Phytogenic flavonoids influence gene expression in liver cells

Reducing inflammatory processes in dairy cows is of high interest because the immune system activation is an energy-demanding process. That necessitates a reallocation of nutrients and energy from dispensable functions such as growth and production.

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Even subclinical inflammation requires extra energy and amino acids, for the production of acute phase proteins. It also has adverse effects on the metabolism, for example by an increase of plasma cortisol. It can safely be assumed that milk production is increased by an inhibition of inflammation. Dietary polyphenols, especially those of the flavonoid subgroup, are known for anti-inflammatory effects in cows.

According to scientific studies, metabolic pathways in the liver can be positively influenced by flavonoid-rich grape seed and grape marc meal extract (GSGME). Among others, supplementing dairy cow diets with GSGME can lead to increased milk yield. Recent studies show beneficial effects of a GSGME based feed additive on various hepatic genes related to inflammation and ER stress in the liver.

The underlying effects remained unclear. Hence, the goal of this study was to explore metabolic pathways in dairy cows receiving phytogenic flavonoids with their feed.

Material and methods

In order to gain insight into how a polyphenol-rich feed additive influences metabolic pathways, a genome-wide transcript profiling of liver tissue and lipid profiling in blood plasma was performed.

The experiment was conducted in dairy cows during the transition period. In total, the study duration was 12 weeks: three weeks before the expected calving until week nine post-partum.

The trial was carried out at the Educational and Research Centre 'Hofgut Neumühle' in the state of Rhineland-Palatinate, Germany.

In total, 28 Holstein cows with an average parity number of 2.8 were used in this experiment. Cows were assigned to two different experimental groups: A negative control group (n=14) and a treatment group (n=14).

During the study time the diet of the treatment group was supplemented with 1% of a GSGME based polyphenol product (Anta Ox by Dr Eckel Animal Nutrition) based on DM content.

The control group instead received 1% of wheat bran as an isoenergetic replacement.

Each experimental group contained 10 multiparous and four primiparous cows.

Average parity number was similar between groups (control group: 2.8, treatment group: 2.9).

Both groups received a total mixed ration (TMR) diet. In the period between week three antepartum and calving, the diet was calculated to meet the demand of a dry cow with a body weight of 650kg and an assumed dry matter intake (DMI) of 12kg per day.

After calving, the diet was calculated to meet the demand of net energy and crude protein requirement for 34kg daily milk yield, with an assumed daily DMI of 22kg.

The chemical composition was comparable (control vs. treatment, per kg DM: 6.5 and 6.8 MJ NEL, 140 and 166g CP, 383 and 356g neutral detergent fibre).

Critical phase in production

Samples were taken one week postpartum because this is the most critical phase in the production cycle concerning liver metabolism and related stress factors. Blood samples and liver biopsies were taken from every cow (n=28, day seven postpartum ± 2 days).

Blood was taken from the vena caudalis. 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 with a sterile 14-G biopsy needle. Approximately 50mg of liver tissue was immediately snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Total RNA was isolated from liver samples.

Concentration and integrity of RNA was analysed using an Agilent 2100 Bioanalyser. The microarray analysis was taken randomly from six samples of each group (n=12, total), consisting of five samples from the multiparous cows and one sample from the primiparous cows.

The 12 RNA samples were selected, according to the GeneChip WT Plus Reagent Kit (Affymetrix, High Wycombe, UK). Samples were hybridised to the Affymetrix GeneChip Bovine Gene 1.0 Sense Target array representing approximately 23,000 bovine transcripts.

Afterwards, hybridisation arrays were washed and stained (Affymetrix GeneChip Fluidics station 450), scanned and computed from the image data (Affymetrix GeneChip Command Console Software) of every sample.

Bioinformatic prediction of mRNA targets for differentially regulated miRNAs was performed using TargetScan release version 7.1.

In order to extract biological meaning from the expressed transcripts and predicted mRNA targets, gene set enrichment analysis (GSEA) was performed to identify enriched Gene Ontology (GO).

GO was divided in three categories, biological process, molecular function and cellular component.

The interpretation on GO was mainly that biological processes or molecular functions and pathways identified as enriched within up-

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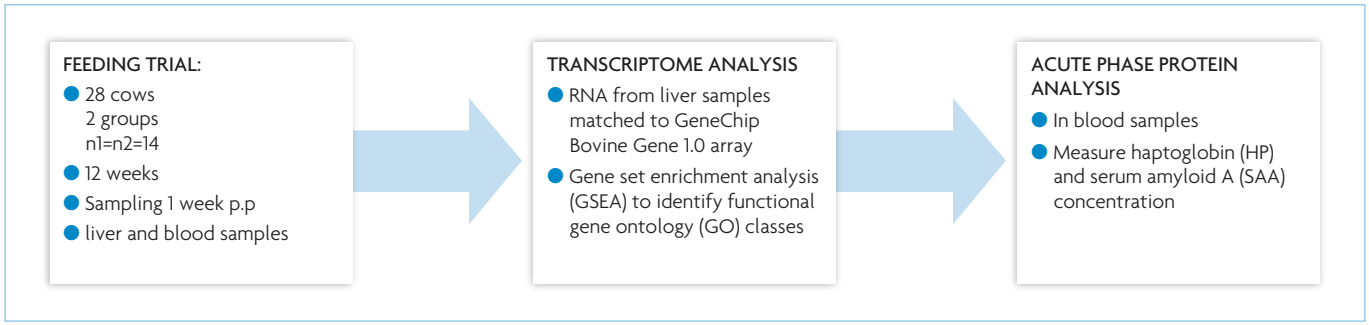
Fig. 1. Overview about the methods used in the study.



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regulated genes are probably activated, whereas down-regulated genes are likely inhibited.

For the qPCR analysis 25 different expressed mRNAs (microarray data) were randomly selected from most strongly up- (n=14) and down-regulated (n=11) mRNAs. The total RNA from all cows (n=14 per group) was for the transcription.

The qPCR protocol is described in Gessner et al. (2017). In blood plasma the acute phase proteins haptoglobin (HP) and serum amyloid A (SAA) were analysed using commercial ELISA kits (CSB-E08585b, CSB-E08592b, Hölzel Diagnostika, Cologne, Germany).

For statistical analysis, data was evaluated by student's t-test (Minitab 13, Minitab Inc, USA) with a multiple testing correction of microarray data.

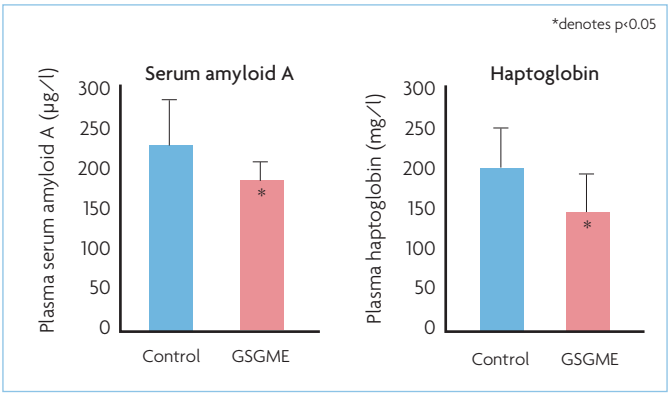


Fig. 2. Plasma concentration of serum amyloid A and haptoglobin in cows of control and treatment group one week post-partum. Mean (bar) and SD (whisker) (Gessner et al 2017).

Results

In total, 207 transcripts were expressed differently in the liver between the control and treatment group. The group with Anta Ox shows 156 up-regulated and 51 down-regulated transcripts.

The up-regulated transcripts were mostly mRNAs (n=155) and one non-protein coding miRNA.

The down-regulated transcripts include 43 mRNAs and eight miRNAs. A striking result was that within the down-regulated genes in the cows from the treatment group, a large number of genes are involved in unfolded protein response (UPR) related to endoplasmatic reticulum stress (ER stress).

For example, X-box binding protein 1 (XBP1) is a critical transcriptional regulator of ER stress. Therefore, the down-regulation of UPR target genes by Anta Ox shows less handling with ER stress factors. UPR target genes aim to restore ER homeostasis. Therefore, typical proteins encoded by UPR target genes, which were identified as down-regulated in the treatment group, are chaperones and co-chaperones. They are implicated in the refolding of proteins, and components of the ER-associated degradation (ERAD) machinery (for example MANF).

Another downregulated ER stress-inducible protein was PHLDA1/TDAG51. It encodes a protein promoting apoptotic cell death when ER stress-induced damage is overwhelming and homeostasis cannot be restored.

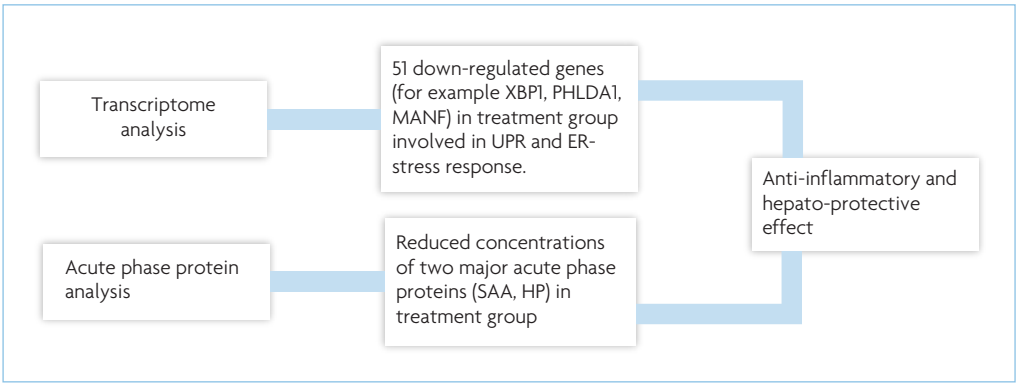
This is important, because other research shows that ER stress-induced UPR genes are up-regulated in the liver of dairy cows from late pregnancy to early lactation, for example XBP1 target genes. It can be assumed that ER stress in the liver of dairy cows has a decisive role in the development of liver diseases. This results in reduced metabolic functions of the liver, health and performance.

Fig. 2 shows the effect on the plasma concentration of the positive acute phase proteins SAA and HP. Acute phase proteins are important inflammation markers.

Concentrations of both acute phase proteins were decreased in the treatment group compared to the control group ($P<0.05$).

The inhibition of hepatic inflammation in the treatment group fits well together with the transcriptome analysis where APP SAA4 was one of the down-regulated genes. Hepatic synthesis of APPs is greatly induced during systemic inflammation triggered by pro-inflammatory cytokines. The acute phase response of the liver, which is determined by the concentrations of SAA and HP in plasma of cows, is inhibited.

Fig. 3. Effects demonstrated by transcriptome analysis and acute phase protein analysis.



Conclusion

Analysis of hepatic transcript profiles revealed important differences between cows receiving phytogenic flavonoids via their diet versus the untreated control group.

In the critical phase one week post-partum, supplementation with dietary polyphenols inhibited ER stress-induced unfolded protein response (UPR) and inflammatory processes on the hepatic cell level.

By inhibiting ER stress and inflammatory processes, Anta Ox reduces the risk of liver associated diseases. This promotes the animal's general health and leads to a better milk performance. ■

References are available from the author on request