carotene (µmol/L)	7.37	6.76	11.3	10.7	14.8	14.3	0.68	0.560	<0.001	0.978

[1]Values are least squares means, n = 14 for each group.

[2]TAG = triacylglycerols; TBARS = thiobarbituric acid-reactive substances; TEAC = Trolox equivalent antioxidant capacity.

in the GSGME group in wk 1 and 3 in comparison with the control group ($P = 0.034$, Table 4). The mRNA abundances of all the other genes considered in wk 1 and 3 did not differ between the 2 groups of cows (Table 4). However, trends were observed toward a reduction of the mRNA abundances of XBP1 and UGT1A1 in the cows supplemented with GSGME at wk 1 and 3 ($P < 0.10$, Table 4).

DISCUSSION

The aim of this study was to investigate the hypothesis that feeding GSGME, a by-product of wine and grape juice processing rich in flavonoids, is able to attenuate inflammation and ER stress in the liver of dairy cows. Considering that most studies dealing with the effects of flavonoids have been performed with monogastric animals, less is known about potential degradation or chemical modification of flavonoids by the microbial flora in the rumen. However, one study performed with sheep shows that the bioavailability of proanthocyanidins from plant extracts is even increased in ruminants in comparison with monogastric animals due to hydrolyzation of polymeric compounds into bioactive monomers by the microbial flora (Gladine et al., 2007). That study also documented the recovery of flavonoids from diet sources in plasma, indicating that at least a part of the flavonoids has been absorbed in the intestine. According to that study, it is expected that at least a part of the flavonoids of GSGME used in the present study is bioavailable and could exert biological effects in the body of cows.

To investigate the effect of GSGME on the inflammatory condition and the occurrence of ER stress in the liver, we considered mRNA abundances of relevant genes in liver samples collected in wk 1 and 3 of lactation. These time points were selected because our previous studies have shown that both inflammation and ER stress reach their maximum in the time interval between wk 1 and 5 of lactation (Gessner et al., 2013a, 2014). We found that mRNA abundances of *TNF*, a pro-inflammatory cytokine, as well as *HP* and *CRP*, 2 acute phase proteins, in the liver were not different between the 2 groups of cows at wk 1 and 3. This finding indicates that supplementation of GSGME did not influence the inflammation process in the liver of cows. We moreover observed that supplementation of GSGME caused a trend toward a downregulation of 2 genes involved in the UPR (*XBP1*, *UGT1A1*). However, mRNA abundances of the majority of hepatic genes involved in UPR such as *HSPA5*, a chaperone which functions as the master regulator of the UPR (Cnop et al., 2012), several downstream genes of the 3 ER stress transducers, including ER chaperones,

Table 4. Concentrations of lipids and hepatic mRNA abundances of genes of the unfolded protein response, Nrf2 target genes, and genes involved in inflammation in the liver of Holstein cows fed the control diet or the diet supplemented with 1% of grape seed and grape marc meal extract (GSGME) at wk 1 and 3[1]

	Wk 1		Wk 3			*P*-value		
Item	Control	GSGME	Control	GSGME	SEM	GSGME	Time	GSGME × time
Lipid concentration (μmol/g)								
TAG	68.9	49.3	81.0	40.7	12.6	0.109	0.884	0.363
Cholesterol	11.8	10.1	10.2	7.91	0.83	0.106	0.027	0.714
Gene expression[2,3]								
Unfolded protein response								
ATF4	1.00	0.70	1.40	1.14	0.19	0.292	0.048	0.900
BAK1	1.00	0.72	1.53	1.25	0.29	0.497	0.001	0.998
BAX	1.00	0.35	0.32	0.24	0.10	0.265	0.002	0.653
CASP3	1.00	0.75	0.90	0.98	0.15	0.670	0.652	0.279
DDIT3	1.00	0.69	0.63	0.53	0.09	0.110	0.005	0.222
EDEM1	1.00	0.62	2.76	2.31	0.40	0.518	0.004	0.980
FGF21	1.00	0.55	1.19	0.29	0.22	0.034	0.888	0.333
HSPA5	1.00	0.38	1.13	0.75	0.22	0.126	0.102	0.396
PDIA4	1.00	0.43	1.19	1.16	0.20	0.281	0.009	0.076
XBP1	1.00	0.41	0.90	0.67	0.15	0.073	0.391	0.071
Nrf2 pathway								
CAT	1.00	0.71	1.82	1.17	0.27	0.233	0.014	0.428
GPX3	1.00	2.30	0.40	0.38	0.34	0.192	0.009	0.132
MGST3	1.00	0.70	1.47	0.96	0.24	0.243	0.052	0.535
NQO1	1.00	0.97	5.57	4.03	0.79	0.478	0.001	0.485
SOD1	1.00	0.82	2.69	2.24	0.47	0.627	0.015	0.810
UGT1A1	1.00	0.45	1.48	0.84	0.24	0.098	0.038	0.800
Inflammation								
CRP	1.00	0.52	1.67	1.56	0.28	0.433	0.008	0.427
HP	1.00	0.35	0.32	0.24	0.21	0.237	0.070	0.176
TNF	1.00	0.49	1.18	1.01	0.20	0.455	0.217	0.536

[1]Values are least squares means, n = 14 for each group. The mRNA abundances of genes are expressed relative to the mRNA concentration at 1 wk postpartum (= 1.00).

[2]Values are least squares means, n = 14 for each group.

[3]*ATF4* = activating transcription factor 4; *BAK1* = BCL2-antagonist/killer 1; *BAX* = BCL2-associated X protein; *CASP* = apoptosis-related cysteine peptidase; *CAT* = catalase; *CRP* = C-reactive protein; *DDIT3* = DNA-damage-inducible transcript 3; EDEM1 = ER degradation enhancer, mannosidase α-like; *FGF21* = fibroblast growth factor 21; *GPX3* = glutathione peroxidase 3; *HSPA5* = heat shock 70 kDa protein 5; *MGST3* = microsomal glutathione S-transferase 3; *HP* = haptoglobin; *HMOX2* = heme oxygenase 2; *NQO1* = NAD(P)H dehydrogenase, quinone 1; *PDIA4* = protein disulfide isomerase family A, member 4; *SOD1* = superoxide dismutase 1; *TNF* = tumor necrosis factor; *UGT1A1* = UDP glucuronosyltransferase 1 family, polypeptide A1; *XBP1* = X-box binding protein 1.

ER-associated protein degradation components (Ron and Walter, 2007; Samali et al., 2010), genes involved in the induction of apoptosis (Wu et al., 2013), and genes of the Nrf2 pathway that are driven by ER stress (Cullinan et al., 2003) remained completely unchanged between both groups of cows. These findings indicate that supplementation of GSGME had less effect on the development of ER stress in the liver of dairy cows in the early postpartal phase.

So far, the effects of plant flavonoids on inflammation and ER stress have been less investigated in ruminants. However, one study is available showing that GS extract is able to reduce the production of pro-inflammatory cytokines and heat shock protein 70, a target gene of the UPR, in heat-stressed jejunum epithelial cells of steers (Li et al., 2014). The results of that study show that plant flavonoids are generally able to attenuate inflammation and ER stress in bovine cells. Three reasons are possible for the finding that supplementation of GSGME failed to attenuate inflammation and ER stress in the liver of dairy cows in this study. First, the basal level of inflammation and ER stress in the liver could have been generally too low to observe potential beneficial effects of GSGME. Second, a large part of the flavonoids could have been degraded in the rumen. Third, concentrations of flavonoids supplied by GSGME could have been insufficient to induce anti-inflammatory effects in the liver.

One interesting finding of this study is that supplementation of GSGME caused a significant downregulation of *FGF21* in the liver at wk 1 and 3 of lactation. The FGF21 was originally identified as an important metabolic hormone that controls fatty acid oxidation and ketogenesis during fasting (Inagaki et al., 2007). Recent studies, however, have shown that FGF21 also acts as a stress hormone that is directly induced by ER

stress, but also by several other stress conditions such as starvation, nutrient excess, autophagy deficiency, mitochondrial stress, exercise, or cold exposure (Kim and Lee, 2014). It has been observed that *FGF21* in the liver of dairy cows is dramatically upregulated during the transition from late pregnancy to early lactation (Carriquiry et al., 2009; Schoenberg et al., 2011; Schlegel et al., 2012), probably as a consequence of various types of stress present in the liver during the periparturient phase. Thus, the finding of a reduced mRNA abundancy of *FGF21* suggests that supplementation of GSGME lowered the generation of stress in the liver during early lactation. This suggestion is in accordance with the observation of an increased plasma retinol concentration observed in the cows supplemented with GSGME. Retinol is released from the liver after binding to retinol-binding protein, a member of the negative acute phase proteins (Bossaert et al., 2012). It has been shown that plasma retinol concentrations are reduced under stress conditions or inflammation, due to a reduced synthesis of retinol-binding protein (Bionaz et al., 2007; Bossaert et al., 2012). Thus, an increased plasma concentration of retinol could be further indication that the level of stress could have been reduced in the liver by supplementation of GSGME.

In the present study, we observed an unexpected increase of milk production, associated with an increased daily milk protein yield, in the cows fed GSGME in comparison with the control group. As DMI was similar in both groups, it is likely that the utilization of energy and crude protein from diet for milk production might have been improved in the cows supplemented with GSGME in comparison with the control cows. As GSGME had less effect on inflammation and ER stress, it is likely that the effects on milk production might have been due to an improved rumen fermentation than rather an altered metabolism. In vitro and in vivo studies have shown that grape products influence ruminal fermentation (Moate et al., 2014; Ishida et al., 2015; Khiaosa-Ard et al., 2015). Grape products are characterized by high concentrations of tannins, which might be most relevant for their influence on ruminal metabolism (Khiaosa-Ard et al., 2015). Tannins are able to form complexes with proteins, leading to a decrease in protein solubility and degradation in the rumen and an increased dietary protein flow to the duodenum (Patra and Saxena, 2011). High dietary levels of tannins can compromise feed intake and digestion in feeds (Patra and Saxena, 2011). However, feeding moderate levels of tannins, which do not affect feed intake, has been shown to improve milk yield, likely due to an increased flux of EAA to the small intestine (Woodward et al., 1999; Bhatta et al., 2000). Recently, feeding a relatively large amount of dried GM (5.0 kg/d) to dairy cows in late lactation slightly increased the milk yield and daily amount of milk protein in comparison with a control group (Moate et al., 2014). In another recent study, feeding polyphenol-rich winery wastes lowered microbial protein degradation in wethers (Ishida et al., 2015). These findings are in accordance with the view that tannins from grape products could increase milk yield by increasing the flux of protein to the small intestine (Moate et al., 2014). Besides their effects on protein degradation in the rumen, tannins from grape products are also able to reduce methanogenesis (Patra and Saxena, 2011). According to an in vitro study, this effect might be mainly due to a change of the microbial population, associated with a moderate depression of nonfiber carbohydrate degradation (Khiaosa-Ard et al., 2015). In contrast, grape products had less effect on the production of individual VFA, both in vitro and in vivo (Moate et al., 2014; Khiaosa-Ard et al., 2015). Based on these studies, the alterations observed in the cows supplemented with GSGME in this study (increased milk yield, increased daily milk protein yield) could be due to a reduced ruminal degradation of crude protein from the diet, leading to an increased amount of protein available in the small intestine. However, as we did not investigate ruminal metabolism in this study, this suggestion remains a matter of speculation. The results of our study with respect to the effect of GSGME feeding on milk yield are, however, contrary to some other studies that investigated the effect of feeding grape products in lactating ruminants. In the study of Santos et al. (2014), inclusion of grape residue silage in amounts up to 10% of food DM decreased total-tract apparent digestibility of DM, CP, ether extract, and fiber fractions in Holstein cows but did not influence milk yield and milk composition. In the study of Nudda et al. (2015), feeding 300 g/d per head of GS did not influence milk yield and milk composition and had less effect on hematological and metabolic parameters in dairy ewes. Both studies indicate that grape products can be included into lactating ruminant diets without adverse effects on milk production. A study in nonlactating multiparous cows showed that feeding of GM in amounts of up to 3 kg of DM per day lowers N digestibility and N excretion in feces (Greenwood et al., 2012). The authors of that study suggested that this effect may be particularly beneficially when feeding diets high in rumen-degradable protein such as in a ryegrass-clover pasture by creating a partitioning of N toward feces instead of absorption.

Plasma NEFA and BHBA are important metabolic parameters in dairy cows during early lactation. Nonesterified fatty acids originate from adipose tissue, and their concentration in plasma mainly reflects mobilization of body fat. However, plasma NEFA concentration

can also be modified by their uptake into tissues by FA transporters (Jorritsma et al., 2003). The finding that plasma NEFA concentration was not influenced by feeding GSGME is in accordance with the observation of a similar energy balance in both groups of cows, and moreover suggests that there might not have been a profound difference in the uptake of NEFA into tissue by FA transporters between the 2 groups of cows. Plasma BHBA concentration in cows during early lactation is an indicator of hepatic ketogenesis. We found that cows supplemented with GSGME had increased plasma concentrations of BHBA in wk 1, 3, and 5 compared with the control group. It is well known that the rate of ketogenesis is dependent on the availability of glucose (van Knegsel et al., 2005). Accordingly, it is likely that the increase in BHBA was due to a lower availability of glucose due to increased lactose synthesis in the GSGME group, which produced 3 to 4 kg more milk than the control group at a similar DMI.

Until recently, few studies dealing with the effects of flavonoids in ruminants have been published. Some of those studies revealed that flavonoids, due to their high antioxidative potential, are able to increase the antioxidative status and reduce lipid peroxidation in plasma of sheep (Gladine et al., 2007), dairy cows (Gobert et al., 2009), or beef cattle (Shabtay et al., 2008). These studies suggested that feeding flavonoid-rich plant extracts could be a strategy to improve health and performance of ruminants by reducing oxidative stress. Our study, demonstrating that feeding of GSGME does not increase the antioxidative capacity and does not reduce the lipid peroxidation in plasma of cows, is in contrast to those studies. Differences in the effects of flavonoids on the antioxidant system between our study and other studies could be at least in part due to different plant sources used in these studies.

CONCLUSIONS

The data of this study indicate that supplementation of dairy cows with GSGME from wk 3 prepartum to wk 9 increased milk production but had no significant effects on inflammation and the occurrence of ER stress in the liver of dairy cows during early lactation. The finding that hepatic mRNA abundancy of *FGF21*, a stress hormone induced by various types of stress, was reduced in the cows supplemented with GSGME suggests an attenuation of stress in the liver of these cows. Consideration of various antioxidative parameters and concentrations of lipid peroxidation products (TBARS) in plasma indicates that supplementation of GSGME did not influence the systemic antioxidative system. Overall, the data of this study indicate that a moderate amount of GSGME could be useful to improve milk performance in dairy cows without negatively affecting energy balance. As less effect of the supplement on metabolism was observed, it is likely that the increased milk performance could be mainly due to effects of GSGME on ruminal metabolism.

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PAPER II

Effects of a plant product consisting of green tea and curcuma extract on milk production and the expression of hepatic genes involved in endoplasmic stress response and inflammation in dairy cows

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Effects of a plant product consisting of green tea and curcuma extract on milk production and the expression of hepatic genes involved in endoplasmic stress response and inflammation in dairy cows

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During the periparturient phase, cows are typically in an inflammation-like condition, and it has been proposed that inflammation associated with the induction of stress of the endoplasmic reticulum (ER) in the liver contributes to the development of fatty liver syndrome and ketosis. In the present study, the hypothesis that supplementation of dairy cows with a plant product consisting of green tea (95%) and curcuma extract (5%) rich in polyphenols attenuates inflammation and ER stress in the liver during early lactation was investigated. Twenty-seven cows were assigned to two groups, either a control group (n = 14) or a treatment group (n = 13). Both groups of cows received a total mixed ration, and the ration of the treatment group was supplemented with 0.175 g of the plant product per kg dry matter from week 3 prepartum to week 9 postpartum. Dry matter intake and energy balance during week 2 to week 9 postpartum were not different between the two groups. However, cows supplemented with the plant product had a greater amount of energy-corrected milk during week 2 to week 9 postpartum and lower concentrations of triacylglycerols and cholesterol in the liver in week 1 and week 3 postpartum than cows of the control group ($p < 0.05$). Cows supplemented with the plant product showed a trend towards a reduced mRNA concentration of haptoglobin ($p < 0.10$), while relative mRNA concentrations of eight genes of the unfolded protein response considered in the liver were not different between the two groups of cows. Relative hepatic mRNA concentration of fibroblast growth factor, a stress hormone induced by various stress conditions, was reduced at week 1 and week 3 postpartum in cows supplemented with the plant product ($p < 0.05$). Overall, the data of this study suggest that – although there were only minor effects on the occurrence of ER stress and inflammation – a supplementation of polyphenols might be useful to improve milk yield and prevent fatty liver syndrome in dairy cows.

Keywords: dairy cows; endoplasmic reticulum stress; inflammation; liver; polyphenols; transition period

1. Introduction

The transition period represents the most critical period in the productive life of high-yielding dairy cows. During this period, the liver experiences pronounced metabolic stress

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due to a marked influx of non-esterified fatty acids (NEFA), which are mobilised from adipose tissue triacylglycerol (TAG) stores due to a severe negative energy balance (NEB). A large part of the whole-body NEFA flux is taken up into the liver and exceeds its oxidation capacity and, thus, promotes the development of fatty liver and ketosis (Drackley et al. 2001). Besides this metabolic stress, the liver of early lactating cows is exposed to diverse inflammatory challenges, like microbial components, proinflammatory cytokines and reactive oxygen species, as a result of infectious diseases, such as mastitis, endometritis, but also subacute rumen acidosis and abomasal displacement (Vels et al. 2009; Zebeli and Metzler-Zebeli 2012), which frequently occur during parturition and/or the onset of lactation. The role of inflammation during transition to lactation for health and performance of dairy cows has been recently highlighted (Bradford et al. 2015).

It has been shown that metabolic stress and inflammation induce the development of stress of the endoplasmic reticulum (ER) in the liver of early lactating cows (Gessner et al. 2014; Ringseis et al. 2014). ER stress is defined as an accumulation of unfolded or misfolded proteins in the ER lumen as a consequence of an imbalance between the protein load and the protein folding capacity (Cnop et al. 2012). Accumulation of unfolded or misfolded proteins leads to a disturbance of the ER function, which in turn causes an activation of the unfolded protein response (UPR), an adaptive response which aims to decrease the unfolded protein load and restore ER function. The activation of the UPR, such as observed in animal models of obesity or diabetes or induced by application of chemical ER stress inducers, leads to a variety of symptoms in the liver, which are similar to those observed in periparturient dairy cows. Therefore, it has been proposed that the ER stress-induced UPR might play an important role in the development of pathophysiologic conditions commonly observed in the liver of periparturient cows, such as the development of fatty liver, ketosis or inflammation (Ringseis et al. 2014).

Numerous studies in humans and animal models have reported that both inflammatory processes and the occurrence of ER stress can be modulated by nutrients. In particular, polyphenols – including those from green tea – as well as curcumin, a polyphenol of *curcuma longa*, have been shown to exert strong antiinflammatory effects under various proinflammatory conditions such as in obese rats, rats fed a high-fat diet or rats challenged with endotoxins (Jobin et al. 1999; Terra et al. 2009). More recently, it has been shown that polyphenols, including curcumin, are also able to suppress the development of ER stress in liver and muscle cells from humans and rodents (Giordano et al. 2014; Rodriguez et al. 2014; Li et al. 2015).

Although many studies in humans and rodents have given evidence that polyphenols are exerting strong antiinflammatory properties, potential anti-inflammatory effects of polyphenols have been given less attention in livestock so far. In the present study, we investigated the hypothesis that supplementation of dairy cows with polyphenols has the potential to attenuate inflammation and ER stress in the liver of dairy cows during early lactation. Therefore, we focused on the expression of genes involved in inflammation and UPR in the liver of dairy cows supplemented with a plant product rich in polyphenols, consisting of green tea and curcuma extract. Our measurements included gene expression of fibroblast growth factor (FGF)-21, a stress hormone which is not only induced by ER stress but also by several other stress factors (Kim and Lee 2014).

2. Materials and methods

The study was carried out at the Educational and Research Centre for Animal Husbandry Hofgut Neumühle (Münchweiler an der Alsenz, Germany). The experimental protocol

was approved by the Provincial Government of Coblenz, Germany (23 177-07/G12-20-074).

2.1. Animals and feeding

The trial included 27 Holstein cows (8 primi- and 19 multiparous cows), which were housed in a freestall barn with straw bedding boxes. The animals were allocated into two groups, either a control group (n = 14, average parity number: 2.8) or a group supplemented with a plant product rich in polyphenols (n = 13, average parity number: 3.1). Multiparous cows were allocated into the groups according to previous performance parameters (lactation number, milk yield, fat- and protein content), while heifers were allocated randomly. Body weights (BW) of the two groups of cows did not differ before parturition (week 8 prepartum: control group, 692 ± 21 kg; treatment group, 695 ± 24 kg; means ± SE) or after parturition (week 2 postpartum: Control group, 632 ± 28 kg; treatment group, 662 ± 20 kg; mean ± SE).

The experimental period comprised the period from week 3 before the expected calving date of the cows until week 9 postpartum. In the period between week 3 prepartum and calving, the total mixed ration (TMR) was calculated to meet the demand of net energy and crude protein (CP) of a dry cow with a BW of 650 kg and an assumed dry matter intake (DMI) of 12 kg/d, for *ad libitum* intake (Table 1). After calving, all animals were offered a basal TMR calculated to meet the demand of net energy and CP

Table 1. Composition of the basal total mixed rations used as experimental diets during dry period and lactation.

	Dry period (day 21–0 before calving)	Lactation (days 1–63 in milk)
Component [% of DM]		
Grass silage	30.9	22.9
Maize silage	35.8	18.1
Pressed beet pulp silage	–	12.8
Straw and hay	6.6	4.9
Barley	6.2	10.6
Grain maize, cracked	6.2	10.6
Soybean meal	–	5.7
Canola meal	12.6	13.1
Calcium carbonate	0.5	0.5
Vitamin and mineral premix[#]	0.4	0.4
Sodium chloride	0.3	0.3
Urea	0.5	0.2
Energy and nutrients contents		
Net energy for lactation [MJ/kg DM][†]	6.5	6.8
Crude fibre [% of DM]	19.1	15.9
Neutral detergent fibre [% of DM]	38.3	35.5
Acid detergent fibre [% of DM]	31.8	19.7
Crude protein [% of DM]	14.0	16.6
Ruminal N balance [g/kg]	1.2	0.5

Notes: [#]Provided per kg total mixed ration (on dry matter basis): calcium, 0.36 g; phosphorus, 0.36 g; sodium, 0.36 g; magnesium, 0.40 g; zinc, 28 mg; manganese, 17 mg; copper, 6.0 mg; cobalt, 0.24 mg; iodine, 0.80 mg; selenium, 0.21 mg; vitamin A, 4.000 IU, vitamin D, 600 IU, vitamin E, 20 mg (RINDAMIN K11 ATG, Schaumann, Pinneberg, Germany). [†]Calculated according to GfE (2001).

for a milk yield of 34 kg, with an assumed daily DMI of 22 kg (Table 1). In the period between week 3 prepartum and week 9 postpartum, the TMR of the treatment group was supplemented with 0.175 g of a plant product rich in polyphenols (Loxidan Dairy Cow B, Lohmann Animal Nutrition GmbH, Cuxhaven, Germany) consisting of green tea (95%) and curcuma extract (5%) per kg DM of product. The plant product had a total polyphenol content of 147 mg gallic acid equivalents per g (according to determination of total polyphenol content by the Folin-Ciocalteau method reported by Singleton and Rossi 1965). The plant product was included into the TMR as a pre-mix in wheat bran (1% of the TMR). The TMR of the control group was supplemented with the identical amount of wheat bran without plant product.

Five days before the expected calving date until 5 days after calving, the cows were kept separately from the herd in straw-bedded calving pens. During this time, feed intake of the individual cows could not be recorded. After transferring the cows to the freestall barn, individual feed intake was recorded by using feed bins, which were equipped with an automatic weighing system (Roughage Intake Control; Insentec B.V., Marknesse, The Netherlands). The TMR were supplied once daily at 07:30 h. All cows had free access to drinking water.

2.2. *Feed samples and analysis*

The provided feed components were sampled fortnightly, samples of TMR were weekly collected and all samples were stored at −20°C for further analysis. The sampled feed components were pooled and the concentrations of crude nutrients were determined according to the official methods in Germany (VDLUFA 2007). Gross energy was determined using a bomb calorimeter (IKA-Calorimeter C5000, Janke & Kunkel IKA Analysentechnik, Staufen, Germany). The net energy content of the used feed components (MJ of NEL) and the utilisable CP at the duodenum were calculated based on digestible nutrients as suggested by the German Society of Nutrition Physiology (GfE 2001).

2.3. *Collection of milk and blood samples and liver biopsies*

Cows were milked twice daily at 05:00 h and 15:30 h in a combined milking parlour offering space for eight cows in the herringbone parlour and a side-by-side parlour for ten cows, manufactured by GEA Farm Technologies (Bönen, Germany). Daily milk yield was recorded electronically via the herd management system Dairy Plan C21 from GEA and stored. From week 1 to week 9 postpartum, once per week milk aliquots of one evening and the next morning milking were taken and pooled for further analysis. Bronopol (2-bromo-2-nitropropane-1,3-diol) preservative-treated milk samples were analysed for milk fat, protein and lactose by infrared spectrophotometry using a MilkoScan FT6000 (Foss Analytical A/S, Hillerød, Denmark) at the laboratory of Landeskontrollverband Rheinland-Pfalz (Bad-Kreuznach, Germany). Energy-corrected milk was calculated according to GfE (2001), which is adjusted to 4% fat and 3.4% protein content.

Blood samples were collected from all cows in the morning before feeding in week 1 (days 5–9), week 3 (days 19–23) and week 5 (days 34–37) postpartum. The blood samples were taken via puncture of the *vena caudalis* into tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (S-Monovette®, Sarstedt, Nümbrecht, Germany) and were centrifuged immediately. After centrifugation of blood at 3500 × *g* for 15 min, plasma was frozen (−20°C) for further analysis. In addition, liver biopsies were taken in week 1 (days 5–9) and week 3 (days 19–23) postpartum directly after blood sampling. For that, the

separated cows were fixed. After shaving and disinfecting of the liver biopsy site on the right side of the cow between the 11th and 12th rib on a line between the olecranon and the tuber coxae, a local subcutaneous anaesthesia with 5 ml Isocaine 2% (procainhydrochloride/epinephrin, Selectavet, Weyarn/Holzolling, Germany) was performed. A small incision was made with a scalpel (carbon steel scalpel blades #11, Aesculap AG, Tuttlingen, Germany), and the biopsies were taken with sterile 14 G biopsy needles (Dispomed Witt oHG, Gelnhausen, Germany). About 50 mg of liver tissue were immediately snap-frozen in liquid nitrogen and stored at −80°C until further analysis. After the biopsy, the puncture site was treated with antiseptic spray (Oxytetracycline spray blue, Bayer Health Care AG, Leverkusen, Germany). All efforts were made to minimise suffering.

2.4. *Analysis of plasma and liver samples*

The thiobarbituric acid reactive substances (TBARS) were measured in plasma using a modified version of the TBARS assay (Sidwell et al. 1954). Sample aliquots were mixed by using thiobarbituric acid reagent (8 g/l thiobarbituric acid with 7% perchloric acid, 2:1, v/v) and heated for 60 min at 95°C. The extraction of TBARS occurred with n-butanol, and the absorption was measured at 532 nm. Concentrations were calculated via a standard curve with 1,1,3,3,-tetraethoxypropane. TAG concentration in plasma was determined by using the enzymatic kits Fluitest® TG (Cat.-No. 5741, Analyticon, Lichtenfels, Germany). For the determination of lipid concentrations in the liver, small samples of the liver biopsies were extracted with a mixture of hexane and iso-propanol (3:2, v/v, according to Hara and Radin 1978). Lipids of the extracts were dissolved in Triton X-100 as described by De Hoff et al. (1978) before enzymatic measurement using commercial kits (Fluitest® TG and Fluitest® CHOL, Analyticon).

The concentrations of retinol, α-tocopherol and β-carotene in plasma were determined by high-performance liquid chromatography (HPLC; L-7100, LaChrom, Merck-Hitachi, Darmstadt, Germany) according to Balz et al. (1993). Samples of 0.2 ml of plasma were mixed with 2 ml of a 10 g/l pyrogallol solution (in ethanol, absolute) associated with a saponification with 300 µl saturated sodium hydroxide solution and heated for 30 min at 70°C. Then, the vitamins were extracted adding 2 ml of n-hexane and 2 ml of distilled water. After centrifugation, an aliquot of the hexane phase was evaporated to dryness under nitrogen and re-dissolved in methanol containing 0.05% of butylated hydroxytoluene. Retinol and tocopherols were separated isocratically by HPLC using a mixture of methanol and water (96:4, v/v) as the mobile phase and a LiChrospher 100 RP18 column (5 µm particle size, 125 mm length, 4.6 mm internal diameter, Merck) and detected by fluorescence (Fluorescence Detector L-7480, Merck-Hitachi; retinol: excitation wavelength, 325 nm, emission wavelength, 475 nm; tocopherols: excitation wavelength, 295 nm, emission wavelength, 325 nm). For measurement of β-carotene, an aliquot of the hexane phase was directly injected on a LiChrospher 100 Diol column (5 µm particle size, 125 mm length, 4.6 mm internal diameter, Merck) with hexane as the mobile phase and detected by absorption (UV-VIS-Detector L4250, Merck-Hitachi, absorption wavelength 455 nm). For the determination of retinol and tocopherols, temperature of the columns was set at 40°C, for β-carotene 30°C using a column oven (L 7360, Merck-Hitachi). The vitamins were calculated by an external calibration based on linear regression.

The Trolox equivalent antioxidative capacity (TEAC) was measured following the protocol of Re et al. (1999). Oxidation of 2,29-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) with potassium persulphate generates the blue/green radical mono cation $ABTS^{\bullet+}$ which is reduced in the presence of hydrogen-donating antioxidants including

hydrophilic and lipophilic antioxidants. Antioxidants contained in the sample reduce $ABTS^{\bullet+}$ to ABTS and therefore cause decolourisation proportional to their concentration. The absorbance was measured in a microplate-reader (Infinite® M200, Tecan, Germany) at a wavelength of 600 nm, and antioxidant capacity of the plasma was calculated against trolox as a standard. TEAC values expressed the amount of trolox [mmol] having the antioxidant capacity corresponding to 1.0 mmol of the test substance (Betancor-Fernandez et al. 2002).

Concentrations of NEFA and β-hydroxy butyric acid (BHBA) in plasma were determined by enzymatic reagent kits (Code No: 436-91995 and 417-73501, Wako Chemicals GmbH, Neuss, Germany).

2.5. *Real-time quantitative polymerase chain reaction*

The total isolation of ribonucleic acid (RNA) from liver biopsies, cDNA synthesis and quantitative polymerase chain reaction (qPCR) were carried out as recently reported by Gessner et al. (2013a, 2013b). According to Vandesompele et al. (2002), the geNorm normalisation factor was used to investigate the expression values of the genes with beta-actin (*ACTB*) (forward: 5′-ACTTGCGCAGAAAACGAGAT-3′ and reverse: 5′-CACCTTCAC CGTTCCAGTTT-3′), mitochondrial ATP synthase, subunit beta (*ATP5B*) (forward: 5′-GGACTCAGCCCTTCAGCGCC-3′ and reverse: 5′- GCC TGGTCTCCCTGCCTTGC-3′) and succinate dehydrogenase complex, subunit A (*SDHA*) (forward: 5′-GCAGAACCTGATGCTTTGTG-3′ and reverse: 5′-CGTAGGAGAGCGTGTGCT T-3′), being the three most stable out of six tested potential reference genes in the liver. Details about gene-specific primer pairs for the target genes C-reactive protein (*CRP*), haptoglobin, tumor necrosis factor α (*TNF*) and activating transcription factor 4 (*ATF4*), BCL2-antagonist/killer 1 (*BAK1*), BCL2-associated X protein (*BAX*), heat shock 70 kDa protein 5 (*HSPA5*), apoptosis-related cysteine peptidase 3 (*CASP3*), DNA-damage-inducible transcript 3 (*DDIT3*), ER degradation enhancer, mannosidase alpha-like (*EDEM1*), protein disulphide isomerase family A, member 4 (*PDIA4*) can be obtained from recent publications (Gessner et al. 2013a, 2014). The mean of week 1 postpartum was set to 1, and relative expression ratios of week 3 and the average of week 1 and week 3 are expressed as fold changes compared to week 1 postpartum. The size of PCR products were verified by the use of 1.5% agarose gel electrophoresis stained with GelRedTM nucleic acid gel stain (Biotium, California, USA) and visualised under UV light with a digital camera (SynGene, Cambridge, England).

2.6. *Calculations and statistical analysis*

The average weekly energy balance was calculated for each cow by using the cows' BW determined automatically twice a day by electronic scales after milking. The energy requirements for maintenance of each cow depending on the weekly mean BW were calculated according to GfE (2001). Additionally, the energy requirements for milk production were determined by using the means of weekly average daily milk yield, milk protein content and milk fat content (GfE 2001). The energy balance was calculated by subtracting calculated requirements for maintenance and milk production from the energy intake, which was determined by feed intake and energy contents of the TMR.

For statistical analysis, initially all data were checked for normality. Data were statistically evaluated by the linear mixed effects model using packages lmertest and lsmeans in R version 3.1.1 (R Core Team 2014) including treatment, week, parity number [1 ($n = 8$), 2–3 ($n = 8$) or ≥4 ($n = 11$)] and the treatment × week interaction as fixed factors and cow as random factor in order to account for repeated measures over the weeks on the

same animal. Pairwise comparisons between the means at each sampling time point were performed by using linear contrasts for least square means. The level of statistical significance was set at $p < 0.05$.

3. Results

No significant difference in deviation of the actual from the expected calving date occurred between the control group and the treatment group (1.1 ± 0.9 d versus 1.3 ± 1.5 d, respectively, mean ± SE). Actual calving dates ranged from 8 d before to 10 d after the expected calving date.

3.1. *Feed intake, milk production and composition*

The animal performance data are shown in Table 2. As expected, DMI and net energy intake, milk yield, milk composition and energy balance were influenced by the week of lactation ($p < 0.05$). However, there were no interactions between treatment and time. Parity number had no effect on DMI and net energy intake (Table 2). However, there was a trend towards a lower milk yield in first parity cows in comparison to cows with two or more parities (data not shown, $p < 0.10$). Moreover, first parity cows had a lower ECM, a lower milk fat and milk protein yield and a less NEB than cows with two or more parities (data not shown, $p < 0.05$). Based on an average feed intake of 17.7 kg DM per day in the time period between week 2 and week 9, the treatment group received an average amount of 3.1 g of the plant product per day. Within the time period between week 2 and week 9,

Table 2. Feed intake, milk production and milk composition of Control cows and cows supplemented with a plant product consisting of green tea and curcuma extract during week 2–9 of lactation.[†]

	Week 2–9			*p*-Value			
	Control ($n = 14$)	Plant product ($n = 13$)	PSEM[#]	Plant product	Time	Plant product × time	Parity
Dry matter intake [kg/d]	17.1	17.7	0.59	0.491	<0.001	0.152	0.316
Net energy intake [MJ/d]	117.8	121.9	4.08	0.491	<0.001	0.152	0.316
Energy balance [MJ NEL/d]	−29.5	−38.1	4.95	0.231	<0.001	0.162	0.043
Milk yield [kg/d]	36.5	39.5	1.21	0.087	<0.001	0.634	0.099
Milk fat yield [kg/d]	1.39	1.56	0.05	0.032	0.684	0.675	<0.001
Milk protein yield [kg/d]	1.13	1.25	0.04	0.031	0.263	0.753	0.008
Energy-corrected milk* [kg/d]	34.5	38.7	1.05	0.010	0.198	0.515	<0.001
Milk composition							
Fat [%]	4.00	3.93	0.10	0.608	<0.001	0.672	0.672
Protein [%]	3.23	3.15	0.05	0.277	<0.001	0.280	0.280
Lactose [%]	4.80	4.81	0.03	0.827	<0.001	0.021	0.179

Notes: [†]Values are least squares means; [#]PSEM, Pooled standard error of the means. *Adjusted to 4% fat and 3.4% protein.

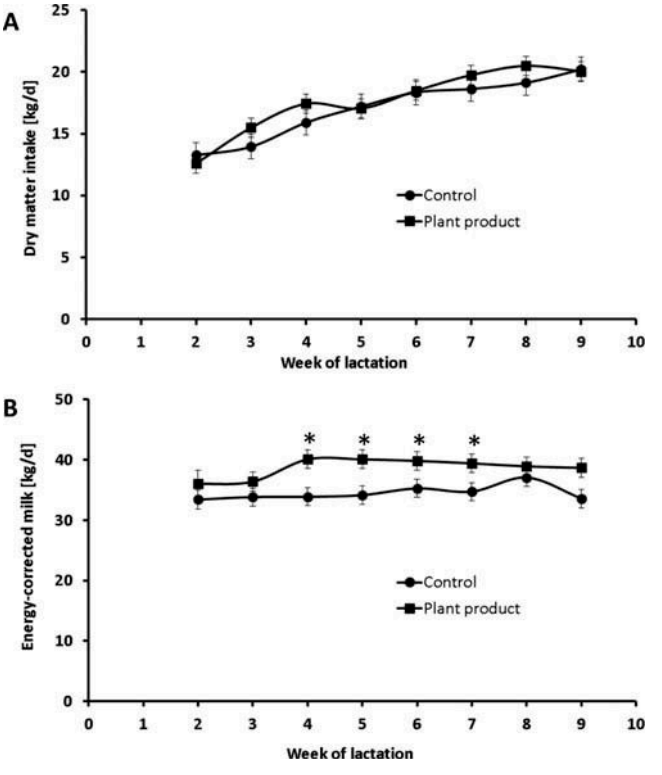

Figure 1. Dry matter intake (panel A) and yield of energy-corrected milk (adjusted to 4% fat and 3.4% protein) (panel B) of control cows and cows supplemented with a plant product (PP) consisting of green tea and curcuma extract (week 2 to week 9 of lactation).
Notes: Values are least square means ± standard errors; control, $n = 14$; Plant product, $n = 13$. *Significant different from control ($p < 0.05$).

DMI, the energy intake and balance of the cows did not differ between groups. Cows receiving the plant product showed a tendency towards an increased milk yield ($p < 0.10$; Table 2) and had a significantly increased daily amount of energy-corrected milk yield ($p < 0.05$; Table 2) from week 2 to week 9 postpartum. The milk composition (concentrations of fat, protein and lactose) was not different between both groups of cows (Table 2). However, daily amounts of milk fat and milk protein were higher in the group of cows receiving the plant product in comparison to the control group ($p < 0.05$, Table 2). The time course of DMI and yield of energy-corrected milk is shown in Figure 1. DMI did not differ between the two groups of cows at any time point (Figure 1A). ECM was significantly increased in the cows supplemented with the plant product in weeks 4–7 in comparison to the control cows ($p < 0.05$, Figure 1B).

3.2. Metabolic and antioxidant parameters in plasma

Metabolic and antioxidant parameters were determined in plasma samples collected in weeks 1, 3 and 5 postpartum. As expected, most of the parameters determined (NEFA, TAG, retinol, α-tocopherol, β-carotene, and TEAC) were influenced by week of lactation (Table 3). Parity number had no effect on any of the parameters considered. The only exception was the

retinol concentration which was lower in cows with four or more parities than in cows with one to three parities ($p < 0.05$, data not shown, Table 3). Concentrations of BHBA, TAG and all the parameters related to the antioxidant system (α-tocopherol, β-carotene, TBARS, TEAC) were not different between the cows supplemented with the plant product and cows of the control group (Table 3). The concentration of retinol in plasma was higher in the cows receiving the plant product than in cows of the control group ($p < 0.05$, Table 3). NEFA concentration in week 1 was lower in cows supplemented with the plant product compared to the control group ($p < 0.05$, Table 3). NEFA concentrations in week 3 and week 5 were not different between the two groups of cows.

3.3. *Liver triacylglycerol and cholesterol concentrations*

Concentrations of TAG and cholesterol in the liver were not influenced by the parity number. TAG concentration in the liver was independent of time of sampling (week 1 vs. week 3), while liver cholesterol concentration was lower in week 3 than in week 1 ($p < 0.05$; Table 4). The concentrations of TAG and cholesterol in the liver, measured in samples of week 1 and week 3 postpartum, were significantly reduced in the group fed the plant product compared to the control group ($p < 0.05$; Table 4).

3.4. *Relative mRNA abundances in the liver*

The relative mRNA concentrations of *FGF21*, inflammatory genes and genes of the UPR in liver biopsy samples were determined in week 1 to week 3 postpartum. Relative mRNA concentration of *FGF21* in liver samples was significantly reduced in the cows supplemented with the plant product in week 1 and week 3 in comparison to control cows (−74% on average of both sampling times, $p < 0.05$, Table 4).

To evaluate the inflammatory status of the liver, we determined relative mRNA concentrations of *TNF*, a proinflammatory cytokine, and the acute phase proteins haptoglobin and *CRP*. While the relative mRNA concentrations of *TNF* and *CRP* were not different between both groups of cows, there was a trend towards a reduction of relative mRNA concentration of haptoglobin (−81%; $p < 0.10$) in the group of cows supplemented with the plant product in comparison to the control group (Table 4).

To assess the occurrence of ER stress in the liver, we determined relative mRNA concentrations of a total of eight genes of the UPR (Table 4). Relative mRNA concentrations of *ATF4* showed a trend towards a reduction in cows supplemented with the plant product in comparison to control cows ($p < 0.10$, Table 4). Relative mRNA concentrations of *BAX, CASP3, DDIT3, EDEM1, HSPA5* and *PDIA* were also numerically reduced (−6 to −43% on average of week 1 and week 3) compared to the control group (Table 4). However, these differences were not statistically significant.

The parity number of the cows had no influence on the expression of any of the genes considered (Table 4).

4. Discussion

4.1. *Plant product used in this study*

The present study was performed to investigate the hypothesis that supplementation of dairy cows with a plant product rich in polyphenols has the potential to suppress the development of inflammation and ER stress in the liver and to improve liver function in

Table 3. Metabolic parameters in plasma of control cows and cows supplemented with a plant product (PP) consisting of green tea and curcuma extract at weeks 1, 3 and 5 of lactation.[†]

	Week 1			Week 3			Week 5			Weeks 1, 3 and 5			*p*-Value			
	Control	PP	PSEM[#]	Control	PP	PSEM	Control	PP	PSEM	Control	PP	PSEM	PP	Time	PP × time	Parity
NEFA[‡] [µmol/l]	0.58[a]	0.33[b]	0.04	0.26	0.23	0.03	0.27	0.21	0.03	0.37	0.27	0.02	0.006	<0.001	0.003	0.848
BHBA* [µmol/l]	407	399	48.3	384	430	37.3	506	424	39.5	433	418	24.6	0.671	0.228	0.255	0.836
TAG[§] [mmol/l]	0.14	0.13	0.01	0.16	0.17	0.01	0.17	0.18	0.01	0.16	0.16	0.01	0.903	<0.001	0.100	0.207
Retinol [µmol/l]	0.68	0.89	0.08	0.87[a]	1.13[b]	0.06	1.03	1.14	0.06	0.86	1.05	0.04	0.001	<0.001	0.435	0.043
α-Tocopherol [µmol/l]	3.82	4.26	0.63	6.56	7.54	0.49	8.52	9.28	0.49	6.30	7.03	0.39	0.199	<0.001	0.857	0.169
β-Carotene [µmol/l]	7.46	6.99	0.96	11.3	11.0	0.78	14.8	14.9	0.80	11.2	11.0	0.68	0.821	<0.001	0.870	0.910
TBARS[Δ] [µmol/l]	6.53	6.42	0.39	6.60	7.20	0.30	7.35	7.01	0.30	6.83	6.88	0.23	0.884	0.082	0.177	0.613
TEAC[♦] [µmol/l]	293	320	15.8	327	349	13.2	337	342	12.9	319	337	9.81	0.204	0.026	0.616	0.590

Notes: [†]Values are least squares means (control group, $n = 14$; Group PP, $n = 13$); [#]PSEM, pooled standard error of the means; [‡]NEFA, non-esterified fatty acids; *BHBA, β-hydroxy butyric acid; [§]TAG, triacylglycerol; [Δ]TBARS, thiobarbituric acid-reactive substances; [♦]TEAC, Trolox equivalent antioxidant capacity; [a,b]Means with different superscripts are significantly different within the respective weeks ($p < 0.05$).

Table 4. Concentrations of lipids and relative hepatic mRNA concentrations of *FGF21* and genes involved in inflammation and unfolded protein response in the liver of control cows and cows supplemented with a plant product (PP) consisting of green tea and curcuma extract at week 1 and week 3.[‡]

	Week 1			Week 3			Weeks 1 and 3			*p*-Value			
	Control	PP	PSEM[#]	Control	PP	PSEM	Control	PP	PSEM	PP	Time	PP × time	Parity
Lipid concentrations [µmol/g]													
Triacylglycerol	64.2	36.7	15.5	76.1[a]	33.6[b]	11.9	70.1	34.9	11.6	0.047	0.680	0.485	0.979
Cholesterol	11.8	8.88	1.01	10.1[a]	7.58[b]	0.84	11.0	8.23	0.80	0.026	0.048	0.796	0.529
Gene expression[†]													
Fibroblast growth factor 21	1.00	0.31	0.32	1.28[a]	0.30[b]	0.29	1.14	0.30	0.25	0.027	0.579	0.564	0.908
Inflammation													
C-reactive protein	1.00	0.47	0.34	1.64	1.11	0.28	1.32	0.79	0.25	0.150	0.023	0.249	0.082
Haptoglobin	1.00	0.19	0.26	0.28	0.06	0.23	0.64	0.12	0.19	0.069	0.066	0.190	0.759
Tumor necrosis factor	1.00	0.97	0.31	1.28	1.47	0.25	1.14	1.22	0.20	0.767	0.155	0.683	0.771
Unfolded protein response													
Activating transcription factor 4	1.00	0.43	0.26	1.47	1.03	0.22	1.24	0.73	0.18	0.060	0.022	0.760	0.226
BCL2-antagonist/killer 1	1.00	1.28	0.46	2.13	1.94	0.43	1.56	1.61	0.36	0.932	0.030	0.527	0.666
BCL2-associated X protein	1.00	0.82	0.15	0.59	0.43	0.13	0.80	0.62	0.11	0.279	0.003	0.957	0.910
Apoptosis-related cysteine peptidase	1.00	0.74	0.17	0.91	0.86	0.15	0.95	0.80	0.14	0.453	0.914	0.382	0.891
DNA-damage-inducible transcript 3	1.00	0.60	0.12	0.64	0.63	0.11	0.82	0.61	0.10	0.154	0.056	0.036	0.626
ER degradation enhancer	1.00	0.59	0.68	3.20	2.50	0.53	2.10	1.54	0.45	0.395	0.002	0.797	0.602
Heat shock 70 kDa protein 5	1.00	0.37	0.23	1.13	0.83	0.21	1.06	0.60	0.20	0.124	0.040	0.216	0.423
Protein disulphide isomerase family A	1.00	0.59	0.42	1.74	1.89	0.34	1.37	1.29	0.29	0.835	0.006	0.289	0.798

Notes: [‡]Values are least squares means (control group, $n = 14$; Group PP, $n = 13$); [#]PSEM, pooled standard error of the means. [†]mRNA concentrations of genes are expressed relative to the mRNA concentration at week 1 postpartum (=1.00); [a,b]Means with different superscripts are significantly different within the respective weeks ($p < 0.05$).

the transition period of high-yielding dairy cows. For this purpose, a commercial product consisting of green tea and curcuma extract (95:5, w/w) was used as a feed supplement. Green tea contains a broad spectrum of polyphenols which in total constitute 30–40% of dry weight (Gaur and Agnihotri 2014). Major polyphenols in green tea are catechins, a class of flavonoids, with epigallocatechin-3-gallate (59% of the total catechins), epigallocatechin (19%), epicatechin-3-gallate (14%) and epicatechin (6%) as major compounds. Minor components in green tea are proteins, mainly enzymes (15–20% of dry weight), carbohydrates (5–7% of dry weight, including cellulose, pectins, glucose, fructose and sucrose), small amounts of lipids, sterols, vitamins and xanthic bases such as caffeine and theophylline (Gaur and Agnihotri 2014). *Curcuma longa* is an Indian spice which has a long history of use in Ayurvedic medicine as a treatment for inflammatory conditions. Curcuma constituents include the three curcuminoids curcumin, demethoxycurcumin and bisdemethoxycurcumin as well as volatile oils, sugars, proteins and resins (Jurenka 2009).

Most studies dealing with effects of polyphenols have been performed with monogastric animals. The potential degradation and the chemical modification of polyphenols by the microbial flora in the rumen have been less investigated so far. One study performed with sheep has shown that the bioavailability of different types of polyphenols from rosemary, grapes, citrus or marigold is even increased in ruminants compared with monogastric animals, probably due to hydrolysation of polymeric compounds into bioactive monomers by the microbial flora (Gladine et al. 2007). In that study, the recovery of polyphenols from diet sources in plasma was reported, indicating that at least a part of the polyphenols has been absorbed in the intestine. Whether the same is true for polyphenols from green tea or curcuma remains unknown. As supplementation of the plant product used in this study induced several biological effects, it is likely that at least a part of the polyphenols of that product was bioavailable. Based on the facts that the plant product had a broad spectrum of polyphenols and that the fate of these compounds in the rumen is unknown, the compounds responsible for the effects observed in this study remain unidentified.

Due to the fact that polyphenols are exerting their biological effects even at very small doses of 0.01% and lower in the diet and lower *in vivo* in the rodent model (Aguirre et al. 2014), a relatively small supplementary dose of the plant product (0.175 g/kg dietary DM) was used in this study, which represents an economically relevant dose.

4.2. Inflammation and stress of the endoplasmic reticulum

In order to assess the effect of the plant product on the inflammatory condition and the occurrence of ER stress in the liver, we considered mRNA concentrations of relevant genes in liver samples collected in week 1 and week 3 of lactation. Those time points were selected as our previous studies have shown that both inflammation and ER stress reach their maximum in the time interval between week 1 and week 5 of lactation (Gessner et al. 2013a, 2014). With respect to inflammation, we observed a trend towards a reduction of the relative mRNA concentrations of haptoglobin ($p < 0.10$), an acute phase protein, in the liver of the cows supplemented with the plant product. This finding indicates that supplementation of the plant product could have – at least moderately – attenuated the proinflammatory condition in the liver of dairy cows, which is commonly observed in early lactation. In order to assess whether a potential attenuation of inflammation by supplementation of the plant product was associated with an improvement of liver function, we determined plasma concentration of retinol, which is released from the liver by retinol-binding protein, a member of the negative acute phase proteins (Bossaert

et al. 2012). Accordingly, the finding of an increased plasma concentration of retinol in cows supplemented the plant product indeed indicates that hepatic production of retinol-binding protein as a carrier of retinol could have been increased in these cows, indicative of an improved liver function.

To assess the occurrence of ER stress in the liver of the cows, the hepatic mRNA concentrations *HSPA5* were determined, a chaperone which function as the master regulator of the UPR (Cnop et al. 2012) and several downstream genes of the three ER stress transducers, including ER chaperones, ER associated degradation components and genes involved in the induction of apoptosis which have been assessed as reliable markers of ER stress (Samali et al. 2010). There were numerical reductions in the mRNA abundances of almost all the UPR genes considered in the cows supplemented with the plant product. However, as all these effects with the exception of few tendencies were not statistical significant, we conclude that the plant product had no or at least only a moderate effect on the occurrence of ER stress in the liver of dairy cows.

4.3. *Concentrations of FGF21 and hepatic lipids*

FGF21 is a hormone, mainly produced in the liver, which was originally identified as an important regulator of energy homeostasis in the state of energy deficiency or fasting (Badman et al. 2007). Recently, it has been found that FGF 21, besides its metabolic function, is an important stress hormone, which is induced by various physiological or pathological stressors (Kim and Lee 2014). One of the key findings of this study is that supplementation of the plant product caused a strong downregulation of *FGF21* in the liver in week 1 and week 3 of lactation. Recent studies have shown that *FGF21* in the liver of dairy cows is dramatically upregulated during the transition from late pregnancy to early lactation (Schoenberg et al. 2011; Schlegel et al. 2012). FGF21 acts as a stress hormone which is directly induced by ER stress, but also by several other stress conditions such as starvation, nutrient excess, autophagy deficiency, mitochondrial stress, exercise or cold exposure (Kim and Lee 2014). Accordingly, the strong increase of *FGF21* observed in dairy cows in the transition period might be caused by various types of stress present in the periparturient phase. Thus, the finding of a reduced *FGF21* mRNA concentration suggests that supplementation of the plant product attenuated the generation of stress condition during early lactation.

Several studies have shown that production of FGF21 is closely related to liver fat content. In patients with steatosis, a direct correlation between plasma FGF21 and liver fat content has been shown (Li et al. 2010; Yan et al. 2011). From these studies, it has been concluded that plasma FGF21 might be a useful biomarker for liver fat accumulation. In agreement with those findings in humans, a positive correlation between plasma concentration of *FGF21* and hepatic TAG concentration has also been observed in dairy cows (Schoenberg et al. 2011). In the present study, we observed a significant reduction of TAG and cholesterol concentration in the liver of the cows supplemented with the plant product, which is in close agreement with reduced hepatic *FGF21* mRNA concentrations in these cows. Accordingly, the present study indicates that feeding a plant product rich in polyphenols might be useful to prevent the development of a fatty liver in dairy cows during early lactation. While studies on the effects of polyphenols on hepatic lipid metabolism are lacking so far, the antilipidogenic effect of polyphenols observed in dairy cows is in close agreement with various studies performed with rats or mice which have shown that various types of polyphenols or polyphenol-rich plant extracts including curcumin or polyphenols from green tea extract are able to prevent the

development of experimental induced steatosis (Um et al. 2013; Aguirre et al. 2014). The antilipidogenic effect observed in these studies has been attributed to a reduced fatty acid and TAG synthesis, an increased rate of β-oxidation and a reduction of oxidative stress and inflammation (Aguirre et al. 2014). As genes of lipid metabolism have not been considered in this study, the molecular mechanism underlying the reduction of TAG and cholesterol concentrations in the liver observed in cows supplemented with the plant product remains unknown.

4.4. Metabolic and antioxidative parameters in plasma

Plasma NEFA and BHBA are important metabolic parameters in dairy cows during early lactation. NEFA are originating from adipose tissue, and their concentration in plasma mainly reflects mobilisation of body fat. However, plasma NEFA concentration can also be modified by their uptake into tissues by fatty acid transporters (Jorritsma et al. 2003). In the present study, we observed that cows supplemented with the plant product had lower concentrations of NEFA in plasma at week 1 than control cows, although their energy balance was not different from that of the control cows. This finding cannot be directly explained by the data of this study. However, it is possible that the reduction of stress – according to reduced *FGF21* mRNA levels in the liver – was associated with an increased fatty acid utilisation in the liver. The finding that plasma BHBA concentrations in plasma were not different between both groups of cows indicates that supplementation of the plant product did not change the rate of ketogenesis.

In order to find out whether supplementation of the plant product rich in polyphenols improved the antioxidative status of the cows, we determined various antioxidative parameters in plasma. Our analyses showed that concentrations of antioxidants, the total antioxidative capacity and concentrations of oxidation products (TBARS) were not influenced, indicating that supplementation of the plant product rich in polyphenols did not improve the systemic antioxidative status. This result is in disagreement with some other studies which observed an increase of the antioxidative status and a reduction of lipid peroxidation in plasma of sheep (Gladine et al. 2007), dairy cows (Gobert et al. 2009) or beef cattle (Shabtay et al. 2008) supplemented with various types of plant sources of polyphenols. Differences in the effects of plant polyphenols on the antioxidant system between our study and other studies could be at least in part due to different plant sources or different doses of polyphenols used in these studies.

4.5. Milk performance

In the present study, we observed that cows supplemented with the plant product rich in polyphenols had an increased amount of energy-corrected milk. As DMI did not differ between the two groups of cows, it is likely that supplementation of the plant product led to an improvement of the utilisation of energy for milk production. The data of our study do not yield a clear explanation for this observation. However, it is possible that a potential reduction of inflammation and metabolic stress in the liver could have contributed to the increased milk production. Inflammatory processes, which are common in the liver of cows during early lactation, not only increase plasma concentrations of catabolic hormones such as cortisol but are also associated with an increased heat production (fever) and the production of proteins involved in the inflammation process (such as acute phase proteins) in liver and immune system tissues (Bertoni et al. 2008). Energy and amino acids required for those processes are not available for milk production which is the

reason why inflammatory conditions are leading to an impairment of milk production (Bionaz et al. 2007). Previously, it has been found that treatment of dairy cows with a low dose of acetylsalicylic acid during the first week of lactation leads to an improvement of liver function and an increase in milk yield (Trevisi and Bertoni 2008). That study confirms the view that attenuation of the inflammatory process might provide a useful strategy to increase milk performance in dairy cows.

5. Conclusions

The data of this study indicate that supplementation of dairy cows with a plant product consisting of green tea and curcuma extract from week 3 prepartum to week 9 had a moderate effect on inflammation but less effect on the occurrence of ER stress in the liver of dairy cows during early lactation. Nevertheless, it was observed that hepatic mRNA concentration of *FGF21*, a stress hormone induced by various types of stress, and hepatic lipid concentrations were reduced in the cows supplemented with the plant product. This suggests that feeding the plant product attenuated metabolic stress in the liver. Consideration of various antioxidative parameters and concentrations of lipid peroxidation products (TBARS) in plasma indicates that supplementation of the plant product did not influence the systemic antioxidative system. Finally, it was observed that supplementation of the plant product increased the amount of energy-corrected milk yield during early lactation. Overall, the data of this study indicate that a plant product consisting of green tea and curcumin could be useful to improve milk performance and prevent fatty liver syndrome in dairy cows during early lactation.

Disclosure statement

No potential conflict of interest was reported by the authors.

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PAPER III

Analysis of hepatic transcript profile and plasma lipid profile in early lactating dairy cows fed grape seed and grape marc meal extract

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RESEARCH ARTICLE Open Access

Analysis of hepatic transcript profile and plasma lipid profile in early lactating dairy cows fed grape seed and grape marc meal extract

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Abstract

Background: It was recently reported that dairy cows fed a polyphenol-rich grape seed and grape marc meal extract (GSGME) during the transition period had an increased milk yield, but the underlying reasons remained unclear. As polyphenols exert a broad spectrum of metabolic effects, we hypothesized that feeding of GSGME influences metabolic pathways in the liver which could account for the positive effects of GSGME in dairy cows. In order to identify these pathways, we performed genome-wide transcript profiling in the liver and lipid profiling in plasma of dairy cows fed GSGME during the transition period at 1 week postpartum.

Results: Transcriptomic analysis of the liver revealed 207 differentially expressed transcripts, from which 156 were up- and 51 were down-regulated, between cows fed GSGME and control cows. Gene set enrichment analysis of the 155 up-regulated mRNAs showed that the most enriched gene ontology (GO) biological process terms were dealing with cell cycle regulation and the most enriched Kyoto Encyclopedia of Genes and Genomes pathways were p53 signaling and cell cycle. Functional analysis of the 43 down-regulated mRNAs revealed that a great part of these genes are involved in endoplasmic reticulum (ER) stress-induced unfolded protein response (UPR) and inflammatory processes. Accordingly, protein folding, response to unfolded protein, unfolded protein binding, chemokine activity and heat shock protein binding were identified as one of the most enriched GO biological process and molecular function terms assigned to the down-regulated genes. In line with the transcriptomics data the plasma concentrations of the acute phase proteins serum amyloid A (SAA) and haptoglobin were reduced in cows fed GSGME compared to control cows. Lipidomic analysis of plasma revealed no differences in the concentrations of individual species of major and minor lipid classes between cows fed GSGME and control cows.

Conclusions: Analysis of hepatic transcript profile in cows fed GSGME during the transition period at 1 week postpartum indicates that polyphenol-rich feed components are able to inhibit ER stress-induced UPR and inflammatory processes, both of which are considered to contribute to liver-associated diseases and to impair milk performance in dairy cows, in the liver of dairy cows during early lactation.

Keywords: Transcriptomics, Lipidomics, Dairy cow, Early lactation, Liver, Polyphenols, Grape seed and grape marc meal extract, Endoplasmic reticulum stress, Inflammation

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Background

The transition period spanning the time period between week 3 prepartum and week 3 postpartum represents the most critical period in the productive life of high-yielding dairy cows. With the onset of lactation, commonly a pronounced negative energy balance (NEB) is emerging due to the fact that feed intake is limited in this phase while energy demand is strongly increasing by milk production. NEB leads to a strong lipolysis of triacylglycerols (TAG) in adipose tissue, leading to the release of a large amount of non-esterified fatty acids (NEFA) into the circulation [1]. Approximately one-third of the whole body NEFA-flux is taken up into the liver. As the capacity of the liver for β-oxidation of fatty acids is limited during this phase, a part of the NEFA is esterified to TAG. Thus, a pronounced NEB during early lactation undoubtedly is involved in the development of liver-associated diseases such as fatty liver and ketosis [2]. Newer studies have shown that, besides metabolic stress induced by NEB, the liver of early lactating cows is also exposed to diverse inflammatory challenges. The inflammatory challenges, which include microbial components, pro-inflammatory cytokines and reactive oxygen species, typically result from infectious diseases, like endometritis and mastitis, but also from gastrointestinal disorders, like subacute rumen acidosis and abomasal displacement [3–5]. Both infectious diseases and gastrointestinal disorders frequently occur during parturition or the beginning of lactation. Due to of this, transition dairy cows develop an inflammation-like condition in the liver [4, 6]. Although this inflammation is mostly of subclinical nature, it is of great impact for health and performance of cows during early lactation [7].

Recently, it has been found that metabolic and inflammatory stress induces stress of the endoplasmic reticulum (ER) in the liver of early lactating cows [8]. ER stress is defined as an imbalance between the folding capacity of the ER and the protein load. As a consequence, unfolded and misfolded proteins accumulate in the ER lumen, thereby, disturbing ER homeostasis [9]. It is known from studies in humans and rodents that this causes activation of an adaptive response, termed unfolded protein response (UPR). While the aim of the UPR is to rapidly restore ER function [9], chronic activation of the UPR, as observed in obese or diabetic rodent models or induced by application of chemical ER stress inducers, causes various hepatic symptoms similar to those observed in periparturient dairy cows. Therefore, it has been proposed that ER stress-induced UPR contributes to the pathophysiologic conditions commonly observed in the liver of periparturient cows, like fatty liver, ketosis or inflammation [10].

Polyphenols are members of a large family of plant-derived compounds classified as flavonoids and non-flavonoids. Numerous studies in humans and rodents have shown that polyphenols are exerting antioxidative, antiinflammatory, cardioprotective, cancer chemopreventive and neuroprotective properties [11, 12]. In a recent study, we investigated the hypothesis that feeding grape seed and grape marc meal extract (GSGME), an inexpensive byproduct of wine and grape juice processing rich in flavonoids, to dairy cows might attenuate inflammation and ER stress in the liver during the transition period [13]. In that study, cows fed GSGME during the transition period had an increased milk yield and had a reduced mRNA concentration of fibroblast-growth factor (FGF)-21, a stress hormone, in the liver at week 1 and week 3 postpartum. Relative mRNA concentrations of various hepatic genes of inflammation and ER stress in the liver were decreased by 20-50% in the cows fed GSGME in comparison to the control group. However, as mRNA concentrations of these genes were not statistically significant different between the two groups of cows, the effect of polyphenols on hepatic inflammation and ER stress remains unclear. As polyphenols are exerting a broad spectrum of metabolic effects [14–16], we hypothesized that feeding of GSGME might influence other metabolic pathways in the liver which could account for the positive effects of GSGME observed in cows during early lactation. In order to investigate this hypothesis, we used a genome-wide transcript profiling technique to explore changes in the hepatic transcriptome of cows supplemented with GSGME during the transition period. A main advantage of large-scale screening technologies like transcriptomics is that changes in the complete transcriptome can be assessed simultaneously, despite only small amounts of tissue, e.g. biopsy samples, being available. Using this technique in dairy cows has strongly increased understanding of the hepatic molecular adaptations occurring in the periparturient period [17–19]. Transcriptomics in combination with the analysis of selected blood metabolites and animal performance parameters facilitates to relate changes in the hepatic transcriptome to alterations of liver function during the periparturient period [17–19].

Recently, a gene-based mapping and pathway analysis of metabolic traits in dairy cows figured out that hepatic genes of glycerophospholipid metabolism (e.g., lysophosphatidylcholine acyltransferase 1) are closely linked to plasma concentrations of NEFA, β-hydroxybutyrate (BHBA) and glucose, three key factors of the metabolic status of dairy cows during early lactation [20]. Moreover, signaling lipids, such as ceramides, are implicated in pathways regulating inflammation [21, 22]. As polyphenols have been shown to exert pronounced effects on hepatic lipid metabolism, particularly under pathological conditions [23], we further aimed to find out whether feeding of GSGME could influence metabolism

of glycerophospholipids and ceramides. Therefore, we performed a lipidomic analysis of plasma samples.

Methods

Animal experiment

For this investigation, we used liver and plasma samples collected at 1 week postpartum of an experiment with dairy cows [13]. At this time point, both metabolic and inflammatory stress markers, such as plasma NEFA, plasma BHBA and hepatic mRNA concentrations of acute phase proteins (APPs), were increased most compared to later sampling time points (week 3 and week 5) in this study and another study [13, 24]. In this experiment, 28 Holstein cows with an average parity number of 2.8 were used as experimental animals. The experiment was conducted at the Educational and Research Centre for Animal Husbandry Hofgut Neumühle in Rhineland-Palatinate (Münchweiler an der Alsenz, Germany); the experimental protocol was approved by the Provincial Government of Coblenz, Germany (23 177–07/G12–20–074). The cows were assigned into 2 experimental groups, either a control group ($n = 14$) or a group supplemented with GSGME (GSGME group; $n = 14$), each consisting of 10 multiparous and 4 primiparous cows and having a similar average parity number (control group: 2.8, GSGME group: 2.9). In the period between week 3 prepartum and calving, a total mixed ration (TMR) was fed which was calculated to meet the demand of net energy (NE) and crude protein (CP) requirement of a dry cow with a BW of 650 kg and an assumed dry matter intake (DMI) of 12 kg/d, according to the German Society of Nutrition Physiology [25]. After calving, all animals were offered a basal TMR calculated to meet the demand of net energy and CP requirement for producing 34 kg of milk, with an assumed daily DMI of 22 kg [13]. Feed components were collected fortnightly and analyzed according to the official methods of Verband der Deutschen Landwirtschaftlichen Untersuchungs- und Forschungsanstalten [26]. The analyzed chemical composition of the TMR offered during dry period and lactation was in average of control and GSGME group as follows (per kg DM): 6.5 and 6.8 MJ NE_L, 140 and 166 g CP, 383 and 356 g neutral detergent fiber. More details on the analytical composition of the TMR have been published recently [13]. In the time period from 3 week before the expected calving date until week 9 postpartum, the basal TMR of the GSGME group was supplemented with 1% of GSGME (Antaox, Dr. Eckel, Niederzissen, Germany) based on DM content. The GSGME product used had a total flavonoid content of 52 mg gallic acid equivalents per gram. The TMR of the control group was supplemented with 1% of wheat bran for an energetic adjustment. Although the NE_L content of the GSGME used in this study was slightly lower (3.64 MJ NE_L/kg DM [27]) than that of wheat bran (4.18 MJ NE_L/kg DM) [28], the NE_L content of the TMR between the two groups was nearly identical due to the small proportion of GSGME and wheat bran, respectively, in the TMR [13].

Blood samples and liver biopsies

Each cow was separated from the herd for blood sampling and liver biopsy procedure. Blood was taken from the vena caudalis at week 1 (day 7 postpartum ± 2 d) using ethylenediaminetetraacetic acid-coated collection tubes (S-Monovette, Sarstedt, Nümbrecht, Germany). Plasma was separated from blood cells by centrifugation, and the plasma samples were stored at −20 °C until analysis. Liver biopsies were taken after sampling of blood according to the protocol recently described [13] and immediately snap-frozen in liquid nitrogen and stored at −80 °C until further analysis.

RNA isolation

Total RNA was isolated from liver samples using Trizol according to the manufacturer's protocol and stored at −80 °C. Prior to sample processing at the Centre of Excellence for Fluorescent Bioanalytics (KFB) at the University of Regensburg, the concentration and integrity of RNA was analyzed using an Agilent 2100 Bioanalyzer (Agilent technologies, Böblingen, Germany). The total RNA concentrations, optical density A260/A280 ratios, RNA integrity number (RIN) values and starting total RNA amounts of all samples were 0.59 ± 0.08 μg/μL, 1.93 ± 0.03, 6.6 ± 0.5 and 3.8 ± 0.5 μg (mean ± SD, $n = 12$), respectively.

Microarray hybridization

For microarray analysis, six RNA samples each of the control group ($n = 6$) and the GSGME group ($n = 6$) were selected. The six RNA samples of each group consisted of five samples randomly selected from the multiparous cows and one sample randomly selected from the primiparous cows. Both groups had a similar average parity number (control group: 2.5, GSGME group: 2.3). Total RNA samples were processed according to the GeneChip WT Plus Reagent Kit (Affymetrix, High Wycombe, UK). In brief, total RNA was transcribed to first strand and second strand complementary DNA (cDNA). Then, complementary RNA (cRNA) was synthesized and amplified by *in vitro*-transcription of the second-stranded cDNA template using T7 RNA polymerase. After purification of cRNA and assessing cRNA yield and quality, single-stranded (ss) cDNA was synthesized by reverse transcription of cRNA using 2^{nd}-cycle primers. The ss cDNA was purified and checked again

for yield and quality. The purified ss cDNA was fragmented and the fragmented cDNA labeled by terminal deoxynucleotidyl transferase using the Affymetrix proprietary DNA labeling reagent that is covalently linked to biotin. Finally, the labeled ss cDNA was hybridized to the Affymetrix GeneChip Bovine Gene 1.0 Sense Target array representing approximately 23,000 bovine transcripts. After hybridization arrays were washed and stained with the Affymetrix GeneChip Fluidics station 450. Finally, arrays were scanned with an Affymetrix GeneChip scanner 3000. The quality of hybridization was assessed in all samples following the manufacturer's recommendations. The microarray data have been deposited in MIAME compliant format in the NCBI's Gene Expression Omnibus public repository ([29]; GEO accession no. GSE86368).

Microarray analysis

After scanning the microarrays, cell intensity files containing a single intensity value for each probe cell were computed from the image data with the Affymetrix GeneChip Command Console Software. Background correction and normalization of probe cell intensity data was performed with Affymetrix Expression Console software using the Robust Multichip Analysis (RMA) algorithm. This algorithm is a log scale multi-chip analysis approach fitting a robust linear model at the probe level to minimize the effect of probe-specific affinity differences. Expression levels of transcripts are measured using log transformed perfect match values, after carrying out a global background adjustment and across microarray normalization [30]. The microarrays were annotated using the Affymetrix BovGene-1_0-st-v1_Probeset_Release 36 annotation file. Transcripts were defined as differentially expressed when the fold-change (FC) between GSGME group and control group was > 1.3 or < −1.3 and the *P*-value of the unpaired Student's t-test (two-tailed distribution, two-sample equal variance) for each transcript was < 0.05. False discovery rates (FDR) according to Benjamini-Hochberg multiple testing correction were also calculated. However, the FDR value was not applied as a cut off criterion, since the FDR-corrected *P*-values for all 23,000 transcripts were > 0.05.

Bioinformatic prediction of mRNA targets of differentially expressed miRNAs

Bioinformatic prediction of mRNA targets for differentially regulated miRNAs was performed using TargetScan release version 7.1 (http://www.targetscan.org/vert_71/) for the species "cow". TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 6 to 8mer sites matching the seed region of each miRNA [31]. In mammals, predictions are ranked based on the predicted efficacy of targeting as calculated using cumulative weighted context++ scores of the sites [32]. A cumulative weighted context++ score < −0.20 was used as cut off criterion for predicting mRNAs targets.

Gene set enrichment analysis

To extract biological meaning from the identified differentially expressed transcripts and predicted mRNA targets, gene set enrichment analysis (GSEA) with a modified Fisher's exact test was performed in order to identify enriched Gene Ontology (GO) terms within GO categories (biological process, molecular function, cellular component) and enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.7 bioinformatic resource [33, 34]. GO terms and KEGG pathways were defined as enriched if the FDR-adjusted *P*-value according to the Benjamini-Hochberg correction was < 0.05. GSEA was performed separately for the up- and down-regulated mRNAs and predicted mRNAs, respectively. The rationale of performing GSEA separately for the up- and down-regulated transcripts and not for all differentially expressed transcripts together is that results from GSEA are better to interpret, i.e. based on this approach it is assumed that biological processes or molecular functions and pathways identified as enriched within up-regulated genes are probably activated, whereas those enriched with down-regulated genes are likely inhibited.

Quantitative real-time polymerase chain reaction (qPCR) analysis

Microarray data of 25 differentially expressed mRNAs were validated by qPCR. For qPCR analysis, total RNA from all cows ($n = 14$ per group) was used to generate cDNA by reverse transcription. The cDNA was synthesized using a Mastermix containing 1.2 μg of total RNA, 100 pmol oligo(dT)18 primer (Eurofins MWG Operon, Ebersberg, Germany), 1.25 μL dNTP mix (10 mM, GeneCraft, Lüdinghausen, Germany), 5 μL 5× RT reaction buffer (Thermo Fisher Scientific, St. Leon-Rot, Deutschland) and 60 units M-MuLV Reverse Transcriptase (Thermo Fisher Scientific). The cDNA synthesis was carried out at 42 °C for 60 min and a final inactivating step at 70 °C for 10 min in a thermocycler (Biometra, Göttingen, Germany). The relative mRNA expression of genes was measured with a Rotor-Gene Q system (Qiagen, Hilden, Germany) using KAPA SYBR FAST qPCR Mastermix (Peqlab, Erlangen, Germany) and gene-specific primer pairs (Eurofins MWG Operon, Ebersberg, Germany) that were designed using Primer3 and BLAST. Primer characteristics of reference genes were recently published [13]. Primer characteristics of target genes are shown in Additional file 1: Table S1. Ct-values of reference

and target genes were obtained using Rotor-Gene Q Software (Qiagen). For normalization of relative expression levels GeNorm normalization factor was calculated from the three most stable (beta-actin, peptidylprolyl isomerase A, ribosomal protein S9) out of six reference genes tested [35]. Raw Ct-values of reference genes were statistically analyzed to ensure that expression levels did not differ between groups. Raw Ct-values were transformed into relative expression values using the $2^{-\Delta Ct}$ equation for the calculation of the normalization factors. The highest relative value of each gene was set to 1. From these values, the normalization factor was calculated as the geometric mean of expression data of the three most stable reference genes. Ct-values of target genes were also transformed into relative expression values using the $2^{-\Delta Ct}$ equation and were normalized with the individual normalization factor resulting in relative gene quantities that were used for the statistical analysis. The mean normalized $2^{-\Delta Ct}$ ratios of the control group was set to 1.0 and the mean and SD of normalized $2^{-\Delta Ct}$ ratios of the GSGME group was scaled proportionally. PCR products were separated electrophoretically using a 1.5% agarose gel stained with GelRed nucleic acid gel stain (Biotium, Hayward, CA, USA) to confirm the expected size of the PCR products.

Plasma concentration of acute phase proteins

Plasma concentrations of bovine haptoglobin (HP) and serum amyloid A (SAA) were analyzed using commercial ELISA Kits (CSB-E08585b, CSB-E08592b, Hölzel Diagnostika, Cologne, Germany). The ELISA procedure was performed based on the instructions provided by the manufacturer and absorbance read in a microplate reader (Infinite® 200, Tecan, Mainz, Germany). According to manufacturer's information, the limits of detection were 7.8 μg HP/L plasma for the HP ELISA kit and 50 μg SAA/L plasma for the SAA kit. All samples were measured in duplicate. Intra-assay coefficients of variability (CV) were < 10% for each sample in both assays. The average of individual CV was 5.8% and 3.7% for the measurement of HP and SAA, respectively.

Lipidomic analysis

Lipid extraction was carried out in the presence of non-naturally occurring lipid species as internal standards according to the protocol of Bligh and Dyer [36]. Determination of plasma lipid species was accomplished by means of direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode as described in [37, 38]. For phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and sphingomyelin (SM) a precursor ion of *m/z* 184 was used [38, 39]. A fragment ion of *m/z* 264 was used to analyze spingosine based ceramides (Cer) and hexosylceramides (HexCer), while a fragment ion of *m/z* 369 was used for the analysis of free cholesterol (FC) and cholesteryl esters (CE) after selective derivatization of FC [38, 40]. Phosphatidylethanolamine species (PE) and phosphatidylinositol (PI) were analysed following neutral loss fragment of 141 and 277 Da, respectively [41, 42]. The analysis of PE-based plasmalogens (PE-P) with 16:0, 18:0 and 18:1 vinylether bonds was performed as described by Zemski-Berry [43]. Data analysis was performed with Mass Lynx software including the NeoLynx tool (Micromass) and results were exported to Excel and further processed by self-programmed Excel Macros [37]. Annotation of lipid species was carried out according to the LipidomicNet proposal for shorthand notation of lipid structures derived from mass spectrometry [44]. Glycerophospholipid species annotation was based on the assumption of even-numbered carbon chains only. Sphingomyelin species were assigned based on the assumption of a sphingoid base with 2 hydroxyl groups.

Statistical analysis

Values presented in the text are means ± SD. All data were evaluated by Student's t test using the Minitab statistical software (Release 13, Minitab Inc., State College, PA, USA). Multiple testing correction of microarray data was performed by Benjamini and Hochberg FDR.

Results

Identification of differentially expressed transcripts

To investigate the effect of GSGME on the transcriptome in the liver of dairy cows, we used a bovine microarray representing approximately 23,000 *Bos taurus* transcripts. Taking into account the criteria FC > 1.3 or FC < −1.3 and $P < 0.05$ a total of 207 transcripts were found to be differentially expressed in the liver between cows fed GSGME and control cows. Substantially more transcripts were up-regulated by GSGME (156), while only 51 transcripts were down-regulated by GSGME in the liver of cows. The up-regulated transcripts included 155 protein-coding transcripts (mRNAs) and 1 non-protein-coding miRNA, whereas the down-regulated transcripts included 43 mRNAs and 8 miRNAs. The 20 most strongly up- and down-regulated mRNAs are presented in Table 1 and Table 2, respectively. The FCs of the most strongly up-regulated mRNAs ranged between 2.91 and 1.90, while those of the most strongly down-regulated mRNAs ranged between −1.66 and −1.39. In Table 3 the differentially regulated miRNAs including FCs and *P*-values are shown.

Validation of microarray data for selected differentially expressed protein-coding transcripts by qPCR

Validation of microarray data was carried out by qPCR analysis for 25 differentially regulated mRNAs. The

Table 1 The 20 most strongly up-regulated mRNAs in the liver of cows fed grape seed and grape marc meal extract (GSGME) versus control cows at 1 week postpartum

Gene symbol	mRNA description	FC[a]	*P*-value
TOP2A	topoisomerase (DNA) II alpha 170 kDa	2.91	0.013
CDKN3	cyclin-dependent kinase inhibitor 3	2.64	0.010
ARHGAP11A	Rho GTPase activating protein 11A	2.61	0.029
STMN1	stathmin 1	2.57	0.015
ECT2	epithelial cell transforming sequence 2 oncogene	2.53	0.023
DEPDC1	DEP domain containing 1	2.51	0.015
CENPA	centromere protein A	2.49	0.006
CENPF	centromere protein F, 350/400 kDa (mitosin)	2.45	0.017
CKAP2	cytoskeleton associated protein 2	2.39	0.024
PRR11	proline rich 11	2.32	0.036
KIF11	kinesin family member 11	2.25	0.007
KIF20A	kinesin family member 20A	2.21	0.017
BUB1B	budding uninhibited by benzimidazoles 1 homolog beta	2.20	0.004
LOC618147	histone cluster 1, H2ai-like	2.17	0.002
KIF4A	kinesin family member 4A	2.13	0.018
LOC787465	histone H2B type 1-like	2.10	0.006
GAS2L3	growth arrest-specific 2 like 3	1.99	0.018
SMC4	structural maintenance of chromosomes 4	1.98	0.011
SMC2	structural maintenance of chromosomes 2	1.96	0.011
RRM2	ribonucleotide reductase M2	1.95	0.046
CASC5	cancer susceptibility candidate 5	1.93	0.029
ESCO2	establishment of cohesion 1 homolog 2	1.93	0.032
HIST2H2BF	histone cluster 2, H2bf	1.91	0.039
HELLS	helicase, lymphoid-specific	1.90	0.013

[a]The FC was calculated from the signal log ratios as follows: $2^{\text{Signal log ratio}}$ if signal log ratio ≥ 0 and $(-1) \times 2^{-(\text{Signal log ratio})}$ if signal log ratio < 0. Signal log ratios were calculated from $n = 6$ microarrays per group

transcripts to be validated by qPCR were randomly selected from the most strongly up- and down-regulated mRNAs. Since the number of transcripts up-regulated was higher than that down-regulated, we validated 14 up- and 11-down-regulated transcripts by qPCR. Table 4 shows that in the case of most mRNAs (19) the effect direction was the same between qPCR and microarray data, but the FCs from qPCR analysis were markedly lower than from microarray analysis. In the case of about half (9) of the differentially regulated mRNAs, qPCR analysis revealed a FC greater than the filter criterion for differential regulation in microarray analysis (>1.3 or < −1.3). In line with this, statistical analysis revealed that only 5 of these 9 mRNAs were differentially regulated according to qPCR analysis at a significance level of $P < 0.05$ (TUBB, PHLDA1) or at least $P < 0.1$ (KIF20A, SAA4, HYOU1). In the case of 15 mRNAs, the FC determined by qPCR analysis was below the filter criterion for differential regulation and the *P*-value was not significant ($P > 0.05$). In the case of one mRNA (HMMR) the effect direction determined by qPCR analysis (FC = −1.34) was contrary to that determined by microarray analysis (FC = 1.87. Regarding these partial inconsistencies between microarray and qPCR data (statistical results, effect size), three causative factors should be noted: 1) The number of biological replicates was different between microarray and qPCR analysis ($n = 6$ vs. $n = 14$) influencing the statistical power of the data. 2) For microarray analysis six cows (1 primiparous and 5 multiparous) were selected from the control group and the GSGME group each consisting of 4 primiparous and 10 multiparous cows. Due to this, the average parity number of the groups used for microarray analysis was slightly lower than of the groups used for qPCR. 3) The detection principle of transcript abundance differs between microarray and qPCR, i.e. for qPCR analysis a 100–250 bp sequence of the transcript is amplified by a single primer pair, whereas for microarray analysis up to 26 unique 25-mer probes are used for each transcript resulting in a high coverage across the entire transcript of up to 650 bp.

Table 2 The 20 most strongly down-regulated mRNAs in the liver of cows fed grape seed and grape marc meal extract (GSGME) versus control cows at 1 week postpartum

Gene symbol	mRNA description	FC[a]	*P*-value
GLCE	glucuronic acid epimerase	−1.66	0.036
TBATA	chromosome 28 open reading frame, human C10orf27	−1.60	0.011
MANF	mesencephalic astrocyte-derived neurotrophic factor	−1.59	0.014
XBP1	X-box binding protein 1, transcript variant 1	−1.59	0.003
LOC618817	olfactory receptor, family 6, subfamily B, member 2-like	−1.55	0.005
SAA4	serum amyloid A4, constitutive	−1.54	0.043
HSPA5	heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	−1.51	0.005
GADD45B	growth arrest and DNA-damage-inducible, beta	−1.51	0.012
WWC1	WW and C2 domain containing 1, transcript variant 2	−1.48	0.037
LOC788587	olfactory receptor, family 4, subfamily D, member 11-like	−1.46	0.001
SOCS3	suppressor of cytokine signaling 3	−1.45	0.042
C15H11orf96	chromosome 15 open reading frame, human C11orf96	−1.44	0.018
PHLDA1	pleckstrin homology-like domain, family A, member 1	−1.43	0.012
SDF2L1	stromal cell-derived factor 2-like 1	−1.41	0.012
IRX3	iroquois homeobox 3	−1.40	0.001
LOC784679	peptidylprolyl isomerase A (cyclophilin A)-like	−1.39	0.023
HYOU1	hypoxia up-regulated 1, transcript variant 1	−1.39	0.018
ALX3	ALX homeobox 3	−1.39	0.010
CFHR2	complement factor H-related 2	−1.39	0.028
LOC520181	olfactory receptor 5-like	−1.39	0.007

[a]The FC was calculated from the signal log ratios as follows: $2^{\text{Signal log ratio}}$ if signal log ratio ≥ 0 and $(-1) \times 2^{-(\text{Signal log ratio})}$ if signal log ratio < 0. Signal log ratios were calculated from $n = 6$ microarrays per group

Identification of enriched annotation terms associated with the differentially expressed protein-coding transcripts

GSEA of the 155 up-regulated mRNAs showed that the GO terms with lowest FDR-adjusted *P*-values (most enriched) from all GO categories (biological process, cellular component, molecular function) were non-membrane-bounded organelle, intracellular non-membrane-bounded organelle, chromosome, cell cycle process, cell cycle, M phase, cell cycle phase, mitotic cell cycle, M phase of mitotic cell cycle, chromosomal part, microtubule cytoskeleton, mitosis, nuclear division, organelle fission, cytoskeletal part, cell division, spindle, microtubule-based process and cytoskeleton. Figure 1 shows the GO terms with FDR-adjusted *P*-values < 0.05 including the number of genes assigned to these terms separately for the GO categories biological process, cellular component and molecular function.

Table 3 The most strongly differentially regulated (FC > 1.3 or FC < −1.3 and *P* < 0.05) miRNAs in the liver of cows fed grape seed and grape marc meal extract (GSGME) versus control cows at 1 week postpartum

Gene symbol	micro RNA description	FC[a]	*P*-value
MIR376C	microRNA mir-376c	1.40	0.027
MIR365-2	microRNA mir-365–2	−1.31	0.010
MIR2345	microRNA mir-2345	−1.33	0.007
MIR2403	microRNA mir-2403	−1.34	0.029
MIR2462	microRNA mir-2462	−1.39	0.006
MIR2359	microRNA mir-2359	−1.41	0.002
MIR2430	microRNA mir-2430	−1.51	0.004
MIR2461	microRNA mir-2461	−1.53	0.010
MIR365	microRNA mir-365	−1.56	0.003

[a]The FC was calculated from the signal log ratios as follows: $2^{\text{Signal log ratio}}$ if signal log ratio ≥ 0 and $(-1) \times 2^{-(\text{Signal log ratio})}$ if signal log ratio < 0. Signal log ratios were calculated from $n = 6$ microarrays per group

For the 43 down-regulated mRNAs GSEA revealed only three enriched GO terms with FDR-adjusted *P*-values < 0.05, namely ER lumen, ER part and ER. These GO terms belonged exclusively to the GO category cellular component.

Identification of enriched regulatory pathways associated with the differentially expressed protein-coding transcripts

To identify regulatory pathways associated with the differentially expressed transcripts GSEA was performed using the KEGG database. The most enriched pathways

Table 4 Validation of microarray data for selected differentially expressed transcripts by qPCR

	Mean FC		*P*-value	
Gene symbol	Microarray	qPCR	Microarray	qPCR
STMN1	2.57	1.37	0.015	0.269
ECT2	2.53	1.45	0.023	0.246
CENPA	2.49	1.16	0.006	0.514
CENPF	2.45	1.07	0.017	0.788
CKAP2	2.39	1.20	0.023	0.557
PRR11	2.32	1.93	0.036	0.181
KIF20A	2.21	1.66	0.017	0.088
BUB1B	2.20	1.22	0.004	0.528
RRM2	1.95	1.56	0.046	0.263
ESCO2	1.93	−1.02	0.032	0.905
SPC25	1.88	−1.19	0.034	0.207
CCNA2	1.87	−1.07	0.030	0.762
HMMR	1.87	−1.34	0.018	0.100
TUBB	1.85	1.38	0.013	0.040
GLCE	−1.66	−1.2	0.036	0.195
MANF	−1.59	−1.23	0.014	0.235
SAA4	−1.54	−1.31	0.043	0.057
SOCS3	−1.45	−1.14	0.042	0.522
PHLDA1	−1.43	−1.34	0.012	0.006
HYOU1	−1.39	−1.33	0.018	0.081
DNAJB11	−1.37	−1.23	0.026	0.170
BAG3	−1.37	1.07	0.032	0.674
UAP1	−1.34	1.05	0.005	0.674
CCNL1	−1.33	−1.26	0.009	0.067
CXCL14	−1.31	−1.16	0.036	0.459

The microarray FC was calculated from the signal log ratios as follows: $2^{\text{Signal log ratio}}$ if signal log ratio ≥ 0 and $(-1) \times 2^{-(\text{Signal log ratio})}$ if signal log ratio < 0. Signal log ratios were calculated from $n = 6$ microarrays per group. The qPCR FC was calculated analogously from normalized $2^{-\Delta Ct}$ ratios. Normalized $2^{-\Delta Ct}$ expression was calculated from $n = 14$ samples per group

with FDR-adjusted *P*-values < 0.05 identified from the 155 up-regulated mRNAs included pathways regulating systemic lupus erythematosus and cell cycle, while no enriched pathways FDR-adjusted *P*-values < 0.05 were identified from the 43 down-regulated mRNAs.

Prediction of mRNA targets of the differentially expressed miRNAs and functional analysis

As described above, several miRNAs were identified as differentially expressed by microarray analysis of the cow livers. In order to identify further protein-coding transcripts that are influenced by feeding GSGME in the liver of cows, we performed bioinformatic target prediction for the 9 differentially regulated miRNAs. Considering a cumulative weighted context++ score < −0.20, a total of 185 target genes were identified for the up-regulated mir-376c, and 2,412 target genes for the highly conserved down-regulated miRNAs (mir-2345, mir-2403, mir-2462, mir-2359, mir-2430, mir-365). Data including gene names, total and 8mer, 7mer and 6mer sites and cumulative weighted context++ score are shown in Additional file 2: Table S2.

To elucidate the biological functions of the predicted target genes we carried out GSEA using GO category "biological process" and KEGG pathways separately for the targets identified for the up- and the down-regulated miRNAs. However, GSEA of the target genes of the up-regulated miRNA revealed no enriched biological process terms and KEGG pathways with FDR-adjusted *P*-values < 0.05.

GSEA of the target genes of the down-regulated miRNAs identified the following enriched GO biological process terms (FDR-adjusted *P*-value < 0.05): intracellular signaling cascade, positive regulation of macromolecule metabolic process, positive regulation of macromolecule biosynthetic process, positive regulation of biosynthetic process, positive regulation of cellular biosynthetic process and positive regulation of transcription. No enriched KEGG pathways with FDR-adjusted *P*-values < 0.05 could be identified by GSEA of target genes of the down-regulated miRNAs.

Plasma concentrations of acute phase proteins

Plasma concentration of the positive APPs SAA and HP were decreased in cows fed the GSGME compared to the control group ($P < 0.05$; Fig. 2).

Plasma lipid profile

Using lipidomic analysis, we were able to detect individual species of major (cholesterol, PC, SM, LPC) and minor (PE, PE plasmalogens, PI, ceramides) lipid classes in plasma samples of the cows. For all these lipid classes, there were no differences between the two groups of cows in the concentrations of any of the individual molecular species ($P > 0.05$, data are shown in Additional file 3: Tables S3-S9). Moreover, for all the lipids analyzed, the concentrations of species with no double bond (SFA), one double bond (MUFA) or two or more double bounds (PUFA) in the fatty acid moieties did not differ between the two groups of cows ($P > 0.05$, Table 5). In the PE plasmalogen fraction, there were moreover no differences in the concentrations of species with 16:0, 18:0 and 18:1 vinyl ether bonds between the two groups of cows ($P > 0.05$, Table 5).

Discussion

Recently, we observed that feeding GSGME to dairy cows from 3 week antepartum to 9 week postpartum increases milk yield and causes some beneficial changes in mRNA concentrations of hepatic genes, such as reduced

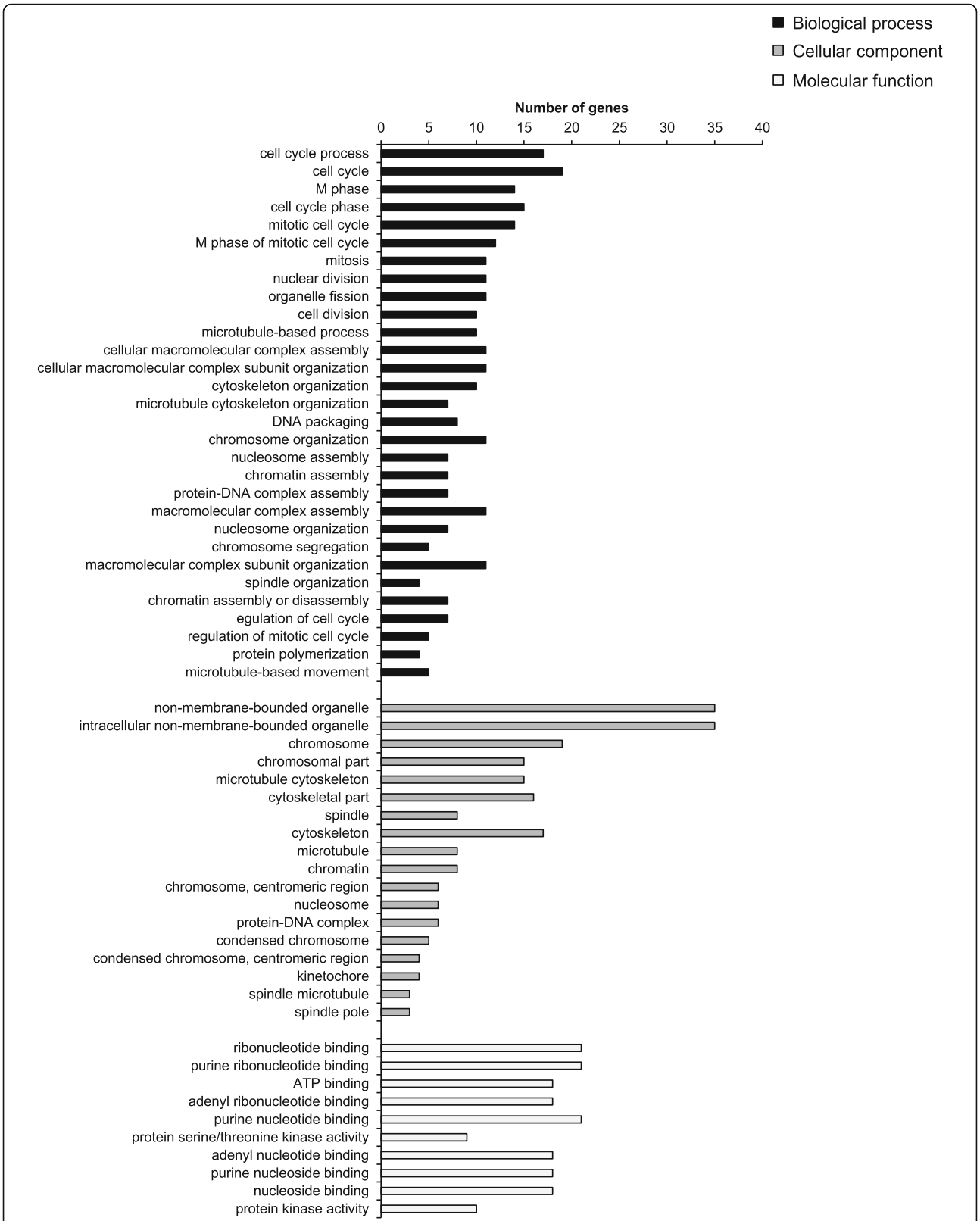

Fig. 1 The most enriched gene ontology (GO) terms assigned to the up-regulated mRNAs including the number of genes. The GO terms were sorted by their enrichment *P*-values (EASE score) (top: lowest *P*-value, bottom: highest *P*-value) within the GO categories biological process, cellular component and molecular function. Only GO terms with FDR-adjusted *P*-values < 0.05 are shown

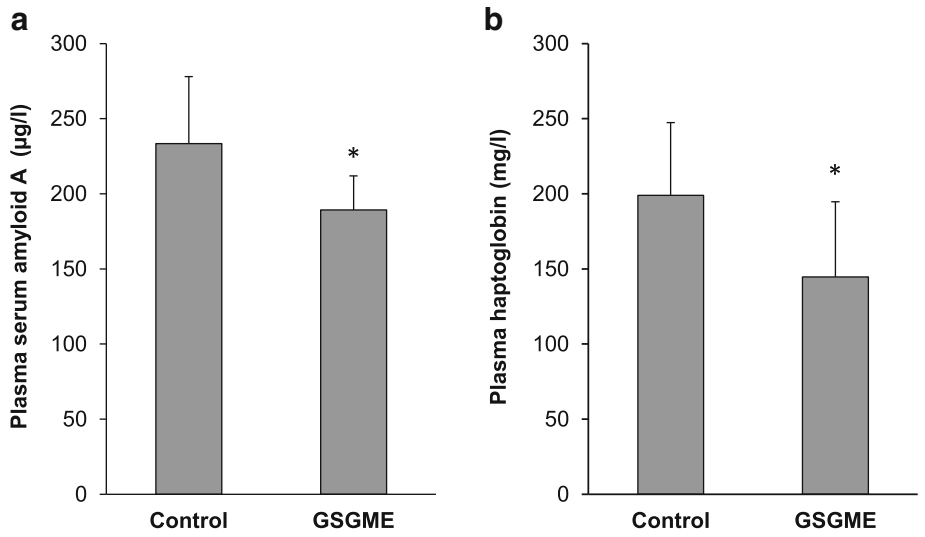

Fig. 2 Plasma concentration of **a** serum amyloid A and **b** haptoglobin in cows fed grape seed and grape marc meal extract (GSGME) and control cows at 1 week postpartum. Bars are means ± SD for $n = 14$ cows per group. Asterisk denotes difference between cows fed GSGME and control cows ($P < 0.05$)

mRNA concentration of FGF21, an indicator of metabolic and ER stress [13]. As the reasons underlying these effects could not be elucidated in the recent study, the aim of the present study was to identify changes in potentially critical signaling or metabolic pathways by using transcriptomic and lipidomic analyses. For this end, we considered liver and plasma samples obtained at 1 week postpartum regarding that metabolic and infectious stress in dairy cows is greatest at this early time after birth [6, 8, 17].

One striking finding of transcriptome analysis in the liver of cows was that within the limited number of protein-coding genes down-regulated by GSGME there was a large number of genes involved in ER stress-induced UPR, such as X-box binding protein 1 (XBP1), heat shock 70 kDa protein 5 (HSPA5)/GRP78, homocysteine inducible ER protein with ubiquitin like domain 1 (HERPUD1), DnaJ (Hsp40) homolog, subfamily C, member 5G (DNAJC5G), calreticulin (CALR), protein disulfide isomerase family A, member 4 (PDIA4), DnaJ (Hsp40) homolog, subfamily B, member 11 (DNAJB11), pleckstrin homology-like domain, family A, member 1 (PHLDA1)/TDAG51, protein phosphatase 1 regulatory subunit 3C (PPP1R3C), growth arrest and DNA damage inducible beta (GADD45B), BCL2-associated anthanogene 3 (BAG3), hypoxia up-regulated 1 (HYOU1) and mesencephalic astrocyte-derived neurotrophic factor (MANF). This is interesting because we have recently reported that ER stress-induced UPR occurs in the liver of dairy cows during early lactation [8] as evident from induction of XBP1, HSP5A, HERPUD1, DNAJC3, PDIA4, inositol-requiring enzyme 1 (IRE1), protein kinase (RNA)-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 ATF6 (ATF6), ER degradation enhancing alpha-mannosidase-like protein 1 (EDEM1), ATF4, BCL2 antagonist/killer 1 (BAK1), BCL2 associated X, apoptosis regulator (BAX), caspase 3 (CASP3), CASP8, CASP9, CASP12, tryptophanyl-tRNA synthetase (WARS) and DNA damage inducible transcript 3 (DDIT3)/C/EBP homologous transcription factor protein (CHOP). In line with this, Loor [45] identified a large number of XBP1 target genes as up-regulated in the liver of dairy cows during the transition from late pregnancy to lactation. The significance of ER stress in the liver of dairy cows is its putative causative role in the development of liver-associated diseases in high-yielding dairy cows [10], which impairs metabolic function of the liver, overall health status, and productive and reproductive performance. UPR target genes encode proteins that mediate protective cellular responses aiming to reduce ER stress and restore ER homeostasis. Therefore, typical proteins encoded by UPR target genes, which were identified as down-regulated by GSGME, are chaperones (e.g. HSPA5, PDIA4, HYOU1, CALR) and co-chaperones (e.g. DNAJC5G, DNAJB1, BAG3), both of which are implicated in the refolding of proteins, and components of the ER-associated degradation (ERAD) machinery (e.g. HERPUD1). The ERAD machinery is involved in the clearance of misfolded proteins that cannot be refolded in the ER and, therefore, are retrotranslocated to the cytosol, where they become degraded by the proteasome after being ubiquitinated by E3 ubiquitin ligases [46]. Down-regulation of these UPR target genes by GSGME is likely mediated by the identified down-regulation of XBP1. The spliced (s) XPB1 is a

Table 5 Plasma lipid profile of cows fed grape seed and grape marc meal extract (GSGME) versus control cows at 1 week postpartum

Lipid class	Group	Total	SFA	MUFA	PUFA
Major lipid classes					
CE, μM	Control	3070 ± 663	135 ± 38.8	206 ± 57.0	2730 ± 574.7
	GSGME	3079 ± 794	131 ± 32.4	204 ± 57.7	2743 ± 714.9
FC, μM	Control	411 ± 111	-	-	-
	GSGME	444 ± 124	-	-	-
LPC, μM	Control	77.0 ± 20.2	55.6 ± 13.9	9.35 ± 3.25	12.1 ± 4.05
	GSGME	80.7 ± 21.2	57.4 ± 15.0	10.0 ± 2.78	13.3 ± 4.18
PC, μM	Control	996 ± 285	22.8 ± 5.80	279 ± 91.8	598 ± 164
	GSGME	999 ± 293	22.6 ± 5.96	285 ± 87.0	594 ± 181
SM, μM	Control	169 ± 46.1	116 ± 27.5	44.4 ± 12.5	8.8 ± 6.0
	GSGME	170 ± 52.2	116 ± 33.1	44.2 ± 13.4	9.9 ± 6.0
Minor lipid classes					
Cer-d18:1, μM	Control	1.28 ± 0.24	1.11 ± 0.23	0.18 ± 0.04	-
	GSGME	1.28 ± 0.27	1.09 ± 0.23	0.19 ± 0.05	-
HexCer-d18:1, μM	Control	0.22 ± 0.05	0.11 ± 0.03	0.11 ± 0.02	-
	GSGME	0.23 ± 0.06	0.12 ± 0.04	0.12 ± 0.03	-
PI, μM	Control	9.65 ± 2.34	0.07 ± 0.01	2.02 ± 0.74	7.57 ± 1.68
	GSGME	9.58 ± 2.89	0.06 ± 0.02	2.08 ± 0.76	7.44 ± 2.18
PE, μM	Control	6.36 ± 1.96	0.19 ± 0.07	0.99 ± 0.30	4.97 ± 1.66
	GSGME	6.36 ± 1.64	0.17 ± 0.07	1.02 ± 0.25	4.95 ± 1.40
PE-P-16:0, μM	Control	6.73 ± 1.27	0.61 ± 0.05	1.01 ± 0.25	5.11 ± 1.06
	GSGME	6.99 ± 1.69	0.59 ± 0.10	1.09 ± 0.29	5.31 ± 1.37
PE-P-18:0, μM	Control	4.09 ± 0.39	0.57 ± 0.06	0.70 ± 0.10	2.82 ± 0.27
	GSGME	3.87 ± 0.52	0.51 ± 0.06	0.67 ± 0.11	2.69 ± 0.38
PE-P-18:1, μM	Control	4.48 ± 0.40	0.59 ± 0.04	0.77 ± 0.14	3.12 ± 0.29
	GSGME	4.41 ± 0.64	0.56 ± 0.08	0.79 ± 0.13	3.06 ± 0.50

Values are means ± SD for $n = 14$ cows per group

critical transcriptional regulator of ER stress response by inducing genes that cope with ER stress factors (ER chaperones, ERAD components) and stimulating phospholipid biosynthesis which leads to an expansion of the ER membrane [9, 47, 48]. Transcriptional regulation of ER stress-responsive genes by sXBP1 and other ER stress-sensitive transcription factors is mediated by binding to ER stress-dependent regulatory promoter motifs [e.g. endoplasmic reticulum stress element (ERSE)]. Functional ERSEs regulated by XBP1 were reported for the MANF and GADD45B genes [49], both of which were identified as down-regulated transcripts in the liver of cows fed GSGME. While GADD45B is localized in the mitochondria and is an activator of pro-survival p38 mitogen-activated protein kinase signaling, MANF is located in the luminal side of the ER and is proposed to help to remove misfolded proteins from the ER by degradation and/or enhancing protein folding [50]. Another ER stress-inducible protein identified as down-regulated by GSGME is PHLDA1/TDAG51, which encodes a protein promoting apoptotic cell death. Apoptosis is induced as consequence of ER stress in the case that ER stress-induced damage is overwhelming and homeostasis cannot be restored [51, 52]. The large proportion of ER stress-induced UPR target genes of total down-regulated transcripts was also reflected by GSEA, according to which ER lumen, ER part and ER were identified as enriched GO annotation terms.

Noteworthy, the chemokine ligands C-X-C motif chemokine ligand 14 (CXCL14) and C-C motif chemokine ligand 3 like 1 (CCL3L1) were also identified as transcripts down-regulated in the liver of cows fed GSGME. These two chemokine ligands belong to a family of about 50 chemokines which as a common feature are key regulators of leukocyte chemotaxis, migration and function, thus playing fundamental roles both in physiological and pathological immune responses, including inflammatory processes [53]. Inflammation is also induced

as a consequence of ER stress through IRE1-mediated activation of nuclear factor kappa B (NF-κB) [9, 54]. NF-κB plays a key role in regulating the transcription of a large set of genes involved in all aspects of inflammation (e.g. chemokines, proinflammatory cytokines, inflammatory enzymes, adhesion molecules and various receptors) [55]. Thus, the observed down-regulation of inflammatory chemokines in the liver of cows fed GSGME is not only an indicator of inhibition of hepatic inflammation but likely also of inhibition of ER stress by GSGME. In line with the assumption of an inhibition of hepatic inflammation by GSGME is a further finding of transcriptome analysis that the APP SAA4 was one of the genes down-regulated by GSGME. Hepatic synthesis of APPs, like SAA, HP, ceruloplasmin, and C-reactive protein, is greatly induced during systemic inflammation [7] triggered by pro-inflammatory cytokines [56]. In line with the view that high-yielding dairy cows suffer from systemic inflammation in the days after parturition, several studies have demonstrated that APPs are elevated in blood of cows during this phase, even in the absence of clinical signs of disease [7, 57, 58]. Thus, in order to substantiate our observation from transcriptome analysis that GSGME is able to attenuate the acute phase response of the liver, we determined the concentrations of SAA and HP in plasma of cows. In fact, the concentrations of both APPs were reduced in plasma of cows fed GSGME confirming our assumption that GSGME inhibits hepatic inflammation.

miRNAs were also identified as differentially regulated transcripts by GSGME in our transcriptome analysis and single miRNAs can regulate the expression of a large number of protein-coding target mRNAs, mainly at the posttranscriptional level. This is mediated by binding to complementary mRNA sequences, thereby causing their degradation or repression of protein translation, and, thus, inhibition of gene expression. Due to the great regulatory potential of miRNAs for regulating gene expression, we performed bioinformatic target prediction. Interestingly, the 185 target mRNAs predicted for mir-376c, which was up-regulated by GSGME, included several inflammatory chemokines, chemokine receptors, interleukins (ILs) and IL receptors [CCL15, CCL28, C-X9-C motif containing 4 (CMC4), CCR9, IL33, IL20RB]. Noteworthy, the target mRNAs predicted for mir-376c also included genes involved critically in the UPR including DDIT3/CHOP, eukaryotic translation initiation factor 2A (EIF2A) and the chaperone HSPD1. Although the predicted UPR target genes were not identical with the UPR target genes identified as differentially regulated, these findings strengthen our observation that GSGME causes down-regulation of ER stress target genes. Interestingly, CHOP is regulated by all branches of the UPR, in particular by ATF6, and is a powerful inducer of apoptosis during ER stress [59], while EIF2A encodes the initiator of protein translation eIF2α and inhibition of eIF2α phosphorylation in response to ER stress has long been known to be a cytoprotective mechanism, because inhibition of translation reduces global protein synthesis and thus work load of the ER [60]. Considering that mRNAs from up-regulated miRNAs are targeted for degradation and thus less transcribed, indicates that expression of genes involved in immune responses and critical genes of the UPR are inhibited by GSGME.

Nevertheless, we have recently reported that hepatic mRNA abundances of UPR target genes determined by qPCR analysis, such as ATF4, BAK1, BAX, CASP3, DDIT3, EDEM1, HSPA5, PDIA4 and XBP1, are not different between cows fed GSGME and control cows [13], because statistical evaluation of these data indicated no significant effect. Despite this, it was particularly striking that qPCR analyses showed a marked and consistent reduction in the mRNA abundances of all UPR target genes by 44% in average of all genes (variation between 25–65%). In addition, our recent study revealed that GSGME causes a strong and significant down-regulation of FGF21 in the liver of these cows. FGF21 is an important metabolic hormone regulating fatty acid oxidation and ketogenesis [61] and recent evidence indicated that FGF21 acts also as a stress hormone and is induced as a consequence of ER stress [62]. Thus, it is not surprising that FGF21 in the liver of dairy cows is dramatically induced during early lactation [63–65], because ER stress and various other stressors (negative energy balance, microbial pathogens) are present during the periparturient phase.

In connection with our results from transcriptome analysis, we are confident to postulate that GSGME is able to inhibit ER stress in the liver of dairy cows. Although we have no direct evidence for this, it is possible that attenuation of ER stress and inflammation was responsible for an increased utilization of energy and nutrients in these cows as reported recently [13]. Immune system activation is an energy-demanding process that necessitates a reallocation of nutrients and energy from dispensable functions such as growth and production [7]. It is well known that even subclinical inflammation increases the requirement of energy and amino acids, e.g. for the production of APPs and moreover has adverse effects on metabolism, e.g. by an increase of plasma cortisol [6, 66, 67]. The hypothesis that milk production is increased by an attenuation of inflammation has been confirmed in several studies in which supplementation of dairy cows with non-steroidal anti-inflammatory drugs during early lactation caused an increased milk yield [68–70]. The observed inhibition of ER stress and the parallel increase of milk yield by

GSGME [13] is noteworthy regarding the relatively low amount of GSGME fed to the cows (1% of DM in the TMR). Other studies dealing with the effect of grape products in ruminants (cows or ewes) used markedly higher concentrations, such as 5 kg dried GM per cow and day [71], 10% grape residue silage of feed DM [72], and 300 g GS per ewe and day [73]. Despite the feeding of much higher amounts of grape products in these studies, only one study observed a slight improvement of milk yield compared to the control group [71]. However, none of these studies investigated the effect of grape products on ER stress and inflammatory signaling pathways, but on methanogenesis and intraruminal and total tract nutrient digestibility. Therefore, further studies dealing with the effects of grape products, with particular consideration of dose–response relationships, on ER stress and inflammatory pathways in the liver of high-yielding dairy cows during the transition period are required, in order to confirm potential beneficial effects of grape products on these pathways and to figure out the optimum supplementary dose.

A further striking observation from transcriptome analysis was that the most enriched GO terms associated with the genes up-regulated by GSGME are dealing with cell cycle regulation, such as M phase, cell cycle phase, mitotic cell phase, microtubule cytoskeleton, mitosis, nuclear and cell division. This is due to the fact that many of the proteins encoded by the up-regulated genes, like topoisomerase (DNA) II alpha (TOP2A), cyclin dependent kinase inhibitor 3 (CDKN3), stathmin 1 (STMN1), epithelial cell transforming 2 (ECT2), DEP domain containing 1 (DEPDC1), centromere protein A (CENPA), CENPF, CENPO, cytoskeleton associated protein 2 (CKAP2), kinesin family member 11 (KIF11), KIF20A, KIF4A, KIF20B, KIF15, BUB1B mitotic checkpoint serine/threonine kinase B (BUB1B), growth arrest specific 2 like 3 (GAS2L3), structural maintenance of chromosomes 4 (SMC4), SMC2, SPC25 NDC80 kinetochore complex component (SPC25), cyclin A2 (CCNA2), NDC80 kinetochore complex component (NUF2), β-tubulin (TUBB) and many others, have important biological functions within mitosis, cell cycle arrest, mitotic spindle organization, cytokinesis, mitotic chromosome condensation, metaphase/anaphase transition, chromosome organization, regulation of cyclin-dependent kinases and nucleosome assembly. For instance, KIF11, KIF20A, KIF4A, KIF20B and KIF15 encode proteins of the kinesin superfamily, a group of microtubule-dependent molecular motors. Proteins of the kinesin superfamily provide force for intracellular transport and cell division and are essential for mitosis and meiosis [74]. An important role during mitosis also plays topoisomerase IIα, encoded by the most strongly up-regulated gene TOP2A (2.9-fold), in resolving anaphase bridges between sister chromatids to ensure that daughter cells receive only one copy of each chromosome [75]. In this regard, centromer proteins, like CENPA, CENPF, CENPO, all of which were also up-regulated by GSGME, are localized to centromeric DNA, also called kinetochores, throughout the cell cycle and ensure correct chromosome attachment to the microtubules, equal segregation of sister chromatids, and their movement to the opposite poles [76]. Also in agreement with the observation that genes involved in mitosis and cell cycle are induced by GSGME is that several genes encoding histone proteins, which play a role for nucleosome assembly and thus affect chromatin structure, were up-regulated by GSGME. Although it is difficult to estimate the precise biological implication of an up-regulation of genes involved in mitosis or cell cycle regulation by GSGME in the context of early-lactating dairy cows, this effect might be explained, at least in part, by the well-described effects of different polyphenolic compounds contained in GSGME on cell cycle regulation and apoptosis, effects that are made responsible for the anti-cancer activities of many polyphenols [77]. For instance, quercetin [78], curcurmin [79], ellagic acid [80], epigallocatechin-3-gallate [81] and resveratrol [82] were found to induce the critical cell cycle regulator p53 and, subsequently, cell cycle arrest and apoptosis in different cancer cells. On the other hand, induction of p53-mediated cell cycle arrest by polyphenolic compounds in normal cells allows complete repair of DNA damage before continuing with cellular division through p53-induced formation of different DNA repair proteins, like mutL homolog 1 and Rad51 recombinase [83, 84]. Due to the central role of p53 for cell cycle regulation and the large number of up-regulated genes involved in this process, it was not surprising to identify cell cycle as an enriched KEGG pathway.

Besides their anti-inflammatory properties, pronounced effects of polyphenols on lipid metabolism have been reported. In rodent models, it has been shown that dietary polyphenols are able to lower plasma lipid concentrations and prevent the development of fatty liver by influencing several pathways of lipid metabolism, including inhibition of lipogenesis and activation of β-oxidation [23, 85]. In dairy cows, hepatic lipid metabolism is a physiological key aspect of health in dairy cows. It has been well established that disturbances of hepatic lipid metabolism, such as a low rate of β-oxidation and a limited capacity of the liver for the secretion of lipids into the blood are critical events in the development of fatty liver and ketosis [86, 87]. Recently, it has been observed that hepatic metabolism of glycerol- and ether phospholipids is closely linked to plasma concentrations of NEFA, BHBA and glucose, three

key factors of the metabolic status of dairy cows during early lactation [20]. Imhashly et al. [88] recently showed, using lipidomic analysis of plasma, that concentrations of some unsaturated PC, LPC and SM species (such as PC 36:4, PC 36:5, PC 36:6, LPC 18:1, LPC 18:2, LPC 18:3, SM 39:1, SM 43:3) in dairy cows are continuously increasing after birth. A common feature of these phospholipids is their requirement for the secretion of hepatic TAG as very low-density lipoprotein particles. Thus, an increased formation and secretion of these phospholipids after birth has been regarded as a means of the liver to prevent accumulation of lipids [88]. In the present study, we observed that concentrations of all the individual phospholipids, and even their molecular species, in plasma of dairy cows in week 1 postpartum are not influenced by feeding GSGME. As the greatest part of plasma phospholipids is synthesized in the liver, this finding strongly suggests that phospholipid metabolism in the liver was not influenced by polyphenols from GSGME. The finding that fatty acid moieties of plasma phospholipids were also not changed in the group of cows supplemented with GSGME indicates that polyphenols also did not influence hepatic desaturation and elongation of fatty acids. This finding is of relevance as the fatty acid composition of phospholipids not only influences properties of cellular membranes [89], but certain phospholipid-bound fatty acids such as arachidonic acid are serving also as precursors for the synthesis of pro-inflammatory eicosanoids [90]. The finding that supplementation of GSGME did not influence the concentrations of free cholesterol and cholesterol esters indicates that polyphenols do not modify hepatic cholesterol metabolism. This finding agrees with our recent study which showed that GSGME does not influence hepatic cholesterol concentration [13]. Ceramide and ceramide-derived sphingolipids are structural components of membranes. In plasma, ceramides are transported as components of low-density lipoproteins of hepatic origin [91]. Ceramides are of physiological relevance as their plasma concentrations have been linked to insulin resistance, oxidative stress, and inflammation [22, 92–94], conditions which are commonly observed in dairy cows during the transition period. Recently, Rico et al. [91] have shown that overweight dairy cows have increased plasma concentrations of ceramides and these are closely linked with the progression of insulin resistance. These authors suggested that ceramides may have a fundamental role in the homeorhetic adaptation to early lactation in dairy cows. Our lipidomic analysis revealed that polyphenols from GSGME do not influence plasma concentrations and the molecular profile of ceramides in plasma. Thus, we conclude that beneficial effects of GSGME on inflammation and ER stress in the liver were independent of metabolism of ceramides.

Conclusion

The present findings from transcriptome analysis of the liver of cows fed GSGME during the transition period at 1 week postpartum indicates that polyphenol-rich feed components, such as GSGME, are able to down-regulate a large set of genes involved in ER stress-induced UPR and inflammatory processes. The observation that GSGME induces specific miRNAs, which are known to bind and thus degrade mRNAs encoding proteins of the UPR and inflammation, indicates that at least some of the GSGME effects on the hepatic transcriptome of dairy cows are mediated by miRNA-mRNA interactions. In contrast, transcriptome analysis of the liver of these cows did not reveal alterations in the expression of genes involved in important metabolic pathways, such as lipid metabolism. This finding is in agreement with our results from plasma lipid profiling demonstrating no differences in the concentrations of individual species of major and minor lipid classes between cows fed GSGME and control cows. Considering that both ER stress and inflammatory processes are considered to contribute to liver-associated diseases, which frequently occur during early lactation in high-yielding dairy cows, and to impair milk performance in dairy cows, it is likely that inhibition of ER stress and inflammation is responsible for the recently observed increase in milk yield of dairy cows fed GSGME.

Additional files

Additional file 1: Table S1. Characteristics of gene-specific primers used for qPCR. (DOCX 17 kb)

Additional file 2: Table S2. Predicted mRNAs of the highly conserved differentially regulated miRNAs including gene names, total and 8mer, 7mer and 6mer sites and cumulative weighted context++ score. (DOCX 298 kb)

Additional file 3: Tables S3-S9. Concentrations of various lipid species in plasma (μM) of cows fed grape seed and grape marc meal extract (GSGME) and control cows at 1 week postpartum. (DOCX 45 kb)

Abbreviations

APP: Acute phase protein; ATF6: Activating trascription factor 6 ATF6; BAG3: BCL2-associated anthanogene 3; BAK1: BCL2 antagonist/killer 1; BAX: BCL2 associated X, apoptosis regulator; BHBA: β-hydroxybutyrate; BUB1B: BUB1B mitotic checkpoint serine/threonine kinase B; CALR: Calreticulin; CASP3: Caspase 3; CCL3L1: C-C motif chemokine ligand 3 like 1; CCNA2: Cyclin A2; CDKN3: Cyclin dependent kinase inhibitor 3; cDNA: Complementary DNA; CE: Cholesteryl esters; CENPA: Centromere protein A; Cer: Ceramides; CHOP: C/EBP homologous transcription factor protein; CKAP2: Cytoskeleton associated protein 2; CMC4: C-X9-C motif containing 4; CP: Crude protein; cRNA: Complementary RNA; CV: Coefficient of variability; CXCL14: Chemokine ligand 14; DAVID: Database for Annotation, Visualization and Integrated Discovery; DDIT3: DNA damage inducible transcript 3 (DDIT3); DEPDC1: DEP domain containing 1; DMI: Dry matter intake; DNAJB11: DnaJ (Hsp40) homolog, subfamily B, member 11; DNAJC5G: DnaJ (Hsp40) homolog, subfamily C, member 5G; ECT2: Epithelial cell transforming 2; EDEM1: ER degradation enhancing alpha-mannosidase-like protein 1; EIF2A: Eukaryotic translation initiation factor 2A; ER: Endoplasmic reticulum; ERAD: ER-associated degradation; ERSE: Endoplasmic reticulum stress elements; ESI-MS/MS: electrospray

ionization tandem mass spectrometry; FC: Fold-change; FC: Free cholesterol; FDR: False discovery rates; FGF-21: Fibroblast-growth factor-21; GADD45B: Growth arrest and DNA damage inducible beta; GAS2L3: Growth arrest specific 2 like 3; GEO: Gene expression omnibus; GO: Gene ontology; GSEA: Gene set enrichment analysis; GSGME: Grape seed and grape marc meal extract; HERPUD1: Homocysteine inducible ER protein with ubiquitin like domain 1; HexCer: Hexosylceramides; HP: Haptoglobin; HSPA5: Heat shock 70 kDa protein 5; HYOU1: Hypoxia up-regulated 1 (HYOU1); IL: Interleukin; KEGG: Kyoto encyclopedia of genes and genomes; KFB: Centre of excellence for fluorescent bioanalytics; KIF11: Kinesin family member 11; LPC: Lysophosphatidylcholine; MANF: Mesencephalic astrocyte-derived neurotrophic factor; NE: Net energy; NEB: Negative energy balance; NEFA: Non-esterified fatty acids; NF-κB: Nuclear factor kappa B; NUF2: NDC80 kinetochore complex component; PC: Phosphatidylcholine; PDIA4: Protein disulfide isomerase family A, member 4; PE: Phosphatidylethanolamine; PE-P: PE-based plasmalogens; PERK: Protein kinase (RNA)-like endoplasmic reticulum kinase; PHLDA1: Pleckstrin homology-like domain, family A, member 1; PI: Phosphatidylinositol; PPP1R3C: Protein phosphatase 1 regulatory subunit 3C; qPCR: Quantitative real-time polymerase chain reaction; RIN: RNA integrity number; RMA: Robust multichip analysis; SAA: Serum amyloid A; SM: Sphingomyelin; SMC4: Structural maintenance of chromosomes 4; SPC25: SPC25 NDC80 kinetochore complex component (SPC25); ss: Single-stranded; STMN1: Stathmin 1; TAG: Triacylglycerols; TMR: Total mixed ration; TPO2A: Topoisomerase (DNA) II alpha; TUBB: β-tubulin; UPR: Unfolded protein response; WARS: Tryptophanyl-tRNA synthetase; XBP1: X-box binding protein 1

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Availability of data and materials
The microarray data set supporting the results of this article is available in the Gene Expression Omnibus repository, GEO Series accession number GSE86368.

Authors' contributions
KE, CK and GD designed research and coordinated the study. AW, CK, and DG performed the experiments. DG and RR analyzed the data and performed functional analysis of microarray data. GL performed lipidomic analysis. DG, RR and KE wrote the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interest.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The experiment was conducted at the Educational and Research Centre for Animal Husbandry Hofgut Neumühle in Rhineland-Palatinate (Münchweiler an der Alsenz, Germany). All cows included in this study originate from the facility's own calf rearing. The experimental protocol was approved by the Provincial Government of Coblenz, Germany (23 177–07/G12–20–074).

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PAPER IV

Determination of polyphenol and crude nutrient content and nutrient digestibility of dried and ensiled white and red grape pomace cultivars

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Determination of polyphenol and crude nutrient content and nutrient digestibility of dried and ensiled white and red grape pomace cultivars

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The present study aimed to determine the nutrient and energy content of fresh and ensiled grape pomace (GP) from different grape varieties originating from Germany, and to estimate the feed value of dried white, dried red and ensiled white GP by calculating nutrient digestibility and the content of metabolisable energy (ME) and net energy lactation (NEL) measured in sheep as a ruminant model. GP from red cultivars had higher contents of organic matter (OM), crude protein (CP), ether extract (EE), crude fibre (CF), total phenolic contents (TPC) and ME, whereas the concentrations of ash and sugar were lower than from white cultivars. Compared with untreated GP, ensiled GP had increased concentrations of CP (+19%), ether extract (EE; +23%) and CF (+12%) and a higher ME content (+7%) and markedly decreased concentrations of sugar (−99.6%) and TPC (−48%). The concentrations of dry matter, OM and ash were not different between ensiled and fresh GP. Compared with dried GP, ensiled GP had a higher nutrient digestibility (OM, +32%; CP, +43%; CF, +46%; neutral detergent fibre [NDF], +54%; acid detergent fibre [ADF], +69%) and higher energy values (ME, +16%; NEL, +19%). The digestibility of OM, CP, EE and CF and the energy content were higher for dried red than for dried white GP, whereas the digestibility of NDF_{OM} and ADF_{OM} was lower for dried red than dried white GP. In conclusion, the results show that both red and white GP are suitable dietary sources for enrichment with TPC. Furthermore, compared with drying ensiling of GP improves the feeding value of GP and is a good possibility of preserving the seasonally produced by-product of winemaking for ruminant feeding.

Keywords: digestibility; drying; ensiling; grape pomace; metabolisable energy; polyphenols sheep

1. Introduction

Germany has a total vineyard area of 102,000 ha on which a total volume of 8.4 million hectolitres wine was produced in 2013 (German Wine Institution 2014). During wine-making, considerable amounts of grape pomace (GP) are produced as the most important by-product, and only a small part of this GP is recycled as fertiliser in the vineyards or is distilled for producing spirituous beverages.

GP consists mainly of skins (about 80% of wet weight, Jiang et al. 2011), but contains also varying amounts of seeds and stems. From a nutritional point of view, GP is interesting because it is an abundant and inexpensive source of polyphenols (El Gengaihi et al. 2013; Toaldo et al. 2013), a class of secondary plant metabolites. According to their structure, polyphenols can be categorised into flavonoids, which is the

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largest group of polyphenols predominantly found in skins, seeds and stems (Bogs et al. 2007), and non-flavonoids, which are most abundant in the berry pulp (Ebrahimi and Schluesener 2012). Polyphenolic compounds, especially flavonoids, have received substantial scientific attention due to important health-promoting effects, such as protection from atherosclerosis and cancer, associated with the intake of flavonoid-rich food (McCullough et al. 2012). Emerging findings indicate that the health-promoting effects of flavonoids are largely based on their anti-inflammatory activities through modulating the activities of key regulators of inflammation, and cell protection is more relevant than the antioxidant properties (Kim et al. 2008). Based on these effects, the pharmaceutical and food industry use GP already as a natural source of flavonoids for the production of nutraceuticals and functional food with the aim to improve human health (González-Centeno et al. 2013; Georgiev et al. 2014).

In contrast to humans, in farm animals the potential health-promoting effects of flavonoids have been investigated scarcely (Santos et al. 2014; Ferlay et al. 2010), despite the fact that inflammatory processes that promote metabolic problems and impair production are a frequent phenomenon in farm animals and thus dietary strategies to combat inflammatory processes are of great demand. At least in monogastric animals it was demonstrated recently that flavonoid-rich feed additives based on grape meal extract or spent hops inhibit markers of inflammation in the intestine, influence the intestinal microflora and improve the gain-to-feed ratio in weaned growing pigs (Gessner et al. 2013; Fiesel et al. 2014). Flavonoid-rich feed additives like GP might be also useful for ruminant farm animals considering that an inflammatory condition is also frequently found in high-producing dairy cows and has been proposed to play a key role in the development of liver-associated diseases such as fatty liver and ketosis (Ringseis et al. 2014). Although it has been already shown that GP, either in the fresh, dried or ensiled form, has a low energy content and can be included in feeding rations for ruminants, especially when fed near to maintenance (Baumgärtel et al. 2007), little is known about the feeding value and the polyphenol content of GP from different grape varieties.

Thus, in light of the scarcity of data to estimate the feeding value of GP in ruminants correctly, the present study had two objectives: (1) to determine the content of crude nutrients, total phenolic contents (TPC) and energy of GP from different grape varieties originating from an important winegrowing area in Rhineland-Palatinate/Germany (in order to investigate the effect of ensiling on the feeding value of GP, these determinations were made for untreated and ensiled GP with or without an ensiling additive); and (2) to determine the feed value of dried white, dried red and ensiled white GP for sheep by calculating nutrient digestibility and the contents of metabolisable energy (ME) and net energy lactation (NEL).

2. Materials and methods

2.1. Determination of the contents of crude nutrients and TPC of different GP cultivars

From the vintage 2012, 5 randomly selected bunches of wine grape cultivars were obtained from 10 different wineries within the winegrowing area "Neustadt an der Weinstraße" (Rhineland-Palatinate, Germany). The selected white grape varieties were Pinot blanc (n = 4) and Riesling (n = 6); the red grape varieties were Dornfelder (n = 5), Pinot noir (n = 5) and Portugais bleu (n = 2). The obtained GP samples were not sieved and the skins were not separated from the seeds.

2.1.1. Sample preparation

Each fresh GP sample was divided into three portions and frozen at −24°C until processing and analysis. After thawing at ambient temperature, one portion of each GP sample was dried for 48 h at 60°C in an Heraeus dehydrator (T 5090 E, Hanau, Germany). The dried GP samples were ground by a laboratory mill (Dietz, Dettingen-Teck, Germany) to pass through a 1 mm mesh screen and were kept at −24°C until use.

2.1.2. Ensiling

After thawing at ambient temperature, the first portion (~4 kg) of each GP sample was divided in two portions of approximately 2 kg each. One portion was mixed with 6.4 g of a chemical ensiling additive (Combisil, Josera, Osterholz-Scharmbeck; 47% sodium chloride, 30% sodium benzoate, 20% calcium formate, 3% sugar beet molasses), put into a plastic bag and sealed hermetically in 5 l buckets. The other portion was prepared similarly without using an ensiling agent. Finally, the closed plastic bags were weighted with stones, stored for 8 weeks and then frozen at −24°C for determination of the content of crude nutrients and TPC.

2.1.3. Determination of the content of crude nutrients and TPC

The concentrations of crude nutrients were determined according to the official analytical methods (VDLUFA 2007). The second portion of each GP cultivar sample and approximately 100 g of each ensiled GP sample were used for determination of dry matter (DM) by drying at 100–105°C for 24 h, ash (method 8.1), crude protein (CP; method 4.1.1), ether extract (EE; method 5.1.1), crude fibre (CF; method 6.1.1) and sugar (method 7.1.2). The third portion of each frozen GP sample and approximately 500 g of each ensiled GP sample were lyophilised and milled by a laboratory mill (Dietz, Dettingen-Teck, Germany). Extraction of TPC from the obtained GP powder was carried out using an ASE 350 (Accelerated Solvent Extraction System, Dionex Corporation, Thermo Scientific, Waltham, MA) as described by González-Centeno et al. (2013). The TPC of the extracted GP cultivars was measured spectrophotometrically in 96-well plates according to the Folin–Ciocalteu method (Singleton and Rossi 1965). The TPC was calculated using gallic acid (GA) as a standard for calibration and expressed as mg GA/g GP. The presented TPC contents are means of six measurements.

2.2. Determination of nutrient digestibility and energy content of dried and ensiled GP

The digestibility trials were conducted in accordance with animal welfare legislation and were approved by the Provincial Government of Coblenz, Germany (no. G12-20-049). The digestibility trials (Trial 1 [July/August 2012]: dried white GP; Trial 2 [September/October 2012]: dried red GP; Trial 3 [November/December 2012]: ensiled GP) were carried out according to the guidelines of Gesellschaft für Ernährungsphysiologie (GfE) (1991) at the Ruminant Research Unit of the University of Applied Sciences Bingen.

2.2.1. Origin and preparation of GP

The dried white GP (Riesling) and dried red GP (Pinot noir) for Trials 1 and 2, respectively, were obtained from a winery in Bingen (Rhineland-Palatinate). Because

seeds and skins were separately supplied, they were blended at a ratio of 70:30 before starting the trials. The GP (Riesling) used for Trial 3 was obtained from a winery in Bingen (Rhineland-Palatinate) from the vintage of 2012 and immediately ensiled in a container without using an ensiling agent.

2.2.2. Animals and housing

Eight male Shropshire sheep were used for the three trials in a 2 (animals) x 4 (treatments) cross-over design (n = 8) with four replicates per dietary treatment. Sheep were kept individually in metabolic cages with *ad libitum* access to water. In Trials 1, 2 and 3, the sheep had an average body weight of 45 ± 3.1 kg, 47 ± 3.4 kg and 53 ± 3.4 kg, respectively. They were randomly assigned to the treatment groups and received diets containing decreasing amounts of chopped hay (grass of second cut; 85%, 70%, 55% and 40%) at the expense of increasing amounts of GP (15%, 30%, 45% and 60%). Thereby, the linearity of digestibility response of the test diets at different supplementation levels could be illustrated by using linear regression analysis (Westreicher-Kristen et al. 2013). Additionally, during the adaptation period and between the three trials each sheep received a vitamin and mineral mixture (VitaMira® Schaf Kristall Plus, BayWa AG, Offenbach, Germany) in an amount of 10 g per sheep and day sufficient to meet the recommendations of GfE (1978). Sheep were weighed at the start and the end of each experimental period. The sheep were fed twice a day at 08:00 and 17:00 h. According to the proportions of GP and hay in the four treatments, rations were mixed daily. The amount of feed was calculated to achieve a daily ME intake of the 1.2-fold of the mean maintenance requirement (GfE 1991), which is equivalent to an amount of 0.9–1.1 kg/d, depending on the weight of sheep. According to GfE guidelines GfE 1991), sheep were adapted to experimental diets, conditions and facilities for 14 d (first week in stable, second week adaptation to metabolic cages) prior to the sampling period. During the sampling period of 7 d, faeces were collected daily, weighted and sub-sampled (from each sheep on each treatment) by taking a 30% aliquot and frozen at −24°C for later analysis. Sub-samples of hay and GP were taken once a day and frozen at −24°C until analysis. At the end of each digestibility trial, all feed samples were pooled, homogenised and analysed for the content of crude nutrients, gross energy and TPC.

2.2.3. Determination of the content of crude nutrients and TPC

The GP and hay batches (dry basis) of each trial were dried in a forced air oven at 60°C for 48 h. The faeces samples (wet basis) were lyophilised, and samples of feed and faeces were ground by a laboratory mill (Dietz, Dettingen-Teck, Germany) to pass through a 1 mm screen and analysed for DM and crude nutrients according to the official analytical methods in Germany as described in Section 2.1.3. (VDLUFA 2007). Neutral detergent fibre (NDF) as measured with a heat-stable amylase and presented without residual ash content, and acid detergent fibre (ADF) and acid detergent lignin (ADL) as determined without correction for ash content were determined according to official methods (VDLUFA 2007). Gross energy was determined using a bomb calorimeter (IKA-Calorimeter C5000, Janke & Kunkel IKA Analysentechnik, Staufen, Germany). The TPC of the previously extracted GP cultivars was quantified as described in Section 2.1.3. The analysed contents of crude nutrients, gross energy and TPC of dried white GP, dried red GP, ensiled GP and hay used in the three trials are shown in Table 1.

Table 1. Analysed chemical composition of grape pomace (GP) and hay used in the three digestibility trials with sheep.*

	Hay	Trial 1 Dried white GP (Riesling)	Trial 2 Dried red GP (Pinot noir)	Trial 3 Ensiled white GP (Riesling)
Dry matter [g/kg]	844	907	901	966
Organic matter [g/kg DM]	928	932	967	918
Crude protein [g/kg DM]	94	109	112	109
Ether extract [g/kg DM]	9	58	50	20
Crude fibre [g/kg DM]	296	241	214	296
Ash [g/kg DM]	69	68	33	82
Neutral detergent fibre [g/kg DM]	622	424	341	401
Acid detergent fibre [g/kg DM]	363	437	346	448
Acid detergent lignin [g/kg DM]	53	320	236	301
TPC† [mg gallic acid/g GP]	n.d.‡	56	87	46
Gross energy [MJ/kg DM]	19.1	21.3	21.7	18.9

Notes: *Values represent three determinations of two pooled samples; †TPC, Total phenolic contents; ‡n.d., Not determined.

2.2.4. *Calculations and statistical analysis*

For the first part of this study, the content of ME was calculated for fresh and ensiled GP (both without and with an ensiling additive) on the basis of digestible nutrients obtained from both digestibility trials of the second part of this study and from Deutsche Landwirtschafts Gesellschaft (DLG) feeding value tables (DLG 1997). The energy values [MJ/kg DM] were calculated as suggested by the GfE (2001). Statistical analysis was carried out using analysis of variance and comparison of means between groups by Tukey's HSD test with Statistica software (Version 8.0, StatSoft). Differences were considered as significant at $p < 0.05$. All experimental data represent means and standard deviations (mean ± SD). For each grape variety, three replicates were used for statistical evaluation (untreated, ensiled with an ensiling agent, ensiled without an ensiling agent).

For digestibility trials, the energy values [MJ/kg DM] of GP and hay were calculated on the basis of digestible nutrients as suggested by the GfE (2001). The digestibility coefficient for nutrients and energy was calculated for each diet and animal based on the differences between quantities of nutrient intake and output (GfE 1991). The nutrient digestibilities were calculated by multiple linear regression based on a common intercept model and individual slopes for test sources according to Kluth et al. (2005) using the equation

$$Y = a + b_n \cdot x_n$$

where Y is the digestibility of GP (at an inclusion rate of 100%), a is the intercept, b_n is the slope for test source n and x_n is a given concentration originating from test source n [%].

To calculate the digestibility of GP at a dietary inclusion rate of 100%, the equation was extrapolated to $x = 100$.

3. Results

3.1. Contents of crude nutrients and TPC of different GP cultivars

3.1.1. Crude nutrients

In the different samples of GP, the content of crude nutrients was influenced by cultivar, treatment and colour (Table 2).

Concerning cultivar, it was observed that the red cultivars Pinot noir, Dornfelder and Portugais bleu had higher concentrations of organic matter (OM), CP, CF and lower concentrations of ash and sugar than the white cultivars Pinot blanc and Riesling, but these effects were significant only for specific cultivars ($p < 0.05$). No effect of cultivar was found with regard to the concentrations of DM and EE.

Regarding colour, it was observed that fresh red GP had higher concentrations of OM, CP, EE, CF and lower concentrations of ash and sugar than the fresh white GP ($p < 0.05$).

With regard to treatment, it was found that the GP ensiled with an ensiling agent had increased concentrations of CP, EE, CF and markedly decreased concentrations of sugar compared to untreated GP ($p < 0.05$). The GP ensiled without additive had only increased concentrations of EE and markedly decreased concentrations of sugar compared to untreated GP ($p < 0.05$). The concentrations of DM, OM and ash were not different between ensiled (with and without additive) and fresh GP.

3.1.2. Total phenolic contents

The TPC of GP was influenced significantly by treatment and colour (Table 2). The ensiled GP had an approximately 50% lower total polyphenol content than the fresh GP ($p < 0.05$), without effect of the ensiling additive. The TPC ranged between 44 mg GA (Riesling) and 65 mg GA (Portugais bleu) per g GP. Although the differences between cultivars were not significant, the average TPC content of all red GP (58 mg GA/g GP) was significantly higher than of all white GP (48 mg GA/g GP).

3.1.3. Metabolisable energy

The ME content of GP was calculated according to two methods. The ME_{DC} was calculated based on the digestible nutrients estimated in the digestibility trials in the second part of this study, thus, considering differences between white and red GP. In contrast, the ME_{DLG} does not distinguish between white and red GP and was calculated on the basis of digestible nutrients presented in the DLG feeding value tables (DLG 1997). It is obvious that the ME_{DC} is generally higher than the ME_{DLG} for all GP regardless of cultivar, treatment and colour.

The ME content of GP was influenced significantly by the cultivar, the treatment and the colour (Table 2). Both ME_{DC} content and ME_{DLG} content were higher in the red GP than in the white GP ($p < 0.05$), but the difference between red and white GP was stronger for ME_{DC} than for the ME_{DLG} content. The differences between red and white GP were also evident when considering the single GP cultivars: both ME_{DC} content and ME_{DLG} content were higher in the red GP cultivars Dornfelder, Pinot noir and Portugais bleu than in the white GP cultivars Pinot blanc and Riesling ($p < 0.05$). Regarding the effect of treatment, it is obvious that ensiling resulted in a higher ME content of GP; for ME_{DC} the difference was significant without additive and for ME_{DLG} with additive.

Table 2. Analysed crude nutrients, total phenolic contents (TPC) and metabolisable energy (ME) of selected grape pomace (GP).

	Dry matter [g/kg]	Organic matter [g/kg DM]	Crude protein [g/kg DM]	Ether extract [g/kg DM]	Crude fibre [g/kg DM]	Ash [g/kg DM]	Sugar [g/kg DM]	TPC [mg gallic acid/g GP]	ME_{DC}* [MJ/kg DM]	ME_{DLG}° [MJ/kg DM]
Effect of cultivar										
Pinot blanc (n = 4)	365 ± 36	879^{ab} ± 31	89^{a} ± 7	31 ± 3	158^{a} ± 24	121^{ab} ± 31	318^{b} ± 109	54 ± 13	5.3^{a} ± 0.2	4.4^{a} ± 0.2
Riesling (n = 6)	363 ± 33	860^{a} ± 36	93^{a} ± 9	27 ± 5	175^{a} ± 24	140^{a} ± 36	283^{b} ± 60	44 ± 11	5.1^{a} ± 0.2	4.3^{a} ± 0.2
Dornfelder (n = 5)	374 ± 66	943^{b} ± 23	138^{b} ± 11	36 ± 6	255^{b} ± 49	57^{b} ± 23	69^{a} ± 99	57 ± 23	6.1^{b} ± 0.1	4.8^{b} ± 0.1
Pinot noir (n = 5)	421 ± 59	912^{ab} ± 60	102^{a} ± 33	38 ± 10	217^{ab} ± 42	88^{ab} ± 60	94^{a} ± 146	56 ± 16	5.9^{b} ± 0.4	4.6^{ab} ± 0.3
Portugais bleu (n = 2)	395 ± 104	953^{ab} ± 19	99^{ab} ± 2	32 ± 9	244^{ab} ± 14	47^{ab} ± 19	213^{ab} ± 1	65 ± 18	6.0^{b} ± 0.3	4.7^{ab} ± 0.2
Effect of colour										
White (n = 10)	364 ± 32	868^{a} ± 34	91^{a} ± 8	29^{a} ± 5	168^{a} ± 24	132^{b} ± 34	297^{b} ± 80	48^{a} ± 6	5.2^{a} ± 0.2	4.3^{a} ± 0.2
Red (n = 12)	397 ± 66	932^{b} ± 43	117^{b} ± 28	36^{b} ± 8	237^{b} ± 43	68^{a} ± 43	103^{a} ± 119	58^{b} ± 13	6.0^{b} ± 0.3	4.7^{b} ± 0.2
Effect of treatment										
Untreated (n = 22)	382 ± 55	903 ± 50	105^{a} ± 25	33^{a} ± 7	206^{a} ± 50	97 ± 50	191^{b} ± 141	56^{b} ± 11	5.6^{a} ± 0.5	4.5^{a} ± 0.3
Ensiled without additive (n = 22)	354 ± 49	907 ± 41	109^{ab} ± 50	41^{b} ± 6	231^{ab} ± 24	93 ± 41	0.7^{a} ± 0.5	32^{a} ± 11	6.0^{b} ± 0.3	4.6^{ab} ± 0.2
Ensiled with additive (n = 22)	371 ± 51	921 ± 27	130^{b} ± 15	43^{b} ± 7	235^{b} ± 24	79 ± 27	4.8^{a} ± 35	29^{a} ± 12	5.8^{ab} ± 0.4	4.7^{b} ± 0.2

Notes: *Calculated value based on digestible nutrients of the digestibility trial in the second part of this study with distinction between white and red GP; °Calculated value based on digestible nutrients according to German feeding tables (DLG 1997) without making differences between white and red GP.
[ab]Means not sharing the same superscript are significantly different ($p < 0.05$).

3.2. Digestibility of nutrients and energy content of dried and ensiled GP

Digestibility of nutrients estimated by multiple regression analysis using a common intercept and individual slopes for test sources, and ME and NEL values calculated on the basis of digestible nutrients are shown in Table 3. The common intercept represents the digestibility of nutrients from a ration consisting of 100% hay.

The digestibility of OM and the crude nutrients CP, CF, NDF_{OM} and ADF_{OM} was higher for ensiled GP (Trial 3) than for the dried GP (Trials 1 and 2), whereas the digestibility of EE did not differ between ensiled and dried GP. The estimated digestibility of OM, CP, EE and CF was slightly higher for dried red GP (Trial 2) than for dried white GP (Trial 1), except NDF_{OM} and ADF_{OM}. Both NDF_{OM} and ADF_{OM} were digested at lower rates from the test diets containing dried red GP (Trial 2) than dried white GP (Trial 1).

According to the differences in nutrient digestibility between ensiled GP and dried GP, the calculated ME and NEL values were highest for the ensiled GP. Calculated ME and NEL values were approximately 0.8 MJ per kg DM higher for dried red GP than for dried white GP (Table 4).

4. Discussion

4.1. Content of crude nutrients and TPC of different GP cultivars

At present, little is known about the feeding value and the TPC content of GP from different grape varieties. Baumgärtel et al. (2007) already demonstrated that the energy value of fresh GP from a German winegrowing area varies greatly between a white and a red variety. In spite of this fact, in the feeding value tables of the DLG (1997) only one energy value of dried GP is provided obviously not considering differences between white and red GP. Although ensiling is an inexpensive conservation technique which can be applied also for fresh GP having a limited storage ability, information on its effect on crude nutrient content, polyphenol stability and nutrient digestibility of GP is scarce.

Our results indicated that the TPC of GP is influenced by grape variety and colour, a finding that is in agreement with investigations of Baumgärtel et al. (2007). But in the present study the TPC content of red GP was significantly higher than in white GP, whereby the findings of Baumgärtel et al. (2007) suggest only a marginal higher TPC content in red than in white GP. In contrast to these results, De La Cerda-Carrasco et al. (2014) reported higher concentrations of TPC in white GP (Sauvignon blanc, Chardonnay) than in red GP (Cabernet sauvignon, Carmenere) obtained from a wine-growing area in Chile, being consistent with a more complete extraction of polyphenols from berries during red winemaking process. It is well-known that the concentration and the composition of TPC depend largely on edaphic, geographic, weather-related factors, viticultural practice and winemaking techniques (Obreque-Slier et al. 2013; De la Cerda-Carrasco et al. 2014). Thus, these variations in TPC content might be due to regional differences in the extraction process in winemaking and differences in the concentration of TPC between grape varieties. Nevertheless, the present findings suggest that both red and white GP are suitable sources for the enrichment of feeding rations with TPC.

Regarding crude nutrients, the present study revealed that GP from red varieties have significantly higher concentrations of OM, CP, EE and CF, and lower concentrations of ash and sugar than GP from white varieties. Similar results were reported for GP from the Saale-Unstrut region in Germany (Baumgärtel et al. 2007) and from a Turkish

Table 3. Nutrient digestibility of dried and ensiled Riesling as well as of Pinot noir grape pomace (GP) based on common intercepts and slopes determined by multiple linear regression analysis.

		Slope			Estimated digestibility [%]		
	Common intercept	Trial 1 Dried white GP (Riesling)	Trial 2 Dried red GP (Pinot noir)	Trial 3 Ensiled white GP (Riesling)	Trial 1 Dried white GP (Riesling)	Trial 2 Dried red GP (Pinot noir)	Trial 3 Ensiled white GP (Riesling)
Organic matter	52.2 ± 1.48	−0.19 ± 0.05	−0.14 ± 0.01	0.00 ± 0.04	34	39	52
Crude protein	44.0 ± 1.89	−0.30 ± 0.05	−0.27 ± 0.04	−0.17 ± 0.12	14	17	27
Ether extract	39.3 ± 1.91	0.57 ± 0.03	0.57 ± 0.05	0.37 ± 0.07	96	97	76
Crude fibre	54.6 ± 1.34	−0.28 ± 0.04	−0.28 ± 0.03	−0.06 ± 0.02	26	27	49
Neutral detergent fibre	55.4 ± 3.17	−0.32 ± 0.10	−0.43 ± 0.02	−0.18 ± 0.08	23	12	38
Acid detergent fibre	44.8 ± 3.70	−0.33 ± 0.11	−0.35 ± 0.00	−0.08 ± 0.11	12	10	36

Table 4. Calculated content of metabolisable energy (ME) and net energy (NE) [MJ/kg DM] * of dried and ensiled Riesling as well as of Pinot noir grape pomace (GP).

	Hay	Trial 1 Dried white GP (Riesling)	Trial 2 Dried red GP (Pinot noir)	Trial 3 Ensiled white GP (Riesling)
ME [MJ/kg DM]	7.22	5.76	6.54	7.36
NEL[MJ/kg DM]	4.20	3.13	3.64	4.16

Notes: *Based on digestible (d) nutrients, equations according to GfE (2001); ME [MJ/kg DM] = 0.0312 · dEE [g/kg DM] + 0.0136 · dCF [g/kg DM] + 0.0147 · (dOM – dEE – dCF) [g/kg DM] + 0.00234 · CP [g/kg DM]; NEL [MJ/kg DM] = 0.6 {1 + 0.004 · (q – 57) · ME [MJ/kg DM]}; GE [MJ/kg DM] = 0.0239 · CP [g/kg DM] + 0.0398 · EE [g/kg DM] + 0.0201 · CF [g/kg DM] + 0.0175 · NfE [g/kg DM]; q = {ME [MJ/kg DM]/ GE [MJ/kg DM]} · 100.

winegrowing area (Basalan et al. 2011). Our data regarding concentrations of OM, CF and ash in fresh red GP are in agreement with those reported by the DLG (1997). In contrast, those in fresh white GP are lower than reported there, but are comparable with those of specific white GP varieties reported in the literature (Spanghero et al. 2009). Thus, these findings indicate that the nutrient contents of GP reported by the DLG (1997) are not suitable to estimate the feeding value of GP from specific white and red varieties correctly. This also applies to the calculation of the ME content, which was found to be higher for GP from red varieties than for GP from white varieties in our study based on different digestibility coefficients for white and red GP.

For efficient utilisation as animal feed, ensiling of GP is besides drying a good possibility of preserving the seasonally produced GP, which is rich in moisture and thus easily perishable. Furthermore, ensiling can be easily performed with GP due to its significant sugar content. On the other hand, the present study demonstrates that the ensiling decreases the TPC content of GP to approximately 50% of the initial content. A decrease in the content of TPC and/or polyphenol classes, like tannins, anthocyanins or saponins, during ensiling of GP has been also reported by Pirmohammadi et al. (2007) and Cardona et al. (2009) and is ascribed to polymerisation and oxidation of TPC, especially tannins (Ben Salem et al. 2005; Ribereau-Gayon et al. 2006). Thus, our findings suggest that an enrichment of TPC in ruminant diets with the aim of inducing health-promoting effects associated with these compounds, e.g. anti-inflammatory effects, can be achieved at low inclusion levels (1%, DM basis) particularly with fresh or dried GP, rather than with ensiled GP. Tannin phenolics (50–75% of TPC content in GP) and other polyphenols (anthocyanins, flavanols) from grapes and fruits are well-known to inhibit digestive enzymes including α-amylase, α-glucosidase and lipase and nutrient digestibility (McDougall et al. 2008; Chamorro et al. 2013). Therefore, adverse effects on animal performance are less likely to occur when ensiled GP, with lower amounts of total and tannin phenolics, is included in rations. Additionally, we found that the usage of an ensiling additive, which improves the ensiling velocity and prevents losses during the ensiling process, increased the concentrations of CP, EE, CF and markedly decreased the sugar content compared to untreated GP. The latter result was expected and in line with recent observations (Alipour and Rouzbehan 2007; Cardona et al. 2009) and is due to sugar fermentation by the epiphytic lactic acid bacteria to lactic acid and other organic acids under moist and anaerobic conditions (Weinberg et al. 1988). While some recent studies are in line with our observations regarding the increase of CP content (Ebrahim Pour et al. 2014;

Yaghoubi et al. 2014) and cell wall fractions (CF or NDF/ADF) of ensiled GP (Makkar and Singh 1993; Alipour and Rouzbehan 2007), other studies reported contradictory results (Alipour and Rouzbehan 2007; Pirmohammadi et al. 2007). Regarding the effect of ensiling on energy content, our study confirms recent findings that ensiling of GP causes a slight increase of ME content (Yaghoubi et al. 2014). However, this finding is in contrast to results of two other studies reporting that the ME content is reduced during ensiling of GP along with a decrease of OM digestibility and in vitro-gas production (Ben Salem et al. 2005; Alipour and Rouzbehan 2007).

4.2. Nutrient digestibility and energy content of dried and ensiled GP

A further aim of this study was to determine the feeding value of GP by calculating nutrient digestibility and energy content using sheep as model ruminants. Data on this topic are very scare; only one study investigating the feed value for sheep of fresh GP from two different grape varieties originating from Germany is known (Baumgärtel et al. 2007). Other published data on the feed value of dried or ensiled GP originate from non-EU countries, such as Iran (Pirmohammadi et al. 2007, 2012), Japan (Ishida et al. 2014), Italy (Spanghero et al. 2009) and Turkey (Basalan et al. 2011). These data are not suitable to estimate the feed value of GP originating from Germany for ruminants with a sufficient correctness. Our study shows that the digestibility of OM and all crude nutrients (except EE) was markedly higher for ensiled GP than for dried GP of both grape varieties. The observed improvement of OM, CP and fibre digestibility by ensiling can be largely explained by the degradation of cell structure in the course of microbial fermentation, thereby increasing the release of anti-nutritive tannins from GP (Ribereau-Gayon et al. 2006) and consequently increasing the OM digestibility of ensiled GP. In line with this, an addition of polyethylene glycol (PEG) forming PEG-tannin complexes and thereby preventing tannins from binding proteins increases the estimated OM digestibility of GP (Alipour and Rouzbehan 2007). Only very few reports investigated the effect of ensiling of pomace from wine grape or other fruits on the digestibility of nutrients (Pirmohammadi et al. 2007; Taasoli and Kafilzadeh 2008). In contrast to our results, these studies revealed either a decrease of OM and/or nutrient digestibility of GP from white varieties or apple pomace or no effect at all of ensiling compared with drying. Regardless of this, in the present study on sheep the calculated ME content of ensiled GP was 0.8 MJ and 1.6 MJ/kg DM higher than for dried red and dried white GP, respectively. This result was in line with the increased digestibility of OM and crude nutrients of ensiled GP.

Concerning the effect of the grape variety/colour of GP, our study showed that the digestibility of OM, CP, EE and CF and, consequently, the ME values for sheep were slightly higher for dried GP from the red (Pinot noir) than the white (Riesling) variety. In contrast, Spanghero et al. (2009) observed no difference between white and red varieties regarding ruminal degradability of GP and grape seeds. While for sheep the CP digestibility of dried GP from red compared with white varieties determined in our study are in a similar range as presented in the DLG feeding tables (DLG 1997), very different results regarding CP digestibility for sheep of dried GP were reported from others (Baumgärtel et al. 2007; Ishida et al. 2014).

But it has to be considered that the presented digestibility trial has one limitation: since the CP content of all GP samples and hay analysed in our pre-investigations reached the recommended dietary CP content of at least 12% (GfE 1991), we did not add any other protein source to the ration during the digestibility trials. However, the analysed CP content of GP and hay of the digestibility trials were slightly below this recommended

value. This indicates that the microbial N-requirement was possibly not fully met during the digestibility study and the amount of utilisable CP at the duodenum was not sufficient to cover the amino acid requirement of the sheep completely and the enzymatic digestive capacity of the sheep was slightly reduced. Due to this the calculated nutrient digestibility found in the present study may be lower compared with other trials. Regardless of this, our data are appropriate to evaluate differences in the nutrient digestibility between dried white GP, dried red GP and ensiled white GP because the CP content of the rations was similar between the three digestibility trials.

5. Conclusions

The present data suggest that both red and white GP are suitable dietary sources for the enrichment with TPC. Compared with drying, ensiling improves the feed value of GP and is a good possibility of preserving the seasonally produced by-product of wine-making. Despite the fact that ensiling causes a 50% loss of TPC content of GP, ensiled GP, which has a low energy content comparable with straw of wheat and oat, might replace other low-quality roughages for ruminants. In addition, our data clearly show that a distinction between white and red GP is required to calculate the ME content of GP correctly.

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3 Summarised discussion

The transition period of high-yielding dairy cows represents the most critical period due to the metabolic load and the inflammation-like conditions which are associated with the development of liver-associated diseases such as fatty liver syndrome and ketosis. It has been found that metabolic and inflammatory stress contributes to the development of stress of the endoplasmic reticulum of early lactating cows (Gessner et al. 2014). Polyphenols of the flavonoid class have been shown to have promising antioxidant as well as strong anti-inflammatory potential. Several studies performed with models of inflammation such as obese rats and mice fed a high-fat diet or rats which livers induced by endotoxins, dietary supplementation of various types of flavonoids decreased inflammation in the liver and reduced the progress of hepatic steatosis (Terra et al. 2009, Bharrhan et al. 2011, Heber et al. 2014). Additionally, Giordano et al. (2014) and Rodriguez et al. (2015) reported that flavonoids contributes to an attenuation of ER stress in the liver and muscle cells of humans. However, to our knowledge, less information is available about effects of polyphenols on antioxidant status as well as on inflammation of periparturient dairy cows. Due to the inflammatory-like condition during the transition period of dairy cows, in **study 1** and **study 2** we investigated the hypothesis that adding flavonoids originating from different plant sources could reduce inflammation and ER-stress in the liver of dairy cows during early lactation. Whereas, the antioxidant as well as the anti-inflammatory potential of several flavonoid-rich plant sources such as GP, green tea and curcumin could be already detected in recent studies (Gessner et al. 2013b, Park et al. 2012, Aggarwal et al. 2005, Lim et al. 2001), as plant products rich in flavonoids, we used a grape seed and grape marc meal extract (GSGME, **study 1**) as well as a plant product consisting of green tea (95%) and curcuma extract (5%) (**study 2**). Therefore, two groups were separated into control and treatment groups, whereby cows of the treatment group fed a diet supplemented with 1% of GSGME or 0.175 g of the plant product (green tea & curcuma extract) per kg dry matter, respectively.

In order to investigate the effects of supplementing GSGME and the plant product consisting of green tea and curcuma extract on the inflammation process, the hepatic mRNA abundances of genes of UPR, Nrf2 pathway and inflammation were determined at week 1 and 3 of lactation. The determined genes involved in the UPR, Nrf2 pathway and inflammation showed significant effects caused by the supplementation of different flavonoid-rich plant sources in both studies.

Trends were observed toward a reduction of mRNA concentrations in **study 1** concerning the genes XBP1 (involved in UPR) and UGT1A1 (involved in Nrf2 pathway) at weeks 1 and 3, and in **study 2** regarding HP, a gene involved in inflammation indicating that adding GSGME can moderately reduce ER stress in the liver of cows during early lactation and that supplementing a plant product consisting of green tea and curcuma extract can moderately attenuate the pro-inflammatory condition in the liver of periparturient dairy cows. Additionally, in **study 2**, the mRNA abundances of almost all the UPR genes were numerically reduced, indicating that the plant product consisting of green tea and curcuma extract had only a moderate effect on the occurrence of ER stress in the liver of dairy cows.

One interesting finding of both studies is that adding the flavonoid-rich plant products to cow´s diet caused a significant down-regulation of FGF21, a gene involved in the UPR, which might be a useful biomarker for liver fat accumulation (Schlegel et al. 2012). These findings indicates that ER stress in the liver during early lactation was attenuated, whereby the hepatic TAG concentration in **study 1** was not positively correlated with the plasma concentration of FGF21, as observed in dairy cows (Schoenberg et al. 2011). This suggestion is in accordance with the observation of an increased plasma retinol concentration observed in cows received the flavonoid-rich plant products (GSGME or green tea and curcuma extract) suggesting that the synthesis of retinol-binding protein, a member of the negative acute phase protein, is enhanced, indicative of an improved liver function.

In order to assess the antioxidant status of dairy cows, α-tocopherol, β-carotene, TEAC, and TBARS were determined in **studies 1** and **2**. In both studies, the concentrations of these antioxidant parameters in plasma were not influenced by the different supplemented flavonoid-rich plant sources indicating that adding polyphenols did not lead to an improvement of the systemic antioxidative status. These findings are in contrast to several other findings (Chis et al. 2009, Choi et al. 2010, Choi et al. 2012) suggesting that further investigations performed with an increased sample number of periparturient dairy cows have to be carried out.

In **studies 1** and **2**, the supplementation of different flavonoid-rich plant sources significantly improved the daily amount of energy-corrected milk yield (adjusted to 4% fat and 3.4% protein) and the daily amount of crude protein from week 2 to week 9 of lactation associated with no differences between the control and treatment group in dry matter and energy intake as well as in energy balance, respectively. Thereby, the increased milk yield might be explained by the effect of condensed tannins which have beneficial effects forming hydrogen bonds

(McSweeney et al. 2001a, Makkar 2003) which are stable between pH 3.5 and 8, whereby these complexes dissociate when the pH falls below 3.5, such as in the abomasum (pH 2.5-3), or is greater than 8, such as in the duodenum (pH 8, Hagerman et al. 1992). Consequently, the effect of tannins on dietary protein decreased their ruminal degradation leading to a greater availability of (mainly essential) amino acids for absorption in the small intestine (Barry and McNabb 1999, Min et al. 2003). Thus, the significantly increased daily amount of milk protein in **study 1** might be explained by a greater availability of intestinal amino acids. Furthermore, a reduced degradability of dietary protein in the rumen by adding tannins to diet is associated with a lower production of ammonia (McSweeney et al. 2001b, Tiemann et al. 2008, Dschaak et al. 2011, Anantasook et al. 2014) in the rumen by the degradation of protein which might reduce the metabolic load of the liver by a reduction of hepatic detoxification of ammonia, which improves liver function, thus promoting greater nitrogen retention by reducing urea excretion. Several studies also reported an increased milk production in sheep grazing *L. corniculatus* compared with sheep treated with polyethylene glycol (Wang et al. 1994, Douglas et al. 1995, Min et al. 1998). It has been reported that milk production enhanced with the increased intake of tannins contained in *L. corniculatus* (Wang et al. 1996, Turner et al. 2005) in ruminants. Anantasook et al. (2014) reported an increased milk production fed tannins to lactating dairy cows. Furthermore, Hymes-Fecht et al (2005) reported that the effects of condensed tannins on retarding forage N-degradation contributes to an increased milk production in dairy cows fed birdsfoot trefoil over alfalfa silage. In contrast, Benchaar et al. (2008) revealed that supplementing tannins to diet have no effects on milk production and composition.

In **studies 1** and **2**, the milk fat concentration was not influenced supplementing flavonoid-rich plant sources which is in line with several other studies (Harris et al. 1998, Woodward et al. 1999).

Despite the fact that recent studies reported that grape seed and grape marc meal extract rich in polyphenols had anti-inflammatory effects on rodent models and pigs (Gessner et al. 2013b, Fiesel et al. 2014), our findings (**study 1**) suggests that feeding GSGME had fewer effects on the ER stress and inflammation in the liver of dairy cows. The mRNA abundances analysed by the qPCR of a total of 3 genes involved in inflammation and 14 genes involved in ER stress response in liver samples of week 1 and 3 postpartum were just numerically reduced in the cows supplemented with GSGME, because of high variations. Therefore in **study 3,** we performed

genome-wide transcript profiling in the liver and lipid profiling in plasma of dairy cows, based on the same sample material used **in study 1**, in order to identify changes in potentially critical signaling or metabolic pathways associated with an explaination of the increased milk yield in **study 1** by feeding GSGME during the transition period at 1 week postpartum. In **study 3**, the findings from transcriptome analysis of the liver of cows fed GSGME during the transition period at 1 week postpartum indicates that polyphenol-rich feed components are able to significantly down-regulate a large set of genes involved in ER stress-induced UPR and inflammatory processes. The observation that GSGME induces specific miRNAs indicates that at least some of the GSGME effects on the hepatic transcriptome of dairy cows are mediated by miRNA-mRNA interactions. In contrast, transcriptome analysis of the liver of these cows did not reveal alterations in the expression of genes involved in important metabolic pathways, such as lipid metabolism. This finding is in agreement with our results from plasma lipid profiling demonstrating no differences in the concentrations of individual species of major and minor lipid classes between cows fed GSGME and control cows. Considering that both ER stress and inflammatory processes are considered to contribute to liver-associated diseases, which frequently occur during early lactation in high-yielding dairy cows, and to impair milk performance in dairy cows, it is likely that inhibition of ER stress and inflammation is responsible for the recently observed increase in milk yield of dairy cows fed GSGME.

The findings in **study 2** were similar concerning the effects on the inflammation process and the occurrence of ER stress in the liver of dairy cows supplementing cow´s ration with green tea and curcuma extract.

Meanwhile, near parturition, the feed intake of dairy cows is reduced, after parturition the demand for energy is progressively increased by the initiation of lactation which is compensated by the mobilisation of NEFA from adipose tissue. During the transition period of dairy cows, the fat mobilisation is increased, associated with the marked formation of acetyl-coenzyme A, leading only to partial metabolism of fatty acids by the tricarboxylic acid cycle. As a consequence, acetyl coenzyme A is converted to acetoacetate which is then reduced to BHBA by BHBA dehydrogenase or spontaneously decarboxylated to acetone. Consequently, high concentrations of ketone bodies indicate that adaptability of metabolism is exceeded. Therefore, plasma NEFA and BHBA are important metabolic parameters in dairy cows during early lactation. Although the plasma NEFA concentration was not influenced by feeding GSGME (**study 1**), which is in accordance with the similar observed energy balance in both groups, in

study 2 the NEFA concentration in plasma was reduced adding a plant product consisting of green tea and curcuma extract indicating that during early lactation the mobilisation of body fat from adipose tissue as a major source of energy to the cow could be reduced. Furthermore, we observed an unexpected increase of BHBA concentration in plasma in weeks 1, 3 and 5 in cows fed the GSGME (**study 1**), indicating that feeding GSGME changes the rate of ketogenesis due to a lower availability of glucose. This was caused by the increased lactose synthesis in the GSGME group which produced 3 to 4 kg more milk compared to the control group at a similar dry matter intake. In contrast, in **study 2**, the BHBA concentration in plasma did not differ between the control and treatment groups, indicating that the rate of hepatic ketogenesis is at a similar level in both groups. During the transition of late pregnancy and early lactation, the enhanced plasma NEFA concentration leads to the increased hepatic uptake of fatty acids, their subsequent esterification, and accumulation of TAG in the liver. In **study 1** of this thesis, the observed TAG and cholesterol concentrations in the liver were not influenced by supplementing GSGME. However, adding a plant product consisting of green tea and curcuma extract (**study 2**) led to reduced concentrations of TAG and cholesterol in the liver of cows, indicating that the development of fatty liver could be prevent. These different findings of **study 1** and **study 2** could be explained by using different substances, whereby the plant product consisting of green tea and curcuma extract might be better combined agents compared to the GSGME.

Finally, both used flavonoids in **studies 1** and **2** had similar effects on the antioxidant system and the inflammation of periparturient dairy cows. Only differences in concentrations of TAG and cholesterol in the liver of cows were observed supplementing two different flavonoid-rich plant sources to cow´s diet.

The anti-inflammatory as well as the antioxidative effects of GP described in the literature are based on their polyphenol contents which strongly varied between different GP varieties associated with scarce information about the differences in polyphenol content of GP originating from German regions. In addition, information about the feeding value of regional GP, especially Rhineland-Palatinate, is scarce and just one study has been concerned with the feeding value of different GP originating from Germany (Saale-Unstrut) (Baumgärtel et al. 2007). Consequently, in **study 4** of this thesis, we determined the crude nutrient contents and total polyphenol contents of GP from different grape varieties originating from Rhineland-Palatinate (Germany) calculating their energy contents. Additionally, in **study 4** we determined the feeding value of dried white, dried red and ensiled white GP for sheep by calculating their

nutrient digestibility and the contents of ME and NEL. The possibility of using GP as dietary supplement in ruminants such as in **studies 1** and **2** of this thesis are associated with considerations concerning the storage of GP. Whereas fresh GP are not storable because of their quick fermentation and drying of GP is too expensive and not practical for a single farmer, ensiling as an inexpensive conservation method can be easily performed due to its high sugar content, thus preserving the seasonally produced GP. Therefore, in **study 4** we investigated the effect of ensiling GP on the content of crude nutrients and total polyphenols as well as on the feeding value.

Only one energy value is provided in the DLG feeding tables, not considering differences between white and red GP. Thus, in order to enrich the feeding rations of ruminants with GP as a suitable source consisting of TPC, the energy of GP has to be correctly calculated by considering differences between white and red GP. Whereas the determined contents of OM, CP, EE and CF in **study 4** were higher in red than in white GP, which is in line with some other investigations (Baumgärtel et al. 2007, Basalan et al. 2011), the TPC of red GP were higher compared to the white GP which is contrary to several other studies (Baumgärtel et al. 2007, De La Cerda-Carrasco et al. 2014). In view of the fact that the inclusion of GP in diets of ruminants contributes to a dilution of energy in ration, information about the energy content of the used GP has to exist. Thus, we calculated the ME based on the digestible nutrients estimated in the digestibility trials of this thesis in order to compare these ME contents with the single value of ME established in the DLG feeding tables. Concerning this, we calculated a higher ME in red GP compared to white GP indicating that differences between white and red GP are quite useful, whereas the contents of sugar and ash were lower in red GP than in white GP. Thus, differences in the contents of crude nutrients between white and red GP exist, indicating that the nutrient contents of GP reported by the DLG (1997) are not suitable to estimate the feeding value of GP from specific white and red varieties correctly.

Regarding the feeding values of dried white and dried red GP, the digestibilities of OM, CP, EE, CF and energy content were higher for dried red than for dried white GP, whereby the digestibility of NDF_{OM} and ADF_{OM} was lower for dried red than dried white GP. Several effects of ensiling GP could be verified such as an increased concentration of CP (+19%) which can be largely explained by the degraded cell structure during microbial fermentation leading to an increased release of anti-nutritive tannins from GP (Ribereau-Gayon et al. 2006). Furthermore, higher EE (+23%) and CF (+12%) and a higher ME content (+7%) as well as markedly

decreased concentrations of sugar (−99.6%) and TPC (−48%) were found in ensiled GP in comparison to untreated GP varieties. Regarding the reduced TPC of ensiled GP, further investigations of supplementing GP in ensiled form to ruminant diets should be carried out in order to prove if the remaining TPC can induce anti-inflammatory effects in periparturient dairy cows. Additionally, ensiled GP had higher nutrient digestibilities [OM, +32%; CP, +43%; CF, +46%; neutral detergent fibre (NDF), +54%; acid detergent fibre (ADF), +69%] and higher energy values (ME, +16%; NEL, +19%).

These findings indicate that either white or red GP are suitable sources for enrichment with TPC. Considering that the inclusion of GP in diets of ruminants contributes to a dilution of energy in the ration, GP might replace other low-quality roughages for ruminants.

4 Summary and Conclusion

During the transition from late pregnancy to early lactation, cows are typically in an inflammation-like condition, and it has been suggested that inflammation associated with the induction of stress of the endoplasmic reticulum (ER) in the liver contributes to the development of fatty liver syndrome and ketosis. In **study 1** and **study 2** of this thesis, we investigated the hypothesis that supplementing a plant extract rich in flavonoids [**study 1**: GSGME, **study 2**: plant product consisting of green tea (95%) and curcuma extract (5%)] attenuates inflammation and ER stress in the liver of dairy cows. For this purpose, two groups of cows received either a total mixed ration as a control diet or the same total mixed ration supplemented with either 1% of GSGME (**study 1**) or 0.175 g plant product per kg dry matter (**study 2**) over the period from wk 3 prepartum to wk 9 postpartum, respectively. In both studies, dry matter intake as well as the energy balance during wk 2 to 9 postpartum was not different between the 2 groups. However, cows fed the diet supplemented with GSGME or the plant product consisting of green tea and curcuma extract had an increased milk yield and an increased daily milk protein yield. Cows added with GSGME and the plant product had a significantly reduced mRNA abundancy of FGF21, a stress hormone induced by various stress conditions, in the liver in wks 1 and 3 postpartum, indicating an attenuation of metabolic stress in the liver of these cows. Meanwhile in **study 1** the mRNA abundances of a total of 3 genes (involved in inflammation) and 14 genes (involved in ER stress response), as well as the concentrations of triacylglycerols and cholesterol, in liver samples from wks 1 and 3 postpartum did not differ between the 2 groups by supplementing GSGME; in **study 2**, cows fed the plant product showed lower concentrations of triacylglycerols and cholesterol and a trend towards a reduction of mRNA concentration of HP as well as no differences of relative mRNA concentrations of 8 genes of the UPR considered in the liver. Additionally, in both studies, the systemic antioxidative system of cows was not influenced by feeding flavonoid-rich plant products. The findings of **study 1** indicate that supplementation of GSGME can moderately reduce inflammation and ER stress in the liver of cows during early lactation, because of high variations. However, the data of **study 2** suggest that supplementation with plant products consisting of green tea and curcuma extract had a moderate effect on inflammation but less of an effect on the occurrence of ER stress in the liver of dairy cows during early lactation and might be useful to improve milk yield and prevent fatty liver syndrome in dairy cows. To verify the assumption that GSME induces a higher milk yield

associated with a moderate reduction of genes involved in inflammation and ER stress response, **study 3** was conducted. We hypothesized that feeding of GSME influences metabolic pathways in the liver which could account for the positive effects of GSGME in dairy cows. Thus, we performed genome-wide transcript profiling in the liver and lipid profiling in plasma of dairy cows fed GSGME during the transition period at 1 week postpartum based on the same sample material used in **study 1**. Analysis of hepatic transcript profile indicates that polyphenol-rich feed components are able to significantly inhibit ER stress-induced UPR and inflammatory processes, both of which are considered to contribute to liver-associated diseases and to impair milk performance in dairy cows, in the liver of dairy cows during early lactation.

Study 4 was conducted to determine the nutrient and energy content of fresh and ensiled GP from different grape varieties originating from Germany. Furthermore, the feed value of dried white, dried red and ensiled white GP was estimated by calculating nutrient digestibility and the content of metabolisable energy (ME) and net energy lactation (NEL) measured in sheep as a ruminant model. The collected data suggest that there are differences in crude nutrients and energy contents between red and white GP cultivars. Consequently, red GP had higher contents of OM, CP, EE, CF, TPC and ME, whereas the concentrations of ash and sugar were lower than from white cultivars. The ensiled GP had increased concentrations of CP (+19%), EE (+23%) and CF (+12%) and a higher ME content (+7%) and markedly decreased concentrations of sugar (−99.6%) and TPC (−48%) caused by the ensiling process. Concerning the digestibility of GP, ensiled GP had a higher nutrient digestibility (OM, +32%; CP, +43%; CF, +46%; NDF, +54%; ADF, +69%) and higher energy values (ME, +16%; NEL, +19%) compared to the dried GP. The digestibility coefficients of OM, CP, EE and CF and the energy content were higher for dried red than for dried white GP, whereas the digestibility of NDF_{OM} and ADF_{OM} was lower for dried red than dried white GP. The findings of **study 4** revealed that red GP should be distinguished from white GP to calculate the ME content of GP correctly and that both red and white GP are suitable sources for the inclusion in diets of ruminants enriched with TPC. Additionally, ensiling contributes to an improvement of the feed value of GP associated with a 50% loss of TPC of GP which might replace low-quality roughages for ruminants.

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I, Anne Winkler, hereby swear that I have completed the present dissertation independently and without inadmissible external support. I have not used any sources or tools other than those indicated and have identified literal and analogous quotations. The thesis in its current version or another version has not been presented to the Martin-Luther-University Halle-Wittenberg or another university in connection with a state or academic examination.

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