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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SUPPLEMENTARY FILE

Supplementary materials and methods

Diet

All cows received the same diet −50 to −21 d before expected calving, [1.40 Mcal of net energy for lactation (NEL)/kg of DM] with no Met. Cows received the same close-up diet −21 d to calving (1.52 Mcal of NEL/kg of DM) and were assigned randomly to each treatment. Diets were fed from −21 ± 2 before expected calving date until 30 days in milk (DIM). The Met supplement was top-dressed once daily at the morning feeding using approximately 50 g of ground corn as a carrier for all treatments. Supplementation of Met (0.08% DM of TMR offered) was calculated daily for each cow. The dosage of Met was based on published studies (Osorio, Ji, Drackley, Luchini, & Loor, 2013). Smartamine M was administered in the form of small beads containing a minimum of 75% DL-Met, physically protected by a pH-sensitive coating, which is considered to have a Met bioavailability of 80% therefore, per 10 g of Smartamine M, the cows received 6 g of Metabolizable Met.

Neutrophil isolation

Blood samples were centrifuged at 600 × g for 30 min at 4 °C. The plasma, buffy coat, and approximately one-third of the red blood cells were discarded. The remaining sample was poured into a 50-mL conical tube (Fisher Scientific). Twenty-five milliliters of deionized water at 4 °C was added to lyse red blood cells, followed by addition of 5 mL of 5 × PBS at 4 °C to restore an iso-osmotic environment. Samples were centrifuged at 200 × g for 10 min at 4 °C and the supernatants were decanted. The pellet was washed with 10 mL of 1 × PBS and centrifuged for 5 min (200 × g at 4 °C) and supernatants were decanted. Eight

milliliters of deionized water at 4 °C was added, followed by addition of 2 mL of 5 × PBS at 4 °C. Samples were centrifuged at 500 × g for 5 min at 4 °C and supernatant was decanted. Two subsequent washings using 10 mL of 1 × PBS at 4 °C were performed with samples centrifuged at 500 × g for 5 min at 4 °C and supernatant was decanted. Although no cell differential was performed, this protocol routinely results in >88 % of isolated cells as neutrophils. Neutrophils were immediately homogenized in 2 mL of Trizol Reagent (Invitrogen, Carlsbad, CA) with 1 μL of liner acrylamide (Ambion Inc., Austin, TX) using a Polytron power homogenizer at maximum speed. The suspension was transferred equally into 2 RNA-free microcentrifuge tubes (2 mL; Fisher Scientific) and stored at −80 °C until further analysis. To ensure good quality samples, an aliquot of 20μl was obtained from the isolation process for quantification and viability assessment. Neutrophil quantification and viability was done using a primary granulocyte antibody (CH138A, Veterinary Microbiology, and Pathology, Washington State University, Pullman, WA) and a second antibody (Goat Anti-Mouse IgM, Human ads-PE Southern Biotech) as described previously (Batistel et al., 2017). All samples collected and used for analysis contained over 80% neutrophils and viability was not less than 90%.

Neutrophil Differential and Viability Analysis

From the aliquot obtained during the PMN isolation process, 20 μL were transferred to a 5-mL falcon tube (Corning Incorporated, Durham, NC), added 150 μL of 1 × PBS at 4°C and 100 μL of a granulocyte primary antibody solution (CH138A, Veterinary Microbiology and Pathology, Washington State University, Pullman, WA), homogenized by vortex and incubated on ice for 15 min. A washing step was performed three times by adding 1 mL of 1 × PBS at 4°C, homogenized by vortexing and centrifuged at 1,012 × *g* for 3 min at 4°C. The supernatant was aspirated using a glass transfer pipette (Fisher Scientific, Pittsburgh, PA) until ~100 μL remained at the bottom of the tube. Then, 50 μL of a second antibody solution (4 μg/mL in 1 × PBS) was added (Goat Anti-Mouse IgM, Human ads-PE; SouthernBiotech, Birmingham, AL) and 50 μL of propidium iodide solution (50 μg/mL in 1 × PBS; Sigma-Aldrich, St. Louis, MO), homogenized by vortex and incubated on ice for 15 min. Two washings were performed as described above. Cells were fixed with 150 μL of 4% paraformaldehyde (Sigma-Aldrich) and preserved at 4°C until flow cytometry reading (LSR II, Becton Dickinson, San Jose, CA).

RNA extraction and cDNA synthesis

Isolated RNA had an RNA integrity value of 7. A portion of the RNA was diluted to 100 ng/µL using DNase/RNase free water before reverse transcription. Complimentary DNA(cDNA) synthesis was performed with 100 ng RNA, 1 ug dT18 (Operon Biotechnologies, AL), 1 µL 10 mmol/L dNTP mix (Invitrogen Corp., CA), 1 µL random primers (Invitrogen Corp), and 10 µL DNase/RNase free water. The mixture was incubated at 65 °C for 5 min and kept on ice for 3 min. A total of 6 µL of master mix composed of 4.5 µL 5X First-Strand Buffer, 1 µL 0.1 M DTT, 0.25 µL (50 U) of SuperScript™ III RT (Invitrogen Corp., CA), and 0.25 µL of RNase Inhibitor (10 U, Promega, WI) was added. The reaction was performed in an Eppendorf Mastercycler® Gradient using the following temperature program: 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. cDNA was then diluted 1:4 (v:v) with DNase/RNase free water. An Eppendorf Mastercycler® Gradient was used to perform the reaction. The temperature program was set as follows: 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. The cDNA was diluted with DNase/RNase free water (1:4 (v:v).

QPCR

The PCR reaction mixture consisted of 4 µL diluted cDNA, 5 µL PerfeCTa SYBR Green FastMix ROX (Quanta Biosciences, Beverly, MA), 0.4 µL each of 10 uM forward and reverse primers, and 0.2 µL DNase/RNase free water in a MicroAmp ™. All samples were carried out in triplicate 10-µl reactions in Optical 384-Well reaction plates (Applied Biosystems, CA). A negative control (NTC) with no cDNA and a 6-point relative standard curve were included in every run. The PCR conditions used was: 5 min at 95 °C, 40 cycles of 1 s at 95 °C for denaturing and 30 seconds at 60 °C (annealing + elongation). Gene expression was normalized using the geometrical mean of three appropriate internal control genes: *GAPDH*, *RPS9*, and *UXT* (Ji, Osorio, Drackley, & Loor, 2012). Genes were considered unexpressed when the standard curve had a slope of $-3.50 > y > -3.00$ and $Ct > 30$.

Primer evaluation

A 20 µL PCR reaction was performed as described previously (Osorio et al. 2013), except for the final dissociation protocol. The amplified PCR products were run on 2 % agarose gel using 5µl of amplified cDNA, 1 µl of six times loading dye (Thermo Scientific) at 100 volts for 30 minutes. Gels were stained with SYBR Safe (2 µL), washed and visualized using Bio-Rad gel documentation system (Bio-Rad Laboratories). The product sizes were compared to the expected

product sizes in Table 1. The 15 µL that remained was cleaned using a QIAquick® PCR Purification Kit (Qiagen) and sequenced.

Galectin ELISA

The concentrations of secreted Galectins (Gal) -1, -2, -3, -4, -8, -9, -12 in plasma were determined using commercial bovine ELISA kits (Gal-1 catalog no. MBS2882620, Gal-2 catalog no. MBS033680, Gal-3 catalog no. MBS017323, Gal-4 catalog no. MBS028694 Gal-8 catalog no. MBS041856, Gal-9 catalog no. MBS033074, Gal-12 catalog no. MBS032400; My BioSource Inc, San Diego, CA) according to the manufacturer's instructions. Absorbance was measured at 450nm using microplate reader (Bio Tek). A standard curve was used to determine the concentration.

Briefly, 100µL of each standard and sample was added to appropriate wells. Wells were covered and incubated for 2.5 hours at room temperature with gentle shaking. The solution was discarded and the plate was washed 4 times with 1X Wash Buffer. One hundred (100) µL of 1X prepared biotinylated antibody was added to each well and Incubated for 1 hour at room temperature with gentle shaking. The solution was discarded and wash step repeated. One hundred (100) µL of prepared Streptavidin- Horseradish peroxidase (HRP) was added to each well and incubated for 45 minutes at room temperature. The solution was discarded and the wash step was repeated. One hundred (100) µL of Tetramethylbenzidine (TMB) Substrate was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. Absorbance was measured at 450nm using a microplate reader (Bio Tek). A standard curve was then used to determine the galectin concentration. The sensitivity of the assays was 0.1 ng/mL, the detection range were: Gal1; 0.31ng/mL & 20.0ng/mL, Gal2; 0.625 ng/mL & 20 ng/mL., Gal3; 10 ng/mL & 0.156ng/mL, Gal4; 0.25 ng/mL & 8 ng/mL, Gal8; 0.5 ng/mL & 16 ng/mL. Gal9; .625 ng/mL & 20 ng/mL, Gal12; 31.2 pg/mL &1000 pg/mL. Both intraassay CV (%) and inter-assay CV (%) for all assays was less than 15%. [CV (%) = SD/mean ×100].

Detection of Cytokines (Interleukin-1β(IL1β) and IL -6), myeloperoxidase (MPO) and glucose in Plasma.

Cytokines were analyzed via ELISA (IL-1β catalog no. ESS0027, IL-6 catalog no. ESS0029; Pierce, Thermo Scientific). Glucose was assessed in a clinical auto-analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA) using a commercial kit (catalog no. 0018250840) from Instrumentation Laboratory (Milano, Italy), as previously described (Jacometo et al., 2016). The MPO concentration was also determined with the ILAB 650, using self-prepared reagents, following the protocol described by Bradley et al. (1982).

Supplementary Table S1 Primers used for real-time qPCR

Ascension number	Gene	*Primer*	*Sequence 5'>3'*	*Product Size (bp)*
NM_175782	*LGALS1*	Forward	GGCAAAGACGACAACAACCT	189
		Reverse	*GGTTAGGTCCGTCTGGTTGA*	
NM_001075494	*LGALS2*	Forward	*CCTCACCAGAGAGCAAGACC*	181
		Reverse	*TGGAAAACACCACAGTTGGA*	
NM_001102341	*LGALS3*	Forward	*GAATGATGTCGCCTTCCACT*	165
		Reverse	*TCAGGTTCAACCAGCACTTG*	
NM_001034768.2	*LGALS4*	Forward	*ATTCACGACTCCTGCAGCTT*	215
		Reverse	*CCCCACCTCGAAGTTTACAA*	
XM_010826300	*LGALS7*	Forward	*TCTACGTGAACCTGCTGTGC*	236
		Reverse	*CCGGAAGTGGTGGTACTCAG*	
NM_001045954.2	*LGALS8*	Forward	*TCGTGATCATGGTCCTGAAA*	232
		Reverse	*TGTGACTCGCCAGACTTTTG*	
NM_001015570.3	*LGALS9*	Forward	*TGTACCCCTCCAAGAGCATC*	233
		Reverse	*ACACATGATCCACACCGAGA*	
XM_015467485	*LGALS12*	Forward	*CAGCTGATCCCACTCTCCTC*	176
		Reverse	*AATGCCAGGCTCTCAGAAGA*	
	GAPDH	Forward	*TTGTCTCCTGCGACTTCAAC*	103
		Reverse	*TCGTACCAGGAAATGAGCTT*	
	UXT	Forward	*TGTGGCCCTTGGATATGGTT*	101

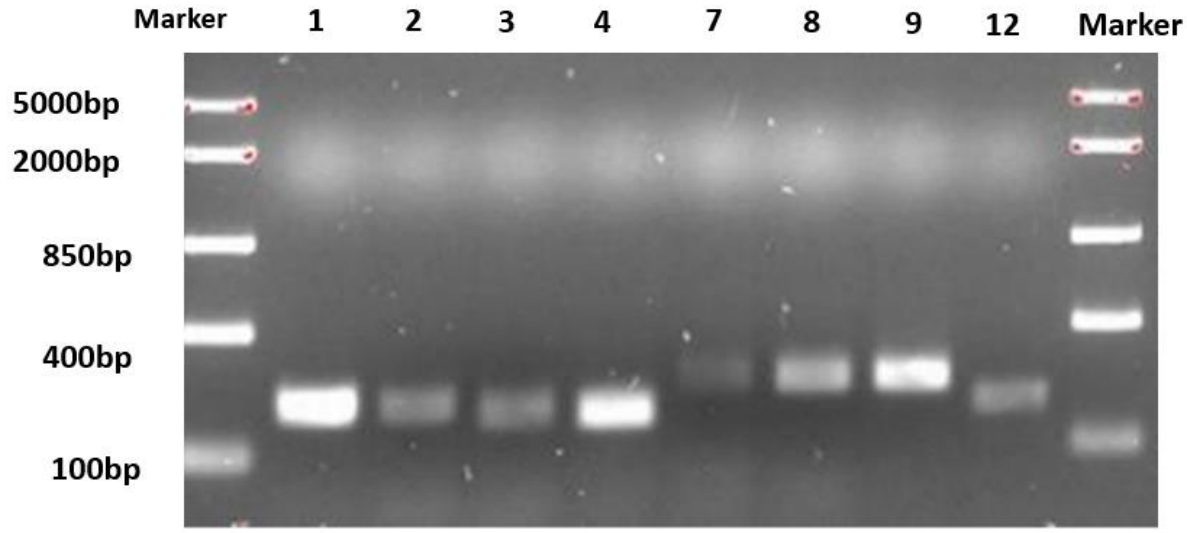
	Reverse	*GGTTGTCGCTGAGCTCTGTG*	
RPS9	Forward	*CCTCGACCAAGAGCTGAAG*	164
	Reverse	*CCTCCAGACCTCACGTTTGT*	

Supplementary Table S2 Effect of Methionine Supplementation on mRNA relative abundance of *LGALS* in bovine neutrophils

	Trt			***P*-values**		
	CON	**Met**	**SEM**	**Trt**	**Day**	**Trt*Day**
LGALS1	1.05 [a]	0.69 [b]	0.18	0.09	0.19	0.36
LGALS2	Undetected					
LGALS3	2.10 [a]	1.03 [b]	0.46	0.03	0.70	0.95
LGALS4	2.19 [a]	1.60 [b]	0.20	0.03	0.31	0.44
LGALS7	Undetected					
LGALS8	1.11	1.07	0.15	0.81	0.54	0.56
LGALS9	1.76	1.09	0.71	0.39	0.82	0.50
LGALS12	0.85	0.75	0.16	0.66	0.65	0.58

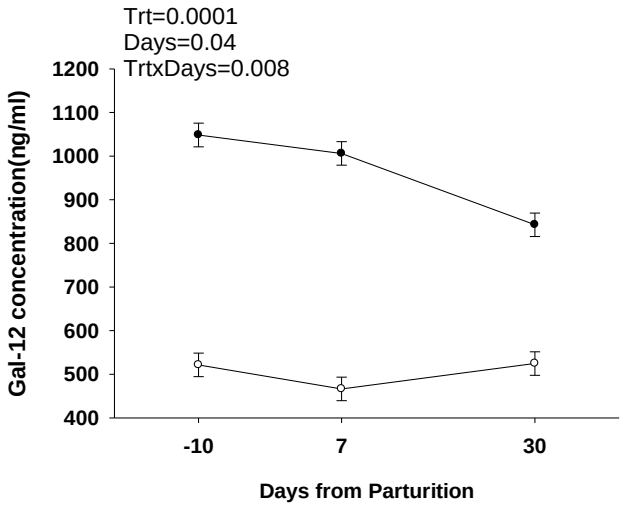
CON = control; Met = rumen-protected Methionine (0.08% of DMI). Means on the same row sharing the same superscript are not significantly different from each other ($P<0.05$).

Supplementary Figure S1:



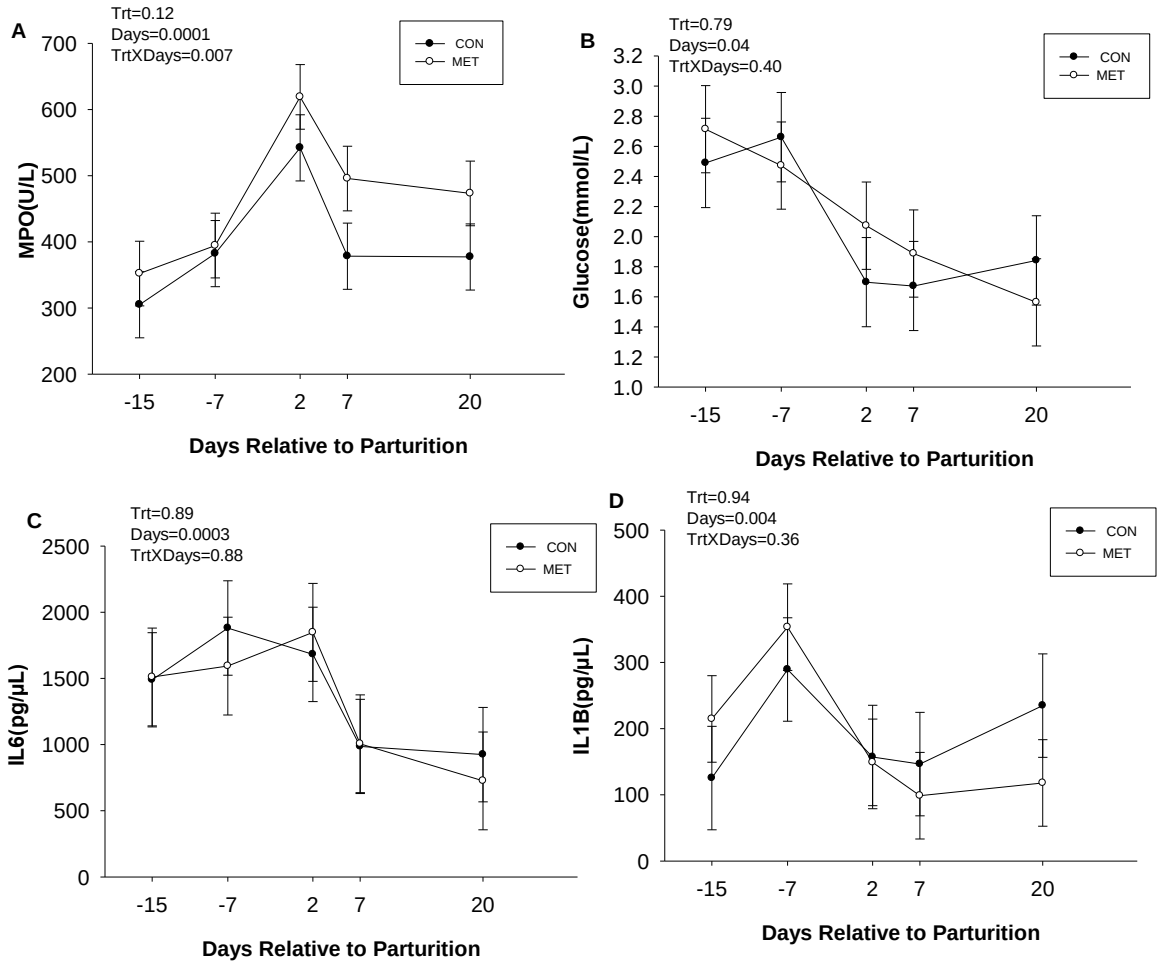
From left to right: M: Marker(100-5000bp), 1: LGALS1(189bp), 2: LGALS2 (181bp), 3: LGALS3 (165bp),4: LGALS4 (215bp), 7: LGALS7 (236bp), 8: LGALS8(232bp), 9: LGALS9 (233bp), 12: LGALS12 (176bp) and M: Marker (100-5000bp).

Supplementary Figure S2:



Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA, Alpharetta, GA) on plasma Gal-12 concentration in blood. Values are means, with standard errors represented by vertical bars

Supplementary Figure S3:



Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA, Alpharetta, GA) on plasma MPO(A), Glucose(B), IL6(C), and IL1β(D) concentration in blood. Error bars represent SEM associated with the model estimate.

References

Batistel, F., Alharthi, A. S., Wang, L., Parys, C., Pan, Y.-X., Cardoso, F. C., & Loor, J. J. (2017). Placentome nutrient transporters and mammalian target of rapamycin signaling proteins are altered by the methionine supply during late gestation in dairy cows and are associated with newborn birth weight. *The Journal of nutrition, 147*(9), 1640-1647.

Bradley, P. P., Priebat, D. A., Christensen, R. D., & Rothstein, G. (1982). Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *Journal of Investigative Dermatology, 78*(3), 206-209.

Jacometo, C., Zhou, Z., Luchini, D., Trevisi, E., Correa, M., & Loor, J. (2016). Maternal rumen-protected methionine supplementation and its effect on blood and liver biomarkers of energy metabolism, inflammation, and oxidative stress in neonatal Holstein calves. *Journal of dairy science, 99*(8), 6753-6763.

Ji, P., Osorio, J., Drackley, J., & Loor, J. (2012). Overfeeding a moderate energy diet prepartum does not impair bovine subcutaneous adipose tissue insulin signal transduction and induces marked changes in peripartal gene network expression. *Journal of dairy science, 95*(8), 4333-4351.

Osorio, J., Ji, P., Drackley, J., Luchini, D., & Loor, J. (2013). Supplemental Smartamine M or MetaSmart during the transition period benefits postpartal cow performance and blood neutrophil function. *Journal of dairy science, 96*(10), 6248-6263.