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Tese

Nutrimetabolômica: efeitos da suplementação materna com minerais orgânicos ou metionina no metabolismo e programação imunometabólica de neonatos bovinos

Carolina Bespalhok Jacometo

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Orientador: Dr. Marcio Nunes Corrêa
Coorientador: Dr. Augusto Schneider
Coorientador no exterior: Dr. Juan J. Loor

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BANCA EXAMINADORA

Prof. Dr. Geraldo Tadeu dos Santos (UEM, Departamento de Zootecnia)

Prof. Dr. Bernardo Garziera Gasperin (UFPel, Faculdade de Veterinária)

Prof. Dr. Tiago Veiras Collares (UFPel, CDTec/Biotecnologia)

Prof. Dr. Marcio Nunes Corrêa (Orientador, UFPel, Faculdade de Veterinária)

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Resumo

JACOMETO, Carolina Bespalhok. **Nutrimetabolômica: efeitos da suplementação materna com minerais orgânicos ou metionina no metabolismo e programação imunometabólica de neonatos bovinos.** 2015. 143f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

O conhecimento de como marcadores do metabolismo imune e hepático são regulados durante o período neonatal e como podem ser influenciados pela dieta materna pré-parto contribui para o desenvolvimento de estratégias nutricionais, buscando reduzir o estresse do nascimento. A transcriptomica é uma ferramenta que tem sido utilizada recentemente com intuito de contribuir para o melhor entendimento dos mecanismos moleculares que regulam o metabolismo durante períodos de estresse. Embora a fisiologia neonatal bovina já seja bem compreendida, os mecanismos pelos quais a nutrição materna no pré-parto pode influenciar o metabolismo hepático e imune dos neonatos bovinos ainda necessitam de maiores investigações. O objetivo desta tese foi avaliar o efeito da suplementação materna no pré-parto com microminerais orgânicos ou metionina no metabolismo e capacidade imune de neonatos bovinos. A suplementação materna com microminerais orgânicos durante os últimos 30 dias de gestação melhorou a função imune dos neonatos, reduzindo o estado pró-inflamatório e controlando o estresse oxidativo, durante as primeiras três semanas de vida. Entretado, a suplementação materna com metionina durante os últimos 24 dias pré-parto não teve efeito sobre o metabolismo imune dos bezerros, do nascimento aos 50 dias de idade. Em relação ao metabolismo hepático, a suplementação materna com metionina durante os últimos 24 dias pré-parto apresentou um efeito benéfico sobre o metabolismo hepático dos bezerros, proporcionando uma maturação mais evidente das rotas metabólicas e também maior eficiência na captação e produção endógena de glicose, do nascimento aos 50 dias de idade. De acordo com a bibliografia atualmente disponível, estes são os primeiros trabalhos a demonstrarem como a suplementação materna com microminerais orgânicos ou metionina atua na regulação do sistema imune e metabolismo hepático de neonatos bovinos, focando em expressão gênica e biomarcadores sanguíneos. Em conclusão, os resultados obtidos evidenciam que a suplementação materna pré-parto com microminerais orgânicos ou metionina pode beneficiar o metabolismo imune e hepático dos neonatos bovinos, contribuindo para uma melhor adaptação ao ambiente extra-uterino.

Palavras-chave: Estresse oxidativo, Fígado, Metabolismo energético, Neutrófilos.

Abstract

JACOMETO, Carolina Bespalhok. **Nutrimetabolomics: effects of maternal supplementation with organic minerals or methionine on metabolism and immunometabolic programming of neonatal calves**. 2015. 143f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Understanding how markers of hepatic and immune metabolism are regulated during the neonatal period and how they could be affected by pre-partum maternal nutrition contributes to the development of nutritional strategies that could ameliorate the initial stress of birth. Transcriptomics is a tool that has been recently applied aiming to better understand the molecular mechanisms during stressful periods. Although the neonatal calf physiology has been well-studied, the mechanisms which pre-partum nutrition can affect the hepatic and immune metabolism of the neonatal calf still warrant further investigations. The aim of this thesis was to evaluate the effect of pre-partum maternal supplementation with organic trace minerals or methionine on neonatal calf metabolism and immune function. Maternal supplementation with organic trace minerals during the last 30 days of pregnancy enhanced neonatal immune function, reducing the pro-inflammatory state and regulating the oxidative stress, during the first 3 weeks of life. However, maternal supplementation with methionine during the last 24 days of pregnancy did not affect calves immune metabolism, from birth through 50 days of age. Regarding hepatic metabolism, maternal supplementation with methionine during the last 24 days of pregnancy improved calves hepatic metabolism, having greater maturation of the metabolic pathways and also higher efficiency in glucose uptake and endogenous production, from birth through 50 days of age. According to the currently available literature, these are the first studies demonstrating how maternal supplementation with organic trace minerals or methionine acts regulating the immune system and hepatic metabolism of neonatal calves, focusing on gene expression and blood biomarkers. In conclusion, the results indicate that materal pre-partum supplementation with organic trace minerals or methionine elicts some beneficial adaptations in the immune and hepatic metabolism of neonatal calves, contributing to a better adaptation to the extra-uterine environment.

Keywords: Oxidative stress, Liver, Energy metabolism, Neutrophil.

Lista de Figuras

Lista de Abreviaturas

Co – Cobalto

Cr – Crômio

Cu – Cobre

DNMT – DNA metiltransferase

EROs – Espécies reativas ao oxigênio

GH – *Growth hormone* (Hormônio do crescimento)

LPS – Lipopolissacarídeos

miR - microRNA

Mn – Manganês

NEFA – *Non esterified fatty acids* (Ácidos graxos não esterificados)

NRC – *National Research Council's Nutrient Requirements of Dairy Cattle* (Requerimentos nutricionais para gado leiteiro)

PMN – Polimorfonucleares

SAM - S-adenosilmetionina

Se – Selênio

SOD – Superóxido dismutase

TLR – *Toll-like receptor* (Receptor do tipo *Toll*)

USDA – *United States Department of Agriculture* (Departamento de Agricultura dos Estados Unidos)

VLDL – *Very Low Density Protein* (Proteína de densidade muito baixa)

Zn – Zinco

Sumário

1 INTRODUÇÃO GERAL

Os benefícios do consumo de leite à saúde humana já são bem conhecidos e disseminados mundialmente. Do ponto de vista nutricional o leite é considerado um alimento completo, sendo o seu consumo indicado a todas as faixas etárias (Fernandez Fernandez et al., 2015). De acordo com o *United States Department of Agricuture* (USDA), em 2012 a produção global foi de 544,1 milhões de toneladas de leite, do qual o Brasil contribuiu com 5% (32,9 milhões de toneladas), sendo o sexto maior produtor mundial de leite bovino.

Em qualquer sistema de criação, medidas que garantam a saúde dos animais são primordiais. Em muitos sistemas comerciais de produção de leite o manejo dos animais no pré-parto (período seco) é negligenciado. Erroneamente os produtores assumem que os requerimentos nutricionais são mínimos, devido ao animal não estar lactando e geralmente fornecem uma dieta de baixa qualidade (Quigley and Drewry, 1998). Pesquisas mostram que um adequado manejo nutricional durante o período seco pode trazer benefícios para a performance produtiva e reprodutiva das vacas durante a lactação (Damgaard et al., 2013, Bernier-Dodier et al., 2011, Janovick et al., 2011, Cardoso et al., 2013). Entretanto, além dos benefícios à vaca, estudos demonstram que intervenções nutricionais no período pré-parto podem afetar o desenvolvimento e metabolismo da prole (Gao et al., 2012, Osorio et al., 2013b). Um outro ponto importante a se destacar é quanto a qualidade do colostro, apesar dos resultados das pesquisas não serem consistentes, a nutrição pré-parto pode influenciar sua composição (Van Saun, 1991, Quigley and Drewry, 1998). Tomando-se em conta o conceito de que “a bezerra de hoje será a vaca de amanhã”

é de extrema importância que o seu bom desenvolvimento seja levado em consideração desde o período intrauterino.

O desenvolvimento fetal é dependente da nutrição materna, que através da circulação sanguínea direciona aminoácidos, glicose e NEFA (do inglês, *non-esterified fatty acids)* para o feto (Zhu et al., 2007). Em ruminantes, os placentomas constituem as unidades de troca materno-fetal e são constituídos da face fetal (cotilédone) e materna (carúncula), portanto o aumento da vascularidade dos placentomas pode aumentar a eficiência do transporte de nutrientes ao feto (Reynolds and Redmer, 1995).

Convencionalmente o período gestacional é dividido em três terços (inicial, médio, final) e, dependendo do período em que as intervenções nutricionais são feitas, as consequências podem ser diferentes. O terço final da gestação é considerado o período em que as interferências nutricionais têm um maior efeito sobre o metabolismo da prole, pois é o período em que a taxa de crescimento do feto e a demanda de nutrientes é maior (Symonds et al., 2007).

Portanto, um manejo nutricional de excelência durante o período de transição entre o final da gestação e início de lactação é de extrema importância. As pesquisas buscam elucidar cada vez mais a função específica de cada nutriente e seu papel na regulação da expressão gênica e modulação de outros aspectos do metabolismo e sistema imune (Seo et al., 2013), bem como avaliar o impacto destes “ajustes finos” da dieta materna sob o metabolismo da prole.

2 REVISÃO BIBLIOGRÁFICA

2.1 Microminerais orgânicos

Os microminerais desempenham papeis críticos na inter-relação entre função imune, metabolismo oxidativo e metabolismo energético em ruminantes. A exemplo, Zn, Cu, Mn, Co, Cr e Se são minerais essenciais e atuam com componentes chaves na ação de enzimas antioxidantes (Overton and Yasui, 2014). A suplementação com estes nutrientes durante o período de transição das vacas leiteiras justifica-se pela alta demanda nutricional e redução da capacidade imunológica durante as últimas semanas que antecedem ao parto e primeiras semanas de lactação (Goff and Horst, 1997).

O Zn está envolvido nos processos de proliferação, diferenciação e apoptose, mecanismos essenciais as células do sistema imune (Haase et al., 2006). Atua também na integridade dos tecidos e, em vacas leiteiras, o Zn é essencial para a produção de queratina, que possui papel importante nos mecanismos de defesa da glândula mamária contra a infestação de patógenos (Andrieu, 2008). É um componente da enzima superóxido dismutase (SOD), responsável pela eliminação dos peróxidos, um dos componentes das espécies reativas ao oxigênio (EROs), produzidas nas células imune (Saker, 2006).

Assim como o Zn, Cu também é um componente da SOD, bem como ceruloplasmina, uma proteína de fase aguda, e há evidencias do seu envolvimento no metabolismo da vitamina A e E, importantes antioxidantes (Sharma et al., 2005). A deficiência de Cu reduz a função de neutrófilos, monócitos e células T (Wintergerst et al., 2007). Após um desafio intra-mamário com *Escherichia coli* a contagem de

bactérias no leite e a ocorrência de mastite clínica foi reduzida em animais suplementados com Cu (Scaletti and Harmon, 2012, Scaletti et al., 2003).

O Co é um componente essencial da vitamina B12, que por sua vez atua como cofator de enzimas envolvidas na gliconeogênese (Overton and Yasui, 2014). Entretanto a suplementação com Co orgânico não demonstra ser efetiva na produção de leite (Akins et al., 2013).

Poucos estudos foram desenvolvidos com intuito de acessar o mecanismo de ação do Mn na função imune e metabolismo de vacas leiteiras, embora Mn seja parte essencial de uma gama de enzimas envolvidas na função imune, metabolismo oxidativo e metabolismo lipídico (Andrieu, 2008).

Em termos de biodisponibilidade, as formas orgânicas dos microminerais, como as formas complexadas com aminoácidos, tem sido comumente usadas em substituição as formas tradicionais, como os sulfatos, minimizando o risco de antagonismo entre microminerais e aumentando a eficiência de absorção (Swecker, 2014). Vacas lactantes suplementadas com Zn orgânico apresentaram uma melhor resposta imune, bem como um aumento na produção de leite (Wang et al., 2013). Em resposta a vacinação, vacas suplementadas com formas orgânicas de Mn, Cu e Zn apresentaram maiores concentrações de anticorpos e os neutrófilos apresentaram maiores taxas de fagocitose quando comparadas com as vacas suplementadas com formas inorgânicas (Nemec et al., 2012). Apesar de encontrarmos na literatura alguns resultados contraditórios quanto a eficiência do uso de microminerais orgânicos, em geral a suplementação pode beneficiar a performance produtiva e reprodutiva de vacas leiteiras (Rabiee et al., 2010).

Com base nestas evidências, justifica-se a suplementação com microminerais orgânicos durante o período de transição das vacas leiteiras. Entretanto ainda não há nenhum resultado de pesquisa disponível que avalie os efeitos da suplementação materna pré-parto com microminerais orgânicos sobre o metabolismo e desenvolvimento dos bezerros.

2.2 Metionina

Após a revisão do *National Research Council's Nutrient Requirements of Dairy Cattle* (NRC), em 2001 grandes avanços foram obtidos sobre o balanceamento de aminoácidos na dieta de vacas leiteiras. Lisina e metionina são os dois aminoácidos mais limitantes na formulação de dietas de vacas leiteiras, pois quando suplementadas contribuem para um aumento da produção de leite, bem como com o aumento de proteína (Rulquin et al., 2006, Appuhamy et al., 2011, Chen et al., 2011, Osorio et al., 2013a, Lee et al., 2015), além de contribuir na redução da ocorrência de transtornos metabólicos relacionados ao periparto, principalmente por proporcionar um aumento na ingestão de matéria seca (Osorio et al., 2013a, Ordway et al., 2009).

Os animais em crescimento também são beneficiados com a ingestão balanceada de aminoácidos. Diversos estudos demonstram como diferentes porporções lisina:metionina podem melhorar a performance de crescimento (Kanjanapruthipong, 1998, Klemesrud et al., 2000, Hill et al., 2008), porém o excesso de aminoácidos pode promover a perda de peso devido a toxicidade (Abe et al., 2000).

Os aminoácidos estão envolvidos em diversos processos biológicos e fisiológicos, incluindo expressão gênica e resposta imune (Wu, 2010). A metionina

atua na síntese da apoproteína B100, componente essencial para a formação de VLDL (do inglês, *very low density lipoprotein*), responsável pelo transporte de triglicerídeos do fígado para tecidos periféricos (Bernabucci et al., 2004). Também é o único aminoácido que desempenha um papel específico na transcrição de RNAm, atua como doador de grupamento metil para a síntese de importantes compostos como a fosfatidilcolina (membranas celulares) e é essencial para a síntese de outros aminoácidos sulfurados, como a cisteína (Metayer et al., 2008). A cisteína por sua vez é necessária para a síntese de glutationa e taurina, compostos essenciais para o mecanismo de defesa contra o estresse oxidativo (Metayer et al., 2008).

A suplementação com metionina em uma apresentação que não permita, ou reduza, a degradação ruminal aumenta sua biodisponibilidade (Graulet et al., 2005), aumentando assim a entrada de metionina no ciclo do metabolismo de 1-carbono (Figura 1), onde inicialmente pode ser convertida a s-adenosilmetionina (SAM), a molécula biológica mais importante em termos de doação de grupamentos metil (Martinov et al., 2010). A disponibilidade de SAM é essencial para o mecanismo de metilação do DNA, um processo biológico importante para a regulação da expressão gênica, desempenhado por DNA metiltransferases (DNMT), como DNMT3A, DNMT3B e DNMT1, responsáveis por estabelecer um padrão de metilação nas “ilhas CpG” do genoma (Li and Zhang, 2014).

Através da rota de transulfuração, a homocisteína é convertida a cisteína, podendo ser direcionada a síntese de taurina ou para o metabolismo da glutationa, atuando no metabolismo oxidativo.

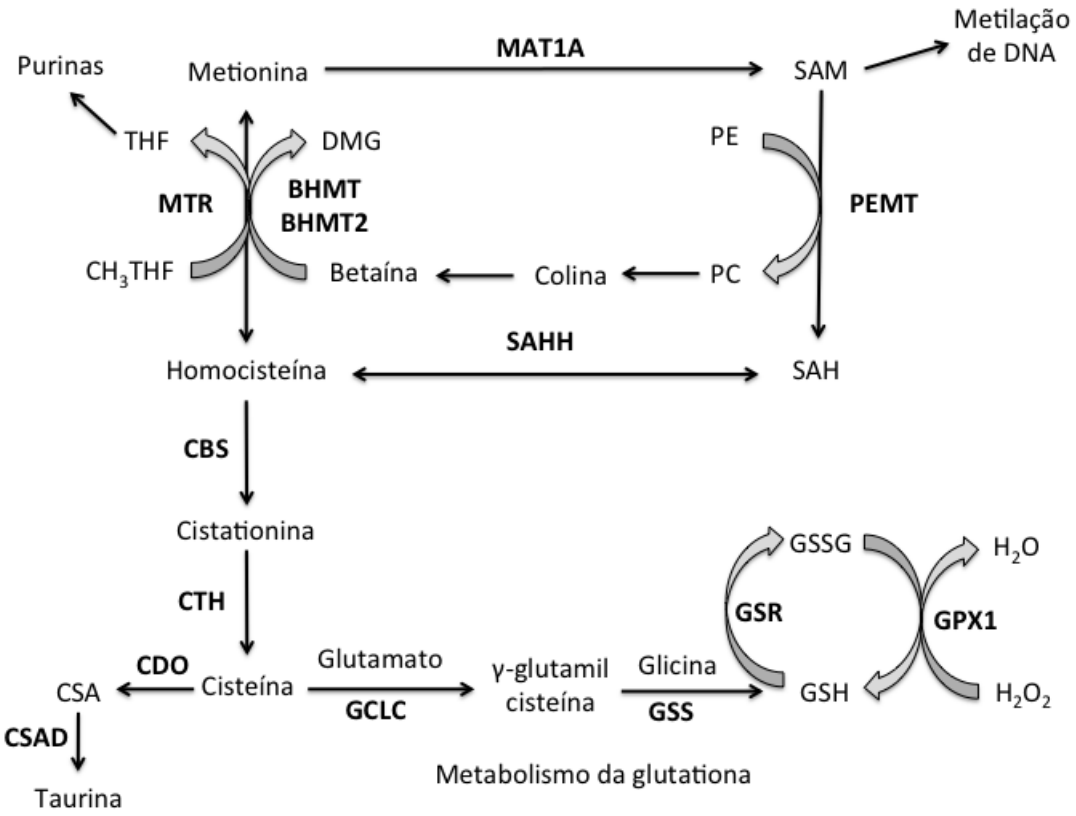

Figura 1. Rota ilustrativa com os principais genes que codificam para as enzimas responsáveis pelo ciclo da metionina (1-carbono) e glutationa. Enzimas: Metionina adenosiltransferase 1A (*MAT1A*), fosfatidiletanolamina metiltransferase (*PEMT*), *S*-adenosilhomocisteína hidrolase (*SAHH*), betaína homocisteína metiltransferase (*BHMT* and *BHMT2*), 5-metilltetraidrofolato-homocisteína metiltransferase (*MTR*), cistationina β-sintase (*CBS*), cistationina β-liase *(CTH),* cisteína dioxigenase *(CDO),* cisteína ácido sulfônico descarboxilase *(CSAD),* glutamato-cisteína ligase *(GCLC),* glutationa sintetase *(GSS),* glutationa redutase *(GSR)* e glutationa peroxidase 1 *(GPX1).* Metabólitos intermediários: SAM = S-adenosilmetionina; PE = fosfatidiletanolamina; PC = fosfatidilcolina; SAH = *S*-adenosilhomocisteína; THF = tetrahidrofolato; CH3THF = 5-metiltetrahidrofolato; DMG = dimetilglicina; CSA = cisteína ácido sulfônico; GSH = glutationa sulfidril; GSSG = glutationa dissulfeto.

Com base nestas evidências justifica-se a suplementação com metionina durante o período de transição das vacas leiteiras. Entretanto ainda não há nenhum resultado de pesquisa disponível que avalie os efeitos da suplementação materna pré-parto com metionina sobre o metabolismo e desenvolvimento dos bezerros.

2.3 Metabolismo imune de neonatos bovinos

A placenta bovina não realiza a transferência de anticorpos para o feto e devido a essa peculiaridade o manejo colostral é de grande importância para reduzir a taxa de mortalidade de neonatos bovinos (Goff, 2006). A imunidade passiva é obtida pelo transporte de macromoléculas encontradas no colostro através do epitélio intestinal, o qual permanece permeável por aproximadamente 24 horas após o nascimento, com máxima capacidade de absorção nas primeiras horas após o nascimento e progressiva redução (Stott et al., 1979). O colostro é extremamente rico em nutrientes, como ácidos graxos essenciais, aminoácidos, microminerais e vitaminas, e também possui componentes não nutricionais como imunoglobulinas, células (epiteliais, eritrócitos e leucócitos), hormônio do crescimento (GH), prolactina, insulina e glucagon (Blum, 2006).

Ao nascimento os bovinos possuem todos os componentes do sistema imune, porém muitas células ainda são imaturas. As células de defesa circulantes são coletivamente chamadas de leucócitos (células brancas) e dentre os polimorfonucleares (PMN) os neutrófilos são as células mais abundantes e as primeiras a responder e fagocitar organismos invasores (Tizard, 2013).

Os neutrófilos exercem sua função antimicrobiana principalmente através de mecanismos dependentes de EROs (Concha et al., 2014). Um intenso estresse oxidativo é produzido durante a fagocitose ou após a estimulação por outros agentes, e durante essas reações são produzidos ânions superóxidos (O_2^-) e peróxido de hidrogênio (H_2O_2) (Robinson, 2009), além disso, os neutrófilos também produzem diversas enzimas, como a gelatinase, lactoferrina, mieloperoxidase, que irão atuar nos mecanismos de defesa (Conejeros et al., 2012). O estresse oxidativo

passa a ser um problema quando há um desequilíbrio entre a produção das EROs e dos mecanismos de neutralização e capacidade antioxidante. Entre as enzimas antioxidantes já identificadas encontram-se a SOD, glutationa peroxidase e vitaminas A e E (Bernabucci et al., 2005).

Os receptores do tipo *Toll* (TLR, do inglês *Tool-like receptors*) pertencem a classe de proteínas transmembranas capazes de responder a uma vasta gama de estímulos, e as isoformas 2 e 4 reconhecem principalmente estímulos de lipopolissacarídeos (LPS), presente na parede de bactérias gram-positivas e gram-negativas, respectivamente (Chen et al., 2015). Amplamente expresso nas células imunes, os TLR quando estimulados, respondem desencadeando uma reposta pró-inflamatória (via MyD88 ou TRIF) (Wolowczuk et al., 2008).

Dentre os mecanismos epigenéticos que potencialmente atuam na regulação do sistema imune, recentemente os microRNAs (miRs) tem sido o foco de estudos (Gantier, 2013). MicroRNAs são pequenas sequências de RNA não codificantes (em média 22 nucleotídeos) que atuam na regulação da expressão gênica e síntese proteica (Hoefig and Heissmeyer, 2008).

Entretanto, os mecanismos pelo qual nutrientes específicos da dieta materna impactam no sistema imune materno durante a gestação e na programação da imunidade fetal ainda necessitam de pesquisas para melhor entendimento.

2.4 Metabolismo hepático de neonatos bovinos

Os neonatos mamíferos precisam se adaptar a mudanças quanto ao fornecimento de nutrientes, pois a nutrição altera da forma contínua parenteral (principalmente glicose, lactato e aminoácidos) através da placenta para uma forma descontínua de

fornecimento de colostro e leite (ou sucedâneo lácteo) que possui lactose e gordura como principal fonte energética (Hammon et al., 2012). Portanto o metabolismo hepático dos neonatos é extremamente desafiado nos primeiros dias de vida.

É comum que os neonatos apresentem um estado de hipoglicemia, devido a glicose ser um nutriente vital para diversos órgãos e tecidos, e a glicose presente no colostro não é suficiente para atender a demanda, portanto há necessidade da ativação do metabolismo gliconeogênico logo após o nascimento (Girard et al., 1992). A capacidade de oxidação de ácidos graxos também aumenta rapidamente logo após o nascimento, principalmente de ácidos graxos de cadeia média, provenientes do leite, contribuindo assim para a manutenção do metabolismo energético (Odle et al., 1995).

Os glicocorticóides e o sistema adrenérgico também são importantes reguladores do metabolismo energético, responsáveis pela maturação dos processos metabólicos e rotas envolvidos no metabolismo energético pós-natal (Fowden and Forhead, 2011, Fowden et al., 1998). Ambos estimulam a produção endógena de glicose, oxidação de ácidos graxos (Letteron et al., 1997) e acúmulo de triglicerídeos no fígado (Rose et al., 2010). Os seus efeitos são mediados por receptores específicos, os receptores adrenérgicos mais abundantes no fígado dos neonatos bovinos são os subtipos α_1 (ADRA1) e β_2 (ADRB2), envolvidos na maturação do metabolismo energético (Carron et al., 2005a, Carron et al., 2005b, Ontsouka et al., 2006), entretanto poucos estudos foram conduzidos investigando a atividade dos receptores de glicocorticóides (GR) no fígado de neonatos bovinos (Schaff et al., 2014).

O fígado desempenha um papel importante também na resposta sistêmica aos processos inflamatórios, com a produção de proteínas de fase aguda positivas, como a haptoglobina e ceruloplasmina, concomitante com a redução da concentração de proteínas de fase aguda negativas, como a albumina e paraoxonase (Bertoni et al., 2008). O estímulo pró-infalamatório contribui para o aumento das concentrações das EROs, e como demonstrado anteriormente, falhas no mecanismo de neutralização e potencial antioxidante podem conduzir ao estresse oxidativo.

O metabolismo hepático é diretamente afetado pela nutrição, portanto qualquer modificação na nutrição durante o periparto pode alterar o fluxo de nutrientes para o feto, e pesquisas são necessárias para verificar como a nutrição materna pode alterar o metabolismo hepático dos neonatos.

3 HIPÓTESE E OBJETIVOS

3.1 Hipótese

A suplementação materna no pré-parto com minerais orgânicos ou metionina é capaz de modular o metabolismo do neonato e beneficiar seu desenvolvimento.

3.2 Objetivo Geral

Avaliar o efeito da suplementação materna com minerais orgânicos ou metionina no metabolismo hepático e capacidade imune dos neonatos, do nascimento até os 50 dias de idade.

3.3 Objetivos Específicos

- Avaliar o desenvolvimento corporal dos animais do nascimento até o desmame em resposta a nutrição materna;

- Avaliar os biomarcadores sanguíneos relacionados ao metabolismo, inflamação e estresse oxidativo em resposta a suplementação materna com microminerais orgânicos ou metionina;

- Avaliar a plasticidade da expressão de genes e microRNAs dos neutrófilos sanguíneos ligados a adesão celular, quimiotaxia, estresse oxidativo e rotas pró-inflamatórias em resposta a suplementação materna com microminerais orgânicos ou metionina;

- Avaliar a regulação do metabolismo hepático através da expressão de genes relacionados ao metabolismo energético, inflamação, sinalização da insulina, eixo GH-IGF1, metabolismo de 1-carbono e da glutationa, em resposta a suplementação materna com metionina.

4 CAPÍTULOS

4.1 Artigo 1– Maternal consumption of organic trace minerals (4-Plex®) enhances neonatal dairy calf growth by alleviating neonatal inflammation and oxidative stress: role of microRNAs in regulating neutrophil function

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Carolina B. Jacometo[1,2], Johan S. Osorio[2], Michael Socha[3], Marcio N. Corrêa[1], Fiorenzo Piccioli-Capelli[4], Erminio Trevisi[4], and Juan J. Loor[2]

[1]NUPEEC, Departamento de Clínicas Veterinária, Programa de Pós-Graduação em Biotecnologia, Universidade Federal de Pelotas, Pelotas, RS, Brazil.

[2]Mammalian NutriPhysioGenomics, Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois, Urbana, IL, USA.

[3]Zinpro Corporation, 10400 Viking Drive, Eden Prairie, MN 55344, USA.

[4]Istituto di Zootecnica, Facoltà di Scienze Agrarie, Alimentari e Ambientali, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy.

Abstract

Organic trace mineral (ORG) supplementation to dairy cows has been associated with improvement in immune function during stressful stages such as the peripartal period. However, the impact of supplemental ORG during pregnancy on the neonatal calf is unknown. Therefore, our aim was to investigate the effects of ORG supplementation during late-pregnancy on the immune system and growth of the neonatal calf. Of particular interest was the evaluation of inflammation-related microRNA (miRNA) and target gene expression in blood neutrophils as indicators of possible nutritional programming. Forty multiparous cows were supplemented for 30

days pre-partum with 40 ppm Zn, 20 ppm Mn, 5 ppm Cu and 1 ppm Co from either organic (ORG) or sulfate (INO) sources (total diet contained, supplemental 75 ppm Zn, 65 ppm Mn, 11 ppm Cu and 1 ppm Co, additional Zn, Mn, and Co provided by sulfates), and a subset of calves (n = 8/treatment) was used for blood immunometabolic marker and polymorphonuclear leukocytes (PMN) gene and miRNA expression analysis. Samples were collected at birth (before colostrum feeding), 1 day (24 h after colostrum intake), 7 and 21 d of age. No differences were detected in body weight, but maternal ORG resulted in greater (P = 0.06) calf withers height. Calves from INO-fed cows had greater concentrations of blood glucose (P = 0.001), AST/GOT (P = 0.015), paraoxonase (P < 0.001), myeloperoxidase (P = 0.003), and reactive oxygen metabolites (ROM) (P = 0.083). Antioxidant capacity (P = 0.043) also was greater in INO compared with ORG calves. PMN expression of TLR pathway genes indicated a greater pro-inflammatory state in INO calves, with higher expression (P < 0.05) of most inflammatory mediators (*MYD88, IRAK1, TRAF6, NFKB* and *NFKBIA*). The greater expression of miR-155 and miR-125b indicated the potential for nutritional programing in regulating the PMN inflammatory response. Expression of *SELL* was greater (P = 0.06) in INO calves, with a marked increase (P < 0.001) from birth to 7 d of life. Expression of *NFE2L2* was greater (P = 0.02) overall in ORG than INO calves. Overall, these results demonstrate that maternal nutrition with organic trace minerals could control the neonatal innate immune response at least in part via changes in gene and miRNA expression. It remains to be determined if such response allows for more nutrients to be utilized for growth during the neonatal period.

Key-words: epigenetic; fetal programming; growth; nutrition; oxidative stress; PMN; TLR.

INTRODUCTION

During the transition period cows are especially challenged as they are in negative energy balance (**NEB**) coupled with intense hormonal and metabolic changes that can lead to a higher risk of developing metabolic disorders (Drackley, 1999). Nutritional supplementation with vitamins, essential amino acids, essential fatty acids and other micronutrients during pregnancy have been intensively studied as effectors for immune system activation, not only for the cow, to face the transition period, but also for the offspring, to adapt to the extra-uterine life (Thornton, 2010).

Trace mineral elements such as Cu, Cr and Zn have important roles in the health and immunity of peripartal dairy cows (Spears and Weiss, 2008). Minerals have been commonly supplemented to cattle in the form of inorganic salts, preferably as sulfates; however, the development of organic forms of trace minerals, such as minerals complexed with amino acids, minimize the risk of mineral antagonism and enhance absorption efficiency (Swecker, 2014). Lactating cows supplemented with organic Zn had a greater immune response as well as improved milk yield (Wang et al., 2013). In addition, greater neutrophil (**PMN**) phagocytosis and antibody titer (0.61 vs 1.07 $\log_{10}$ arbitrary units) in response to rabies vaccination were observed in early lactation cows supplemented with organic forms of Mn, Cu and Zn compared with inorganic forms (Nemec et al., 2012).

The implications of trace mineral deficiency or impaired placental transfer of these minerals to fetal and neonatal ruminant metabolism have been studied for more than 30 years (Hidiroglou, 1980). For instance, dairy calves supplemented with an injectable trace mineral complex containing Se, Cu, Zn, and Mn increased PMN and glutathione peroxidase activity, while reducing incidence of diarrhea, pneumonia,

and otitis (Teixeira et al., 2014). In addition, it has been observed that epigenetic modifications through microRNA (**miRNA**) are an important part of the fine-tuning regulation of several cellular process (Aguilera et al., 2010) that modulate PMN function including regulation of senescence, differentiation, adherence capacity and cytokine production (Gantier, 2013). These observations could be of interest if linked to the fact that epigenetic marks are candidates for bearing the memory of specific intrauterine nutritional exposure causing alterations in long-term gene expression programming, and consequently inducing developmental adaptations in physiology and metabolism (Attig et al., 2010).

Mature miRNA are non-protein-coding small RNAs (~20 nucleotides length) that repress translational activity, promote destabilization of target mRNA, and regulate the abundance level of mRNA target genes; however, these mechanisms remain under debate (Eulalio et al., 2008). It was reported in-vitro using mouse P19 embryonal carcinoma cells that miR125-b can regulate mammalian neuronal differentiation by downregulating both translational efficiency and mRNA abundance of *lin-28* (Wu and Belasco, 2005). Using a microarray approach with miR-transfected HeLa cells (miR-1 and miR-124), it was demonstrated that miRNA could reduce the levels of many of their target transcripts, not just the amount of protein deriving from these transcripts (Lim et al., 2005).

The general hypothesis of the present study was that maternal supplementation with organic trace minerals would improve neonatal calf metabolism and immune function, which would be reflected in better measures growth and performance during the neonatal period. The specific objectives were to examine residual effects of organic trace mineral supplementation during late-pregnancy on

the immune system and growth of the neonatal calf, and the possible epigenetic regulation through the action of miRNA in the pro-inflammatory signaling pathway.

MATERIAL AND METHODS

All the procedures for this study were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois (protocol #12097).

Maternal Treatments

The experiment was conducted as a randomized complete blocked design with 40 multiparous Holstein cows blocked according to parity, previous lactation milk yield, and expected day of parturition. All cows received a common lactation diet (1.76 Mcal/kg DM and 16.7% CP) during the last 60 d of lactation prior to dry-off, and a common early-dry period diet (1.1 Mcal/kg DM, 14.5% CP) from -50 to -30 d relative to parturition. Both diets were supplemented at 100% NRC (NRC, 2001) requirements with Zn, Mn, Cu, and Co in the form of an inorganic trace mineral mix. All cows received the same diet (1.5 Mcal/kg DM, 15% CP) from -30 d to parturition ("close-up" period), and from parturition through 30 d post-partum (1.76 Mcal/kg DM and 18% CP). Close-up and lactation diets were partially supplemented with an inorganic trace mineral mix of Zn, Mn, and Cu to supply 35, 45, and 6 ppm, respectively, of the total diet. Cows were randomly assigned to an oral administration of a bolus containing a mix of either inorganic (**INO**, n = 20) or organic (Availa®Zn Zn amino acid complex**;** Availa®Mn Mn amino acid complex; Availa®Cu Cu amino acid complex and CoPro® cobalt glucoheptonate; Zinpro Corporation, Eden Prairie, MN) (**ORG**, n = 20) Zn, Mn, Cu, and Co in order to achieve 75, 65, 11, and 1 ppm,

respectively, in diet DM. After birth, calves were fed a common diet and managed similarly. Hence, any observed treatment effects are attributed to maternal nutrition during the last 30 d of gestation.

Animal management and calf enrollment criteria

During the dry period, cows were housed in a ventilated, sand-bedded free-stall barn, with a photoperiod of 8 h of light and 16 h of dark. Diets were fed for ad-libitum intake as a total mixed ration (**TMR**) once daily using an individual gate feeding system (American Calan, Northwood, NH) and dry matter intake (**DMI**) was recorded daily. As cows began demonstrating signs of impending parturition, they were moved to an individual maternity pen bedded with straw. After parturition, cows were moved within 2 h to an individual chute and then milked with a porta-milker vacuum pump (Nasco, Fort Atkinson, WI; Cat. no. Z15664N). Colostrum volume was recorded and IgG content was estimated based on specific gravity with a bovine colostrometer (Nasco, Fort Atkinson, WI; Cat. no. C10978N).

A subset of calves was selected (n = 8/group) if they fulfilled all the following criteria: 1) single calf; 2) calving difficulty score <3; 3) colostrum quality assessed by a bovine colostrometer of >60 mg/L of IgG; 4) dam produced at least 3.8 L of a good quality first colostrum; 5) calf birth weight >36 kg (Johnson et al., 2007). On d 7 and 21 calves were bled ~3 h after the morning feeding.

After birth, calves were weighed, had the navel disinfected with a 7% tincture of iodine soln (First Priority Inc., Elgin, IL), vaccinated with TSV II (Pfizer Inc., New York, NY) via nostril application, and received 3.8 L of first milking colostrum from the respective dam within 2 h after birth. Calves were offered first milking colostrum again on the second feeding at 4 h after birth if colostrum intake had not reached the

3.8 L required. Calves were housed in individual outdoor hutches bedded with straw, fed twice daily with a milk replacer (Advance Excelerate, Milk Specialities, Carpentersville, IL; 28.5% CP, 15% fat) (from 1 to 10 d of age: 520 g/days, 11 to 20 d of age: 680 g/d, 21 to 35 d of age: 840 g/d, and from 36 to 42 d of age: 420 g/d in a single feeding) and had ad libitum access to a starter grain mix (19.9% CP, 13.5% NDF). Health checks including fecal score were recorded daily until weaning, while rectal temperature was recorded daily until 21 d of age. Growth performance including body mass (**BW**) and withers height (**WH**) were recorded weekly. Calves were weaned at 42 d of age.

Sample collection

Blood samples were collected from the jugular vein using 20-gauge BD Vacutainer needles (Becton Dickinson, Franklin Lakes, NJ) before receiving colostrum (baseline), 24 h after receiving colostrum, 7 d and 21 d after birth. At each time point, a total of 120 mL of total blood were collected in vacutainer tubes (10 mL, BD Vacutainer, Becton Dickinson) containing serum clot activator, sodium heparin, or soln A of trisodium citrate, citric acid, and dextrose (**ACD**). After blood collection, tubes with ACD and sodium heparin were placed on ice and tubes with clot activator were kept at room temperature until centrifugation (~30 min). Serum and plasma were obtained by centrifugation of clot activator and sodium heparin tubes, respectively, at 1,900 × *g* for 15 min. Serum and plasma were aliquoted and stored at -20°C until further analysis according to the manufacturer's specifications for determination of blood metabolites, oxidative stress biomarkers and acute-phase proteins (**APP**). The RNA from PMN was harvested from 100 mL of blood collected in ACD vacutainers tubes.

Blood metabolites, APP and oxidative stress biomarkers

Blood samples were analyzed for albumin, cholesterol, bilirubin, creatinine, urea, GOT, GGT, glucose, haptoglobin (**HP**), ceruloplasmin, nitric oxide (**NOx**) and constituents [nitrite (NO_2^-) and nitrate (NO_3^-)], antioxidant potential (**FRAP**), paraoxonase (**PON**), myeloperoxidase (**MPO**), and reactive oxygen metabolites (**ROM**) using kits purchased from Instrumentation Laboratory (IL Test). Non-esterified fatty acids (**NEFA**) and BHBA were measured using kits from Wako Chemicals and Randox Laboratories Ltd., respectively, following the procedures described previously (Bionaz et al., 2007, Trevisi et al., 2012, Osorio et al., 2013) using a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA). Serum amyloid A (**SAA**) concentration was assessed with a commercial ELISA immunoassay kit (Tridelta Development Ltd., Many- nooth, Co. Kildare, Ireland). Total antioxidants were assessed through the oxygen radical absorbance capacity (**ORAC**) assay. This method measures a fluorescent signal from a probe (fluorescein) that decreases in the presence of radical damage (Cao and Prior, 1999). Retinol and tocopherol were determined as previously described (Bionaz et al., 2007). Bovine interleukin-6 (**IL-6**) plasma concentration was determined by a colorimetric sandwich ELISA using a Bovine IL-6 Screening Set (#ESS0029 Endogen, Pierce, Rockford, IL).

Minerals were extracted adding 0.3 mL of tricloracetic acid (10% v/v) to 0.3 mL of plasma, sample was mixed and centrifuged at 3500 × g for 10 min. A 0.3 mL of supernatant was added to 2.7 mL of Millipore® water and mixed. Copper, Fe, Mn and Zn in final soln were determined by inductively coupled plasma optical emission spectrometry (ICP-OES 5100, Agilent Technologies, Victoria, Australia) fitted with cyclonic chamber in which samples were introduced with SeaSpray nebulizer (Agilent

Technologies, Victoria, Australia). Tricloracetic acid solution at the same concentration of the samples was used as a blank and to prepare the calibration curve diluting external standard (Merck, Darmstadt, Germany) to 5, 10, 50, 100 ppb. Instrumental detection limits were 0.35, 0.10, 0.06, 0.25 ppb for Cu, Fe, Mn and Zn, respectively. Accuracy of results was verified using a mineralized soln of SRM 1577b (National Institute of Standards and Technology - NIST).

PMN isolation

Neutrophils were isolated according the procedures described by Osorio et al. (2013) with minor modifications. Blood (100 mL), collected in ACD soln A vacutainer tubes, was mixed well by inversion and placed on ice until PMN isolation (~1 h). Tubes were combined into three 50-mL conical tubes (Fisher Scientific, Pittsburgh, PA) and centrifuged at 918 × *g* for 30 min at 4°C. The plasma, buffy coat, and approximately one-third of the red blood cells (**RBC**) were removed and discarded. Cells were lysed with 25 mL of deionized water at 4°C, homogenized gently by inversion, and then 5 mL of 5 × phosphate-buffered saline (PBS; pH 7.4) at 4°C was added, in order to restore an iso-osmotic environment. The cell suspension was centrifuged at 330 × *g* for 10 min at 4°C and the supernatants were decanted. Ten mL of 1 × PBS at 4°C was added in each tube, homogenized until there was nothing attached to the bottom of the tube, and then the three tubes were combined in one. The cell suspension was centrifuged at 663 × *g* for 5 min at 4°C and the supernatants were discarded. The remaining RBC were lysed with 8 mL of deionized water at 4°C, homogenized gently by inversion and 2 mL of 5 × PBS at 4°C was added. The samples were centrifuged at 663 × *g* for 5 min at 4°C and the supernatant was discarded. Two subsequent washings using 10 mL of 1 × PBS at 4°C were performed, centrifuged at 663 × *g* for 5 min at 4°C and supernatant discarded. Prior

to the last centrifugation, 100 µL of the cell suspension were aliquoted for further PMN concentration and cell viability analysis.

The neutrophil pellet was homogenized with 1.5 mL of 1 × PBS at 4°C and transferred to a 2 mL RNase-DNase free reinforced o-ring tube (Omni International, Kennesaw, GA, USA) containing one stainless steel bead, 5 mm (Qiagen, Hilden, Germany), centrifuged at 1,435 × *g* for 5 min at 4°C, and then the supernatant was discarded. Immediately, 1 mL of Qiazol (Qiagen, Hilden, Germany) was added to the samples and homogenized twice in a Beadbeater (Biospec Products, Bartlesville, OK; Cat. no. 607) for 30 s. During all the isolation process samples were kept on ice while outside of the centrifuge. The homogenized soln was transferred to a 2 mL DNase-RNase free tube (USA Scientific Inc., Ocala, FL) and stored in -80°C until further analysis.

Neutrophil concentration 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 soln (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 soln (4 µg/mL in 1 × PBS) was added (Goat Anti-Mouse IgM, Human ads-PE; SouthernBiotech, Birmingham, AL) and 50 µL of propidium iodide soln (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, St. Louis, MO) and preserved at 4°C until flow cytometry reading (LSR II, Becton Dickinson, San Jose, CA). These procedures were performed only to ensure good quality samples, and were excluded from the study samples with less than 80% of neutrophils and 90% of viability.

mRNA and miRNA isolation

For the PMN extraction the miRNeasy kit (Qiagen, Hilden, Germany) was used following the manufacturer's protocols, allowing the recovery of both mRNA and miRNA. Samples were treated on-column with DNaseI (Qiagen, Hilden, Germany), quantification was accessed using the NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE), and RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Target gene cDNA synthesis and qPCR

All the details for cDNA synthesis, qPCR and primer design can be found in the supplementary material published by Osorio et al. (2013). For this study *GOLGA5*, *SMUG1* and *OSBPL2* were used as internal control genes, and their geometric mean was used to normalize the expression data.

miRNA procedures

For cDNA synthesis we used the qScript miRNA cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). Each reaction started with 500 ng of total RNA, mixed with 2 µL of Poly (A) Tailing Buffer (5x), 1 µL of Poly (A) Polymerase and 7 µL of RNase/DNase free water. The mixture was incubated at 37°C for 20 min and then 70°C for 5 min. A second mix containing 9 µL of miRNA cDNA Reaction Mix and

1 µL qScript Reverse Transcriptase was added and incubated at 42°C for 20 min and then 85°C for 5 min. A pool of cDNA samples was used to prepare the 6-point standard curve (dilution 1:4). Then cDNA samples were also diluted 1:4 with DNase/RNase free water. A combination of 1 µL of diluted cDNA with 9 µL of the mix containing of 5.25 µL of Perfecta SYBR Green Fast Mix (Quanta Biosciences, Gaithersburg, MD, USA), 0.45 µL each of 10 µM sequence-specific forward primer and Universal PCR Primer (Quanta Biosciences, Gaithersburg, MD, USA) and 3.85 µL of DNase/RNase free water were added to each well of a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems, Grand Island, NY, USA). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, Grand Island, NY, USA) under the following thermocycler conditions: 95°C for 2 min, and 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by a dissociation curve step (95°C for 15 s, 60°C for 15 s and 95°C for 15 s). miR-let7a, miR-103 and miR-191 were used as internal controls, and their geometric mean was used to normalized the miRNA expression data.

All the select target genes and miRNA evaluated in this study are listed in Table 1, and primers, PCR products and PCR efficiencies are reported in the supplementary material. The model of the TLR pathway under miRNA control is illustrated on Figure 1.

Statistical analysis

Data were analyzed with the Proc MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Fixed effects in the model were treatment (Trt), d (D) or wk, and their interaction (Trt (T)×D, T×wk) . Random effect was calf within diet. The exponential correlation covariance structure SP for repeated measures was used for analysis of

blood metabolites and gene expression. Blood metabolites and gene expression results were log_2-scale transformed if needed to comply with normal distribution of residuals, and subsequently back-transformed. Least squares means separation between time points was performed using the PDIFF statement.

RESULTS

Growth performance and health

Colostral IgG was not affected ($P = 0.87$) by diet and averaged 96.7 ± 6.2 mg of IgG/mL. Similarly, apparent efficiency of absorption was not affected ($P = 0.93$) by maternal diet, averaging 35.9 ± 3.1%.

Maternal supplementation with ORG did not affect ($P > 0.05$) BW at birth or throughout the experiment. However, regardless of treatment, there was a linear increase in BW ($P < 0.001$) in both groups (Figure 2). In contrast, WH was greater overall ($P = 0.063$) in ORG calves than INO. An overall increase ($P < 0.001$) across time was observed for WH (Figure 2).

No treatment effect ($P > 0.05$) was observed for starter intake despite the increase in consumption through time ($P < 0.001$) in both groups (Table 2). Rectal temperature decreased ($P = 0.032$) from birth to 2 wk in both groups; however, it was overall greater ($P = 0.069$) in INO calves (Table 2). Although there was no overall treatment effect ($P = 0.69$), INO calves had greater (T×W $P = 0.060$) fecal score than ORG calves at 4 wk of age. Regardless of treatment effect, calves experienced the greatest ($P < 0.001$) fecal score during wk 1 of age, followed by a gradual decrease ($P < 0.001$) until 7 wk (Table 2).

Blood immunometabolic biomarkers

Main effects of diet, time, and interactions for blood immunometabolic biomarkers are presented in Table 3. Overall calves born to dams supplemented with ORG had lower glucose concentrations ($P = 0.001$) and this was particularly evident at birth (T×D, $P = 0.017$). Subsequently, change in glucose concentrations were similar with a rapid increase from birth to 1 d of life followed by stabilization after one wk of age ($P < 0.001$).

NEFA and creatinine decreased from birth to 1 d of age ($P < 0.001$), with no treatment effect ($P > 0.05$), while BHBA concentration increased over time ($P < 0.001$) and at 21 d of age ORG calves had lower ($P = 0.05$) concentration (T×D, $P = 0.112$). Urea decreased from birth to 7 d of age ($P < 0.001$) in both groups. The ORG calves had an abrupt decrease in urea from birth to 1 d of age, and INO calves had a gradual decrease until 7 d of age (T×D, $P = 0.039$). There was no difference from birth to 1 d of age in bilirubin concentrations ($P > 0.05$), and a gradual decrease regardless of treatment was observed until 21 d ($P < 0.001$).

The hepatic enzymes AST/GOT and GGT had a marked increase from birth to 1 d of age, followed by a gradual decrease ($P < 0.001$). An overall treatment effect ($P = 0.015$) was observed in AST/GOT associated with lower concentration in ORG calves, which had a more pronounced decrease from 1 to 7 d of age.

Regarding the inflammation and oxidative biomarkers, compared with INO, ORG calves had lower paraoxonase ($P < 0.001$), MPO ($P = 0.003$) and FRAP ($P = 0.043$) concentrations, and tended ($P = 0.083$) to have lower ROM, albumin ($P = 0.113$) and ORAC ($P = 0.109$). No treatment effect was observed for ceruloplasmin, cholesterol, HP, SAA and IL-6 ($P > 0.05$). However, ceruloplasmin and cholesterol

increased over time ($P < 0.001$), and SAA and IL-6 had a marked increase from birth to 1 d of age ($P < 0.001$ and $P = 0.056$, respectively). Concentration of ORAC, retinol, tocopherol, NOx and constituents (NO_2^- and NO_3^-) increased over time ($P < 0.05$).

There was no effect ($P > 0.05$) of maternal diet on the evaluated blood mineral concentrations. At birth, concentrations of all minerals were numerically greater in ORG than INO and Zn concentrations tended to have an interaction effect (T×D, $P = 0.091$), with higher concentration ($P = 0.07$) in INO than ORG calves at 1 d of age. A time effect was observed for Cu ($P < 0.001$) and Zn ($P < 0.001$) concentrations because of an increase over time, whereas concentration of Fe decreased ($P < 0.001$).

Gene and miRNA gene expression

TLR pathway. In agreement with the blood immunomarkers, evaluation of components of the TLR pathway indicated that INO had a greater inflammatory status during the first three wk of life. Even though there was an overall greater expression in ORG calves of *TLR2* ($P = 0.018$) and *TLR4* (T×D, $P = 0.033$) at 21 d of life, there was an overall decrease of several genes that participate in the pro-inflammatory cytokine response. The calves in ORG had lower expression of *MYD88* ($P = 0.059$), *IRAK1* ($P = 0.050$), *TRAF6* ($P = 0.063$), *NFKB1* ($P = 0.031$) and *NFKBIA* ($P = 0.047$). Although, no treatment effect was observed for *IL1B* ($P = 0.103$), the expression of *TNF* had an interaction (T×D, $P = 0.056$) namely due to lower ($P = 0.017$) expression in ORG calves at 21 d of age. An overall increase from birth to 1 d of life was observed for the expression of *IRAK1* ($P = 0.007$), *TRAF6* ($P = 0.017$),

NFKB1 (*P* = 0.006), *TNF,* (*P* < 0.001) and *IL1B*, (*P* < 0.001), while *NFKBIA* (*P* < 0.001) expression decreased during the same period (Figure 3).

miRNA related to the TLR signaling pathway. Maternal supplementation had an effect on miR-155 (*P* = 0.024) and miR-125b (*P* = 0.036) by down-regulating their expression in ORG calves. The expression of miR-155, miR-146a, and miR-9 was up-regulated (*P* < 0.004) from birth to 7 days of age. The expression of miR-223 was not affected by maternal treatment (*P* = 0.707) or time (*P* = 0.164) (Figure 3).

Cell receptors, pathogen recognition and oxidative stress. Expression of *SELL* was greater (*P* = 0.057) in INO calves, with a marked increase (*P* < 0.001) from birth to 7 days of life. Overall expression of *ZBP1* also increased (*P* < 0.001) during the first week of life but no treatment effect (*P* = 0.185) was detected (Figure 4).

Expression of *NFE2L2* had a significant interaction (T×D, *P* = 0.017) due to greater (*P* = 0.02) expression in ORG calves than INO at 7 and 21 days of age. An overall time effect (*P* = 0.002) also was observed mainly due to a linear down-regulation in its expression from 1 to 21 days in the INO calves. There was no treatment effect on *MPO* expression (*P* = 0.206), but there was a significant interaction (T×D, *P* = 0.026), where greater (*P* = 0.009) expression was observed in INO calves at birth. Expression of *SOD1* and *NOS2* was not affected (*P* = 0.264 and 0.361) by maternal treatment. Expression of *SOD1* increased from birth to 7 days (*P* < 0.001), while *NOS2* decreased (*P* = 0.005) during the same period (Figure 4).

DISCUSSION

An improved health status during the neonatal period is essential for optimal growth under common stressors of early life including colostrum deprivation, transportation stress, environmental adaptations and even residual effects of maternal nutrition (Osorio et al., 2012). Trace minerals play a critical role in the function of the immune system, especially when bioavailability is enhanced through supplementation as chelates of minerals within amino acids or organic forms. Using those approaches in dairy cattle during stressful times (e.g. the periparturient period) has resulted in better health status (Nocek et al., 2006, Andrieu, 2008).

Growth performance and health

As already well-established in poultry (Bao et al., 2010, Rao et al., 2013), the greater withers height in calves born to dams supplemented with organic trace minerals could be a direct effect on bone growth. In mammals, intrauterine bone development is strongly and directly affected by growth factors such as insulin-like growth factor 2 (IGF-2), and epigenetic modifications modulate this pathway (Constancia et al., 2002, Tabano et al., 2010). Female rats that were exposed to intrauterine growth restriction had a negative long-term effect on bone size and mineral content and strength in part due to a decrease in endochondral ossification responsiveness (Chen et al., 2013). Maternal vitamin D consumption affects bone accretion during the intrauterine period and influences bone size through increased 25-hydroxyvitamin D and parathyroid hormone concentration in the circulation (Morley et al., 2006, Viljakainen et al., 2010). A review about keratin formation also highlighted the role of Mn on cartilage and bone formation, as it is needed for activation of galactotransferase and glycosyltransferase enzymes, needed for the

synthesis of chondroitin-sulfate side chains of proteoglycan molecules (Tomlinson et al., 2004). Whether the same mechanisms account for the greater withers height in calves from cows fed ORG remains to be determined.

Compared with calves from cows fed a control diet, neonatal dairy calves receiving a higher-energy diet including organic trace mineral supplementation (Zn, Mn, Cu, and Co) had greater rates of growth during the pre-weaning period (Osorio et al., 2012). Our results indicate that in-utero organic trace mineral nutrition also can contribute to enhancing post-natal calf growth, even without postnatal supplementation. The lower oxidative stress and inflammatory status of these calves, discussed below, also could play a role in the growth response.

Although rectal temperature was greater in INO than ORG calves, it did not surpass the normal physiological threshold (Scibilia et al., 1987). The negligible increase in temperature of 0.5°C in INO calves could be of biological significance and, as suggested by the *TNF* and *NFKB1* expression could reflect a transient inflammatory response. The higher body temperature and inflammatory response in INO calves could have increased maintenance energy needs, hence, impairing bone growth (Mundy, 1993; Lacey et al., 2009). Calves supplemented with an injectable trace mineral supplement containing Se, Cu, Zn, and Mn had lower diarrhea incidence during the first mo of life (41.7% vs. 49.7%), and it was associated with an enhanced immune response (Teixeira et al., 2014). In contrast to a human study (Bhandari et al., 2008), calves treated for diarrhea with an oral rehydration soln enriched with Zn did not have a better recovery than controls (Glover et al., 2013). The effectiveness of Zn to reduce diarrhea in children was associated with an improvement of enzymatic function in the intestinal cells and also with enhanced intestinal mucosa repair (Sazawal et al., 1995, Strand et al., 2002); however, children

are obviously kept under a more controlled environment than calves, in terms of temperature, humidity, feeding plan, which could contribute to increase the efficacy of diarrhea treatments. The mechanisms of Zn on calf diarrhea are unclear, but as Zn is associated with many immunological functions, feeding ORG to dams could elicit better postnatal health status.

Blood biomarkers of energy, protein and inflammatory metabolism

Both innate and adaptive immune systems are important to prevent or limit infections, but such important regulatory functions are energy-demanding (Calder, 1995). Glucose is considered the most quantitatively important energy source for immune cells, in addition to glutamine, ketone bodies and fatty acids, and these nutrients are essential to allow rapid cellular responses within hours after stimulation, e.g. for phagocytosis and oxidative burst (Wolowczuk et al., 2008). Neutrophils contain relatively few mitochondria, and derive most of their energy from glycolysis, including for neutrophil extracellular traps release (Rodriguez-Espinosa et al., 2014). Greater glucose concentrations in INO calves could be indicative of a more pronounced pro-inflammatory status in response not only to an infection, but also to a stressful cellular environment. For instance, the neonate has to activate gluconeogenesis after birth partly via greater glucocorticoid production, which in the long-term promotes maturation of metabolic pathways (Hammon et al., 2012).

The decrease in NEFA concentrations after birth underscored the depletion of these metabolites that via placental transfer where identified in the calf bloodstream. The increase in AST/GOT and GGT concentrations after colostrum intake could have been a result of intestinal absorption as immunoglobulins, in fact as for immunoglobulins their content is higher in colostrum (Bertoni et al., 2009; Ontsouka

et al., 2011), and not necessarily related with liver damage. The activity of GOT in colostrum is 400-800 times higher than in serum of adult animals (Parish et al., 1997). As indices of liver damage, AST/GOT and GGT in the newborn calf could reflect a metabolic disorder of the dam such as subclinical acidosis (Lechowski, 1996), but based on previous work this increase appears to be a normal physiological response in the calf (Bertoni et al., 2009).

The neonatal capacity for fatty acid oxidation and ketone body production in liver is low, but increases markedly during the first 24 h of life in response to colostrum and milk replacer feeding (Blum, 2006), thus, explaining the gradual increase over time in BHBA concentration. Compared with adult dairy cattle, concentration of BHBA in young calves is typically lower (Hammon et al., 2012). The increase of BHBA through the time of weaning is associated with the gradual intake of solid feed which allows for rumen development and fermentation capacity leading to production of volatile fatty acids of which butyrate can be metabolized by rumen epithelium to the ketone bodies BHBA and acetoacetate (Naeem et al., 2012).

The acute-phase response is characterized by the concomitant increase in concentration of positive APP such as ceruloplasmin, haptoglobin and SAA, and the decrease of negative APP such as paraoxonase and albumin (Bertoni et al., 2009; Eckersall and Bell, 2010). Regardless of maternal nutrition, paraoxonase concentration remained within physiological levels for healthy calves, and the increase over time agrees with a previous study (Giordano et al., 2013). The increase in IL-6 concentration right after birth is indicative of an activation of the immune system. These responses together could be related with maturation of hepatic metabolic pathways, immune stimulation, and colostrum intake (Orro et al., 2008). Despite the APP serving as non-specific markers of inflammation, the increase of the

positive and/or decrease of negative APP also could be related with subclinical disease, thus, also serving as indicators of a mild chronic state of inflammation. Under that scenario, if the calf is challenged with a stressor, the response could be exacerbated and potentially lead to an imbalance of the immune system.

The cellular antioxidant capacity of the cow after parturition is inadequate to neutralize all the ROM generated by the normal increase in metabolic rate leading to a state of oxidative stress that could directly impair the animal's immune function, hence, increase the susceptibility to disease (Sordillo and Aitken, 2009). Assuming the calf experiences the same condition soon after birth, the lower concentrations of MPO, ROM, and FRAP in ORG calves suggest a positive effect of maternal supplementation with ORG in helping to reduce the oxidative stress response of the calf after birth. Antioxidant activity has been positively correlated with increased FRAP (Benzie and Strain, 1996) and ORAC, thus, the higher FRAP and ORAC concentration in INO calves could be related to a greater need for neutralizing ROS production. Whether such an effect was induced in utero or via colostrum is difficult to ascertain. However, the results revealed a clear activation of the oxidative stress response right after birth regardless of maternal treatment.

In a previous study, excess maternal energy did not alter plasma concentrations of ROM or MPO in calves during the neonatal period (Osorio et al., 2013). In another study, however, calves born to cows that were energy-restricted during the prepartal period had impaired immune and antioxidant functions, with lower total antioxidant capacity and superoxide dismutase activity at birth (Gao et al., 2012). Thus, it appears that prepartal energy nutrition of the cows could alter immune function of the neonatal calf. In terms of mineral nutrition, the fact that dietary organic Zn supplementation to the cow resulted in greater blood Zn concentration in the calf

(Wang et al., 2013) suggests a higher rate of transfer to the calf. Feeding a high level of Se not only increased its concentration in blood of the dam but also the calf (Gunter et al., 2003). In addition, a recent study reported that calves supplemented with minerals via injection had greater neutrophil function and antioxidant capacity during the first 2 wk of life (Teixeira et al., 2014).

In order to control the intracellular redox environment and protect themselves against the constant oxidative challenge, cells need to develop mechanisms to ensure a proper balance between pro- and antioxidant molecules (Forman and Torres, 2001). Cu/Zn superoxide dismutase (SOD-1) is a key enzyme in the dismutation of superoxide radicals, and already has been reported as an important regulator of the inflammatory process (Marikovsky et al., 2003). The fact that expression of *SOD1* increased concomitantly with serum concentrations of Cu and Zn underscores an improvement in immune function in the calves.

It is concluded that supplementing the mother with organic trace minerals can benefit the development and capacity of the immune cells during the last stages of intra-uterine life as reflected in lower glucose, APP and oxidative stress biomarkers. In turn, it could be envisaged that availability of energy for physiological functions under those conditions is used more “efficiently” for growth. Similarly, increased trace mineral availability to the mammary gland as the result of supplementing the pregnant cow with ORG likely allows reducing pathogen infection in the mammary gland and consequently a lower colostrum pathogen load. In addition, it enhances the transfer of these essential nutrients to calves (via placenta and colostrum) and could be one factor that enhances their immune response.

PMN gene expression regulation of the innate immune response

The pro-inflammatory cascade can be initiated via different pathways, but both TLR2 and TLR4 are essential for neutrophil pathogen recognition and defense, including adhesion, generation of ROM, and release of chemokines (Sabroe et al., 2005). Even though calves in the ORG treatment had greater TLR expression, that response was not associated with upregulation of the pro-inflammatory pathway as indicated by the lower expression of the cytokine mediators *MYD88*, *IRAK1*, *TRAF6* and *NFKB1* and also the lack of change in *IL1B*. It could be possible that ORG calves have a more “efficient” immune system such that they maintain higher expression of TLR and do not need to upregulate expression of the pro-inflammatory signaling genes, i.e. this subtle response is sufficient to mount a response against pathogens or other challenges to the immune system. In contrast, INO calves which have a more “fragile” immune system, in an attempt to respond to a challenge have to reiterate the mechanisms of defense (including inflammation). As such, the pro-inflammatory signaling genes are upregulated.

In the context of the TLR system, previous work detected an upregulation of expression of *TLR2* in cow blood PMN after lipopolysaccharide (LPS) stimulation (Moyes et al., 2014), and maternal prepartal energy overnutrition increased *TLR4* expression in neonatal calf PMN (Osorio et al., 2013). Serum concentrations of Ca, Cu, Mo and Zn were lower in cows with endometritis and retained placenta (Bicalho et al., 2014), highlighting the beneficial effects of trace minerals on the immune system. Therefore, it is plausible that both increased availability of trace minerals during pregnancy (via the blood) or via colostrum can improve calf immunity, hence, reducing the pro-inflammatory signaling during the first three wk of life.

The lower expression of *SELL* in ORG calves could be related to increased turnover rate, as L-selectin-deficient neutrophils are more susceptible to apoptosis

(Matsuba et al., 1997). In addition, the marked increase in *SELL* expression during the first wk of life, also observed previously (Osorio et al., 2013), likely reflects the colostrum potential to trigger inflammation and prime the innate immune response. The same stimulus could be responsible for the expression profile of *ZBP1*, because ZBP1 is a cytosolic pathogen DNA recognition molecule that acts as a sensor to activate the innate immune response (Takaoka et al., 2007).

The transcription factor NFE2L2 can regulate the expression of genes associated with detoxification, antioxidant, and anti-inflammatory cellular responses, playing an important protective role in the development of disease (Cardozo et al., 2013). Thus, the greater expression of *NFE2L2* in ORG calves suggests that they benefitted from the antioxidant and anti-inflammatory regulation controlled via NFE2L2. As opposed to the blood markers related to oxidative stress (MPO, ROM and FRAP), the fact that no treatment effect was observed in the expression of the genes related to the oxidative stress (*SOD1*, *MPO* and *NOS2*) suggests the existence of post-transcriptional regulation.

miRNA expression and its relationship with the TLR pathway

Although miR-223 expression was neither affected by maternal diet nor time, it was the most abundant among all miRNA evaluated (Supplementary material). Several lysosomal proteases essential for the immune defense are regulated by miR-233 (Gantier, 2013). This miRNA is one of the master regulators of neutrophil proliferation and activation (Lindsay, 2008), as suggested by miR-223 knockout mice which had greater numbers of granulocyte progenitors in the bone marrow and hypermature neutrophils in the bloodstream. Thus, miR-223 has a negative effect on maturation but not on differentiation of granulocytes. However, the absence of miR-

223 expression did not lead to any detrimental effects on extravasation, migration, or phagocytosis (Johnnidis et al., 2008).

The greater miR-155 expression, especially at 21 d of life, in the calves born to INO cows could be related with the enhanced pro-inflammatory response observed in these animals. This miRNA is strongly induced by TLR pathogen sensing and inflammatory cytokines, with a broad range of mediators, including viral and bacterial responses (O'Connell et al., 2007). Furthermore, when induced by LPS miR-155 can enhance TNF translation (Tili et al., 2007). The greater expression of miR-155 in INO calves was associated with greater expression of *NFKB1* and *TNF* (at 21 d). As the miRNA acts to repress the protein translation process, it is possible that enhanced mRNA synthesis (or greater half-life) is a compensatory response to counterbalance this inhibitory effect. Taken together, it is plausible that when calves received intrauterine ORG trace minerals, the miR-155 expression was downregulated, and consequently reduced the requirement to upregulate the expression of pro-inflammatory mediators such as NFKB1 and TNF. In contrast to miR-155, miR-125b was suppressed by exogenous LPS stimulus (Tili et al., 2007), which is consistent with our results where ORG calves had concomitant greater *TLR4* expression at 21 d of age while lower miR-125b expression.

The expression of miR-146a can be induced by bacterial activation of cell surface TLRs (TLR2, TLR4, TLR5) and also by TNF and IL1B treatment in an NFKB-dependent manner (Taganov et al., 2006). The genes *IRAK1* and *TRAF6* are direct targets of miR-146a, and their downregulation via the miRNA may function as a negative feedback regulatory mechanism, i.e. miR-146a expression may be critical to prevent excessive inflammation. Despite the lack of a maternal nutrition effect, the increased expression from birth to the first wk of life indicates an important function of

miR-146a on the neonatal immune system. The increased expression of miR-9 during the first wk of life is likely related with TLR4 activation in an NFKB dependent-manner, operating in a negative feedback control by exerting close control on the expression of key members of the NFKB pathway (Bazzoni et al., 2009).

To the authors' knowledge, this is the first study demonstrating the plasticity of miRNA expression in bovine neutrophils during the neonatal period, as well as how maternal diet supplementation with organic trace minerals can influence growth and the immune response. Our results indicate that calves from dams fed ORG during the last 30 d of gestation improved immune function, as they had a lower pro-inflammatory status concomitant with a better control of the oxidative stress during the neonatal period. If the improved immune function spared energy for growth, it would partly explain the positive response in bone growth, reflected in greater withers height, around weaning time. Additional research in this area to clarify the mechanisms at play seems warranted.

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Table 1. Genes and microRNA selected for transcript profiling in bovine neutrophils.

Gene name	HUGO gene symbol
Interleukin 1, beta	*IL1B*
Interleukin-1 receptor-associated kinase 1	*IRAK1*
Myeloperoxidase	*MPO*
Myeloid differentiation primary response gene (88)	*MYD88*
Nuclear factor (erythroid-derived 2)-like 2	*NFE2L2*
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	*NFKB1*
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	*NFKBIA*
Nitric oxide synthase 2, inducible	*NOS2*
Selectin L	*SELL*
Superoxide dismutase 1, soluble	*SOD1*
Toll-like receptor 2	*TLR2*
Toll-like receptor 4	*TLR4*
Tumor necrosis factor- α	*TNF*
TNF receptor-associated factor 6	*TRAF6*
Z-DNA binding protein 1	*ZBP1*
MicroRNA mir-125b	*MIR125b*
MicroRNA mir-146a	*MIR146a*
MicroRNA mir-155	*MIR155*
MicroRNA mir-223	*MIR223*
MicroRNA mir-9	*MIR9*

Table 2. Rectal temperature, fecal score and starter intake in calves born to dams fed a conventional diet supplemented with only inorganic (INO) or inorganic and organic (ORG) trace minerals during the last 30 d pre-partum.

	Treatment (T)			P-value[1]		
Week	ORG	INO	SEM	Trt	wk	T×W
Rectal temperature, °C						
0	38.15	38.41	0.14	0.069	0.032	0.973
1	37.94	38.32	0.14			
2	37.78	38.11	0.14			
Fecal score (1-4)						
0	1.41^{b}	1.40^{b}	0.12	0.694	<0.001	0.060
1	1.83^{a}	1.89^{a}	0.12			
2	1.42^{b}	1.25^{c}	0.12			
3	1.21^{bc}	1.28^{bc}	0.12			
4	1.09^{c}	1.40^{b}	0.12			
5	1.15^{c}	1.10^{d}	0.12			
6	1.04^{d}	1.00^{d}	0.12			
7	1.00^{d}	1.08^{d}	0.12			
Starter intake (kg/day)						
1	0.04	0.01	0.13	0.818	<0.001	0.406
2	0.13	0.17	0.13			
3	0.45	0.53	0.13			
4	0.69	0.77	0.13			
5	0.86	0.95	0.13			
6	1.57	1.55	0.13			
7	2.57	2.58	0.13			

[a,b,c]Differences (P< 0.05) between time points within treatment

[1]*P* value for treatment (Trt), wk, or their interaction (T×W)

Table 3. Blood immunometabolic biomarkers in calves born to dams fed a conventional diet supplemented with only inorganic (INO) or inorganic and organic (ORG) trace minerals during the last 30 d pre-partum.

		Day of age					P-value[1]		
Item	Trt	0	1	7	21	SEM[2]	Trt	Day	T×D
Glucose, mmol/L	ORG	2.06^{Bc}	8.03^{a}	6.12^{b}	5.58^{b}	0.37	0.001	<0.001	0.017
	INO	4.09^{Ac}	8.95^{a}	5.86^{b}	6.39^{b}	0.34			
NEFA, mmol/L	ORG	1.00	0.32	0.40	0.17	0.08	0.808	<0.001	0.781
	INO	1.10	0.30	0.39	0.16	0.07			
BHBA mmol/L	ORG	0.04	0.06	0.08	0.11	0.01	0.361	<0.001	0.112
	INO	0.04	0.06	0.08	0.15	0.01			
Creatinine (Log2), µmol/L	ORG	7.63	6.78	6.64	6.42	0.08	0.445	<0.001	0.756
	INO	7.77	6.79	6.71	6.43	0.07			
Urea, mmol/L	ORG	4.53^{a}	3.25^{b}	3.21^{b}	3.96^{ab}	0.36	0.968	<0.001	0.039
	INO	3.79^{b}	3.67^{b}	2.85^{c}	4.58^{a}	0.34			
Bilirubin, µmol/L	ORG	4.03	4.28	3.16	2.20	0.20	0.170	<0.001	0.756
	INO	4.07	4.34	3.56	2.46	0.19			
AST/GOT, U/L	ORG	5.40	6.72	5.38	5.77	0.13	0.015	<0.001	0.611
	INO	5.61	6.79	5.81	6.02	0.12			
GGT, U/L	ORG	3.87	11.22	8.74	6.88	0.29	0.422	<0.001	0.444
	INO	3.78	11.40	9.16	7.37	0.29			
Cholesterol, mmol/L	ORG	0.74	1.00	1.58	2.58	0.13	0.268	<0.001	0.345
	INO	0.79	1.02	1.69	3.00	0.12			
Albumin, g/L	ORG	29.7	25.9	29.2	31.5	0.6	0.113	<0.001	0.192
	INO	31.2	26.6	30.4	32.8	0.6			
Ceruloplasmin, µmol/L	ORG	0.03	0.86	2.76	2.66	0.26	0.345	<0.001	0.468
	INO	0.29	1.24	2.91	2.60	0.22			
Haptoglobin, g/L	ORG	0.40	0.40	0.51	0.39	0.07	0.894	0.251	0.768
	INO	0.46	0.41	0.47	0.33	0.07			

Paraoxonase, U/mL	ORG	6.3[c]	12.1[bc]	17.2[b]	42.5[Ba]	2.6	<0.001	<0.001	0.053
	INO	10.2[c]	15.6[c]	23.7[b]	56.9[Aa]	2.7			
Myeloperoxidase (Log2), U/L	ORG	ND[3]	4.62	5.04	5.86	0.38	0.003	0.013	0.822
	INO	ND[3]	5.38	5.83	6.28	0.27			
SAA, µg/mL	ORG	41.94	243.9	267.2	149.6	22.56	0.398	<0.001	0.652
	INO	51.49	270.5	307.6	141.9	24.24			
IL-6, pg/mL	ORG	275.7	453.3	300.2	285.6	97.04	0.796	0.056	0.607
	INO	133.5	493.9	275.2	294.8	103.2			
ROMt (Log2), mg H_2O_2/100 mL	ORG	ND[2]	-0.09	2.08	2.22	0.26	0.083	<0.001	0.712
	INO	ND[2]	0.40	2.57	2.40	0.21			
FRAP, µmol/L	ORG	256	198	165	165	19	0.043	<0.001	0.746
	INO	293	220	218	213	19			
NOx, µmol/L	ORG	14.44	20.89	16.95	26.54	5.49	0.229	0.006	0.852
	INO	15.03	28.05	22.04	27.65	4.71			
NO_2^-, µmol/L	ORG	6.07	8.91	6.62	11.98	2.87	0.216	0.016	0.857
	INO	6.92	13.51	9.65	11.75	2.45			
NO_3^-, µmol/L	ORG	8.35	11.97	9.67	14.59	2.67	0.228	0.002	0.712
	INO	8.70	16.11	12.38	15.81	2.27			
ORAC, TE mol/L	ORG	8.89	10.59	11.57	11.04	0.58	0.109	<0.001	0.382
	INO	8.96	11.55	12.56	12.79	0.58			
Retinol, µg/100mL	ORG	5.45	10.12	13.75	15.64	3.38	0.335	<0.001	0.529
	INO	6.18	14.61	18.52	24.78	3.31			
Tocopherol, µg/mL	ORG	0.58[b]	1.67[b]	2.75[a]	3.31[a]	0.43	0.119	<0.001	0.086
	INO	0.67[b]	1.36[b]	1.24[b]	3.08[a]	0.46			
Cu, µmol/L	ORG	3.13	5.38	11.40	11.51	0.86	0.952	<0.001	0.624
	INO	2.93	6.25	11.40	11.05	0.89			
Mn, µmol/L	ORG	0.12	0.13	0.10	0.11	0.01	0.722	0.678	0.147
	INO	0.10	0.11	0.12	0.13	0.01			
Fe, µmol/L	ORG	20.3	20.5	11.5	14.2	2.5	0.478	<0.001	0.127

	INO	16.8	25.2	14.4	17.5	2.7			
Zn, µmol/L	ORG	15.3[a]	8.7[b]	18.6[a]	19.6[a]	2.7	0.633	<0.001	0.091
	INO	12.2[c]	15.7[bc]	16.9[b]	22.6[a]	2.7			

[A,B]Differences (P< 0.05) between treatments within time point

[a,b,c]Differences (P< 0.05) between time points within treatment

[1]*P* value for treatment (Trt), d (D), or their interaction (T×D)

[2]Greatest standard error of the mean (SEM) is shown

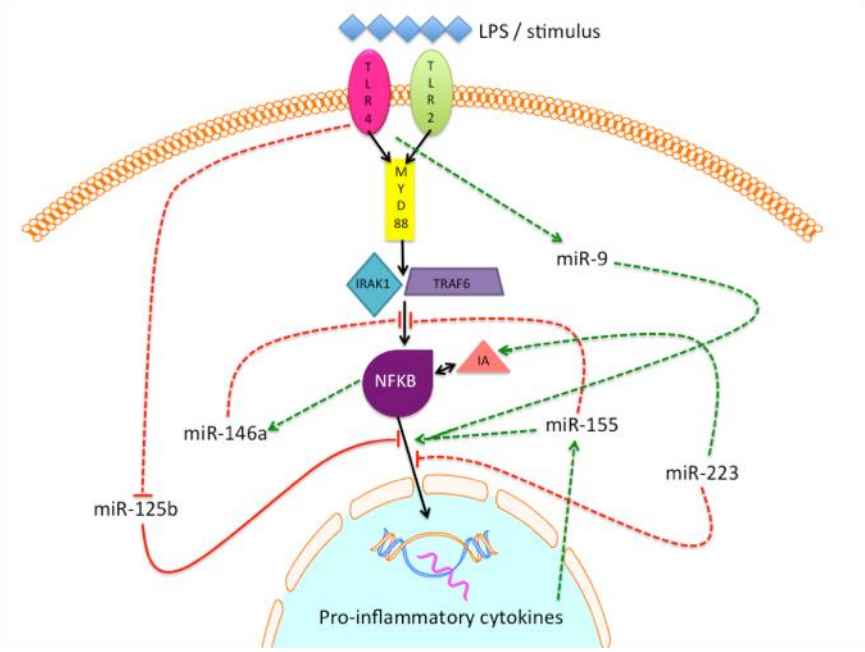

Figure 1. Model of the regulation of the innate immune response by microRNA. Green lines indicate activation and red lines indicate a suppressor effect. Broken lines indicate indirect effects, continuous line direct effects.

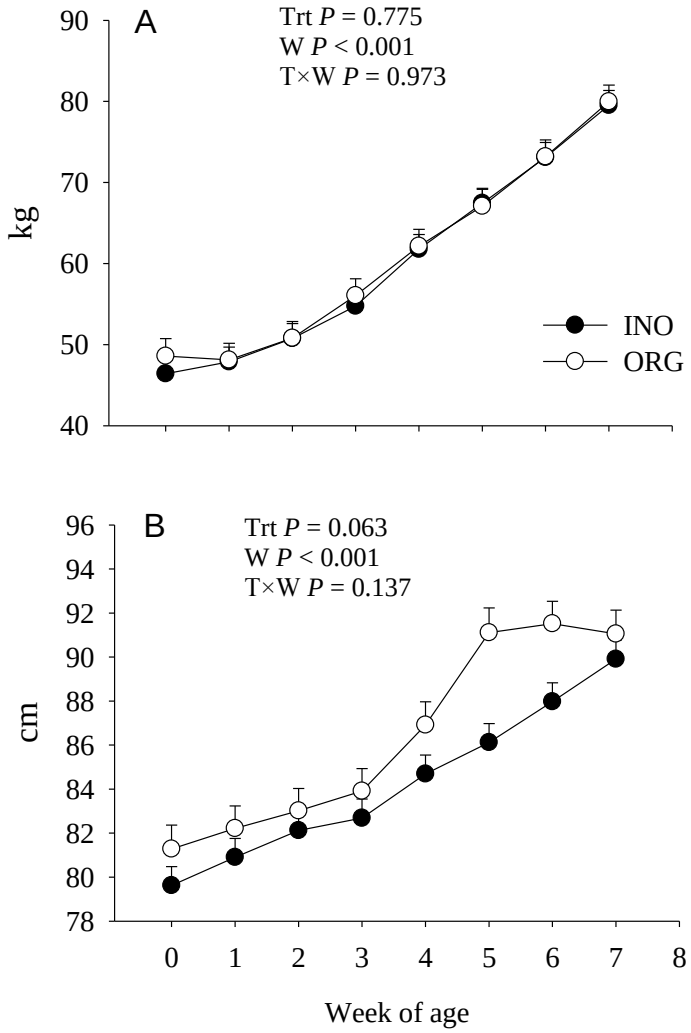

Figure 2. Body weight (panel A) and withers height (B) of calves born to dams fed a conventional diet supplemented with only inorganic (INO) or inorganic and organic (ORG) trace minerals during the last 30 d pre-partum. Shown are P values for main effects of treatment (Trt) and day (D), and their interaction (T×D).

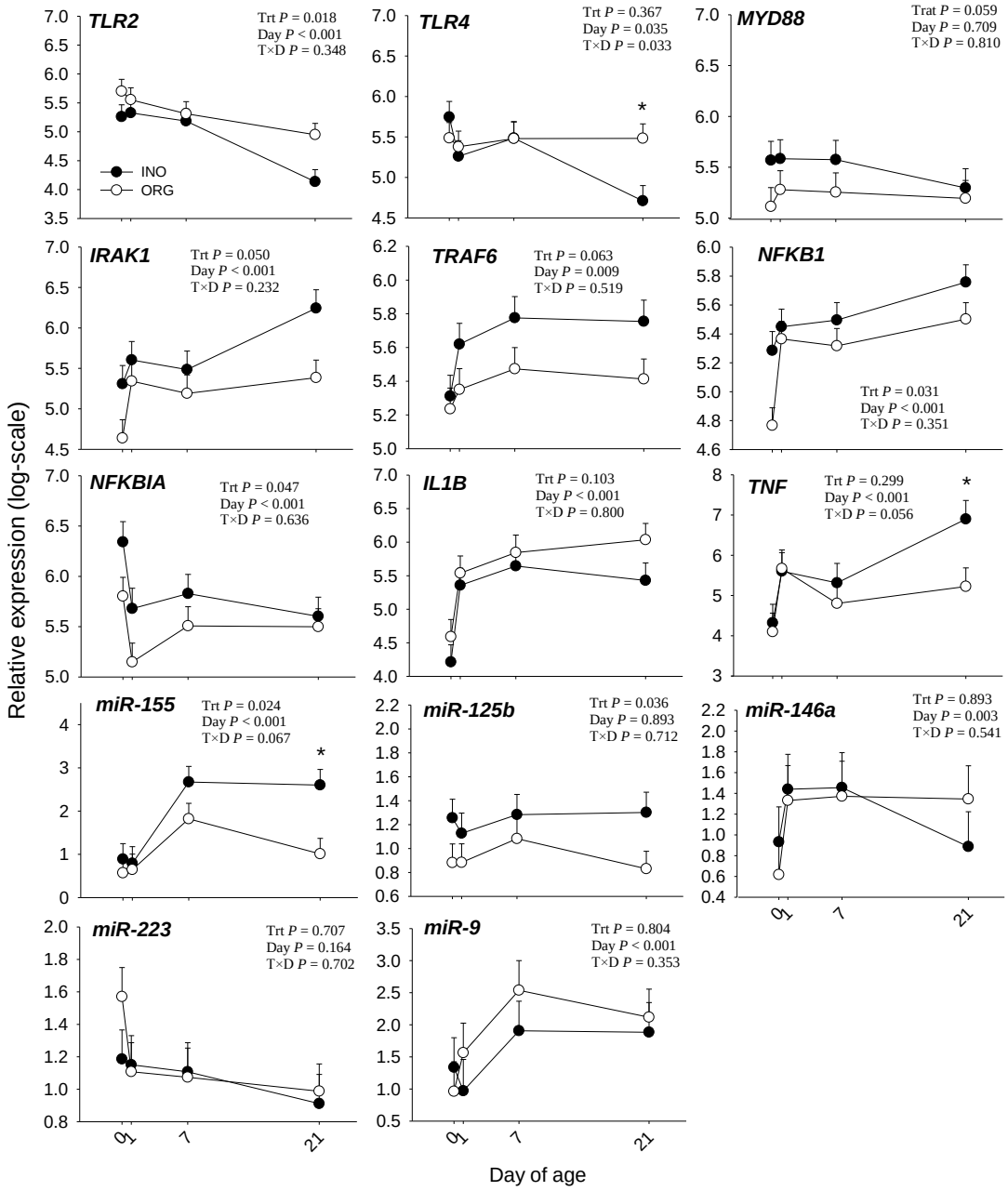

Figure 3. Expression of genes related to the pro-inflammatory pathway activated by TLRs and related microRNA from calves born to dams fed a conventional diet supplemented with only inorganic (INO) or inorganic and organic (ORG) trace minerals during the last 21 d pre-partum. Shown are P values for main effects of treatment (Trt) and day (D), and their interaction (T×D). *Means differ ($P < 0.05$) between treatments.

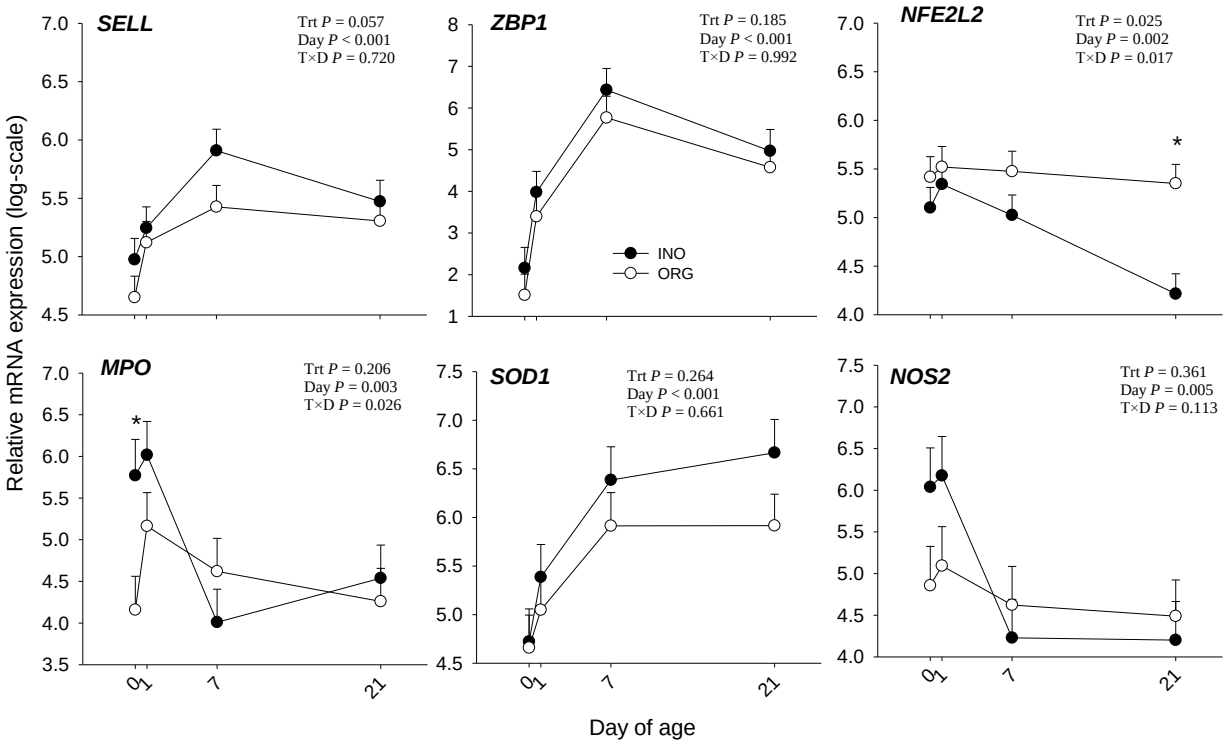

Figure 4. Expression of genes related to PMN adhesion, pathogen recognition and oxidative stress from calves born to dams fed a conventional diet supplemented with only inorganic (INO) or inorganic and organic (ORG) trace minerals during the last 21 d pre-partum. Shown are P values for main effects of treatment (Trt) and day (D), and their interaction (T×D). *Means differ (P < 0.05) between treatments.

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4.2 Manuscrito 1 - Regulation of blood and liver biomarkers of inflammation, oxidative stress, energy metabolism and dairy calf performance by maternal pre-partum methionine supplementation

Carolina B. Jacometo[1,2], Zheng Zhou[2], Daniel Luchini[3], Erminio Trevisi[4], Marcio N. Corrêa[1] and Juan J. Loor[2*]

[1]NUPEEC, Departamento de Clínicas Veterinária, Programa de Pós-Graduação em Biotecnologia, Universidade Federal de Pelotas, Pelotas, RS, Brazil.

[2]Mammalian NutriPhysioGenomics, Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois, Urbana, IL, USA.

[3]Adisseo USA Inc., Alpharetta, GA, USA.

[4]Istituto di Zootecnica, Facoltà di Scienze Agrarie, Alimentari e Ambientali, Università Cattolica del Sacro Cuore, Piacenza, Italy.

INTRODUCTION

After birth, the greatest challenge that calves have is to deal with the change in the nutrient intake flow. It changes from a continuous and parenteral feeding, mainly with glucose, lactate and amino acids, to an enteral feeding, rich in lactose and fat as energy source (Hammon et al., 2012). During intrauterine period, glucose is the main energy source for fetal energy supply, there is a linear relation between maternal and fetal plasma glucose concentrations, and maternal fasting or undernutrition for several days induces to fetal hypoglycemia in sheep (Bell and Ehrhardt, 2002).

Dairy cows experience a challenging period during the transition from pregnancy to lactation with a high risk of developing metabolic disorders (Drackley, 1999). Research in ruminant animals supports the hypothesis that increasing the supply of methionine could enhance the liver capacity to export triacylglycerol (TAG) in the form of very low density lipoproteins (VLDL) and help to ameliorate the negative effects of fatty acid accumulation in the liver (Auboiron et al., 1994, Auboiron et al., 1995).

Besides being an essential amino acid for milk protein synthesis it also has a role for antioxidant reactions and the immune function, mainly through lipoprotein synthesis (Soder and Holden, 1999, Chen et al., 2007). The regulation of the oxidative stress status also could be influenced by methionine, hence it could serve as a substrate for glutathione synthesis via homocysteine (Martinov et al., 2010). Glutathione is one of the most abundant natural antioxidants produced within the liver, and a reduction in its synthesis in rodents leads to steatosis, mitochondrial damage, and marked increases in lipid peroxidation (Chen et al., 2007). Also a reduction in the inflammatory response was observed when supplementing with methionine during the peripartal period (Osorio et al., 2014b).

Our hypothesis was that rumen-protected methionine supplementation, besides providing cows all the benefits reported in the literature, would benefit calf liver metabolism during the neonatal period. The specific objectives were to identify the residual effects of methionine supplementation during late-pregnancy on calves blood immunometabolic markers and liver expression of genes related to gluconeogenesis, fatty acid oxidation, lipoprotein metabolism, GH-IGF1 axis, insulin signaling, inflammatory response, glucocorticoid and adrenergic receptors, methionine cycle and glutathione metabolism.

MATERIAL AND METHODS

All the procedures for this study were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois (protocol #13023).

Maternal Treatments

The experiment was conducted as a randomized complete blocked design with 40 multiparous Holstein cows blocked according to parity, previous lactation milk yield, and expected day of parturition. All cows received a common early-dry period diet ("far-off diet") from -50 to -25 d relative to parturition, with low energy and high straw designed to meet and not greatly exceed 100% of energy requirements. During the late-dry period diet ("close-up diet"), from -24 d until calving day, cows received a high-energy diet. Cows were randomly assigned to a top-dressed on the total mixed ration (TMR) supplementation with rumen-protected methionine (MET, n = 20 – 0.08% of the dry matter of the diet/d methionine, Smartamine® M Adisseo, Alpharetta, GA, USA, ~2.9:1 Lys:Met), or with no supplementation (CON, n = 20, ~3.35:1 Lys:Met). After birth, calves were fed a common diet and managed similarly. Hence, any observed treatment effects are attributed to maternal nutrition during the last 24 d of gestation.

Animal management and calf enrollment criteria

During the dry period, cows were housed in a ventilated, sand-bedded free-stall barn, with a photoperiod of 8 h of light and 16 h of dark. Diets were fed for ad-libitum intake as a TMR once daily using an individual gate feeding system (American Calan, Northwood, NH) and dry matter intake (DMI) was recorded daily. As cows began demonstrating signs of impending parturition, they were moved to an

individual maternity pen bedded with straw. After parturition, cows were milked at the end of the farm's next milking period (4 AM, noon or 8 PM). Colostrum volume was recorded and IgG content was estimated based on specific gravity with a bovine colostrometer (Nasco, Fort Atkinson, WI; Cat. no. C10978N).

Calves were kept in the experiment if they fulfilled all the following criteria: 1) single calf; 2) calving difficulty score <3; 3) dam's colostrum quality assessed by a bovine colostrometer of >50 mg/L of IgG; 4) dam produced at least 3.8 L of a good quality first colostrum; 5) calf birth weight >36 kg (Johnson et al., 2007).

After birth, calves were weighted, had the navel disinfected with a 7% tincture of iodine solution (First Priority Inc., Elgin, IL), vaccinated with TSV II (Pfizer Inc., New York, NY) via nostril application, and received 3.8 L of first milking colostrum from the respective dam within 8 h after birth. If voluntary colostrum intake had not reached the 3.8 L required, calves were tubed with an oesophageal groove. Calves were housed in individual outdoor hutches bedded with straw, fed twice daily with a milk replacer (Advance Excelerate, Milk Specialities, Carpentersville, IL; 28.5% CP, 15% fat) (from 1 to 10 d of age: 520 g/d, 11 to 20 d of age: 680 g/d, 21 to 35 d of age: 840 g/d, and from 36 to 42 d of age: 420 g/d in a single feeding) and had ad libitum access to a starter grain mix (19.9% CP, 13.5% NDF). Health checks, as fecal score (scale 1 – 4) was recorded daily until weaning, while rectal temperature was recorded daily until 21 d of age. Growth performance including body weight (BW) and withers height (WH) were recorded weekly. Calves were weaned at 42 d of age.

Sample collection

Blood samples were collected from the jugular vein using 20-gauge BD Vacutainer needles (Becton Dickinson, Franklin Lakes, NJ) before receiving

colostrum (baseline), 24 h after receiving colostrum, 14, 28 and 50 d (n = 12 / group). At each time point, a total of 40 mL of total blood were collected in vacutainer tubes (10 mL, BD Vacutainer, Becton Dickinson) containing serum clot activator or sodium heparin. After blood collection, tubes with sodium heparin were placed on ice while tubes with clot activator were kept at room temperature until centrifugation (~30 min). Serum and plasma were obtained by centrifugation of clot activator and sodium heparin tubes, respectively, at 1,900 × *g* for 15 min. Serum and plasma were aliquoted and stored at -80°C until further analysis.

Liver was sampled via puncture biopsy (adapted from (Dann et al., 2006) from calves under local anesthesia previously to the afternoon feeding, at approximately 3 PM, on days 4, 14, 28 and 50 of age (n = 8 / group). Tissue specimens were stored in liquid N_2 until further analysis.

Apparent Efficiency of Immunoglobulin Absorption

Apparent efficiency of absorption was estimated by measuring the IgG content in both first milking colostrum and calf serum at 24 h after colostrum ingestion. Apparent efficiency of absorption was calculated as plasma IgG (g/L) × plasma volume (L)/IgG intake (g) (Quigley et al., 2002).

Blood metabolites, APP and oxidative stress biomarkers

Blood samples were analyzed for albumin, cholesterol, bilirubin, creatinine, urea, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), glucose, beta-hydroxybutyrate (BHBA), haptoglobin (HP), ceruloplasmin, antioxidant potential (FRAP), paraoxonase (PON), myeloperoxidase and reactive oxygen metabolites (ROMt) using kits purchased from Instrumentation Laboratory (IL Test). Retinol and tocopherol were determined as previously described (Bionaz et al.,

2007). Non-esterified fatty acids (NEFA) was measured using kits from Wako Chemicals, following the procedures described previously (Bionaz et al., 2007, Trevisi et al., 2012, Osorio et al., 2013) using a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA). Bovine interleukin-6 (IL-6) and interleukin-1 beta (IL1B) plasma concentration were determined by a colorimetric sandwich ELISA using a bovine screening set (Thermo Fisher Scientific, Waltham, MA). IgG concentration (colostrum and serum) was measured using a quantitative bovine IgG ELISA kit (ZeptoMetrix Corporation, Buffalo, NY).

mRNA isolation

For liver total RNA extraction the miRNeasy kit (Qiagen, Hilden, Germany) was used following the manufacturer's protocols. Samples were treated on-column with DNaseI (Qiagen, Hilden, Germany), quantification was accessed using the NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE), and RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

cDNA synthesis and qPCR

Complementary DNA was synthesized using 100 ng RNA. Firstly random primers (10 mM) (Invitrogen Corp., CA) and DNase/RNase free water were mixed and incubated at 65 °C for 5 min and kept on ice for 3 min. Then a second mix containing DNase/RNase free water, first strand buffer (5X),oligo dT18 (Operon Biotechnologies, AL), dNTP mix (10 mM) (Invitrogen Corp., CA), RevertAidReverse Transcriptase (200 U/µL) (Fermentas Inc., MD) and RNase Inhibitor (20 U/ µL)(Promega, WI) was added. The reaction was performed in an Eppendorf Mastercycler® Gradient using the following temperature program: 25 °C for 5 min, 42 °C for 60 min and 70 °C for 5 min. cDNA was then diluted 1:3 with DNase/RNase

free water.

Quantitative PCR was performed using 4 µL diluted cDNA combined with 6 µL of a mixture composed of 5 µL of SYBR Green master mix (Quanta Biosciences, Gaithersburg, MD), 0.4 µL each of 10 mM forward and reverse primers, and 0.2 µL DNase/RNase free water in a MicroAmpTM Optical 384-Well Reaction Plate (Applied Biosystems, CA). Each sample was run in triplicate and a 6 points relative standard curve plus the non-template control were used. The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, CA) using the following conditions: 5 min at 95 °C, 40 cycles of 1 s at 95 °C (denaturation) and 1 min at 60 °C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15 s, 65 °C for 15 s plus 95 °C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems, CA).

Primers were designed using Primer Express 2.0 with minimum amplicon size of 80 bp (when possible amplicons of 100-120 bp were chosen) and limited 3' G+C (Applied Biosystems, CA). When possible, primer sets were designed to fall across exon– exon junctions. Primers were aligned against publicly available databases using BLASTN at NCBI (Nucleotide BLAST, 2008) and UCSC's Cow (Bos taurus) Genome Browser Gateway. Prior to qPCR primers were tested in a 20 µL PCR reaction using the same protocol described for qPCR except for the final dissociation protocol. For primer testing we used a universal reference cDNA (RNA mixture from different bovine samples) to ensure identification of desired genes. Five µL of the PCR product were run in a 2% agarose gel stained with SYBR safe. Only those primers that did not present primer-dimers and a single band at the expected size in the gel, and had the right amplification product (verified by sequencing) were used for

qPCR. The accuracy of a primer pairs also was evaluated by the presence of a unique peak during the dissociation step at the end of qPCR.

For this study *GAPDH*, *UXT* and *RPS9* were used as internal control genes, and their geometric mean was used to normalize the expression data. All evaluated genes and its related function are presented in the supplemental material.

Statistical analysis

Data were analysed with the Proc MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Fixed effects in the model were treatment (T), day (D) or week (wk), and their interaction (T×D, T×wk). Random effect was calf within diet. The exponential correlation covariance structure SP for repeated measures was used for analysis of blood metabolites and gene expression. Blood metabolites and gene expression results were $\log_2$-scale transformed if needed to comply with normal distribution of residuals, and subsequently back-transformed. Least squares means separation between time points was performed using the PDIFF statement. Statistical significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

Apparent Efficiency of IgG absorption, Growth Performance and Health

Colostrum IgG was not affected ($P = 0.52$) by maternal diet and averaged 75.6±6.6 mg/dL for CON cows and 81.8±6.7 mg/dL for MET cows. Similarly, calves apparent efficiency of IgG absorption was not affected ($P = 0.36$), averaging 26.1±3.2% for CON calves and 30.3±3.2% for MET calves. Calf birth weight was similar between groups (CON: 42.9±1.5 kg, MET: 44.2±1.6 kg, $P = 0.55$), as well as

body weight, weekly gain and withers height (P > 0.05) until 7 weeks of life, however a linear increase (*P* < 0.001) was observed in all parameters for both groups (Figure 1). Regardless of maternal treatment, starter intake increased (*P* < 0.001) over time (Table 1). Also rectal temperature and fecal score were not affected by maternal treatment (*P* < 0.05), although both groups had a higher fecal score at week 2, and then reduced until weaning time (*P* < 0.001) (Table 1).

Blood biomarkers

Metabolites

Main effects of diet, day, and interactions for blood metabolites are shown in Figure 2. A T×D (*P* = 0.04) was observed in glucose concentration, where MET calves had lower (*P* = 0.007) glucose at birth, however there was no overall maternal treatment effect (*P* = 0.18). Both groups had a marked glucose increase (*P* < 0.001) from birth to 24 h after colostrum intake, and then reduced (*P* < 0.001) at 14 d and kept constant until 50 d. Cholesterol increased over time (*P* < 0.001) and was not affected (*P* = 0.94) by maternal diet. NEFA concentrations decrease (*P* < 0.001) over time, with a marked reduction from birth to 24 h after colostrum intake, while BHBA concentrations increased (*P* < 0.001) over time, with higher concentrations at 50 d of age, and both markers were not affected by maternal diet (*P* > 0.05). The hepatic enzymes, AST and GGT had a time effect (*P* < 0.001) mainly due the increased response at 24 h after colostrum intake. AST also had a T×D (*P* = 0.05), and at birth MET calves had lower (*P* = 0.009) AST concentration. At birth MET calves also had higher (*P* = 0.01) creatinine, but no overall maternal diet effect was observed (*P* = 0.35), and its concentration had a marked decreased (*P* < 0.001) after colostrum intake. Bilirubin concentration start decreasing at 24 h after colostrum intake (*P* <

0.001) until 50d, with no maternal diet effect (*P* = 0.70). A T×D (*P* = 0.002) was observed for urea concentration, MET calves had higher (P = 0.02) concentrations at 24 h and then lower at 14 d of age, compared to CON calves, with no overall treatment effect (*P* = 0.82). Also a day effect (P < 0.001) was observed, mainly due a marked increase at 50 d of age.

Inflammation

Main effects of diet, day, and interactions for inflammation markers are shown in Figure 3. No overall maternal treatment effect was observed (*P* > 0.05) for all these markers. Haptoglobin had a day effect (P < 0.001) mainly due a peak observed at 28 d of age. Paraoxonase, albumin and ceruloplasmin increased (*P* < 0.001) their concentration over time. Albumin and ceruloplasmin tended (*P* = 0.09 and 0.11) to be lower in MET calves. Ceruloplasmin also had a T×D (*P* = 0.003) with lower (*P* = 0.004) concentration in MET calves at 14 d of age. IL1B and IL6 had a marked decrease (*P* < 0.001) from birth to 24 h after colostrum intake. IL1B had its higher concentration at birth, while IL6 was at 14 d of age.

Oxidative stress

Main effects of diet, day, and interactions for oxidative stress markers are shown in Figure 4. No overall maternal treatment effect was observed (*P* > 0.05) for all these markers. A T×D was observed for tocopherol (*P* = 0.05) and a trend for ROMt (*P* = 0.06), with lower concentrations (*P* = 0.02 and 0.001, respectively) for MET calves at 14 d of age. Regardless maternal treatment, ROMt and myeloperoxidase had their higher concentrations at 14 d of age, while FRAP was in its lower level at the same time (*P* < 0.001). Tocopherol had its higher concentration at 28 d (*P* < 0.001) and retinol had a consistent increase over time (*P* < 0.001).

Gene expression

Gluconeogenesis

Main effects of diet, day, and interactions for genes related to gluconeogenesis are shown in Figure 5. There was a trend (P = 0.09) for overall higher expression of *FBP1* in MET calves. Regardless of maternal treatment, *PCK1*, *PC* and *FBP1* increased the expression over time (P < 0.001). *PC* expression was not affected.

Fatty acid oxidation

Main effects of diet, day, and interactions for genes related to fatty acid oxidation are shown in Figure 6. Maternal supplementation with methionine reduced (P = 0.02) *ACOX1* and increased (P < 0.001) *CPT1A* expression. *PPARA* and *CPT1A* expression reduced (P < 0.001) from 4 d until 28 d and then increased (P < 0.001) at 50 d of age, while *ACOX1* expression consistently increase over time (P = 0.02). *HMGCS2* expression was not affected.

Lipoprotein metabolism

Main effects of diet, day, and interactions for genes related to lipoprotein metabolism are shown in Figure 7. No maternal diet effect was observed in these genes, only an overall increased expression (P = 0.03), regardless of treatment, was observed for *APOB*. *MTTP* expression was not affected.

GH-IGF1 axis

Main effects of diet, day, and interactions for genes related to the GH-IGF1 axis are shown in Figure 7. No maternal diet effect was observed in these genes,

only an overall increased expression, regardless of treatment, for *IGF1* (*P* = 0.001) and *GHR1A* (*P* = 0.03).

Insulin signaling pathway

Main effects of diet, day, and interactions for genes related to insulin signaling are shown in Figure 8. Maternal supplementation with methionine increased *AKT2* (*P* = 0.02) and *SLC2A2* (*P* = 0.002) expression. Regardless of maternal diet, *INSR*, *IRS1*, *AKT2* and *SREBF1* expression increased (*P* < 0.05) over time, while *SLC2A2* tended (*P* = 0.06) to decrease. *FOXO1* expression was not affected.

Inflammatory response

Main effects of diet, day, and interactions for genes related to the inflammatory response are shown in Figure 9. Maternal supplementation with methionine increased (*P* = 0.002) *SOD2* expression, and tended to increase the overall expression of *NFKB* (*P* = 0.10) and *SOD1* (*P* = 0.10). *SOD1* expression increased over time (*P* < 0.001) while *SOD2* had a reduction (*P* = 0.04), mainly due the reduction observed from 4 d to 14 d of age.

Glucocorticoid receptors

Main effects of diet, day, and interactions for glucocorticoid receptors genes are shown in Figure 9. Maternal supplementation with methionine increased (*P* = 0.02) *GR* expression. A T×D trend (P = 0.08) for *ADRB2* was observed, with higher expression for MET calves at 4 d of age (*P* = 0.05). Regardless of maternal treatment, *GR* and *ADRB2* expression increased over time (*P* < 0.001). *ADRA1* expression had a time effect (*P* = 0.02) mainly due a marked reduction at 28 d of age.

Methionine cycle

Main effects of diet, day, and interactions for genes related to methionine cycle are shown in Figure 10. Maternal supplementation with methionine increased (P = 0.01) *BHMT2* expression and decreased *CDO* (P = 0.01) and *DNMT1* (P < 0.001). Regardless of maternal diet, a day effect was observed for *MAT1A*, *CBS*, *PEMT*, *CSAD* and *DNMT3B* (P < 0.001, for all), mainly due a marked increase at 50 d of age. *DNMT1* increased (P = 0.003) and *DNMT3A* tended to increase (P = 0.09) their expression over time. *CDO*, *MTR* and *BHMT* increased (P < 0.05) their expression from 4 d to 14 d of age, while *CTH* decreased (P = 0.007). Also, the day effect observed in *BHMT2* expression (P = 0.02) was mainly due a decrease from 14 to 28 d of age.

Glutathione metabolism

Main effects of diet, day, and interactions for genes related to glutathione metabolism are shown in Figure 11. No maternal diet effect was observed in the evaluated genes related to this pathway. Regardless of maternal treatment, GCLC, GSR and GPX1 increased their expression over time (P < 0.001 for all), while GSS expression decreased from 4 d to 14 d of age and then increased from 28 d to 50 d of age (P < 0.001 for both).

DISCUSSION

For a dairy system, to be economically viable it is important that calves have a maximum growth rate. More than the percentage of the crude protein (CP), the amino acid balance of the milk replacers can affect calf growth performance, however the optimal ratios are not clearly defined yet and have been estimated in a

limited number of studies. Feeding calves 0.68 kg of a whey-based MR with synthetic Lys and Met that was 26% CP, 17% fat, 2.34% lysine, 0.72% methionine, 1.27% methionine+cystine, and 1.8% threonine maximized ADG and efficiency, while feeding calves 4 to 11% more CP and all essential AA at a minimal rate did not improve ADG and efficiency (Hill et al., 2008). Our results indicate that the maternal supplementation with rumen-protected methionine (reducing the lysine:methionine ratio) during the last 3 weeks of pregnancy was not effective in enhance dairy calves growth performance. As there is no other literature reference, it remains to be defined if another supplementation period could be more efficient in helping calves growth.

An adequate colostrum management is essential to ensure calf health. An alternative to low quality or unavailable maternal colostrum there are commercial plasma-derived or colostrum-derived colostrum replacer. However calves fed with 3.8 L of maternal colostrum had higher serum total protein, serum IgG, weaning weights and body weight gain and lower mortality than calves fed commercial colostrum formulas, although no difference was observed in the apparent IgG absorption efficiency (Priestley et al., 2013). Another study demonstrated that maternal heat stress during the last 6 weeks of gestation negatively affects calf to acquire passive immunity, regardless of colostrum source (Monteiro et al., 2014). Despite our results demonstrate a 6-mg/dL increase in MET colostrum IgG concentration and 4% increase in calves IgG absorption efficiency it did not reach statistical differences.

Although an effect of handling neonatal calves on biomarkers can not be discounted, the similar metabolic profiles at birth in MET and CON calves suggest a similar degree of nutrients flow from the mother to the calf through umbilical cord and placenta. The changes observed at 24 h after feeding are likely induced by colostrum intake, either through components in the colostrum or the calf response to those

components (e.g., insulin, cortisol, prolactin, IGF-1 and progesterone) are present in colostrum at a wide range of concentrations (Blum, 2006), and over time (until about one week after weaning) there is a clear maturation of the calf metabolic pathways.

At birth, MET calves had lower glucose than CON calves. Cortisol is positively and directly correlated with glucose (Vannucchi et al., 2015), then this lower concentration could be related with less stress right before and at calving. Also lower AST and higher creatinine was observed at birth in MET calves. As indices of liver damage, AST/GOT and GGT in the newborn calf could reflect a metabolic disorder of the dam such as subclinical acidosis (Lechowski, 1996), and higher creatinine, an indicative of skeletal muscle catabolism (Osorio et al., 2014b). However, when supplementing peripartal cows with methionine Osorio et al. (2014b) demonstrated that skeletal muscle catabolism was reduced, but regardless treatment, it increases around parturition.

The interactions observed at 14 d of age are of special interest in a physiological point of view. MET calves, compared to CON, had lower ceruloplasmin, a positive acute phase protein, lower ROMt, metabolites that are produced during the oxidative stress, lower myeloperoxidase, enzyme produced in response to ROMt, and lower tocopherol, an antioxidant molecule. This scenario is concomitant with increase in fecal score, in response to an increase in the feeding plan. At this moment calves are more likely to metabolic disorders, and having a more controlled oxidative metabolism could reflect in increased health (Ranade et al., 2014).

At the hepatic transcriptomic level, the increased expression of genes related to energy metabolism (gluconeogenesis, fatty acid oxidation, glucocorticoid and adrenergic receptors, lipoprotein metabolism, GH-IGF1 axis and insulin signaling)

illustrate the maturation of the metabolic pathways, essential for calves growth and development (Hammon et al., 2012). Surprising, a little effect of maternal was observed in the enzymes from the methionine cycle, while glutathione metabolism was not affected, differently to what was observed in a swine model, where sows supplemented throughout gestation with 3 g/day of betaine increased most of the one-carbon metabolism enzymes, and also regulated gluconeogenic genes through DNA and histone methylation and microRNAs (Cai et al., 2014).

Maternal dietary protein restriction programs hepatic G6PC gene in newborn piglets, which is associated with hypomethylation of G6PC gene promoter (Jia et al., 2012). Controversially the promoters of PEPCK2 and FBP1 genes were hypomethylated in the liver of piglets prenatally exposed to betaine (Cai et al., 2014). Therefore, the mechanism which maternal supplementation with methionine increased *FBP1* expression remains to be elucidated, even though there is any specific mechanism in ruminant species.

The reduction in *ACOX1* expression at the same time as *CPT1A* expression increased in likely the same mechanism observed in a rat model, when feeding essential fatty acids (Jacometo et al., 2014), a cellular mechanism of regulation in the equilibrium between fatty acid transfer from cytoplasm to the mitochondria and b-oxidation, a manner to regulate fatty acid oxidation when the uptake overload fatty acid oxidation capacity. Higher *SLC2A2* and *AKT2* expression could be linked with higher efficiency in glucose uptake. Calves fed colostrum compared to formula-fed ones had higher efficiency in glucose uptake (Steinhoff-Wagner et al., 2014), so we could speculate that the differences observed in both genes, besides being related to intrauterine programming, the colostrum composition could be an important player.

The function of GR in neonatal calves liver is not well elucidated, it seems to be linked to adrenergic receptor function (Schaff et al., 2014), thus higher *GR* expression in MET calves could indicate a higher maturation of the metabolic pathways in response to ageing (Schaff et al., 2015).

About inflammation markers, the higher *NFKB1* and *SOD2* expression in MET calves can indicate a more activated immune response. Cows fed rumen-protected methionine during the transition period also had these markers of inflammation increase, however between the 2 isozymes of SOD (SOD1 and SOD2), *SOD1* was the only one affected, suggesting that any effect of Met supplementation was partly compartmentalized to the cytosol (*SOD1*) rather than mitochondria (*SOD2*) (Osorio et al., 2014a), indicating different effects between dam and offspring.

As far as author's knowledge this is the firs report linking maternal pre-partum supplementation with rumen-protected methionine and its effect on calves blood metabolites and hepatic transcriptomics. Overall, the data suggested that maternal treatment elicited some changes that could benefit calves metabolism from birth through weaning. However, additional research in this area seems warranted to clarify some effects.

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Table 1. Rectal temperature, fecal score and starter intake from calves born to dams supplemented with rumen-protected methionine (MET) or no supplementation (CON) during the last 24 d pre-partum.

	Treatment (T)			P-value		
Week	MET	CON	SEM	Trt	wk	T×W
Rectal temperature, °C						
0	39.49	38.48	0.06	0.428	0.356	0.192
1	38.61	38.44	0.06			
2	38.43	38.46	0.06			
Fecal score (1-4)						
1	2.40	2.24	0.10	0.191	<0.001	0.995
2	2.79	2.69	0.10			
3	2.40	2.33	0.10			
4	2.14	2.01	0.10			
5	2.07	2.03	0.10			
6	1.82	1.69	0.10			
Starter intake (kg/day)						
1	0.01	0.02	0.01	0.686	<0.001	0.94
2	0.07	0.10	0.07			
3	0.27	0.23	0.07			
4	0.40	0.44	0.07			
5	0.57	0.65	0.07			
6	1.31	1.34	0.07			

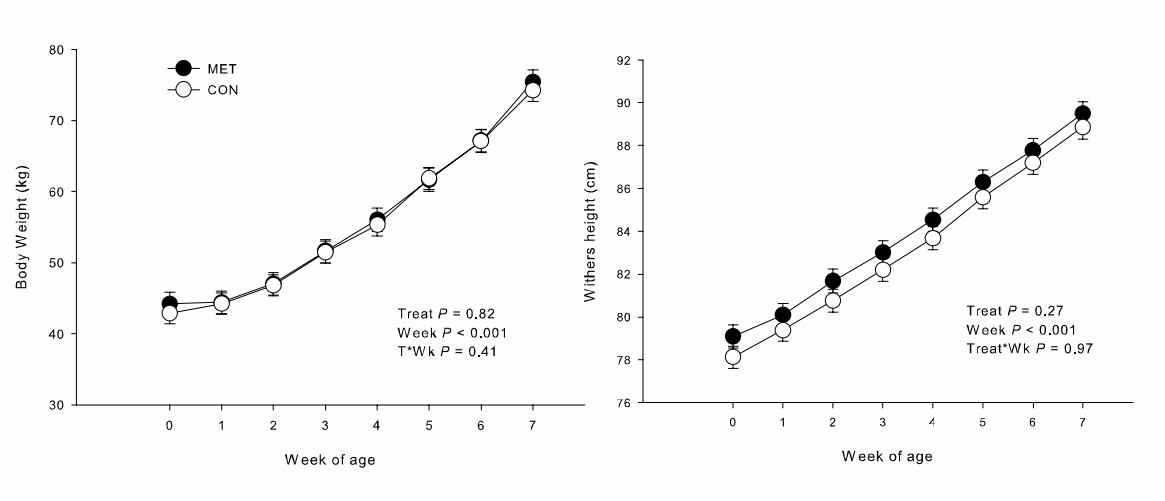

Figure 1. Body weight and withers height of calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and week, and their interaction (T×Wk).

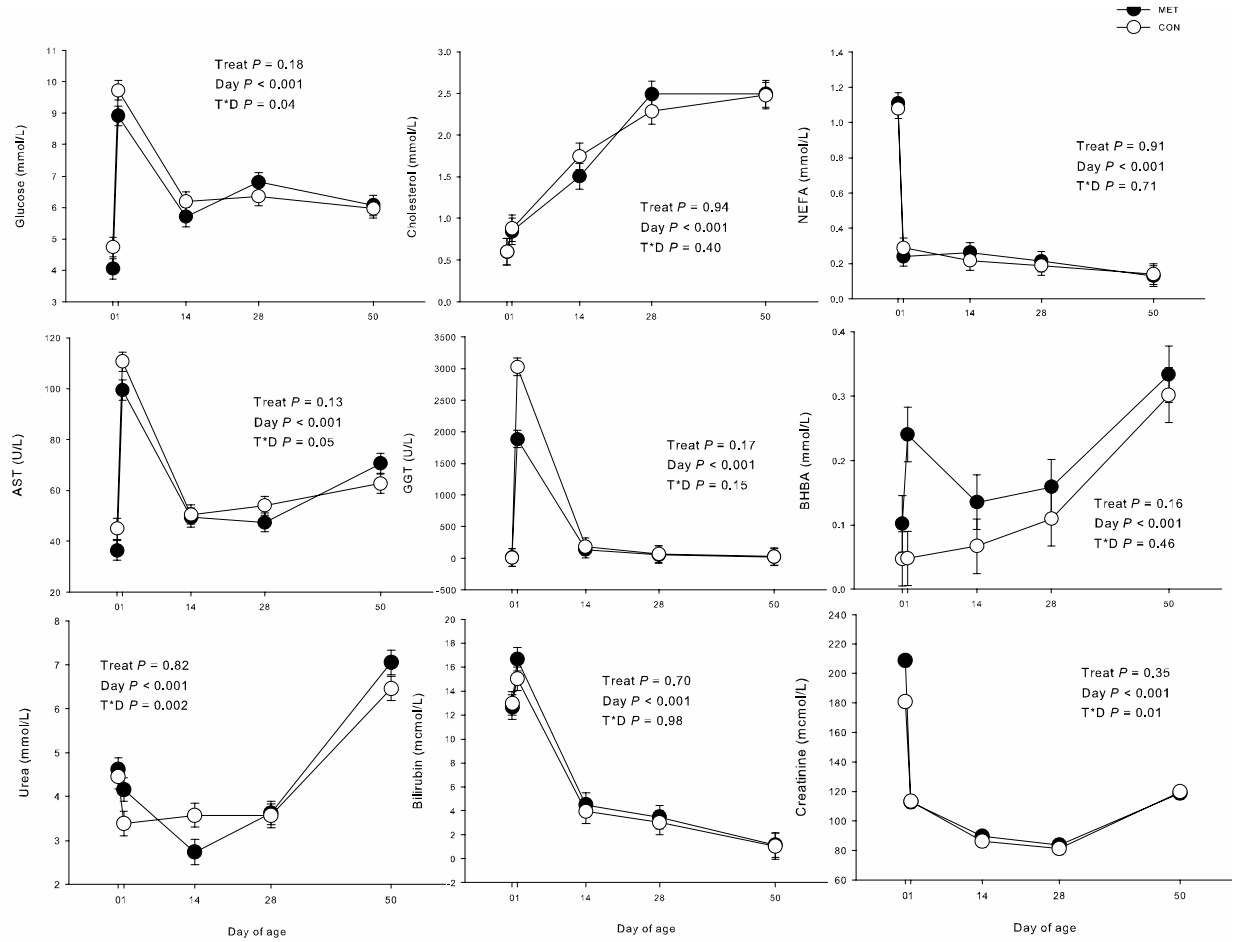

Figure 2. Blood metabolites of calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

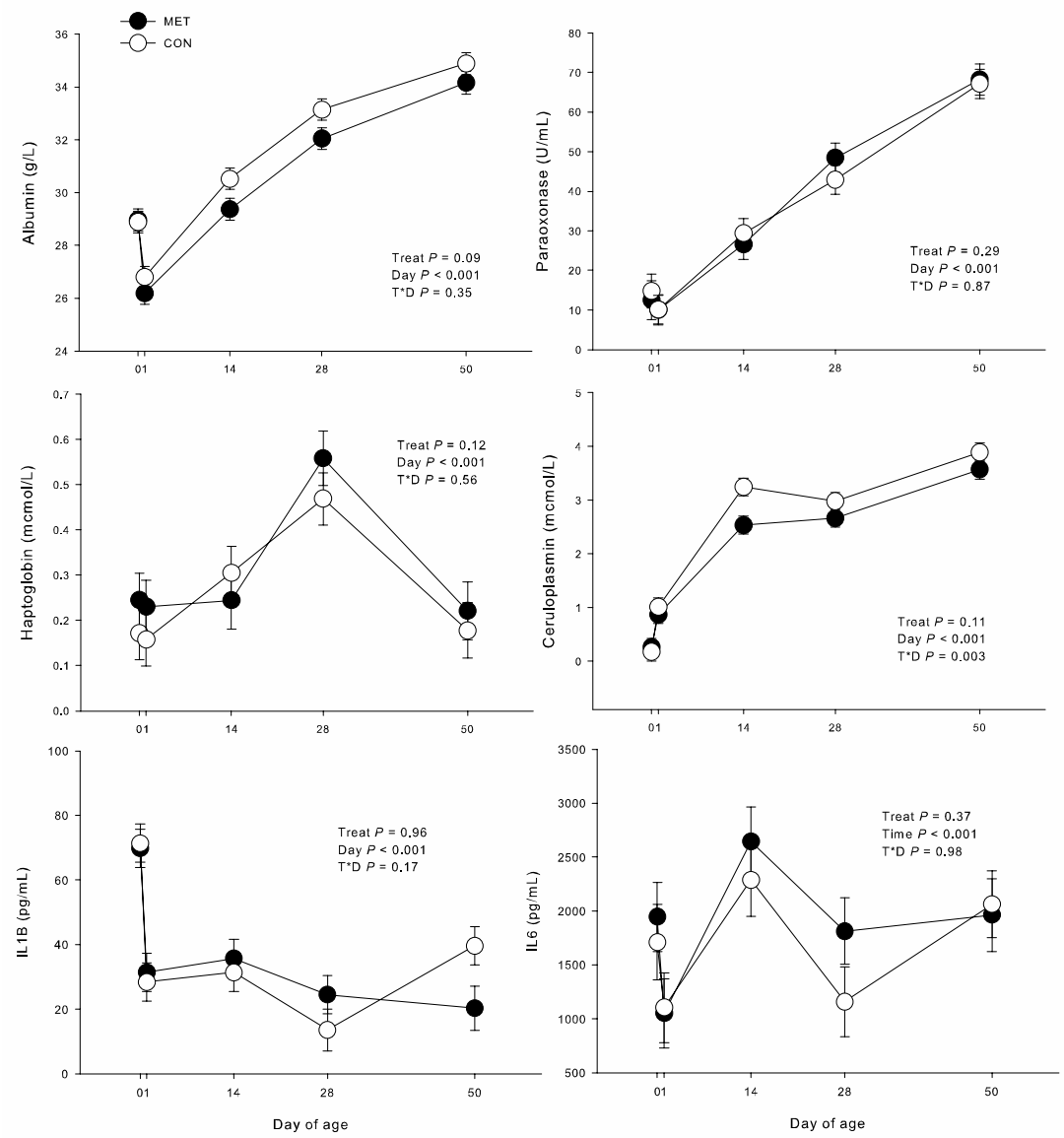

Figure 3. Blood biomarkers of inflammation from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

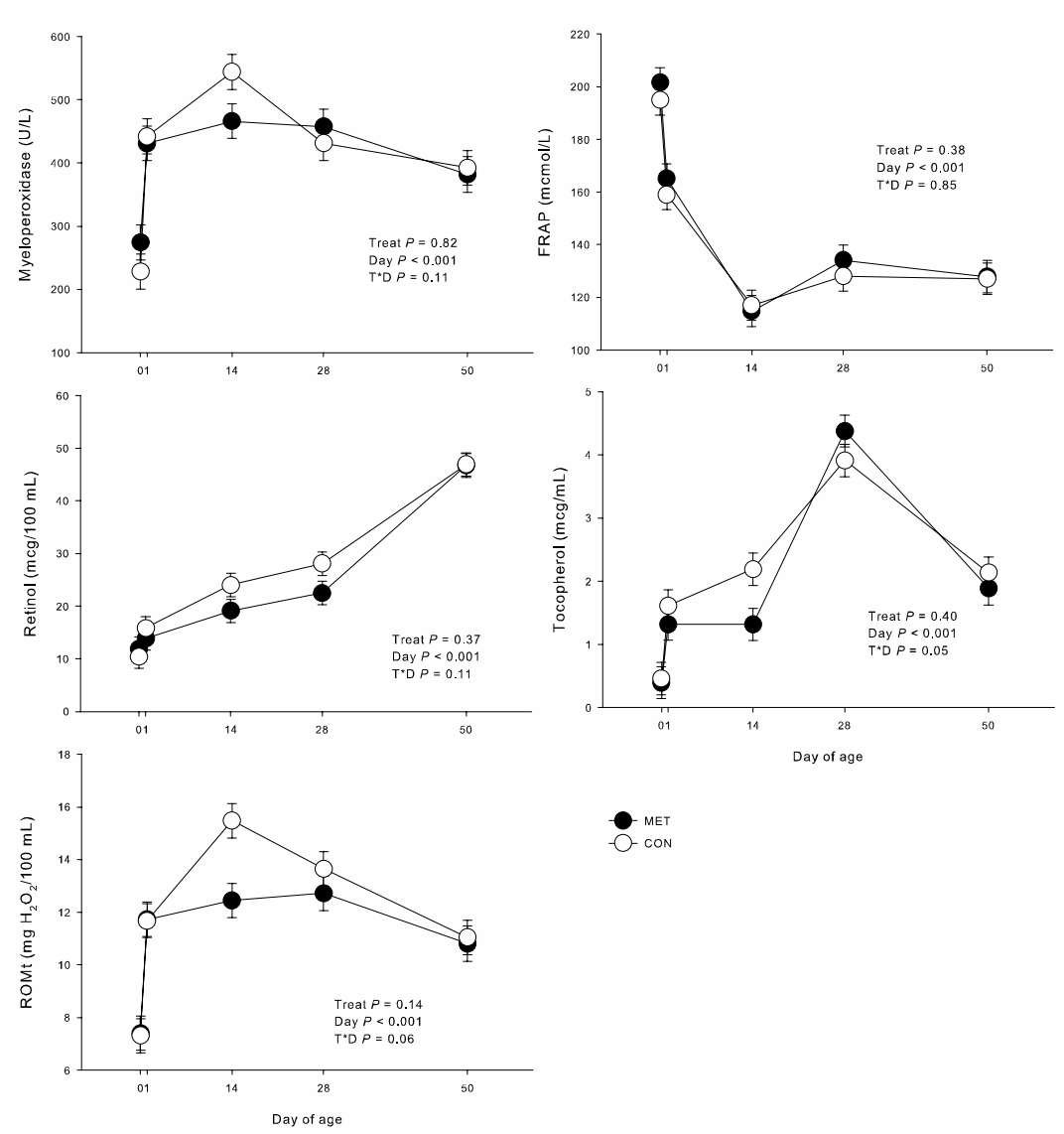

Figure 4. Blood biomarkers of oxidative stress from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

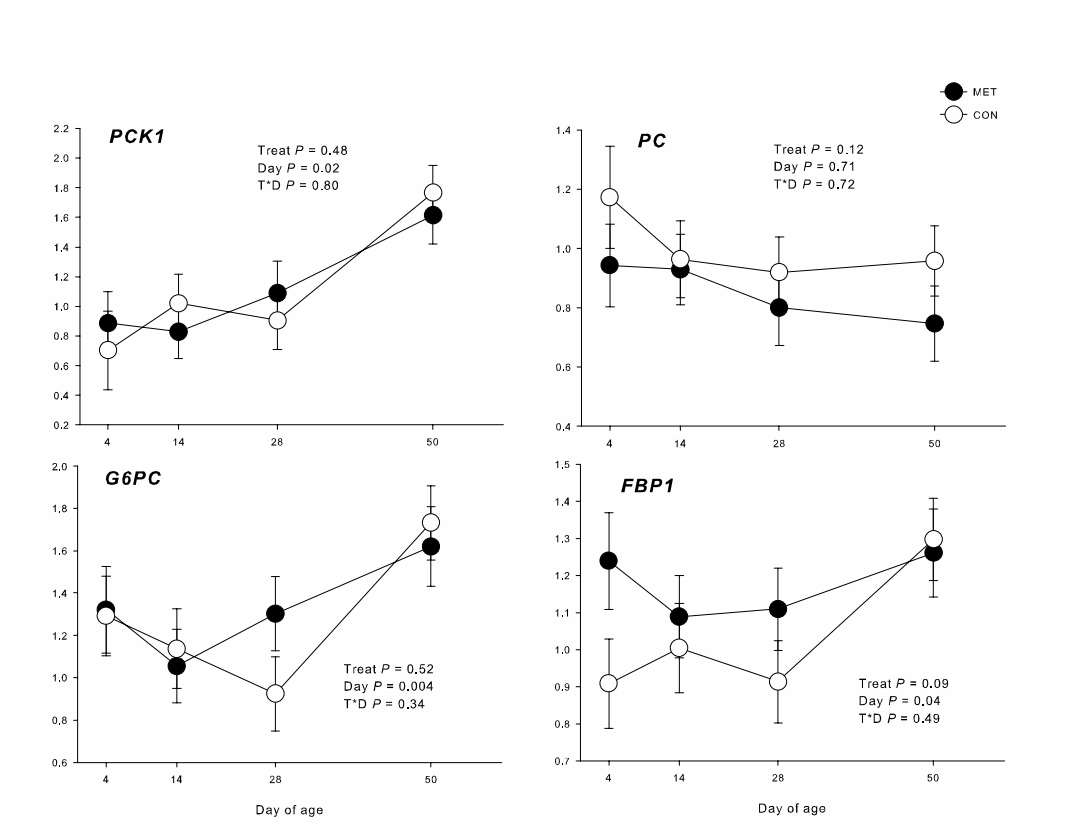

Figure 5. Expression of genes (R.U.) related to gluconeogenesis from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

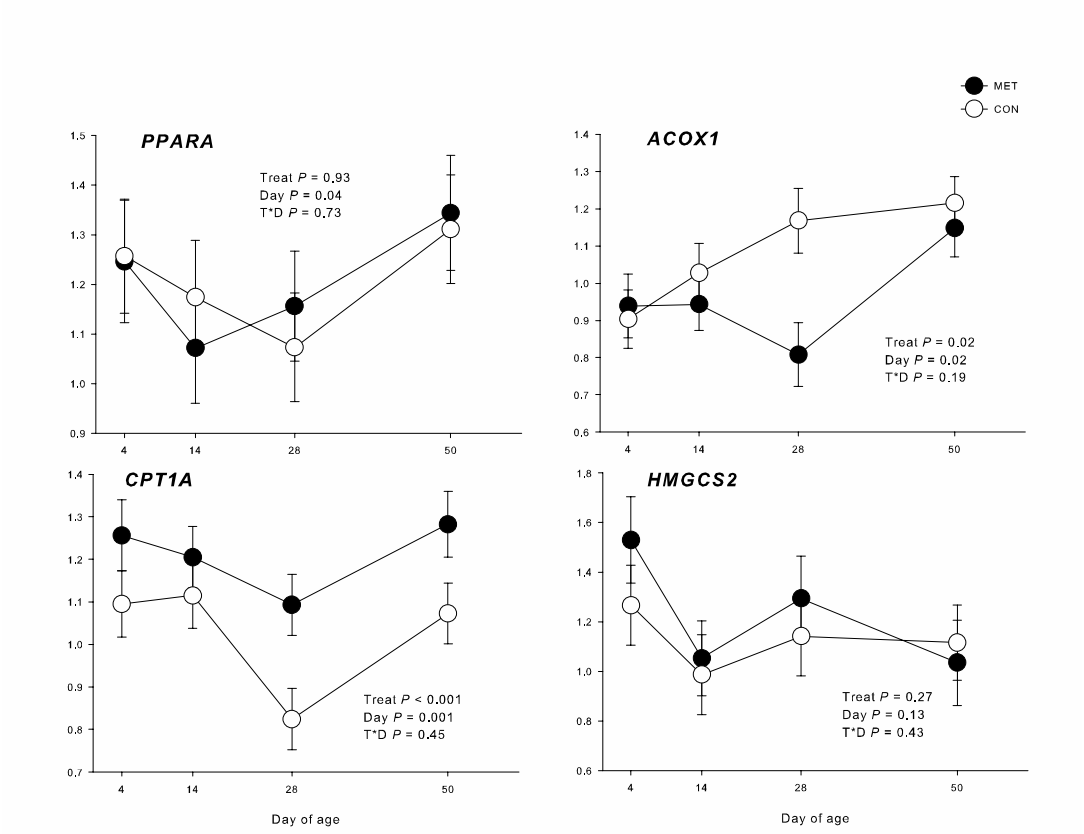

Figure 6. Expression of genes (R.U.) related to fatty acid oxidation from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

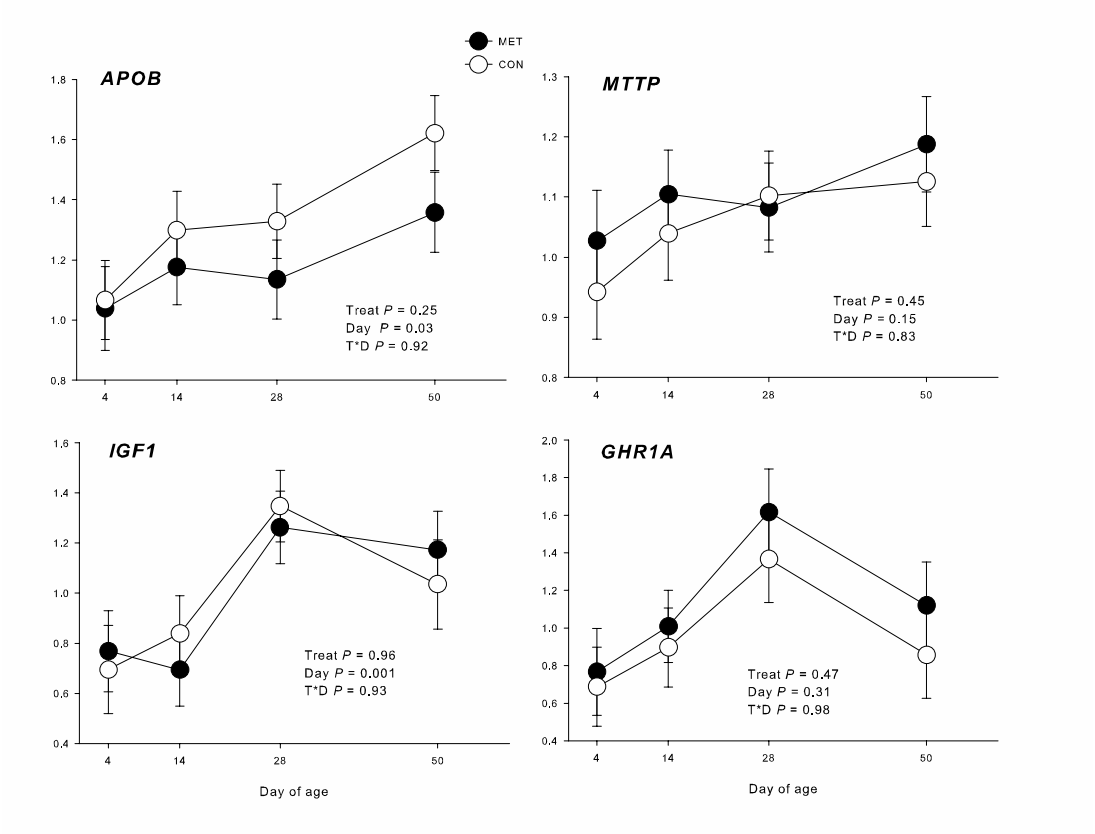

Figure 7. Expression of genes (R.U.) related to lipoprotein metabolismo and GH-IGF1 axis from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

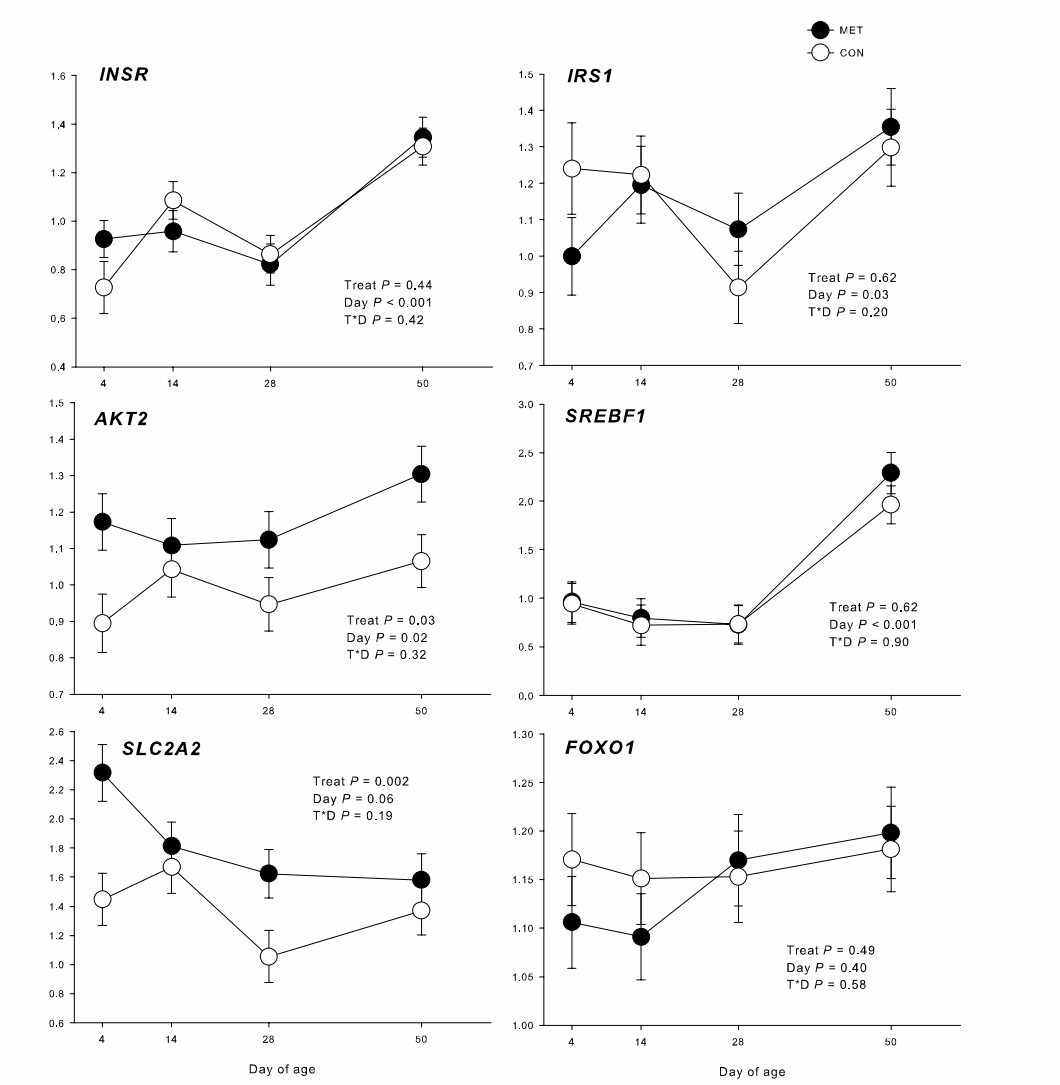

Figure 8. Expression of genes (R.U.) related to the insulin signaling pathway from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

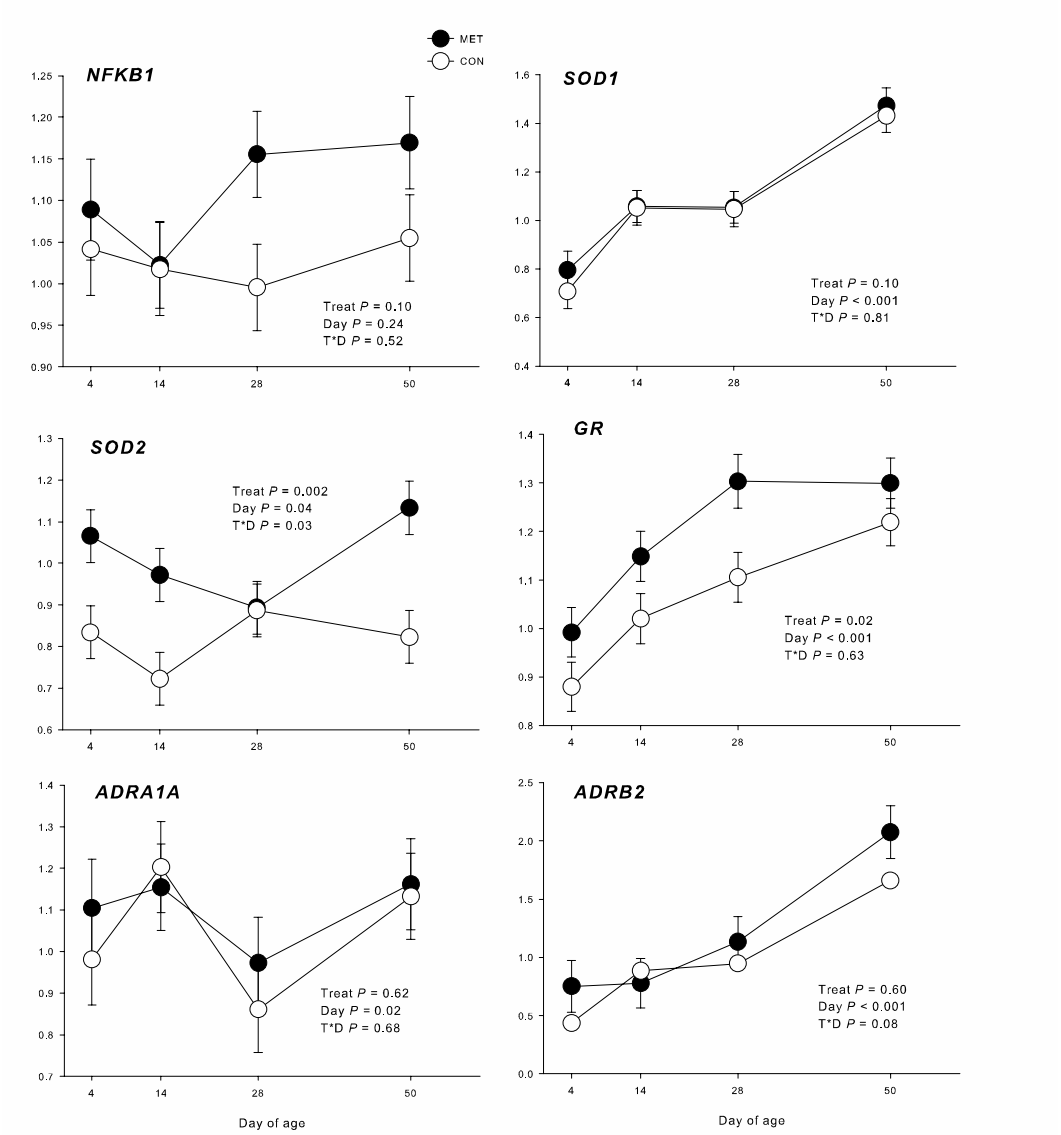

Figure 9. Expression of genes (R.U.) related to the inflammation resoponse and glucocortocoid and adrenergic receptors from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

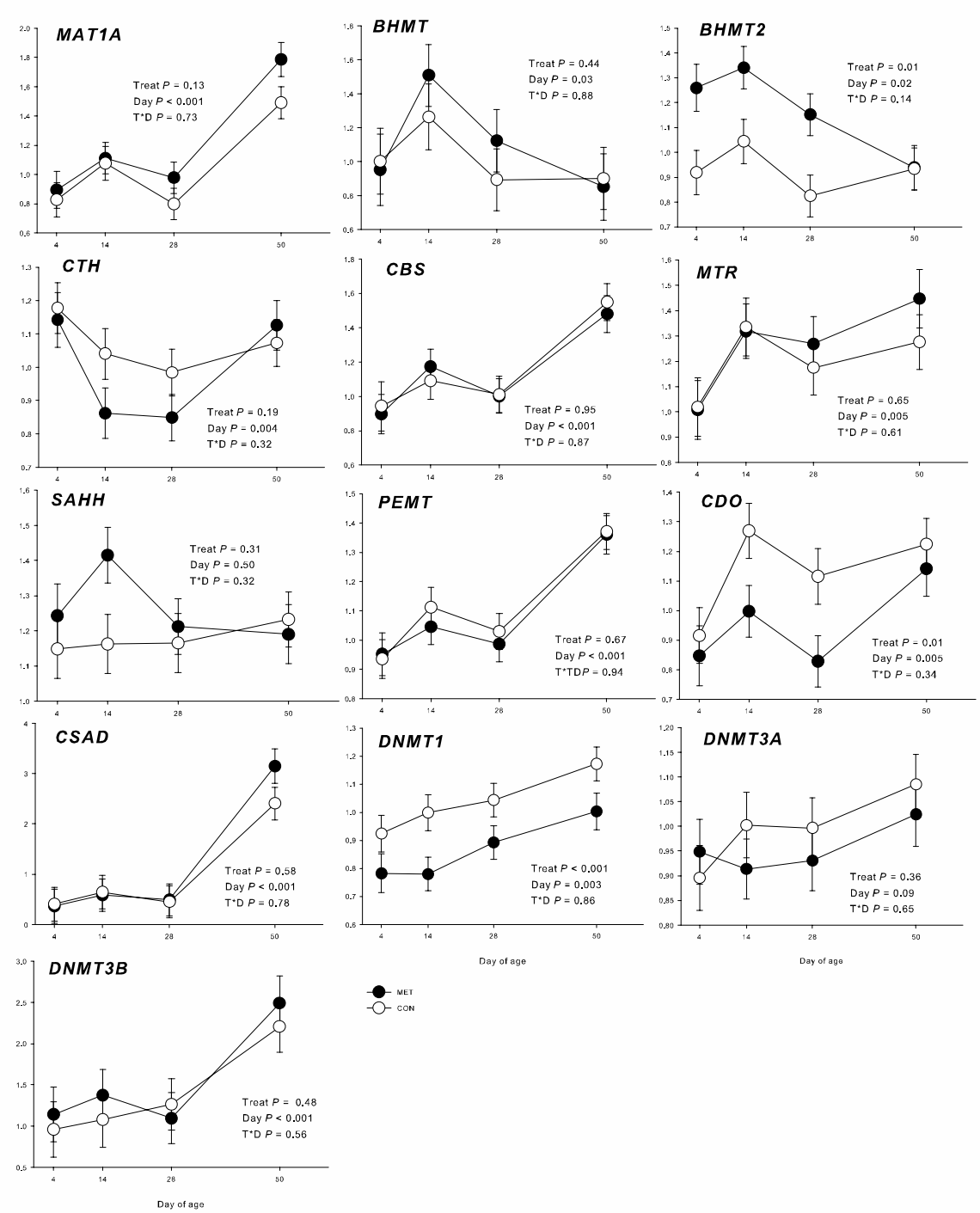

Figure 10. Expression of genes (R.U.) related to methionine cycle from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

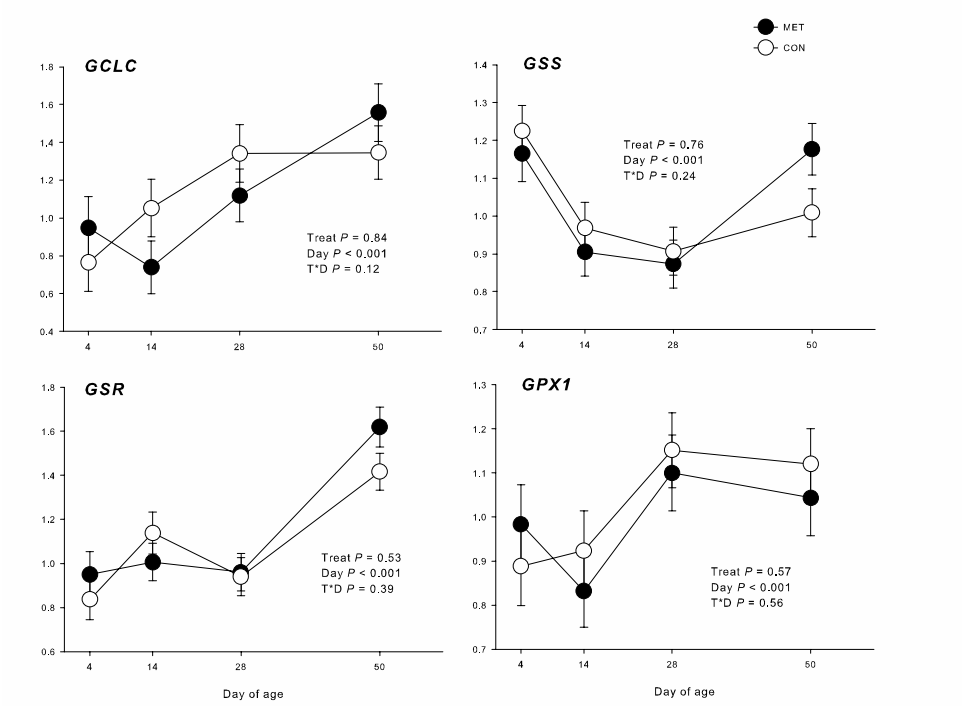

Figure 11. Expression of genes (R.U.) related to glutathione metabolism from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

Supplemental material

Function of the genes selected for transcriptional profile on calves liver.

Source: NCBI (http://www.ncbi.nlm.nih.gov/gene)

GLUCONEOGENESIS

PC – Pyruvate carboxylase

Located exclusively in the mitochondrial matrix, it catalyse the carboxylation of pyruvate to oxaloacetate. The active enzyme is involved in gluconeogenesis, lipogenesis, insulin secretion and synthesis of the neurotransmitter glutamate.

PCK1 - Phosphoenolpyruvate carboxykinase 1

This gene is a main control point for the regulation of gluconeogenesis. The cytosolic enzyme encoded by this gene, along with GTP, catalyzes the formation of phosphoenolpyruvate from oxaloacetate, with the release of carbon dioxide and GDP. The expression of this gene can be regulated by insulin, glucocorticoids, glucagon, cAMP, and diet. A mitochondrial isozyme of the encoded protein also has been characterized.

G6PC - Glucose-6-phosphatase, catalytic subunit

Glucose-6-phosphatase (G6Pase) is a multi-subunit integral membrane protein of the endoplasmic reticulum that is composed of a catalytic subunit and transporters for G6P, inorganic phosphate, and glucose. Glucose-6-phosphatase catalyzes the hydrolysis of D-glucose 6-phosphate to D-glucose and orthophosphate and is a key enzyme in glucose homeostasis, functioning in gluconeogenesis and glycogenolysis.

FBP1 - Fructose-1,6-bisphosphatase 1

Fructose-1,6-bisphosphatase 1, a gluconeogenesis regulatory enzyme, catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate. Fructose-1,6-diphosphatase deficiency is associated with hypoglycemia and metabolic acidosis.

FATTY ACID OXIDATION

PPARA - Peroxisome proliferator-activated receptor alpha

Peroxisomes are subcellular organelles found in plants and animals that contain enzymes for respiration and for cholesterol and lipid metabolism. The action of peroxisome proliferators is thought to be mediated via specific receptors, called PPARs, which belong to the steroid hormone receptor superfamily. PPARs affect the expression of target genes involved in cell proliferation, cell differentiation and in immune and inflammation responses. Three closely related subtypes (alpha, beta/delta, and gamma) have been identified. This gene encodes the subtype PPAR-alpha, which is a nuclear transcription factor.

CPT1A - Carnitine palmitoyltransferase 1A

The mitochondrial oxidation of long-chain fatty acids is initiated by the sequential action of carnitine palmitoyltransferase I (which is located in the outer membrane and is detergent-labile) and carnitine palmitoyltransferase II (which is located in the inner membrane and is detergent-stable), together with a carnitine-acylcarnitine translocase. CPT I is the key enzyme in the carnitine-dependent transport across the mitochondrial inner membrane and its deficiency results in a decreased rate of fatty acid beta-oxidation.

ACOX1 - Acyl-CoA oxidase 1, palmitoyl

The protein encoded by this gene is the first enzyme of the fatty acid beta-oxidation pathway, which catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs. It donates electrons directly to molecular oxygen, thereby producing hydrogen peroxide.

HMGCS2 - 3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)

The protein encoded by this gene belongs to the HMG-CoA synthase family. It is a mitochondrial enzyme that catalyzes the first reaction of ketogenesis, a metabolic pathway that provides lipid-derived energy for various organs during times of carbohydrate deprivation, such as fasting.

LIPID/LIPOPROTEIN METABOLISM

APOB - Apolipoprotein B

This gene product is the main apolipoprotein of chylomicrons and low density lipoproteins, is responsible for carrying fat molecules (lipids), including cholesterol,

around the body (within the water outside cells) to all cells within all tissues. It occurs in plasma as two main isoforms, apoB-48 and apoB-100: the former is synthesized exclusively in the gut and the latter in the liver.

MTTP - Microsomal triglyceride transfer protein

This gene encodes the large subunit of the heterodimeric microsomal triglyceride transfer protein. Protein disulfide isomerase (PDI) completes the heterodimeric microsomal triaglyceride transfer protein, which has been shown to play a central role in lipoprotein assembly. Apoprotein B48 on chylomicra and Apoprotein B100 on LDL, IDL, and VLDL are important for MTP binding.

GH-IGF1 AXIS

IGF1 - Insulin-like growth factor 1 (somatomedin C)

IGF-1 is a hormone similar in molecular structure to insulin. It plays an important role in childhood growth and continues to have anabolic effects in adults. A synthetic analog of IGF-1 is used for the treatment of growth failure. It is produced primarily by the liver as an endocrine hormone as well as in target tissues in a paracrine/autocrine fashion. Production is stimulated by growth hormone (GH) and can be retarded by undernutrition, growth hormone insensitivity, lack of growth hormone receptors, or failures of the downstream signalling pathway post GH receptor including SHP2 and STAT5B. Approximately 98% of IGF-1 is always bound to one of 6 binding proteins (IGF-BP).

GHR1A – Growth hormone receptor 1 A

This gene encodes a protein that is a transmembrane receptor for growth hormone. Binding of growth hormone to the receptor leads to receptor dimerization and the activation of an intra- and intercellular signal transduction pathway leading to growth.

INSULIN SIGNALING PATHWAY

INSR - Insulin receptor

After removal of the precursor signal peptide, the insulin receptor precursor is post-translationally cleaved into two chains (alpha and beta) that are covalently linked. Binding of insulin to the insulin receptor (INSR) stimulates glucose uptake.

IRS1 - Insulin receptor substrate 1

This gene encodes a protein which is phosphorylated by insulin receptor tyrosine kinase. Mutations in this gene are associated with type II diabetes and susceptibility to insulin resistance.

AKT2 – v-akt murine thymoma viral oncogene homolog 2 (Protein kinase B)

Akt2 is an important signaling molecule in the Insulin signaling pathway. It is required to induce glucose transport. In a mouse which is null for Akt1 but normal for Akt2, glucose homeostasis is unperturbed, but the animals are smaller, consistent with a role for Akt1 in growth. In contrast, mice which do not have Akt2, but have normal Akt1, have mild growth deficiency and display a diabetic phenotype (insulin resistance), again consistent with the idea that Akt2 is more specific for the insulin receptor signaling pathway.

SLC2A2 - Solute carrier family 2 (facilitated glucose transporter), member 2

This gene encodes an integral plasma membrane glycoprotein of the liver, islet beta cells, intestine, and kidney epithelium. The encoded protein mediates facilitated bidirectional glucose transport. It is the principal transporter for transfer of glucose between liver and blood, and has a role in renal glucose reabsorption.

FOXO1 - Forkhead box O1

FOXO1 is a transcription factor that plays important roles in regulation of gluconeogenesis and glycogenolysis by insulin signaling, and is also central to the decision for a preadipocyte to commit to adipogenesis. FOXO1, through increasing transcription of glucose-6-phosphatase, indirectly increases the rate of hepatic glucose production.

SREBF1 - sterol regulatory element binding transcription factor 1

The protein is synthesized as a precursor that is attached to the nuclear membrane and endoplasmic reticulum. Following cleavage, the mature protein translocates to the nucleus and activates transcription by binding to the SRE1. It regulates genes required for glucose metabolism and fatty acid and lipid production and its expression is regulated by insulin.

INFLAMMATORY RESPONSE AND OXIDATIVE STRESS

NFK1 -Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1

NFKB is a transcription regulator that is activated by various intra- and extra-cellular stimuli such as cytokines, oxidant-free radicals, ultraviolet irradiation, and bacterial or viral products. Activated NFKB translocates into the nucleus and stimulates the expression of genes involved in a wide variety of biological functions. Inappropriate activation of NFKB has been associated with a number of inflammatory diseases while persistent inhibition of NFKB leads to inappropriate immune cell development or delayed cell growth.

SOD1 - Superoxide dismutase 1, soluble

The protein encoded by this gene binds copper and zinc ions and is one of two isozymes responsible for destroying free superoxide radicals in the body. The encoded isozyme is a soluble cytoplasmic protein, acting as a homodimer to convert naturally-occuring but harmful superoxide radicals to molecular oxygen and hydrogen peroxide.

SOD2 - Superoxide dismutase 2, mitochondrial

This gene is a member of the iron/manganese superoxide dismutase family. It encodes a mitochondrial protein that forms a homotetramer and binds one manganese ion per subunit. This protein binds to the superoxide byproducts of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen.

GLUCOCORTICOID AND ADRENERGIC RECEPTOS

GR – Glucocorticoid receptor (NR3C1)

This gene encodes glucocorticoid receptor, which can function both as a transcription factor that binds to glucocorticoid response elements in the promoters of glucocorticoid responsive genes to activate their transcription, and as a regulator of other transcription factors. This receptor is typically found in the cytoplasm, but upon ligand binding, is transported into the nucleus. It is involved in inflammatory responses, cellular proliferation, and differentiation in target tissues.

ADRA1A - Adrenoceptor alpha 1A

Alpha-1-adrenergic receptors (alpha-1-ARs) are members of the G protein-coupled receptor superfamily. They activate mitogenic responses and regulate growth and proliferation of many cells.

ADRB2 - Adrenoceptor beta 2, surface

This gene encodes beta-2-adrenergic receptor, which is a member of the G protein-coupled receptor superfamily, in the liver induces glycogenolysis and gluconeogenesis.

METHIONINE CYCLE

MAT1A - Methionine adenosyltransferase I, alpha

This gene catalyzes a two-step reaction that involves the transfer of the adenosyl moiety of ATP to methionine to form S-adenosylmethionine and tripolyphosphate, which is subsequently cleaved to PPi and Pi. S-adenosylmethionine is the source of methyl groups for most biological methylations.

BHMT – betaine-homocysteine S-methyltransferase

This gene encodes a cytosolic enzyme that catalyzes the conversion of betaine and homocysteine to dimethylglycine and methionine, respectively.

BHMT2 - betaine-homocysteine S-methyltransferase 2

Homocysteine is a sulfur-containing amino acid that plays a crucial role in methylation reactions. Transfer of the methyl group from betaine to homocysteine creates methionine, which donates the methyl group to methylate DNA, proteins, lipids, and other intracellular metabolites. The protein encoded by this gene is one of two methyl transferases that can catalyze the transfer of the methyl group from betaine to homocysteine.

CTH - Cystathionine gamma-lyase

Cystathionine gamma-lyase (CSE) (or cystathionase) is an enzyme which breaks down cystathionine into cysteine and α-ketobutyrate.

CBS - Cystathionine-beta-synthase

The protein encoded by this gene acts as a homotetramer to catalyze the conversion of homocysteine to cystathionine, the first step in the transsulfuration pathway.

MTR - 5-methyltetrahydrofolate-homocysteine methyltransferase

This gene encodes the 5-methyltetrahydrofolate-homocysteine methyltransferase. This enzyme, also known as cobalamin-dependent methionine synthase, catalyzes the final step in methionine biosynthesis.

SAHH – adenosylhomocysteinase (AHCY)

It catalyzes the reversible hydrolysis of S-adenosylhomocysteine (AdoHcy) to adenosine (Ado) and L-homocysteine (Hcy). Thus, it regulates the intracellular S-adenosylhomocysteine (SAH) concentration thought to be important for transmethylation reactions.

PEMT - Phosphatidylethanolamine N-methyltransferase

Phosphatidylcholine (PC) is the most abundant mammalian phospholipid. This gene encodes an enzyme which converts phosphatidylethanolamine to phosphatidylcholine by sequential methylation in the liver.

CDO - Cysteine dioxygenase 1, cytosolic

It catalyzes the conversion from cysteine to cysteine sulfinic acid.

CSAD - Cysteine sulfinic acid decarboxylase

This gene encodes a protein taht plays a role in multiple biological processes as the rate-limiting enzyme in taurine biosynthesis, catalyzing the decarboxylation of cysteinesulfinate to hypotaurine.

DNMT1 - DNA (cytosine-5-)-methyltransferase 1

DNA (cytosine-5-)-methyltransferase 1 has a role in the establishment and regulation of tissue-specific patterns of methylated cytosine residues.

DNMT3A - DNA (cytosine-5-)-methyltransferase 3 alpha

This gene encodes a DNA methyltransferase that is thought to function in de novo methylation, rather than maintenance methylation. The protein localizes to the cytoplasm and nucleus and its expression is developmentally regulated.

DNMT3B - DNA (cytosine-5-)-methyltransferase 3 beta

This gene encodes a DNA methyltransferase that is thought to function in de novo methylation, rather than maintenance methylation. The protein localizes to the cytoplasm and nucleus and its expression is developmentally regulated.

GLUTATHIONE METABOLISM

GCLC - Glutamate-cysteine ligase, catalytic subunit

Glutamate-cysteine ligase, also known as gamma-glutamylcysteine synthetase is the first rate-limiting enzyme of glutathione synthesis.

GSS - Glutathione synthetase

The protein encoded by this gene functions as a homodimer to catalyze the second step of glutathione biosynthesis, which is the ATP-dependent conversion of gamma-L-glutamyl-L-cysteine to glutathione.

GSR - Glutathione reductase

This gene encodes a member of the class-I pyridine nucleotide-disulfide oxidoreductase family. This enzyme is a homodimeric flavoprotein. It is a central enzyme of cellular antioxidant defense, and reduces oxidized glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant.

GPX1 - Glutathione peroxidase 1

This gene encodes a member of the glutathione peroxidase family. Glutathione peroxidase functions in the detoxification of hydrogen peroxide, and is one of the most important antioxidant enzymes in humans.

4.3 Manuscrito 2 - Effect of maternal supplementation with methionine on neutrophil gene and microRNA network expression, biomarkers of oxidative stress and inflammation in Holstein calves

Carolina B. Jacometo[1,2], Zheng Zhou[2], Daniel Luchini[3], Erminio Trevisi[4], Marcio N. Corrêa[1] and Juan J. Loor[2*]

[1]NUPEEC, Departamento de Clínicas Veterinária, Programa de Pós-Graduação em Biotecnologia, Universidade Federal de Pelotas, Pelotas, RS, Brazil.

[2]Mammalian NutriPhysioGenomics, Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois, Urbana, IL, USA.

[3]Adisseo USA Inc., Alpharetta, GA, USA.

[4]Istituto di Zootecnica, Facoltà di Scienze Agrarie, Alimentari e Ambientali, Università Cattolica del Sacro Cuore, Piacenza, Italy.

INTRODUCTION

The negative energy balance (NEB) experienced by cows soon after calving has been associated with impaired neutrophil migration, phagocytosis, and oxidative burst capacity (Goff, 2006, Sordillo et al., 2009). The mechanisms that lead most cows experience immunosuppression around the time of calving still is not well understood, but the metabolic challenges associated with the onset of lactation are the main factors capable of interact with the immune function (Goff, 2006).

Supplementing transition cows with rumen-protected methionine increased dry matter intake (DMI), which is extremely important to reduce the metabolic disorders characteristic from peripartum period (Osorio et al., 2013a). Also a reduction in the

pro-inflammatory response has been previously associated with methionine supplementation during the peripartal period (Osorio et al., 2014).

Understanding how markers of stress and inflammation change during the neonatal period and are affected by maternal nutrition can help researchers develop strategies to reduce the initial stress of birth. Transcriptomics have been used in recent years to enhance the knowledge of the molecular mechanisms that acts regulating basic functions of immune cells during stressful periods (Weber et al., 2006, Moyes et al., 2010). Though the nutritional physiology of the neonatal calf has been well-studied (Blum, 2006), the extent to which prenatal maternal supplementation with methionine affects the profiles of stress and inflammatory markers, metabolites, and polymorphonuclear leukocyte (PMN) function and gene expression in neonatal calves are yet to be defined.

Our hypothesis was that increased maternal methionine intake during late gestation could affect neonatal immune and metabolic markers. The objective of this study was to evaluate the effect of supplementing a rumen-protected methionine during the last 24 days of pregnancy on blood inflammation and oxidative stress biomarkers, gene and microRNA expression profiles of blood PMN and link that information with PMN function of Holstein calves, from birth through weaning.

MATERIAL AND METHODS

All the procedures for this study were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois (protocol #13023).

Maternal Treatments

The experiment was conducted as a randomized complete blocked design with 40 multiparous Holstein cows blocked according to parity, previous lactation milk yield, and expected day of parturition. All cows received a common early-dry period diet (“far-off diet”) from -50 to -25 d relative to parturition, with low energy and high straw designed to meet and not greatly exceed 100% of energy requirements. During the late-dry period diet (“close-up diet”), from -24 d until calving day, cows received a high-energy diet. Cows were randomly assigned to atop-dressed on the total mixed ration (TMR) supplementation with rumen-protected methionine (MET, n = 20 – 0.08% of the dry matter of the diet/d methionine, Smartamine® M Adisseo, Alpharetta, GA, USA, ~2.9:1 Lys:Met), or with no supplementation (CON, n = 20, ~3.35:1 Lys:Met). After birth, calves were fed a common diet and managed similarly. Hence, any observed treatment effects are attributed to maternal nutrition during the last 24 d of gestation.

Animal management and calf enrollment criteria

During the dry period, cows were housed in a ventilated, sand-bedded free-stall barn, with a photoperiod of 8 h of light and 16 h of dark. Diets were fed for ad-libitum intake as a TMR once daily using an individual gate feeding system (American Calan, Northwood, NH) and dry matter intake (DMI) was recorded daily. As cows began demonstrating signs of impending parturition, they were moved to an individual maternity pen bedded with straw. After parturition, cows were milked at the end of the farm’s next milking period (4 AM, noon or 8 PM). Colostrum volume was recorded and IgG content was estimated based on specific gravity with a bovine colostrometer (Nasco, Fort Atkinson, WI; Cat. no. C10978N).

Calves were kept in the experiment if they fulfilled all the following criteria: 1) single calf; 2) calving difficulty score <3; 3) dam's colostrum quality assessed by a bovine colostrometer of >50 mg/L of IgG; 4) dam produced at least 3.8 L of a good quality first colostrum; 5) calf birth weight >36 kg (Johnson et al., 2007).

After birth, calves were weighted, had the navel disinfected with a 7% tincture of iodine solution (First Priority Inc., Elgin, IL), vaccinated with TSV II (Pfizer Inc., New York, NY) via nostril application, and received 3.8 L of first milking colostrum from the respective dam within 8 h after birth. If voluntary colostrum intake had not reached the 3.8 L required, calves were tubed with an oesophageal groove. Calves were housed in individual outdoor hutches bedded with straw, fed twice daily with a milk replacer (Advance Excelerate, Milk Specialities, Carpentersville, IL; 28.5% CP, 15% fat) (from 1 to 10 d of age: 520 g/d, 11 to 20 d of age: 680 g/d, 21 to 35 d of age: 840 g/d, and from 36 to 42 d of age: 420 g/d in a single feeding) and had ad libitum access to a starter grain mix (19.9% CP, 13.5% NDF). Health checks including fecal score (scale 1 – 4) weas recorded daily until weaning, while rectal temperature was recorded daily until 21 d of age. Growth performance including body weight (BW) and withers height (WH) were recorded weekly. Calves were weaned at 42 d of age.

Sample collection

Blood samples were collected from the jugular vein using 20-gauge BD Vacutainer needles (Becton Dickinson, Franklin Lakes, NJ) before receiving colostrum (baseline), 24 h after receiving colostrum, 14 d and 28 d and 50 d after birth (n = 12 / group). At each time point, a total of 120 mL of total blood were collected in vacutainer tubes (10 mL, BD Vacutainer, Becton Dickinson) containing serum clot activator, sodium heparin, or solution A of trisodium citrate, citric acid, and

dextrose (ACD). After blood collection, tubes with ACD and sodium heparin were placed on ice and tubes with clot activator were kept at room temperature until centrifugation (~30 min). Serum and plasma were obtained by centrifugation of clot activator and sodium heparin tubes, respectively, at 1,900 × *g* for 15 min. Serum and plasma were aliquoted and stored at -20°C until further analysis. The RNA from PMN cells was harvested from 100 mL of blood collected in ACD vacutainers tubes.

Apparent Efficiency of Immunoglobulin Absorption

Apparent efficiency of absorption was estimated by measuring the IgG content in both first milking colostrum and calf serum at 24 h after colostrum ingestion. Apparent efficiency of absorption was calculated as plasma IgG (g/L) × plasma volume (L)/IgG intake (g) (Quigley et al., 2002).

Blood immuno biomarkers

Blood samples were analyzed for albumin, haptoglobin (HP), ceruloplasmin, antioxidant potential (FRAP), paraoxonase (PON), myeloperoxidase and reactive oxygen metabolites (ROMt) using kits purchased from Instrumentation Laboratory (IL Test), using a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA). Retinol and tocopherol were determined as previously described (Bionaz et al., 2007). Bovine interleukin-6 (IL-6) and interleukin-1 beta (IL1B) plasma concentration were determined by a colorimetric sandwich ELISA using a bovine screening set (Thermo Fisher Scientific, Waltham, MA). IgG concentration (colostrum and serum) was measured using a quantitative bovine IgG ELISA kit (ZeptoMetrix Corporation, Buffalo, NY).

Whole blood / neutrophil phagocytosis

The phagocytic capacity of PMN isolated from heparinized whole blood was determined using the Phagotest kit (Orpegen Pharma, Heidelberg, Germany) following the manufacturer's instructions. Briefly, 20 µL of stabilized and opsonized FITC-labeled E. coli suspension (1 x 10^9 bacteria/mL) was added to 2 whole-blood samples (100 µL) in test tubes (Falcon, Becton Dickinson). After vortexing all samples, the test tube was incubated at 38°C in a water bath for 10 min; the control tube remained on ice. Phagocytosis was stopped by putting the tube back into ice. To eliminate the fluorescence of nonphagocytosed bacteria, 100 µL of quenching solution was added. The cells were washed twice with 3 mL of washing solution and pelleted by centrifugation (250 x g for 5 min, 4°C). Cells were resuspended and incubated in 2 mL of lysing solution for lysis of erythrocytes and fixation of phagocytes for 20 min at room temperature. After centrifugation, cells were recovered by resuspending in 3 mL of washing solution, followed by a final centrifugation step. The cells were resuspended in 100 µL of DNA-staining solution, and analyzed by flow cytometry, performed on a BD LSR system (Becton Dickinson Biosciences, San Jose, CA).

In vitro whole blood lipopolysaccharide (LPS) challenge

The blood collected in a lithium-heparin tube remained on a hot water bath (38 - 39°C) until start as soon as possible the LSP challenge (30 minutes). Briefly, 980 µL of homogenized whole blood were transferred to endotoxin free 2 mL tubes and added the *E. coli* LPS solution (strain 0111:B4, cat. no. L4391, Sigma Al- drich, St. Louis, MO), in duplicates. Three doses were tested: control (0 µg LPS/mL blood), dose 0.01 (0.01 µg LPS/mL blood) and dose 5 (5 µg LPS/mL blood). Samples were homogenized and incubated in a rotated water bath (3.5 hours, 38°C), centrifuged (4,000 x g for 15 min, 4°C) and then the supernatant plasma was harvested and

store at -80°C until analysis. Bovine interleukin-6 (IL-6) and interleukin-1 beta (IL1B) plasma concentration were determined by a colorimetric sandwich ELISA using a bovine screening set (Thermo Fisher Scientific, Waltham, MA).

Polimorfonuclear (PMN) cells isolation

Neutrophils were isolated according the procedures described by Osorio et al. (2013b) with minor modifications. Blood (100 mL), collected in ACD solution A vacutainer tubes, was mixed well by inversion and placed on ice until PMN isolation (~1 h). Tubes were combined into three 50-mL conical tubes (Fisher Scientific, Pittsburgh, PA) and centrifuged at 918 × *g* for 30 min at 4°C. The plasma, buffy coat, and approximately one-third of the red blood cells (RBC) were removed and discarded. Cells were lysed with 25 mL of deionized water at 4°C, homogenized gently by inversion, and then 5 mL of 5 × phosphate-buffered saline (PBS; pH 7.4) at 4°C was added, in order to restore an iso-osmotic environment. The cell suspension was centrifuged at 330 × *g* for 10 min at 4°C and the supernatants were decanted. Ten mL of 1 × PBS at 4°C was added in each tube, homogenized until there was nothing attached to the bottom of the tube, and then the three tubes were combined in one. The cell suspension was centrifuged at 663 × *g* for 5 min at 4°C and the supernatants were discarded. The remaining RBC were lysed with 8 mL of deionized water at 4°C, homogenized gently by inversion and 2 mL of 5 × PBS at 4°C was added. The samples were centrifuged at 663 × *g* for 5 min at 4°C and the supernatant was discarded. Two subsequent washings using 10 mL of 1 × PBS at 4°C were performed, centrifuged at 663 × *g* for 5 min at 4°C and supernatant discarded. Prior to the last centrifugation, 100 µL of the cell suspension were aliquoted for further PMN concentration and cell viability analysis.

The neutrophil pellet was homogenized with 1.5 mL of 1 × PBS at 4°C and transferred to a 2 mL RNase-DNase free reinforced o-ring tube (Omni International, Kennesaw, GA) containing one stainless steel bead, 5 mm (Qiagen, Hilden, Germany), centrifuged at 1,435 × *g* for 5 min at 4°C, and then the supernatant was discarded. Immediately, 1 mL of Qiazol (Qiagen, Hilden, Germany) was added to the samples and homogenized twice in a Beadbeater (Biospec Products, Bartlesville, OK; Cat. no. 607) for 30 s. During all the isolation process samples were kept on ice while outside of the centrifuge. The homogenized solution was transferred to a 2 mL DNase-RNase free tube (USA Scientific Inc., Ocala, FL) and stored in -80°C until further analysis.

Neutrophil concentration 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 soln (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, St. Louis, MO) and preserved at 4°C until flow

cytometry reading (LSR II, Becton Dickinson, San Jose, CA). These procedures were performed only to ensure good quality samples, and were excluded from the study samples with less than 80% of neutrophils and 90% of viability.

mRNA and miRNA isolation

For the PMN extraction the miRNeasy kit (Qiagen, Hilden, Germany) was used following the manufacturer's protocols, allowing the recovery of both mRNA and miRNA. Samples were treated on-column with DNaseI (Qiagen, Hilden, Germany), quantification was accessed using the NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE), and RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Target gene cDNA synthesis and qPCR

Complementary DNA was synthesized using 100 ng RNA. Firstly random primers (10 mM) (Invitrogen Corp., CA) and DNase/RNase free water were mixed and incubated at 65 °C for 5 min and kept on ice for 3 min. Then a second mix containing DNase/RNase free water, first strand buffer (5X),oligo dT18 (Operon Biotechnologies, AL), dNTP mix (10 mM) (Invitrogen Corp., CA), RevertAidReverse Transcriptase (200 U/μL) (Fermentas Inc., MD) and RNase Inhibitor (20 U/ μL)(Promega, WI) was added. The reaction was performed in an Eppendorf Mastercycler® Gradient using the following temperature program: 25 °C for 5 min, 42 °C for 60 min and 70 °C for 5 min. cDNA was then diluted 1:3 with DNase/RNase free water.

Quantitative PCR was performed using 4 μL diluted cDNA combined with 6 μL of a mixture composed of 5 μL of SYBR Green master mix (Quanta Biosciences, Gaithersburg, MD), 0.4 μL each of 10 □M forward and reverse primers, and 0.2 μL

DNase/RNase free water in a MicroAmpTM Optical 384-Well Reaction Plate (Applied Biosystems, CA). Each sample was run in triplicate and a 6 points relative standard curve plus the non-template control were used. The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, CA) using the following conditions: 5 min at 95 °C, 40 cycles of 1 s at 95 °C (denaturation) and 1 min at 60 °C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15 s, 65 °C for 15 s plus 95 °C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems, CA).

Primers were designed using Primer Express 2.0 with minimum amplicon size of 80 bp (when possible amplicons of 100-120 bp were chosen) and limited 3' G+C (Applied Biosystems, CA). When possible, primer sets were designed to fall across exon– exon junctions. Primers were aligned against publicly available databases using BLASTN at NCBI (Nucleotide BLAST, 2008) and UCSC's Cow (Bos taurus) Genome Browser Gateway. Prior to qPCR primers were tested in a 20 µL PCR reaction using the same protocol described for qPCR except for the final dissociation protocol. For primer testing we used a universal reference cDNA (RNA mixture from different bovine samples) to ensure identification of desired genes. Five µL of the PCR product were run in a 2% agarose gel stained with SYBR safe. Only those primers that did not present primer-dimers and a single band at the expected size in the gel, and had the right amplification product (verified by sequencing) were used for qPCR. The accuracy of a primer pairs also was evaluated by the presence of a unique peak during the dissociation step at the end of qPCR.

For this study *GOLGA5*, *SMUG1* and *OSBPL2* were used as internal control genes, and their geometric mean was used to normalize the expression data. All the

select genes evaluated in this study and the functions they are related are listed in the supplemental material.

miRNA procedures

For cDNA synthesis we used the qScript miRNA cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). Each reaction started with 500 ng of total RNA, mixed with 2 µL of Poly (A) Tailing Buffer (5x), 1 µL of Poly (A) Polymerase and 7 µL of RNase/DNase free water. The mixture was incubated at 37°C for 20 min and then 70°C for 5 min. A second mix containing 9 µL of miRNA cDNA Reaction Mix and 1 µL qScript Reverse Transcriptase was added and incubated at 42°C for 20 min and then 85°C for 5 min. A pool of cDNA samples was used to prepare the 6-point standard curve (dilution 1:4). Then cDNA samples were also diluted 1:4 with DNase/RNase free water. A combination of 1 µL of diluted cDNA with 9 µL of the mix containing of 5.25 µL of Perfecta SYBR Green Fast Mix (Quanta Biosciences, Gaithersburg, MD, USA), 0.45 µL each of 10 µM sequence-specific forward primer and Universal PCR Primer (Quanta Biosciences, Gaithersburg, MD, USA) and 3.85 µL of DNase/RNase free water were added to each well of a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems, Grand Island, NY, USA). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, Grand Island, NY, USA) under the following thermocycler conditions: 95°C for 2 min, and 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by a dissociation curve step (95°C for 15 s, 60°C for 15 s and 95°C for 15 s). miR-let7a, miR-103 and miR-191 were used as internal controls, and their geometric mean was used to normalized the miRNA expression data. In this study the expression of miR-155, miR-223, miR-125b, miR-146a and miR-9-5p were evaluated.

Statistical analysis

Data were analysed with the Proc MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Fixed effects in the model were treatment (T), day (D) or week (wk), and their interaction (T×D, T×wk). Random effect was calf within diet. The exponential correlation covariance structure SP for repeated measures was used for analysis of blood metabolites and gene expression. Blood metabolites and gene expression results were log_2-scale transformed if needed to comply with normal distribution of residuals, and subsequently back-transformed. Least squares means separation between time points was performed using the PDIFF statement. Statistical significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

Apparent Efficiency of IgG absorption, Growth Performance and Health

Colostrum IgG was not affected ($P = 0.52$) by maternal diet and averaged 75.6±6.6 mg/dL for CON cows and 81.8±6.7 mg/dL for MET cows. Similarly, calves apparent efficiency of IgG absorption was not affected ($P = 0.36$), averaging 26.1±3.2% for CON calves and 30.3±3.2% for MET calves. Calf birth weight was similar between groups (CON: 42.9±1.5 kg, MET: 44.2±1.6 kg, $P = 0.55$), as well as body weight, weekly gain and withers height ($P > 0.05$) until 7 weeks of life, however a linear increase ($P < 0.001$) was observed in all parameters for both groups. Regardless of maternal treatment, starter intake increased ($P < 0.001$) over time (Table 1). Also rectal temperature and fecal score were not affected by maternal treatment ($P < 0.05$), however both groups had a higher fecal score at week 2, and then reduced until weaning time ($P < 0.001$) (Table 1).

Blood biomarkers

Inflammation

Main effects of diet, day, and interactions for inflammation markers are shown in Figure 2. No overall maternal treatment effect was observed ($P > 0.05$) for all these markers. Haptoglobin had a day effect ($P < 0.001$) mainly due a peak observed at 28 d of age. Paraoxonase, albumin and ceruloplasmin increased ($P < 0.001$) their concentration over time. Albumin and ceruloplasmin tended ($P = 0.09$ and 0.11) to be lower in MET calves. Ceruloplasmin also had a T×D ($P = 0.003$) with lower ($P = 0.004$) concentration in MET calves at 14 d of age. IL1B and IL6 had a marked decrease ($P < 0.001$) from birth to 24 h after colostrum intake. IL1B had its higher concentration at birth, while IL6 was at 14 d of age.

Oxidative stress

Main effects of diet, day, and interactions for oxidative stress markers are shown in Figure 3. No overall maternal treatment effect was observed ($P > 0.05$) for all these markers. A T×D was observed for tocopherol ($P = 0.05$) and a trend for ROMt ($P = 0.06$), with lower concentrations ($P = 0.02$ and 0.001, respectively) for MET calves at 14 d of age. Regardless maternal treatment, ROMt and myeloperoxidase had their higher concentrations at 14 d of age, while FRAP was in its lower level at the same time ($P < 0.001$). Tocopherol had its higher concentration at 28 d ($P < 0.001$) and retinol had a consistent increase over time ($P < 0.001$).

Whole blood / neutrophil phagocytosis

Main effects of diet, day, and interactions for neutrophil phagocytosis are shown in Figure 4. No overall maternal treatment effect was observed ($P = 0.47$), only a gradual increase ($P < 0.001$) over time. Despite there was no T×D ($P = 0.52$),

observing the pre and post-colostrum measurement, MET calves increased (P = 0.04) phagocytosis from 82 to 87%, while CON calves did not change (P = 0.89) (84 to 83%).

In vitro whole blood LPS challenge

Main effects of diet, day, and interactions for IL1B and IL6 concentrations after LPS challenge are shown in Figure 5. No overall maternal treatment effect was observed and control samples (no stimulated) had a similar response over time (P > 0.05). For both cytokines, the response observed with the higher dose (5 µg LPS/mL blood) was 2 fold higher than to the lower dose (0.01 µg LPS/mL blood) (P < 0.001). From birth until 14 d of age IL1B and IL6 response was similar for both LPS doses, a decrease from birth to 24 h after colostrum intake followed by an increase at 14 d of age (P < 0.001 for all comparisons). Only IL1B concentration, at the higher dose of LPS stimulation, also increased from 14 to 24 d and then decreased to 50 d of age (P < 0.001).

Gene expression

Cell adhesion and chemotaxis

Main effects of diet, day, and interactions for genes related to cell adhesion and chemotaxis are shown in Figure 6. No overall maternal treatment effect was observed (P > 0.05) for all these genes. There was a trend (P = 0.07) for *ZPB1*, with lower (P = 0.04) expression in MET calves at 50 d of age and a trend (P = 0.06) to higher expression of MET calves at 28 d of age. Regardless of maternal treatment, expression of *CADM1*, *LCP1* and *CYBA* had a similar pattern, an increase from birth to 24 h after colostrum intake, then a gradual decrease until 28 d of age, followed by an increase at 50 d of age (P < 0.05 for all comparisons). The day effect (P = 0.002)

observed for *SELL* expression was mainly due a reduction from 14 d to 28 d of age. *CXCR2* expression also decreased from 14 d to 28 d, but had an increase from 28 d to 50 d of age too ($P < 0.001$). *ZBP1* had higher expression at 28 d of age, while *CASP8* at 50 d of age ($P < 0.001$).

Oxidative stress

Main effects of diet, day, and interactions for genes related to oxidative stress are shown in Figure 7. No overall maternal treatment effect was observed ($P > 0.05$) for all these genes. Regardless of maternal diet, SOD1 expression increased ($P < 0.001$), while SOD2 decreased ($P < 0.001$) over time. NFE2L2 also had a marked decrease ($P < 0.001$) from 24 h after colostrum intake to 14 d of age. MPO and NOS2 had a peak of their expression at 24 h after colostrum intake and at 50 d of age ($P < 0.001$ for all comparisons).

Pro-inflammatory (TLR) pathway

Main effects of diet, day, and interactions for genes related to oxidative stress are shown in Figure 8. No overall maternal treatment effect was observed ($P > 0.05$) for all these genes. Due a T×D ($P = 0.04$) MET calves had lower ($P = 0.002$) *TLR2* expression at 14 d of age. Regardless of maternal treatment, *IRAK1*, *NFKB1*, *TNF* and *SLAMF7* increased the expression over time ($P < 0.001$ for all), while *TLR2*, *TLR4*, *MYD88* and *NFKBIA* had some marked decrease in their expression ($P < 0.001$ for all). *TRAF6* and *IL1B* expression were not affected.

Methionine cycle

Main effects of diet, day, and interactions for genes related to methionine cycle are shown in Figure 9. No overall maternal treatment effect was observed ($P > 0.05$) for all these genes. Regardless of maternal treatment, *CTH* and *SAHH*

increased ($P < 0.001$ for both) the expression at 24 h after colostrum intake, while *CBS* decreased ($P < 0.001$). Then at 14 d of age *SAHH* expression was reduced ($P < 0.001$) and *CBS* ($P < 0.001$) increased to birth level. *SAHH* expression also had another increase ($P < 0.001$) at 50 d of age. *MAT1A*, *BHMT* and *BHMT2* were not expressed in the neutrophils.

Glutathione metabolism

Main effects of diet, day, and interactions for genes related to glutathione metabolism are shown in Figure 9. No overall maternal treatment effect was observed ($P > 0.05$) for all these genes. Regardless of maternal treatment, *GCLC* had an increasing ($P < 0.001$) expression profile over time. *GSR*, *GSS* and *GPX1* expression increased from birth to 24 h after colostrum intake and from 28 d to 50 d of age ($P < 0.001$ for all comparisons). However, *GPX1* and *GSR* expression decreased ($P < 0.001$ for both) to 14 d of age.

microRNAs expression

Main effects of diet, day, and interactions for microRNA expression are shown in Figure 10. No overall maternal treatment effect was observed ($P > 0.05$) for all these genes. Regardless of maternal treatment, only *miR-223* expression decreased ($P < 0.001$) over time. *miR-9-5p* expression increased ($P < 0.001$) from birth to 28 d of age. *miR-155* expression increased ($P < 0.001$) from 24 h after colostrum intake to 14 d of age, while *miR-146a* increased ($P < 0.001$) from 14 d to 28 d of age. Lastly, *miR-125b* expression increased from birth to 24 h after colostrum intake and then from 14 d to 28 d of age ($P < 0.001$) for both.

DISCUSSION

In the present study, we attempted to integrate blood PMN function and gene network expression with whole-animal performance, blood metabolic indicators of inflammation and oxidative stress in calves born from dams that were supplemented with methionine during the last 24 days of pregnancy. However, our results demonstrate a lack of interaction of mother's feeding methionine and calves immunometabolism.

As demonstrated by Osorio et al. (2013a), besides of no effect on blood metabolites (e.g. NEFA, BHBA, insulin, glucose) cows supplemented with two different methionine presentations (MetaSmart – 0.19% DMI/day; Smartamine – 0.07% DMI/day) during the last three weeks pre-partum had greater neutrophil phagocytosis compared to the no supplemented ones (55.1% and 45.8% vs 38.5%). With calves, maternal overnutrition (1.47 Mcal of NE_L/kg of DM) during the last three weeks pre-partum increased calves neutrophil phagocytosis during the first week of life, with a marked increase after colostrum intake (Osorio et al., 2013b). Our results also demonstrated a more pronounced increase in neutrophil phagocytosis from the MET calves after colostrum intake, however was not enough to be statistically detectable, besides being biologically important any increase in neonatal calves immune function.

The same *in vitro* LPS challenge were done with the cows from this trial, and preliminary results demonstrated that methionine supplementation were efficient in enhancing the IL1B response (MET: 1812 pg/mL; CON: 1043 pg/mL), evaluated at days -15, -7, 2, 7, and 20 relative to parturition (Vailati Riboni et al., 2014). This result confirmed the responsiveness of blood cells to an inflammatory challenge even in a

period of immune suppression (peripartum) and that supplemental methionine during the transition period enhances the pro-inflammatory cytokine response, hence, potentially enhancing the responsiveness to invading pathogens. However, we were not able to detect the response of maternal supplementation of methionine on calves' cytokines response after a LPS challenge.

Recently, Osorio et al. (2014b) published complementary results to Osorio et al. (2013), demonstrating the effectiveness of supplementing methionine during the transition period in enhance inflammatory and oxidative response. MET cows had lower ceruloplasmin and haptoglobin, classical positive acute phase proteins, higher ORAC, a measurement of antioxidant capacity, and also higher liver concentration of glutathione. Liver expression of genes related to one-carbon metabolism, glutathione metabolism, inflammation and oxidative stress also demonstrated to be modulated by methionine supplementation (Osorio et al., 2014a).

A study evaluating how lactating cows in induced negative or positive energy balance respond to an intramammary infection (IMI) with *Streptococcus uberis* reported that among the PMN genes that were upregulated by negative energy balance during the IMI, TLR2 and TL4 were two of the most affected (Moyes et al., 2010). The toll-like receptors (TLR), expressed in immune cells such as neutrophils and macrophages (Akira et al., 2001), recognize pattern molecules on invading pathogens and play a major role in activating innate immunity, mainly trhough the pro-inflammatory response (Lee and Hwang, 2006). Bacteria contain unique motifs, such as lipopolysaccharides (LPS) that are potent stimulators of innate immunity. The TLR activate the NFKB pro-inflammatory signaling cascade that encompasses the activation of several mediators, such as MYD88, TRAF6, IRAK1, and the IKK

complex, which culminates with the secretion of the pro-inflammatory cytokines, such as TNF-α, IL6, and IL1B (Akira et al., 2001).

Maternal overnutrition (1.47 Mcal of NE_L/kg of DM) during the last three weeks pre-partum also had an effect on neutrophil expression of genes related to cell adhesion (Osorio et al., 2013b). In agreement with our study, Osorio et al. (2013b) demonstrated an increased expression, specially of SELL, during the first week of life. L-Selectin is an important glycoprotein for adhesion of leukocytes to endothelial cells (Abdelrahman et al., 2005) and the marked increase in *SELL* expression right after colostrum intake likely reflected the potential for immune-related proteins in colostrum to trigger inflammation and prime the innate immune responses.

Overall, our results demonstrate that maternal supplementation with methionine during the last 24 days of pregnancy did not affect calves immune metabolism, from birth through weaning. Due the fact that previous research results are consistent in demonstrate the beneficial effect of supplementing methionine during the transition period on cow's immune function, the mechanisms of intrauterine nutrition with methionine and calves immune metabolism is worthy of further investigation.

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Table 1. Rectal temperature, fecal score and starter intake from calves born to dams supplemented with rumen-protected methionine (MET) or no supplementation (CON) during the last 24 d pre-partum.

	Treatment (T)			P-value		
Week	MET	CON	SEM	Trt	wk	T×W
Rectal temperature, °C						
0	39.49	38.48	0.06	0.428	0.356	0.192
1	38.61	38.44	0.06			
2	38.43	38.46	0.06			
Fecal score (1-4)						
1	2.40	2.24	0.10	0.191	<0.001	0.995
2	2.79	2.69	0.10			
3	2.40	2.33	0.10			
4	2.14	2.01	0.10			
5	2.07	2.03	0.10			
6	1.82	1.69	0.10			
Starter intake (kg/day)						
1	0.01	0.02	0.01	0.686	<0.001	0.94
2	0.07	0.10	0.07			
3	0.27	0.23	0.07			
4	0.40	0.44	0.07			
5	0.57	0.65	0.07			
6	1.31	1.34	0.07			

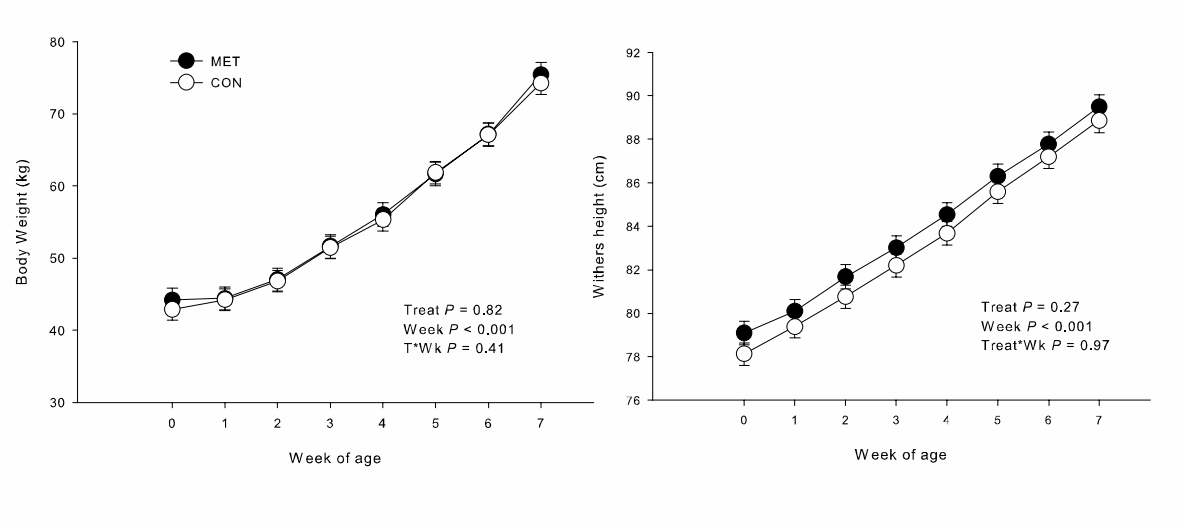

Figure 1. Body weight and withers height of calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and week, and their interaction (T×Wk).

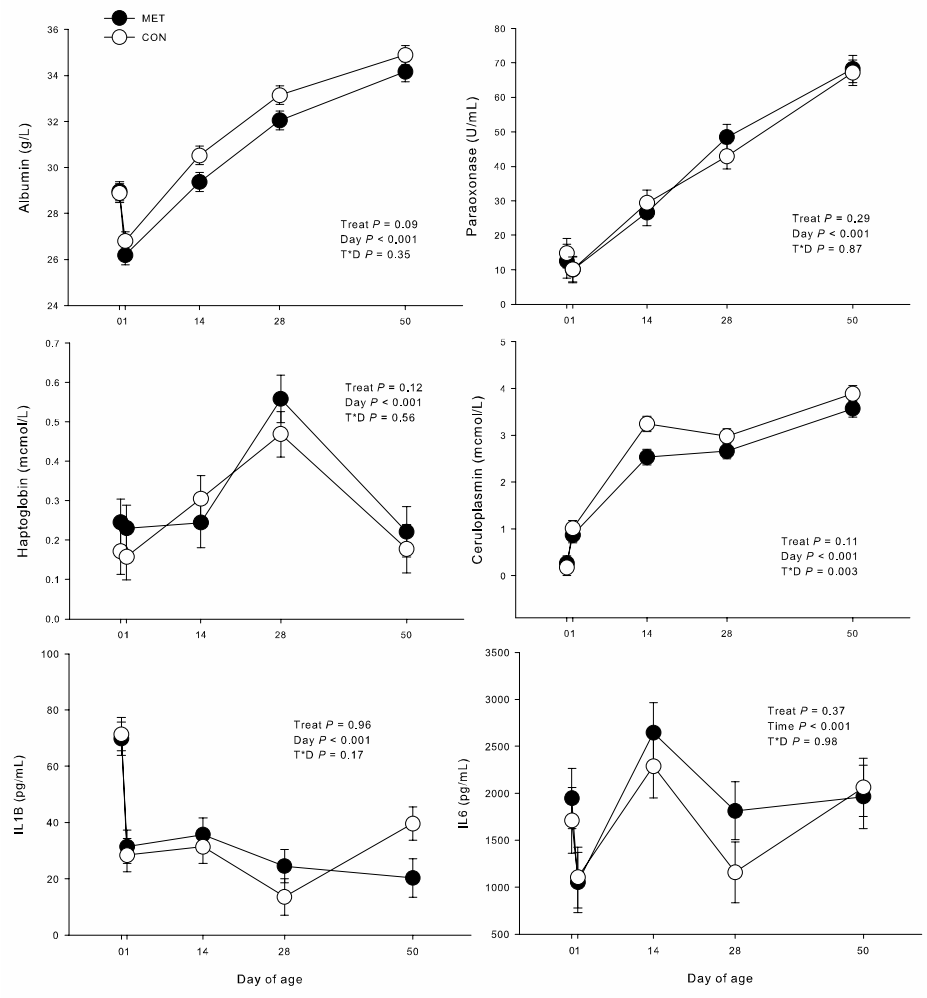

Figure 2. Blood biomarker of inflammation from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

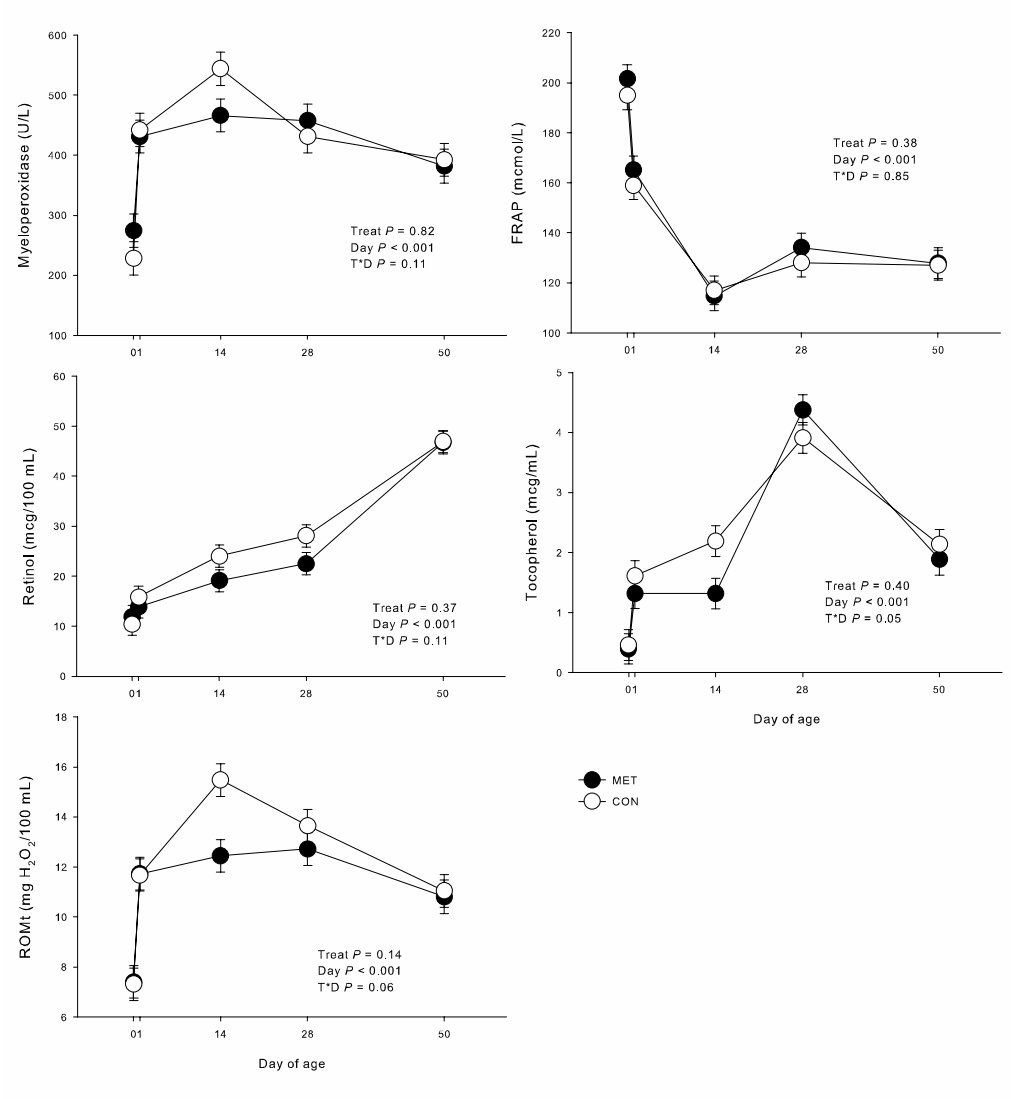

Figure 3. Blood biomarkers of oxidative stress from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

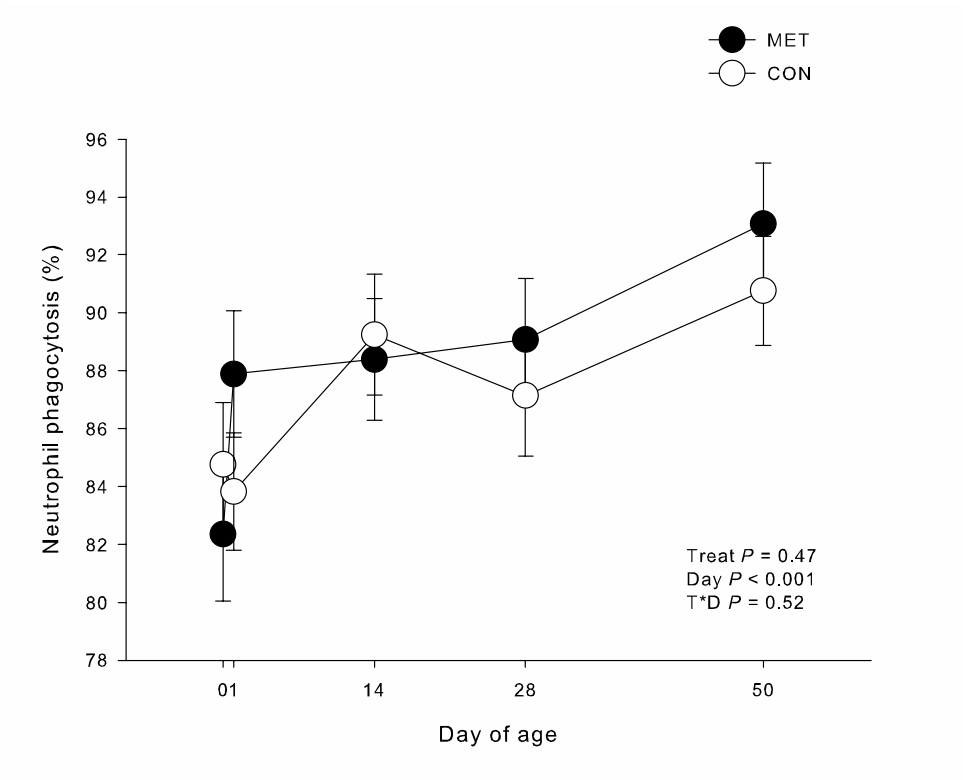

Figure 4. Blood neutophil phagocytosis (%) from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

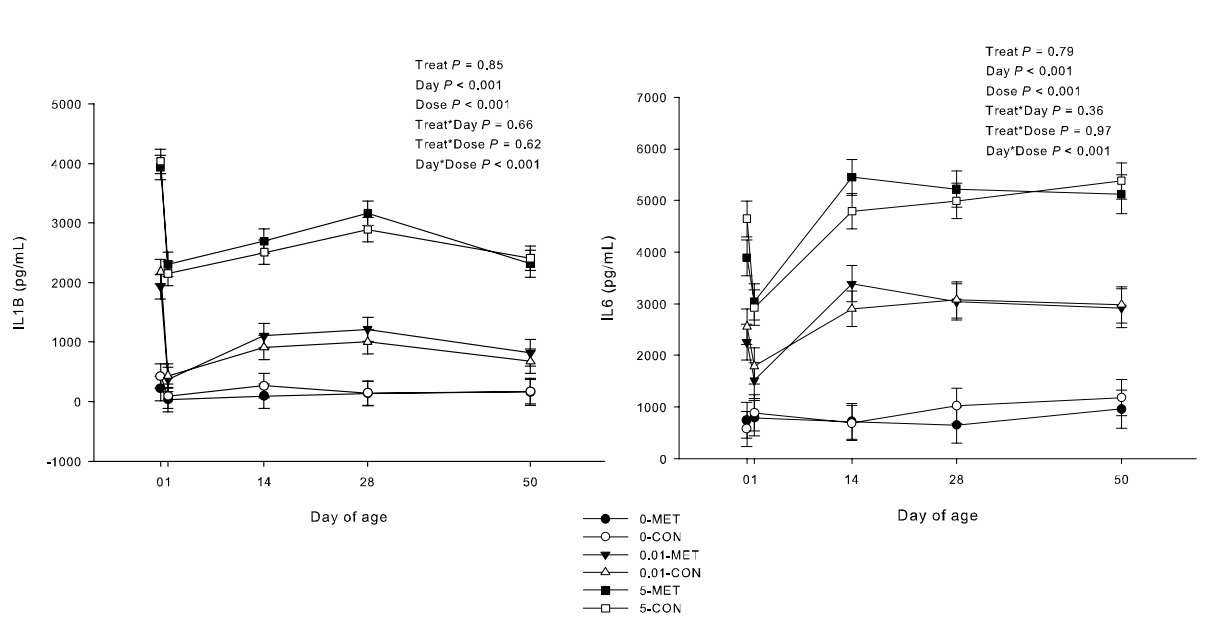

Figure 5. Blood IL1B and IL6 concentrations from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum after *in vitro* LPS challenge (Doses: 0 µg LPS/mL blood, 0.01 µg LPS/mL blood and 5 µg LPS/mL blood. Shown are P values for main effects of treatment (Treat) day and dose, and their interaction (Treat×Day, Treat×Dose, Day×Dose).

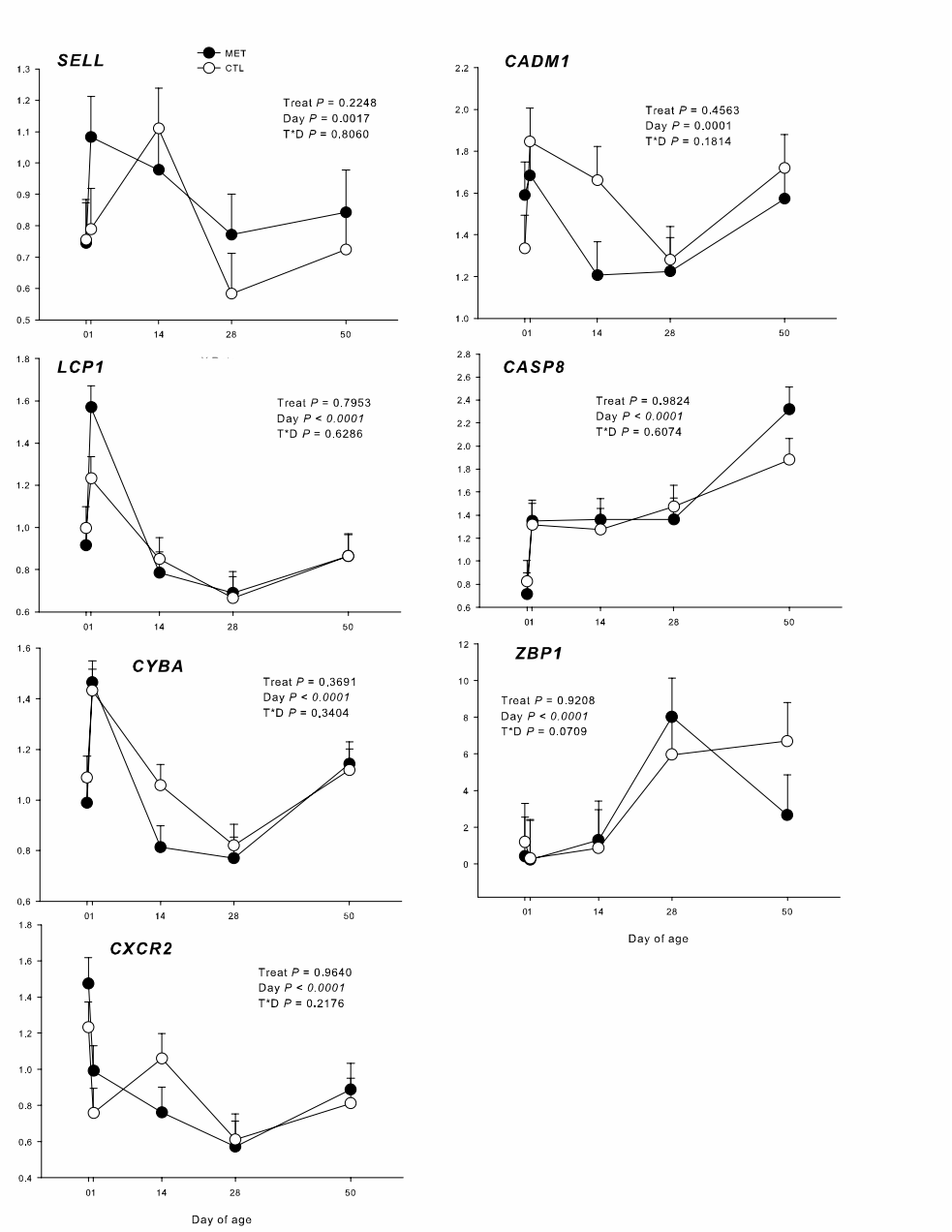

Figure 6. Expression of genes (R.U.) related cell adhesion and chemotaxis from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

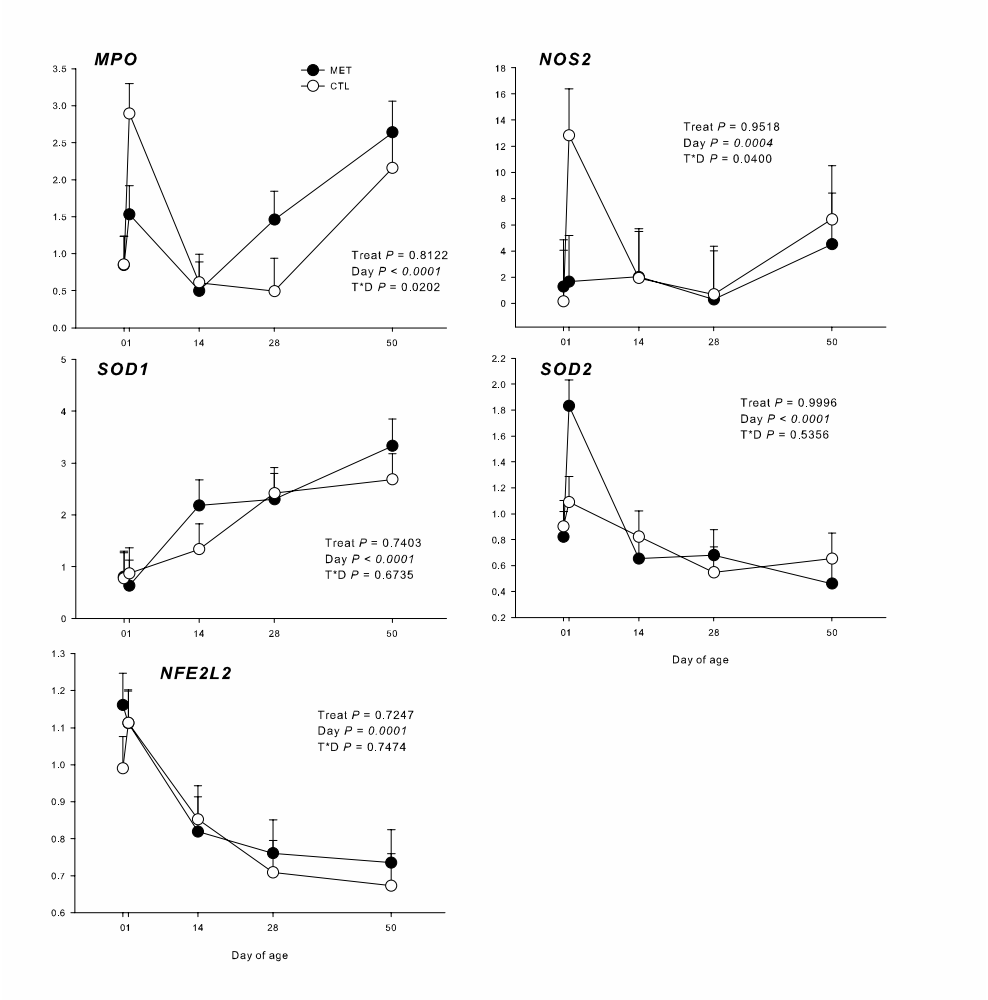

Figure 7. Expression of genes (R.U.) related to oxidative stress from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

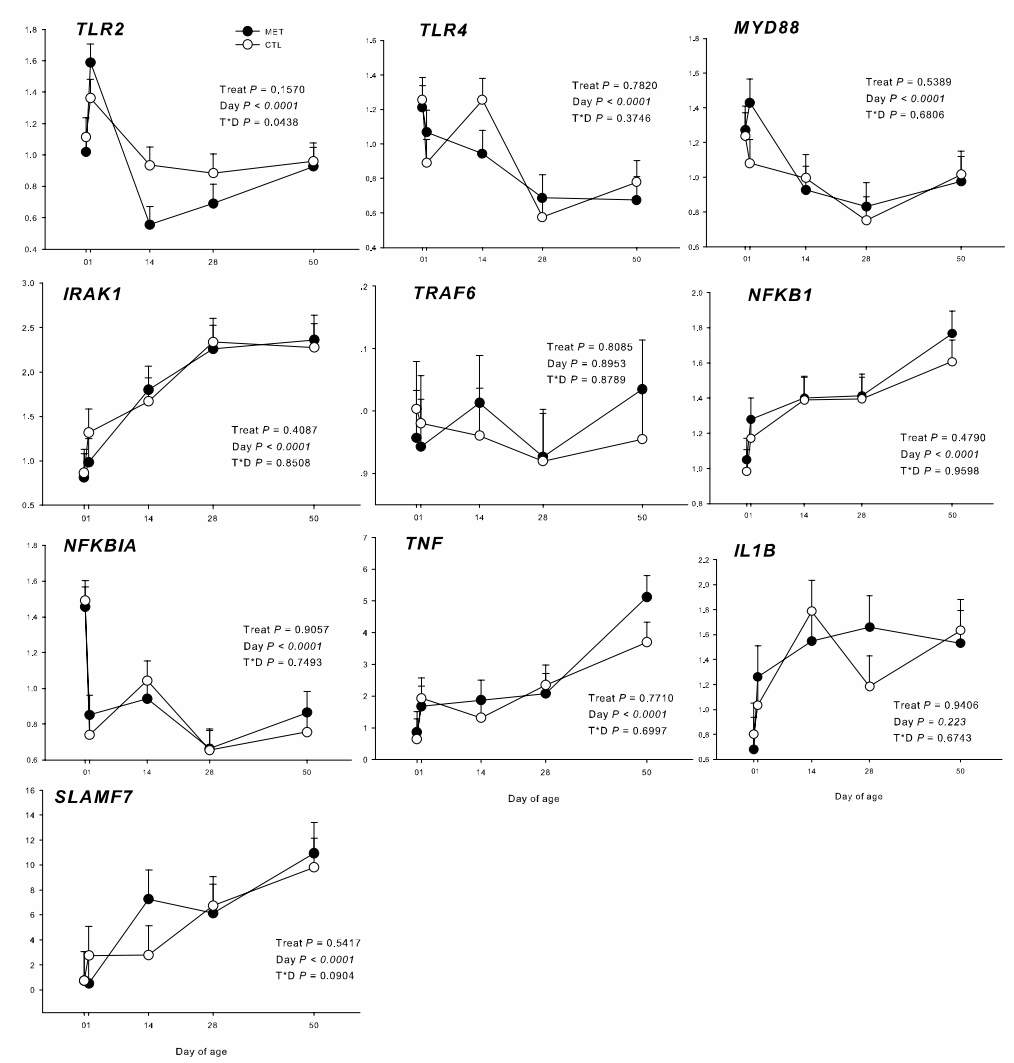

Figure 8. Expression of genes (R.U.) related to a pro-inflammatory pathway (TLR pathway) from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

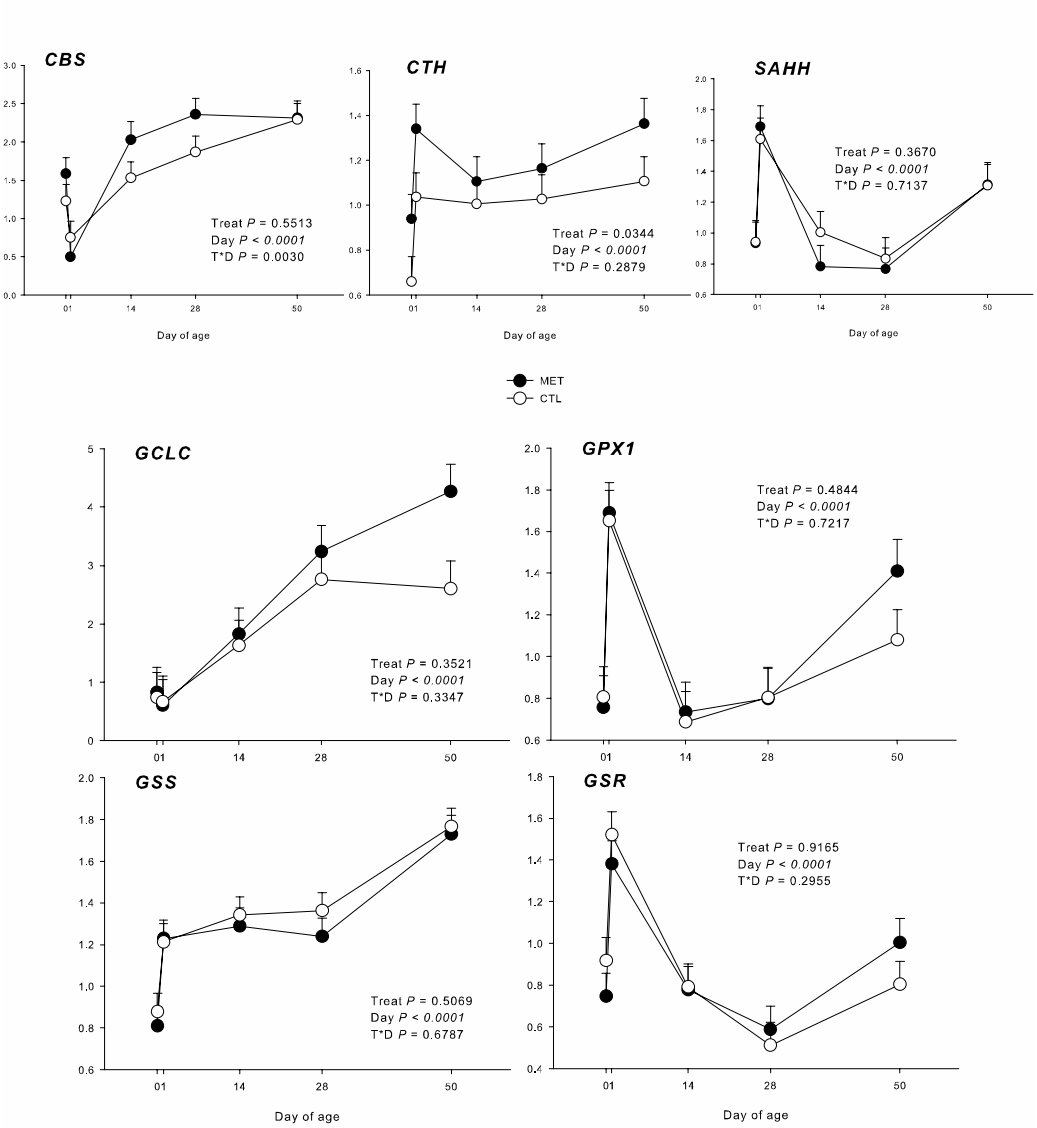

Figure 9. Expression of genes (R.U.) related to methionine cycle and glutathione metabolismo from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

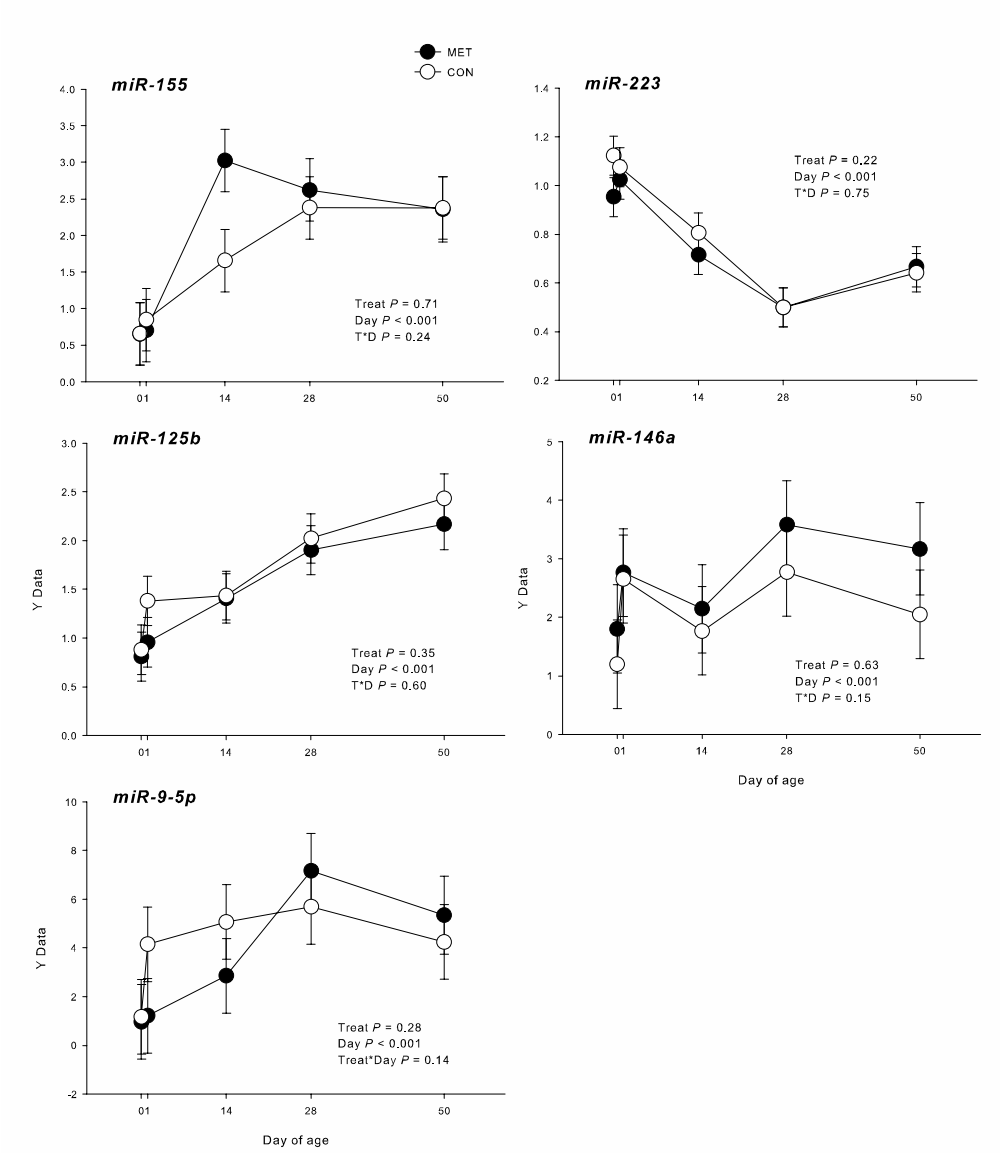

Figure 10. Expression of microRNAs (R.U.) from calves born to dams supplemented with methionine (MET) or no supplementation (CON) during the last 24 d pre-partum. Shown are P values for main effects of treatment (Treat) and day, and their interaction (T×D).

Supplemental material

Function of the genes selected for transcriptional profile on calves neutrophils.

Source: NCBI (http://www.ncbi.nlm.nih.gov/gene)

CELL ADHESION AND CHEMOTAXIS

SELL - Selectin L

This gene encodes a cell surface adhesion molecule that belongs to a family of adhesion/homing receptors. The encoded protein contains a C-type lectin-like domain, a calcium-binding epidermal growth factor-like domain, and two short complement-like repeats. The gene product is required for binding and subsequent rolling of leucocytes on endothelial cells, facilitating their migration into secondary lymphoid organs and inflammation sites.

CADM1 - Cell adhesion molecule 1

This gene encodes for a protein that acts as a cell adhesion molecule essential for several functions of neutrophils.

LCP1 - Lymphocyte cytosolic protein 1 (L-plastin)

This gene encodes for an actin-binding protein. Plays a role in the activation of immune cells.

CASP8 - Caspase 8, apoptosis-related cysteine peptidase

This gene encodes a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis.

CYBA - Cytochrome b-245, alpha polypeptide

Cytochrome b is comprised of a light chain (alpha) and a heavy chain (beta). This gene encodes the light, alpha subunit which has been proposed as a primary component of the microbicidal oxidase system of phagocytes.

ZBP1 - Z-DNA binding protein 1

This gene encodes a Z-DNA binding protein. The encoded protein plays a role in the innate immune response by binding to foreign DNA and inducing type-I interferon production.

CXCR2 - Chemokine (C-X-C motif) receptor 2

The protein encoded by this gene is a member of the G-protein-coupled receptor family. This protein is a receptor for interleukin 8 (IL8). It binds to IL8 with high affinity, and transduces the signal through a G-protein activated second messenger system. This receptor mediates neutrophil migration to sites of inflammation.

OXIDATIVE STRESS

MPO – Myeloperoxidase

Myeloperoxidase (MPO) is a heme protein synthesized during myeloid differentiation that constitutes the major component of neutrophil azurophilic granules. This enzyme produces hypohalous acids central to the microbicidal activity of neutrophils.

NOS2 - Nitric oxide synthase 2, inducible

Nitric oxide is a reactive free radical which acts as a biologic mediator in several processes, including neurotransmission and antimicrobial and antitumoral activities. This gene encodes a nitric oxide synthase which is expressed in liver and immune cells and is inducible by a combination of lipopolysaccharide and certain cytokines.

SOD1 - Superoxide dismutase 1, soluble

The protein encoded by this gene binds copper and zinc ions and is one of two isozymes responsible for destroying free superoxide radicals in the body. The encoded isozyme is a soluble cytoplasmic protein, acting as a homodimer to convert naturally-occuring but harmful superoxide radicals to molecular oxygen and hydrogen peroxide.

SOD2 - Superoxide dismutase 2, mitochondrial

This gene is a member of the iron/manganese superoxide dismutase family. It encodes a mitochondrial protein that forms a homotetramer and binds one manganese ion per subunit. This protein binds to the superoxide byproducts of

oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen.

NFE2L2 - Nuclear factor, erythroid 2-like 2

This gene encodes a transcription factor which is a member of a small family of basic leucine zipper (bZIP) proteins. The encoded transcription factor regulates genes which contain antioxidant response elements (ARE) in their promoters; many of these genes encode proteins involved in response to injury and inflammation which includes the production of free radicals.

TLR PATHWAY

TLR2 - Toll-like receptor 2

The protein encoded by this gene is a member of the Toll-like receptor (TLR) family which plays a fundamental role in pathogen recognition and activation of innate immunity. They recognize pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. The various TLRs exhibit different patterns of expression. This gene is expressed most abundantly in peripheral blood leukocytes, and mediates host response to Gram-positive bacteria and yeast via stimulation of NFKB.

TLR4 - Toll-like receptor 4

The protein encoded by this gene is a member of the Toll-like receptor (TLR) family which plays a fundamental role in pathogen recognition and activation of innate immunity. They recognize pathogen-associated molecular patterns that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. The various TLRs exhibit different patterns of expression. This receptor has been implicated in signal transduction events induced by lipopolysaccharide (LPS) found in most gram-negative bacteria.

MYD88 - Myeloid differentiation primary response 88

This gene encodes a cytosolic adapter protein that plays a central role in the innate and adaptive immune response. This protein functions as an essential signal transducer in the interleukin-1 and Toll-like receptor signaling pathways. These

pathways regulate that activation of numerous proinflammatory genes.

IRAK1 – interleukin-1 receptor-associated kinase 1

This gene encodes the interleukin-1 receptor-associated kinase 1, one of two putative serine/threonine kinases that become associated with the interleukin-1 receptor (IL1R) upon stimulation. This gene is partially responsible for IL1-induced upregulation of the transcription factor NF-kappa B.

TRAF6 - TNF receptor-associated factor 6, E3 ubiquitin protein ligase

The protein encoded by this gene is a member of the TNF receptor associated factor (TRAF) protein family. TRAF proteins are associated with, and mediate signal transduction from, members of the TNF receptor superfamily. This protein mediates signaling from members of the TNF receptor superfamily as well as the Toll/IL-1 family. This protein functions as a signal transducer in the NFKB pathway that activates IKB kinase (IKK) in response to pro-inflammatory cytokines.

NFKB1 - Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1

NFKB is a transcription regulator that is activated by various intra- and extra-cellular stimuli such as cytokines, oxidant-free radicals, ultraviolet irradiation, and bacterial or viral products. Activated NFKB translocates into the nucleus and stimulates the expression of genes involved in a wide variety of biological functions.

NFKBIA - Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

This gene encodes a member of the NF-kappa-B inhibitor family, which contain multiple ankrin repeat domains. The encoded protein interacts with REL dimers to inhibit NFKB/REL complexes which are involved in inflammatory responses.

TNF - Tumor necrosis factor

This gene encodes a multifunctional pro-inflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. This cytokine is mainly secreted by macrophages. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation.

IL1B - Interleukin 1, beta

The protein encoded by this gene is a member of the interleukin 1 cytokine family. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.

SLAMF7 - SLAM family member 7

Signaling lymphocyte activation molecules (SLAM) family receptors are expressed on hematopoietic cells and play important role in immune regulation, acting as an inhibitor of pro-inflammatory cytokine production.

METHIONINE CYCLE

CTH - Cystathionine gamma-lyase

Cystathionine gamma-lyase (CSE) (or cystathionase) is an enzyme which breaks down cystathionine into cysteine and α-ketobutyrate.

CBS - Cystathionine-beta-synthase

The protein encoded by this gene acts as a homotetramer to catalyze the conversion of homocysteine to cystathionine, the first step in the transsulfuration pathway.

SAHH – adenosylhomocysteinase (AHCY)

It catalyzes the reversible hydrolysis of S-adenosylhomocysteine (AdoHcy) to adenosine (Ado) and L-homocysteine (Hcy). Thus, it regulates the intracellular S-adenosylhomocysteine (SAH) concentration thought to be important for transmethylation reactions.

GLUTATHIONE METABOLISM

GCLC - Glutamate-cysteine ligase, catalytic subunit

Glutamate-cysteine ligase, also known as gamma-glutamylcysteine synthetase is the first rate-limiting enzyme of glutathione synthesis.

GSS - Glutathione synthetase

The protein encoded by this gene functions as a homodimer to catalyze the second step of glutathione biosynthesis, which is the ATP-dependent conversion of gamma-L-glutamyl-L-cysteine to glutathione.

GSR - Glutathione reductase

This gene encodes a member of the class-I pyridine nucleotide-disulfide oxidoreductase family. This enzyme is a homodimeric flavoprotein. It is a central enzyme of cellular antioxidant defense, and reduces oxidized glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant.

GPX1 - Glutathione peroxidase 1

This gene encodes a member of the glutathione peroxidase family. Glutathione peroxidase functions in the detoxification of hydrogen peroxide, and is one of the most important antioxidant enzymes in humans.

5 CONCLUSÃO GERAL

- A suplementação de vacas leiteiras com microminerais orgânicos durante os últimos 30 dias de gestação beneficia a função imune dos bezerros, pois reduz o estado pró-inflamatório e proporciona um melhor controle do estresse oxidativo durante o período neonatal.
- A suplementação de vacas leiteiras com metionina durante do últimos 24 dias de gestação exerce efeito positivo sobre o metabolismo hepático dos bezerros durante o período neonatal, há uma maturação mais evidente das vias metabólicas e também maior eficiência no uso da glicose.
- A suplementação de vacas leiteiras com metionina durante do últimos 24 dias de gestação não influencia o mestabolismo imune inato dos bezerros durante o período neonatal, não foi possível observar efeitos da dieta materna nos marcadores sanguíneos relacionados a inflamação e estresse oxidativo, bem como na expressão de genes dos neutrófilos sanguíneos e microRNAs.

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