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Rumen-protected methionine supplementation during the peripartal period alters the expression of galectin genes associated with inflammation in peripheral neutrophils and secretion in plasma of Holstein cows

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Abstract

The work described in this research communication aimed to investigate whether rumen-protected methionine (Met) supplementation during the periparturient period would affect the expression of galectins in blood-derived neutrophils, and secretion of galectins, IL (interleukin)-1β, IL-6, myeloperoxidase (MPO), and glucose in plasma. Because supplementation of rumen-protected Met would alleviate inflammation and oxidative stress during the peripartal period, we hypothesized that enhancing Met supply would benefit the innate immune response at least in part by altering the expression of galectin genes associated with neutrophil activity and inflammation. Galectins (Gal) have an immuno-modulating effect acting like cell-surface receptors whose activation often results in signaling cascades stimulating cells such as neutrophils. This study revealed an association between Met supplementation and galectin expression and secretion. This implies that galectin expression and secretion can be modulated by Met supplementation. Further studies are needed to evaluate the regulation of galectin gene expression for therapeutic and dietary intervention in the peripartal cow.

The periparturient period of dairy cows (which we define here as from 3 weeks before to 3 weeks after parturition) is characterized by significant metabolic and physiological changes that contribute to a dysfunctional immune system (Sordillo *et al.*, 2009). These metabolic and physiological changes occur concurrently with a high incidence of infectious diseases such as mastitis, metritis and ketosis (Esposito *et al.*, 2014). This has been attributed in large part to reduced neutrophil function. In the quest to reduce cow health disorders, numerous studies have been conducted to better understand the underlying causes of diseases to design effective management practices (Mitchell *et al.*, 2014). It has been established that methionine (Met) supplementation during the periparturient period can improve the health of the dairy cow by boosting the immune response of neutrophils and antioxidant status and by increasing dry matter intake (DMI). Milk yield is also increased. Taken together, Met supplementation during the periparturient period is beneficial to the dairy cow.

Galectins are glycan-binding proteins that are involved in the regulation of vital cellular processes like cell adhesion, migration, proliferation, differentiation, survival, and death. It has been well-established that galectins modulate both survival and apoptotic signaling pathways (Stowell *et al.*, 2007). This makes galectins important candidates in the quest to elucidate the molecular mechanisms underlying immunosuppression in periparturient dairy cows. Because galectin expression and Met supply are both closely linked to immunomodulation, glucose metabolism, inflammation, and the maintenance of overall health, we sought to investigate whether *LGALS* gene expression in neutrophils and concentration in plasma responds to Met supply during the periparturient period. We hypothesized that enhancing Met supply during the periparturient period would benefit the innate immune response at least in part by altering the expression of galectin genes associated with neutrophil activity and inflammation. Objectives of this study were to evaluate mRNA expression of 8 galectin genes in bovine neutrophils, and also assess galectin secretion, IL (interleukin)-1β, IL-6, myeloperoxidase (MPO), and glucose concentrations in plasma during the periparturient period.

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Materials and methods

All protocols for animal handling (Protocol no. 13023) were approved by the Institutional Animal Care and Use Committee of the University of Illinois (Urbana-Champaign, IL, USA).

Animals and management

Fourteen Holstein cows supplemented with Met ($n = 7$) or without (CON; $n = 7$) were used for this study.

Experimental design

The cows used were a subset from a previous experiment (Zhou *et al.*, 2016). In the main experiment, a total of 81 cows were used in a randomized, complete, unbalanced block design with 2×2 factorial arrangement of Met and choline (CHOL) level (with or without). Treatments were control (CON, $n = 20$), with no Met or CHOL supplementation; smartamine (SMA, $n = 21$), CON plus Met at a rate of 0.08% of DM; Reashure (REA, $n = 20$), CON plus CHOL at 60 g/d; or Smartamine and Reashure (MIX, $n = 20$), CON plus Met plus CHOL. Dosage of Met was based on Osorio *et al.* (2014), whereas CHOL was supplemented following the manufacturer's recommendations. Blocking of the cows was done according to expected calving date, body condition score, parity, and previous lactation milk yield. Blocking had no effect ($P < 0.05$). Per Institutional Animal Care and Use Committee guidelines, a subset of 14 periparturient cows (7 cows/treatment) were used for this portion of the study.

Because choline had no effect on production, health status, and little effect on blood biomarkers (Zhou *et al.*, 2016), the focus of the present study was on a subset of the cows ($n = 7$) supplemented with Met (Smartamine M, Adisseo NA, Alpharetta, GA) or controls (CON, $n = 7$). Animals were not blocked in the current study since blocking had no significant effect in the main experiment ($P > 0.05$).

Blood sampling

Complete details of the blood sampling were reported elsewhere (Zhou *et al.*, 2016). Briefly, whole blood (10 ml) was aseptically sampled from the coccygeal vein at −10, 7 and 30 d relative to actual calving date into vacutainer tubes containing a solution of trisodium citrate, citric acid and dextrose (ACD) and lithium heparin (BD Vacutainer). Following blood collection, the tubes were gently mixed, placed on ice, and transported to the Mammalian NutriPhysioGenomics laboratory (Urbana-Champaign, IL, USA) for further analysis. The lithium heparin tubes were centrifuged at $1900 \times \boldsymbol{g}$ for 15 min at 4 °C to obtain the plasma. Plasma was aliquoted and stored at −80 °C until further analysis.

Neutrophil isolation and viability analysis

Neutrophils were isolated based on procedures described by Abdelmegeid *et al.* (2017) with slight modifications (online Supplementary File Materials and Methods).

RNA extraction and cDNA synthesis

Total RNA was isolated using Qiazol according to the manufacturer's instructions (Qiagen, Hilden, Germany). The extraction with Qiazol was done in combination with miRNeasy kits to remove genomic DNA from the harvested RNA (Qiagen, Hilden, Germany). A NanoDrop ND-1000 spectrophotometer was used to measure RNA concentration and purity (NanoDrop Technologies Wilmington, DE) (Asiamah *et al.*, 2016). The purity of RNA (A_{260}/A_{280}) for all samples was above 1.9. RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) (Supplementary Materials and Methods).

Quantitative real-time PCR (qPCR)

RT-PCR (qPCR) was performed in QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems) as described previously (Batistel *et al.*, 2017). Data was analyzed with QuantStudio™ 6 and 7 Flex Real-Time PCR software (version 1.3 Applied Biosystems, CA). The geometric mean of the internal control genes was used to normalize the expression data (online Supplementary File Materials and Methods).

Primer evaluation and design

Primer-3 online tool was used to design the forward and reverse primers for cow galectins *LGALS1, 2, 3, 4, 7, 8, 9, 12* and *15*. Primers specific for *LGALS1, LGALS2, LGALS3, LGALS4, LGALS7, LGALS8, LGALS9, LGALS12* were purchased from IDT (Coralville, IA) (Supplementary Table S1). *GAPDH*, *RPS9,* and *UXT* were used as internal controls and for normalization. Primers were confirmed by using the BLASTN tool to align sequences against the publicly available database at NCBI. Primer testing was performed as described previously by Osorio *et al.* (2014). Amplified PCR products were run on a 2% agarose gel stained with SYBR Safe (2 µl)(online Supplementary Fig. S1).

Detection of galectins -1, -2, -3, -4, -8, -9, -12 in Plasma

Galectin concentrations in plasma from treated and control groups were evaluated using a commercial enzyme-linked immunosorbent assay following the manufacturer's instructions (MyBioSource) (online Supplementary File Materials and Methods).

Detection of cytokines (interleukin-1β (IL1β) and IL-6), myeloperoxidase (MPO) and glucose in plasma

Plasma was analyzed for IL-6 and IL-1β, myeloperoxidase (MPO) and glucose using commercial ELISA kits (Pierce, Thermo Scientific) (online Supplementary File Materials and Methods).

Statistical analysis

All data from Galectin qPCR were log 2 transformed before statistical analysis. Proc MIXED in SAS 9.4 was used to analyze both qPCR and ELISA (MPO, glucose and galectin concentration) data (SAS Institute Inc., Cary, NC). Treatment (T), day (D), and their interaction (T × D) were the fixed effects in the model. Random effect was cow within treatment. Significant differences were declared at $P < 0.05$. Correlation analysis of MPO, cytokines, and galectin concentrations in plasma was done using PROC CORR in SAS 9.4.

Results and discussion

All galectins tested were detected in neutrophils except for *LGALS2* and *LGALS7* (online Supplementary Table S2). Also, all galectins tested were detected in plasma including *Gal2*,

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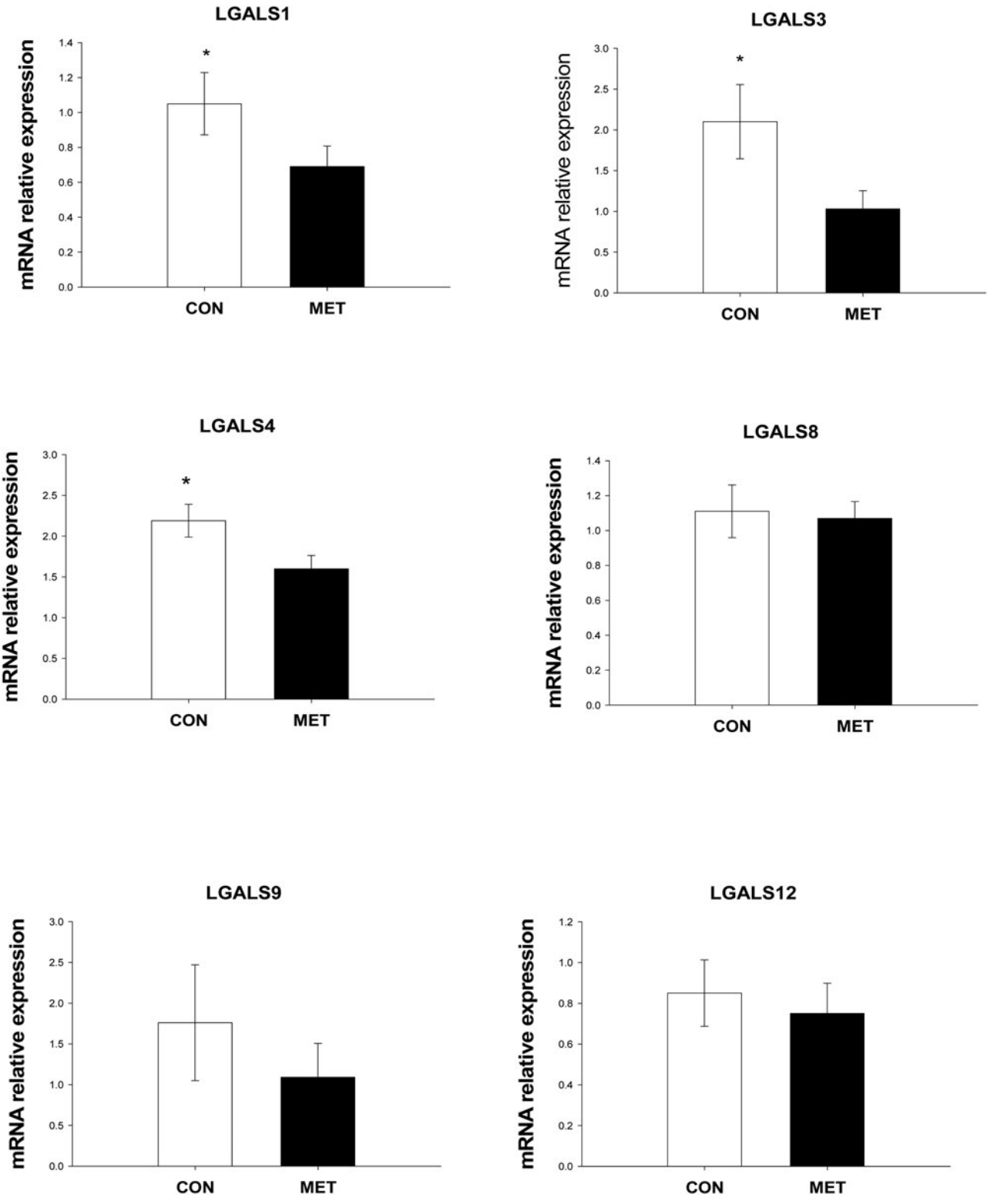

Fig. 1. Transcript abundance of galectin genes (*LGALS*-1, -3, -4, -8, -9, and -12) in Neutrophils of cows supplemented with Methionine (MET, $n = 7$) or Control (CON, $n = 7$) during the pre- and post-partal period.

which was previously undetected in neutrophils. This corroborates previous reports that galectin expression and secretion are cell-specific (Barondes *et al.*, 1994).

The expression of proinflammatory galectins, *LGALS-1, -3, -4* was reduced in neutrophils of Met supplemented cows ($P < 0.05$) (Fig. 1). This may imply that Met reduced inflammation in periparturient cows in part by reducing the expression of these galectins in neutrophils. The improved neutrophil function by Met supplementation in cows which is reported earlier could be as a result of the reduction of these proinflammatory galectins in

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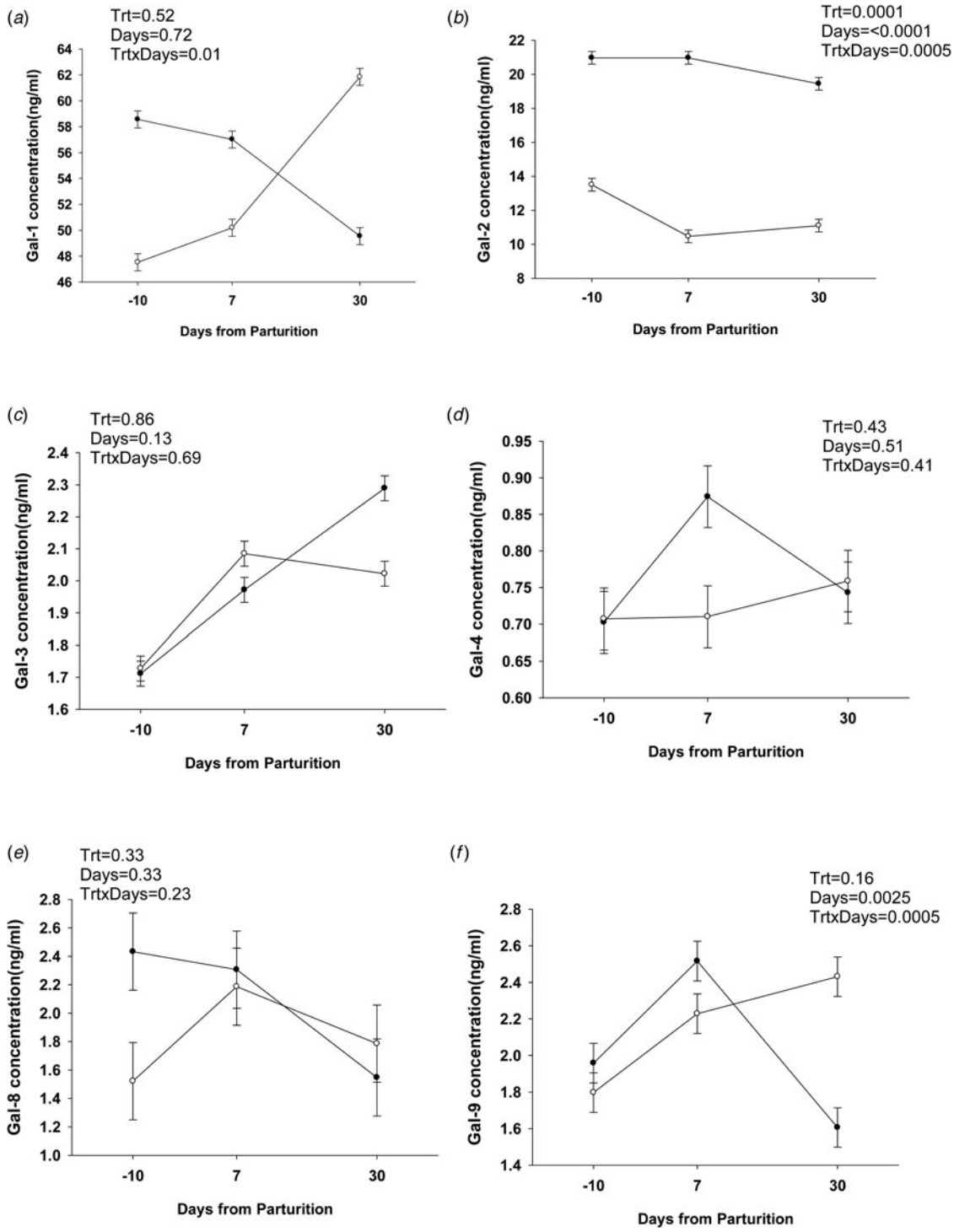

Fig. 2. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA, Alpharetta, GA) on plasma Gal-1, -2, -3, -4, -8, -9 concentration in blood. Values are means, with standard errors represented by vertical bars.

addition to the other inflammatory markers reported previously (Osorio *et al.*, 2014; Zhou *et al.*, 2016). It will be essential to investigate whether reducing transcription of *LGALS1, -3, -4*, in overexpressed conditions, would deliver important therapeutic effects for the cow.

Although Met did not affect MPO, glucose or IL1β and IL6 concentrations in plasma (online Supplementary Fig. S3), Gal secretions were affected. Met reduced *Gal2*(Fig. 2b) and *Gal12* (online Supplementary Fig. S2) concentrations ($P < 0.005$). The reduction in *Gal12* is particularly important *post-partum* because its increase has been implicated in reduced insulin sensitivity post-partum (Drackley *et al.*, 2001). The reduction of *Gal12* by Met ,merits further investigation because it could be vital for the improvement of immune and metabolic function of the cow during the periparturient period. A positive correlation between *Gal2* secretion and IL-1β production was also observed ($P <$

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0.05, $r = 0.49$). IL-1β is a cytokine that is only secreted upon induction and, therefore, not generally expressed in healthy cells or tissues (Borthwick, 2016). The effect of *Gal2* secretion and IL-1β production also merits further investigation with increased sample size for their potential use as biomarkers for inflammatory and metabolic disorders in cows.

The increase in Galectin 9 by Met observed at 30 d (Fig. 2f) could also be attributed to an attempt to restore homeostasis by increasing apoptotic activity in the cow post-calving. This is because *Gal9* induces apoptosis and play a vital role in the regulation of inflammation (Chen *et al.*, 2005).

In conclusion, this study provides new experimental evidence for a crucial role of Met in galectin expression and suggests a potential therapeutic approach for restoring immune tolerance during the periparturient period. Met supplementation could reduce inflammation in cows, and the downregulation of various galectins provides evidence for an association between Met supplementation, inflammation, and alterations in galectin gene expression in neutrophils. Elucidation of the role of galectins in immunoregulation and inflammation will shed more light on how the regulation of galectin expression and their activities can be used for therapeutic purposes in the dairy industry.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029919000736.

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