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Suplementação com metionina, colina e ácidos graxos poli-insaturados no periparto e seus efeitos imunonutricionais e transgeracionais

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Para meu noivo, pais e irmãos, com carinho e gratidão.

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“Viver é acalentar sonhos e esperanças.
É buscar nas pequenas coisas, um grande motivo para ser feliz!”
Mário Quintana

Resumo

MONTAGNER, Paula. **Suplementação com metionina, colina e ácidos graxos poli-insaturados no periparto e seus efeitos imunonutricionais e transgeracionais**. 2017. 144f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Atualmente, os alimentos imunonutriconais ganham ênfase, pois suprem as necessidades nutricionais básicas, apresentam papel modulador da resposta imune, além de prevenirem ou tratarem de distúrbios metabólicos e doenças. Neste contexto, destacam-se a metionina (MET) e a colina (COL) e, também, os ácidos graxos poli-insaturados ômega-3 e ômega-6. Durante a gestação, a limitação dos nutrientes exerce efeitos diretos na mãe e na prole, principalmente no fígado e nas células polimorfonucleadas, porque estes são os primeiros a responderem à limitação de nutrientes, bem como à adição de novos nutrientes à dieta. Portanto, o objetivo deste estudo foi avaliar os efeitos de diferentes estratégias alimentares na expressão de genes relacionados ao sistema imune na mãe e na prole, utilizando como modelos vacas e ratas. Esse estudo baseia-se na compilação de quatro trabalhos com foco nessa temática. O primeiro trabalho avaliou a funcionalidade hepática durante o período de transição em vacas leiteiras e sua relação com a produção, reprodução e resposta de fase aguda. Foi observada uma relação direta entre a funcionalidade hepática e as proteínas de fase aguda produzidas pelo fígado. O segundo estudo visou avaliar os efeitos da suplementação com MET e COL durante o período de transição das vacas (desde 24 dias anteriores ao parto até o dia 30 após o parto) na expressão gênica de células polimorfonucleadas. Animais que receberam MET e COL tiveram maior expressão de genes relacionados a adesão celular (CADM1 e SELL) sugerindo uma capacidade de resposta frente a antígenos mais rápida do que o grupo controle. O terceiro trabalho utilizou bezerras, filhas das vacas provenientes do experimento anterior, e avaliou o efeito da nutrição materna com MET e COL sobre a expressão hepática de enzimas relacionadas a rota da MET e COL e marcadores sanguíneos imunes e energéticos. Foi observado que a suplementação materna com MET e COL modificou a expressão de enzimas envolvidas na rota da transulfuração (BHMT e MTR). O quarto trabalho propôs avaliar dietas com diferentes relações de ômega-3 e ômega-6 sobre a expressão hepática em três sucessivas gerações, a fim de investigar os efeitos imunonutricionais transgeracionais dos ácidos poli-insaturados. A suplementação com ômega-3 modulou a expressão de gene envolvidos na rota GPR120 – β-Arrestina, promovendo um efeito anti-inflamatório ao longo das gerações. As estratégias adotadas demostraram ser benéficas para a mãe, contribuindo para uma melhor adaptação durante o periparto, bem como apresentou efeitos positivos sobre a prole.

Palavras-chave: periparto, imunossupressão, células polimorfonucleadas, expressão gênica, funcionalidade hepática.

Abstract

MONTAGNER, Paula. **Supplemation with methionine, choline and polyunsaturated fatty acids in the peripartum and their immunonutritional and transgenerational effects.** 2017. 144f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Nowaday, immunonutrition foods gain emphasizes by supply basic nutritional requires, have a role in modulating the immune response, and prevent or treat metabolic disorders and diseases. In this context, we highlight methionine (MET) and choline (CHOL), and also the polyunsaturated fatty acids omega-3 and omega-6. During the gestation, the nutrient limitation has direct effects on the mother and offspring, especially on the liver and polymorphonucleated cells, because they are the first to respond to nutrient limitation as well, the addition of new nutrients to the diet. Therefore, the aim of this study was to evaluate the effects of different feeding strategies on the expression of genes related to the immune system in the mother and offspring, using as cows and rats models. This study is the compilation of four papers with focus on this subject. The first paper evaluated the hepatic function during the transition period in dairy cows and its relationship with production, reproduction, and acute phase response. A direct relationship was observed between liver function and the acute phase proteins produced by the liver. The second paper aimed at evaluating the effects of MET and CHOL supplementation during the transitional period of cows (from 24 days before calving to 30 days postpartum) on the gene expression of polymorphonucleated cells. Animals that received MET and CHOL had higher expression of genes related to cell adhesion (CADM1 and SELL) suggesting faster ability to respond to antigens than control group. The third paper used the calves, daughters of the cows from the previous experiment, and evaluated the effect of maternal nutrition with MET and CHOL on the hepatic expression of enzymes related to MET and CHOL and in the blood analyzed immune and energetic biomarker. It was observed that maternal supplementation with MET and CHOL modified the expression of enzymes involved in the transsulfuration pathway (BHMT and MTR). The fourth paper proposed to evaluate diets with different omega-3 and omega-6 relationships on liver expression in three successive generations in order to investigate the transgenerational immunonutritional effects of polyunsaturated acids. Omega-3 supplementation modulated a gene expression involved in the GPR120 - β-arrestin pathway, promoting an anti-inflammatory effect over the generations. The strategies adopted proved to be beneficial for the mother, contributing to a better adaptation during the peripartum, as well have positive effects on the offspring.

Keywords: Peripartum, imunussupression, polymorphonuclear cells, gene expression, hepatic functionality.

Lista de Figuras

Lista de Abreviaturas

AA – Aminoácidos

AG – Ácidos graxos

AGNE – Àcidos graxos não esterificados

COL – Colina

HAP – Haptoglobina

IFG1 – *Insulin-like growth factor 1* (Fator de crecimento semelhante a insulina)

IL-1β – Interleucina 1 beta

IL-6 – Interleucina 6

IMS – Ingestão de matéria seca

LFI – *Liver funionality index* (Indice de funcionalidade hepatica)

LPS – Lipopolissacarídeos

MET – Metionina

PON – Paraoxonase

SAM -S-adenosilmetionina

SOD –Superóxido dismutase

TNF-α – *Tumor de nercosis factor alfa* (Fator de necro tumoral)

Sumário

1 INTRODUÇÃO GERAL

Durante o período de transição, que compreende as três semanas pré-parto e as três semanas pós-parto, as vacas leiteiras sofrem intensas variações hormonais seguidas pelo decréscimo da ingestão de matéria seca (IMS) e o aumento dos ácidos graxos não esterificados (AGNE) (Goff and Horst, 1997). Essas mudanças alteram o metabolismo e promovem uma condição de imunossupressão, reduzindo a capacidade do sistema imune frente aos desafios do periparto (Sordillo et al., 2009).

Essas variações também afetam o metabolismo hepático, que possui uma relação direta com a condição imune durante o periparto (Contreras and Sordillo, 2011; Seo et al., 2014). Isso pode ser avaliado pelo aumento acentuado das proteínas de fase aguda positiva (PFA+), bem como a redução das proteínas de fase aguda negativa (PFA-) no periparto (Bionaz et al., 2007; Trevisi, et al., 2011). A fim de demostrar e quantificar essa relação (fígado-imunidade), alguns modelos que utilizam marcadores sanguíneos foram gerados, considerando os modelos mais utilizados em vacas leiteiras: o índice de atividade hepática e o índice de funcionalidade hepática (LFI – do inglês Liver Funcionality Index) (Bertoni and Trevisi, 2013a).

Diante desta temática, relação periparto/imunossupressão, algumas estratégias nutricionais vêm sendo implementadas para amenizar as consequências da imunossupressão e, com isso, maximizar os benefícios tanto para a vaca e sua prole, como para o produtor de leite. O ajuste do nível de energia e da IMS preveniu que as vacas ganhassem ou perdessem muito peso, promovendo assim, a redução dos níveis de AGNE (Ingvartsen, 2006). Da mesma forma, a restrição moderada de energia na dieta durante período seco, promoveu o aumento da fertilidade, produção de leite e nos parâmetros indicativos de saúde (Beever, 2006; Cardoso et al., 2013; Huang et al., 2014). Por outro lado, em animais com dieta sem restrição energética, apresentaram redução da função imunológica, comprometendo a saúde dos animais (Dann et al., 2006; Graugnard et al., 2013; Zhou et al., 2015).

Além dessas estratégias, são necessárias ferramentas adicionais capazes de modular a resposta inflamatória, evitando os aspectos negativos e promovendo efeitos

positivos. Nesse contexto, surgem como candidatos os ácidos graxos poli-insaturados, em particular de cadeia longa da série ômega-3, e da metionina (MET) e colina (COL).

Aos olhos da nutrição humana, esses alimentos são considerados imunonutricionais; esse termo é empregado no contexto de nutrientes específicos ou nas combinações de nutrientes utilizados para tratar pacientes cirúrgicos e criticamente doentes que estão em risco devido a atividade imune prejudicada ou inadequada (Pérez-Cano et al., 2012). Da mesma forma, devido aos desafios que ocorrem durante o periparto, as vacas leiteiras passa ser modelo de estudos com os alimentos imunonutricionais.

Assim, considerando que a dieta é fundamental para manter a função imune ideal (Field et al., 2002), a aplicação de uma alimentação com nutrientes imunonutricionais apresenta benefícios para a mãe e que se estendem para a prole, pois o desenvolvimento fetal é dependente da nutrição materna, que por meio da circulação sanguínea direciona os nutrientes para o feto (Zhu et al., 2007).

Em conclusão, a utilização de ingredientes imunonutricionais durante o período de transição, entre o final da gestação e início de lactação, é de extrema importância tanto para a mãe quanto para a sua prole. Assim, esse estudo busca elucidar, á nível molecular, a função específica de cada nutriente no sistema imune.

2 REVISÃO BIBLIOGRÁFICA

2.1 Os impactos da imunossupressão sobre a funcionalidade hepática da vaca leiteira

A resposta inflamatória durante o periparto é caracterizada pelo aumento da produção de proteínas de fase aguda positiva, como a haptoglobina (HAP), e, concomitantemente, pela diminuição da produção de proteínas de fase aguda negativa, como a albumina (ALB) e a paraoxonase (PON) (Bertoni et al., 2008). O fígado é responsável pela maior parte da produção destas proteínas; os desencadeadores para o aumento da síntese das referidas proteínas ou a sua inibição são as citosinas pró-inflamatórias: IL-6, IL-1β e TNF-α (Brodzki et al., 2015). Além desse efeito no fígado, essas citosinas são responsáveis pela redução da ingestão alimentar e, consequentemente, da produção de leite, febre e mobilização de gordura (Chaplin, 2010).

Uma fórmula para quantificar a resposta inflamatória a nível hepático e auxiliar na predição de doenças foi desenvolvida por Bertoni & Trevisi (2013). Definida como índice de funcionalidade hepática (LFI - do inglês Liver Funcionality Index), essa fórmula utiliza as mudanças plasmáticas nas concentrações de albumina, colesterol e bilirrubina, no terceiro e 28º dia pós-parto. Ao utilizar essa fórmula, se uma vaca apresentar um valor baixo de LFI, indica pronunciada resposta inflamatória, sugerindo de um período de transição mais difícil, enquanto um alto valor LFI é indicativo de uma transição mais suave (Bossaert et al., 2012).

O cálculo LFI é realizado em dois passos: o primeiro considera os valores de concentração (V) dos três parâmetros detectados no terceiro dia após o parto (V3) e as variações nas concentrações entre o dia 3 e o dia 28 (V28). Os índices de albumina e colesterol são calculados seguindo a fórmula *0,5*V3 + 0,5*(V28 - V3).* O índice de bilirrubina é calculado como a seguinte equação: *0,67*V3 + 0,33* (V28 - V3)*, essa diferença se deve ao fato que o nível de bilirrubina no terceiro dia pós-parto representa 67% e a redução entre o dia 3 e o dia 28 pós-parto, os 33%. Na segunda etapa, esses índices parciais foram padronizados de acordo com os valores médios observados em vacas saudáveis, e o LFI foi calculado de acordo com a seguinte fórmula: LFI = *[(índice*

*de albumina - 17,71) / 1,08 + (índice de colesterol - 2,57) / 0,43 - (índice de bilirrubina - 6,08) / 2,17] (*Bertoni & Trevisi 2013).

Diversos trabalhos fizeram uso do LFI em sistemas de produção intensiva de bovinos, demostrando sua relação com a incidência de doenças e as alterações metabólicas durante o periparto (Bossaert et al., 2012; Trevisi et al., 2012). Entretanto dados em sistema semi-extensivo ainda não foram demonstrados. Como também ainda não foi verificada a utilização do LFI para a reprodução, uma vez que o fígado apresenta uma relação direta com a reprodução, devido a síntese de IGF-1 (Fator de crescimento semelhante a insulina do inglês - *Insulin-like growth factor 1*). Durante o periparto ocorre diminuição da síntese de IGF-1, afetando o desenvolvimento folicular e a ovulação (Webb et al., 2004). Segundo Lucy et al. (2011) o aumento nas concentrações séricas de IGF-1 pode ser obtido por meio de uma melhor saúde hepática e melhor *status* nutricional.

Os alimentos imunonutricionais apresentam uma relação direta com o LFI; estudos demostraram que a suplementação com metionina (MET) auxiliou no aumento dos níveis de albumina (Osorioet al., 2014b; Zhou et al., 2016). No experimento realizado por Zhou et al. (2016), 35% dos animais sem suplementação de MET tiveram baixos valores de LFI, enquanto no grupo que recebeu MET somente 10% das vacas apresentaram baixo LFI, sugerindo um efeito positivo da MET sobre a função hepática. Já a suplementação com ômega-3, demostrou um aumento mais rápido dos níveis de colesterol e albumina no pós-parto, promovendo um maior valor de LFI (Trevisi et al., 2011).

Diante do exposto, a utilização do LFI é uma ferramenta interessante na avaliação dos sistemas de produção de bovinos, pois pode servir tanto para predizer doenças, quanto para relacionar a retomada da capacidade reprodutiva após o parto.

2.2 O papel imunonutricional da metionina e colina

Os principais doadores do grupo metil (metionina (MET) e colina (COL)) sofrem degradação microbiana no rúmen (Sharma and Erdman, 1989; Girard and Matte,

2005), gerando necessidade de promover suplementação com desses alimentos protegidos da ação ruminal, para satisfazer as exigências da vaca. Animais suplementados com doares do grupo metil apresentaram aumento da produção leiteira e dos benefícios para a saúde durante o período de transição (Osorio et al., 2014a; Osorio et al., 2014b; Zom et al., 2011).

A MET, juntamente com a lisina são os aminoácidos (AA) mais limitantes para a síntese de leite (*Nutrient Requirements of Dairy Cattle*, 2001). A MET é o único AA que possui enxofre em sua estrutura, atuando assim como precursor de outros AA que contém enxofre, tais como cisteína, homocisteína e taurina (Brosnan and Brosnan, 2006). Como agente lipotrópico, a MET é diretamente envolvida na síntese de lipoproteínas de muito baixa densidade (VLDL) por meio da geração de S-adenosilmetionina (SAM), o qual é o doador de metil mais importante (Martinov et al., 2010). Por sua vez, o SAM pode ser utilizado para metilar fosfatidiletanolamina e gerar fofatidilcolina, que é essencial para a síntese de VLDL (Auboiron et al., 1995).

A COL também apresenta ação lipotrófíca, pois participa da síntese de fosfatidilcolina, que é indispensável para a síntese e liberação de quilomicrons e VLDL (Pinotti et al., 2002). De tal modo, a suplementação de MET e/ou COL protegida da ação ruminal aumentou a exportação de triacilglicerol hepático (TAG), um dos componentes do VLDL e, consequentemente, diminui a lipidose hepática (Zom et al., 2011). O metabolismo da COL e MET está demostrado na Figura 1.

Além do efeito lipotrófíco direto no fígado, estudos demostraram que a suplementação de MET durante o periparto apresentou efeito positivos nos leucócitos, aumentando a capacidade de fagocitose de neutrófilos (Osorio et al., 2014a), que é a primeira linha de defesa na imunidade animal (Paape et al., 2003). Zhou et al. (2016), detectou que a suplementação de MET, promoveu maior capacidade de *burst oxidativo* (explosão respiratória) do primeiro dia do pós-parto até ao dia 28° pós-parto.

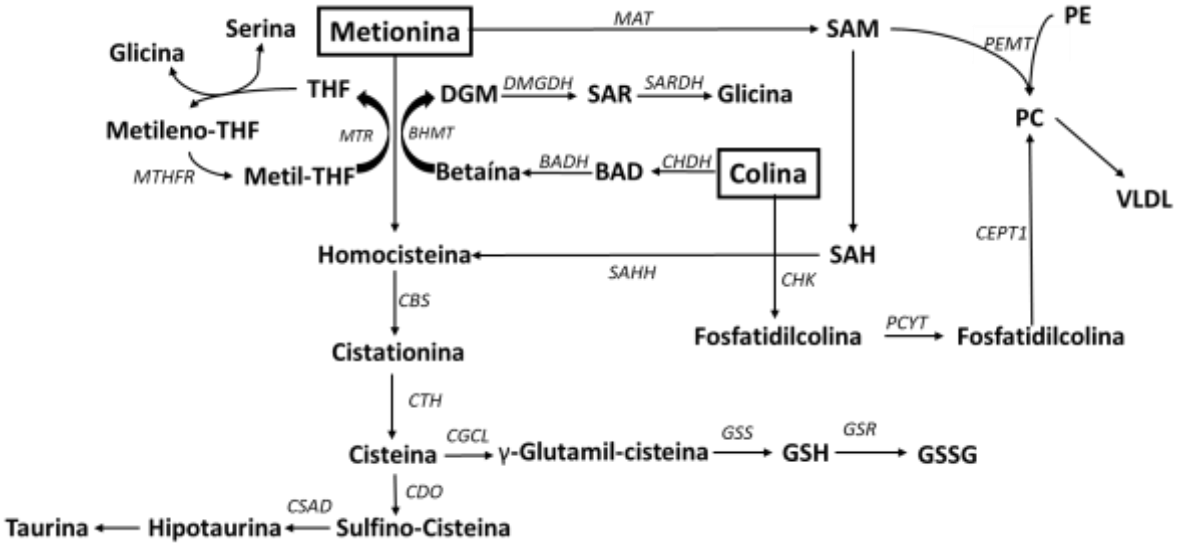

Figura 1: Rota ilustrativa com os principais genes que codificam para as enzimas responsáveis pelo metabolismo da colina e metionina. Metabolitos intermediários: SAM = S-adenosilmetionina, PE = fosfatidiletanolamina, PC = fosfatidilcolina, VLDL = *lipoproteínas de muito baixa densidade,* SAH = *S*-adenosilhomocisteína, THF = tetrahidrofolato; DMG = dimetilglicina, SAR = sarcosina, BAD = aldeído de betaína, GSH = glutationa sulfidril; GSSG = glutationa dissulfeto. Em *itálico* genes que codificam enzimas: MAT = Metionina adenosiltransferase, PEMT = fosfatidiletanolamina metiltransferase, SAHH = *S*-adenosilhomocisteína hidrolase, CHDH = colina desidrogenase, BADH= betaína aldeído desidrogenase, BHMT = betaína homocisteína metiltransferase, MTR = 5-metilltetraidrofolato-homocisteína metiltransferase, DMGDH = dimetilglicina desidrogenase, SARDH = sarcosina desidrogenase, MTHFR = metilenotetrahidrofolato redutase; CHK = colina quinase, PCYT = fosfato citidiiltransferase, CEPT1 = Colina / etanolamina fosfotransferase 1, CBS = cistationina β-sintase, CTH = cistationina β-liase , CDO = cisteína dioxigenase, CSAD = cisteína ácido sulfônico descarboxilase, CGCL = glutamato-cisteína ligase, GSS = glutationa sintetase, GSR = glutationa redutase

Ao encontro desses dados, verifica-se que a suplementação com MET protegida otimizou a resposta aos lipopolissacárideos (LPS) (componente das paredes das células das bactérias), controlando a capacidade inflamatória das células imunitárias (Vailati-Riboni et al., Inédito). Isto é muito relevante durante o período de transição, uma vez que as vacas podem produzir uma resposta inflamatória excessiva aos patógenos, criando mais danos do que benefícios (Jahan et al., 2015). Um possível mecanismo poderia estar relacionado à capacidade da MET em influenciar o estado oxidativo das vacas periparturientes, uma vez que é um precursor da glutationa e da taurina (Atmaca, 2004).

Apesar da conexão entre MET e COL (por meio do metabolismo de um carbono demostrado na Figura 1), há uma escassez de dados sobre os efeitos da COL na resposta imune bovina. As células imunes não têm a capacidade de converter COL em MET pela via da betaína, como demostrado na Figura 1, a qual está presente nos bovinos apenas no fígado e no rim (Lambert et al., 2002). No entanto, existem dados gerados usando outros modelos animais, em que a suplementação de COL na dieta melhorou os índices imunes em peixes (Wu et al., 2013) e ratos (Lewis et al., 2016), o que fez os autores acreditarem que o modo de ação mais provável é o efeito indireto da betaína (derivado de colina). Dados de frangos de corte revelaram que a suplementação com betaína na dieta melhorou a saúde intestinal e induziu um aumento na resposta imune intestinal ao desafio de coccidiose (Klasing et al., 2002).

O sistema imunológico tem dois componentes distintos, mas cooperativos. A resposta inicial, imediata, mas não específica, é a resposta imune inata (Parkin and Cohen, 2001). As células imunes inatas infiltram o tecido lesado ou infectado e induzem sinais para atrair outras células imunes, que ingerem patógenos e detritos e apresentam os antígenos (Chaplin, 2010). As células imunes inatas, especialmente os neutrófilos (PMN), são responsáveis pela produção de respostas inflamatórias agudas (Delves and Roitt, 2000). A resposta secundária é a resposta imune adaptativa, que leva mais tempo a ser ativada, mas é mais específica (Parkin and Cohen, 2001).

Como o sistema imune inato produz uma resposta mais rápida, e está envolvido na inflamação em uma extensão maior do que o sistema imune adaptativo, este se torna mais importante para pesquisas sobre o período de transição. Desta forma, os neutrófilos são frequentemente considerados os elementos mais importantes do sistema imunitário inato, pois são os primeiros a responderem aos sítios de inflamação e constituem 25% dos leucócitos circulantes em bovinos (Paape et al., 2003).

As pesquisas centradas em PMN demonstram adaptações imunes durante o período de transição (Hammon et al., 2006; Rinaldi et al., 2008; Zerbe et al., 2000). Na expressão gênica, foram observadas reversões nas prioridades, da migração, funções bactericidas e apoptose, para foco na sobrevivência prolongada e remodelação do tecido reprodutivo (Burton et al., 2005; Madsen et al., 2004). Foi

demostrado também, que o estado metabólico altera a expressão de genes associados à inflamação em PMN; pois as vacas em balanço energético negativo apresentaram uma regulação negativa de genes-chave em PMN envolvidos na resposta inflamatória (ex, IRAK1 e TNF (Moyes et al., 2014)).

Os efeitos da suplementação com MET durante o periparto da vaca leiteira sobre a expressão gênica de neutrófilos são apenas parcialmente conhecidos; porém, já foi demonstrado o aumento da expressão do gene superóxido dismutase 2 (SOD2) (Li et al., 2016), o que confere proteção contra o estresse oxidativo celular (Olsson et al., 2011). Entretanto, estudos avaliando o efeito da COL ainda não foram caracterizados.

Portanto, uma caracterização da expressão gênica em PMN durante o pariparto em animais suplementados com MET e COL é necessária para evidenciar de forma mais abrangente a atuação nas células imunes.

2.3 O papel imunonutricional dos ácidos graxos poli-insaturado de cadeia longa (ômega 3 e ômega 6)

Os lipídios oferecidos na dieta são fontes cruciais de energia, fazem parte essencial de estruturas físicas e funcionais da membrana celular, além de participarem da formação de hormônios esteroides e sais biliares (Tessaro et al., 2015). Devido à falta da enzima dessaturase, não há ação desta a partir do 9º carbono da cadeia acil, de modo que alguns ácidos graxos (AG) não são produzidos pelo organismo de mamíferos. Esses AG são denominados ácidos graxos essenciais (AGE). Em ruminantes, entre os que não podem ser produzidos endogenamente se inclui o linolêico (LA C18:2, ω-6), o qual pode ser obtido a partir de óleos vegetais; o ácido linolênico (LNA, 18:3, ω-3), que predomina nos lipídios de forrageiras e na linhaça; e os ácidos eicosapentaenoico (20:5, ω-3) e docosahexanóico (DHA, 226, ω -3), os quais são encontrados principalmente em peixes e óleo de peixe (Cheng, 2001). Esses ácidos são caracterizados como ácidos graxos poli-insaturados (PUFA

do inglês - Polyunsaturated Fatty Acids), devido a apresentarem mais de uma ligação dupla.

Para suprir a demanda nutricional, deve haver suplementação na dieta dos ácidos graxos. Entretanto, em bovinos, dados apontam que a suplementação com gordura sofre biohidrogenação (adição de moléculas de hidrogênio em AGs insaturados, tornando-os AG saturados, sem a presença da dupla ligação). Aproximadamente 70% do LA (C18:2 ω-6) e 85% do LNA (C18:3, ω-3) fornecidos para vacas em lactação são biohidrogenados no rúmen quando adicionados na forma de óleos ou alimentos desprotegidos da ação ruminal (Jenkins et al., 2008), o que leva a redução dos efeitos benéficos esperados, pois a biohidrogenação completa destes AGs por bactérias ruminais resulta principalmente na produção de ácido esteárico (18: 0) (Jenkins, 1993).

Para prevenir esse *deficit*, a utilização de ácidos protegidos da ação ruminal ganhou ênfase nas duas últimas décadas, como demostrado por diversos trabalhos, em que se observou que o aumento da densidade energética obtido por meio da adição de gordura melhorou o desempenho da lactação e também a eficiência reprodutiva (Gonzalez et al., 2015; Juchem et al., 2010).

Contudo, além do fornecimento desses AG, é importante salientar que a proporção de PUFA ômega-6 para ômega-3 na dieta tem sido reconhecida como uma influência importante na patogênese da inflamação, especialmente os derivados de PUFA - ômega-6 que iniciam e exacerbam exacerbar a resposta inflamatória (Raphael and Sordillo, 2013) Desta forma, a alimentação rica em ácidos graxos ômega-3, por exemplo, resultou numa diminuição no nível de ácido araquidônico nos fosfolipídios das membranas celulares, e consequente, diminuição nos eicosanoides derivados de ômega-6 (prostaglandina E2 e o leucotrienos da série 4 – potente ação pró-inflamatória) e aumentou os derivados de ômega-3 (prostaglandina E3 e leucotrinos da série 5), os quais possuem atividade biológica muito inferior às primeiras (Raphael and Sordillo, 2013). Os efeitos desta rota, são bem conhecidos, pois foi demostrado que vacas suplementadas com PUFA ômega-3 tiveram alteração na expressão dos tecidos adiposos dos genes PPAR (*Peroxisome Proliferator-Activated Receptor* –

Recepetor ativado por proliferadores de peroxissomos) e NFκB (*Nuclear Factor Kappa* B – Nuclear fator kappa B) (Elis et al., 2016).

Além disso, recentemente foi demostrado, que o PUFA n- ômega-3 pode também ligar-se ao receptor de ácidos graxos de superfície acoplados a proteínas G (GPR120), ativando, assim, as suas vias de sinalização (Calder, 2013). Na presença de ômega-3 o GPR120 gera um efeito anti-inflamatório, inibindo a via inflamatória do NFκB (Miyamoto et al., 2016). Como demostrado da Figura 2:

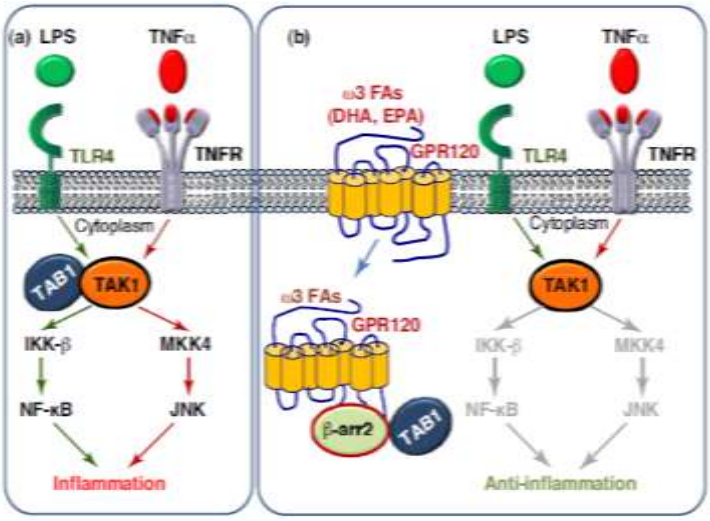

Figura 2: Rota inflamatória ativada pelo LPS e TNF e a atuação do Ômega 3 por meio do GPR120, promovendo uma efeito anti-inflamatório. Fonte Talukdar et al., (2011)

Diante do exposto, mais dados precisam ser demonstrados, para avaliar os efeitos de dietas ricas em ômega 3 na via de ativação do GPR120 no tecido hepático.

2.4 Os efeitos da nutrição materna com doadores dos grupos metil e ácidos graxos poli-insaturados

O conceito de programação fetal afirma que qualquer estimulo materno ou lesão ocorrida durante o desenvolvimento fetal pode ter efeitos de longo prazo sobre a saúde do indivíduo após o nascimento (Barker and Clark, 1997). Neste contexto, a preocupação com a adequação da nutrição desde a vida intrauterina, visando o

equilíbrio entre os elementos para atingir o melhor rendimento possível, torna-se uma temática importante.

O tecido hepático responde de maneira eficaz à regulação da nutrição materna, já que a massa hepática e suas funções são essencialmente estabelecidas durante o desenvolvimento fetal, sendo regulada pelo ambiente intrauterino (Hyatt et al., 2008). De tal modo que a nutrição materna altera a expressão gênica a nível hepático (Clayton et al., 2015; Jacometo et al., 2014). Dados epidemiológicos sugerem que a organogênese hepática é suscetível à reprogramação nutricional e que o comprometimento do desenvolvimento hepático no útero pode resultar em consequências funcionais duradouras, aumentando o risco de doenças desse tipo mais tarde, na vida (Hyatt et al., 2008).

Durante a gravidez, a ingestão de colina materna pode afetar a função metabólica e fisiológica da prole através de uma variedade de mecanismos inter-relacionados (Jiang et al., 2014). Estudos demostraram que o fornecimento de COL durante a gravidez modificou o padrão de expressão gênica do fígado (Kovacheva et al., 2007) e do cérebro (Davison et al., 2009) dos fetos. A COL disponível na circulação materna é absorvida pela placenta; o mesmo ocorre com a MET e os derivados de grupo metil (SAM). Dentro da placenta, a COL é usada para sintetizar a acetilcolina, enquanto a MET pode ser utilizada para metilar o genoma placentário (Jiang et al., 2014), como demostrado na Figura 3.

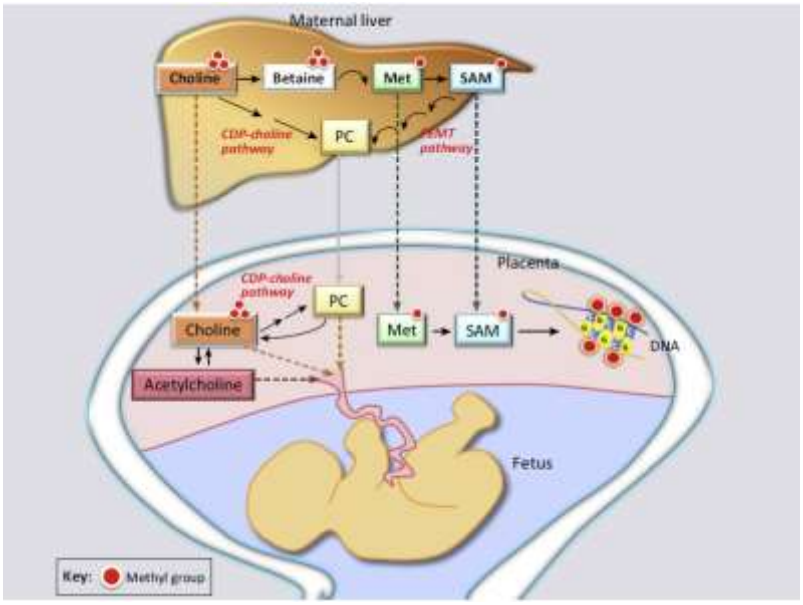

Figura 3: Metabolismo da colina e metionina na transferência entre compartimentos materno, placentário e fetal. -. No fígado materno, a colina (na figura em inglês c*holine*) fornece grupos metil para a síntese de metionina (Met) e S-adenosilmetionina (SAM). A fosfatidilcolina (PC) é sintetizada via difosfato citidina (CDP) - e a partido do SAM na via e fosfatidiletanolamina N-metiltransferase (PEMT). Os metabólitos da colina são transportados para a placenta, onde o SAM é utilizado para reações de metilação, incluindo metilação do DNA e a colina é usada para sintetizar PC e acetilcolina (na figura em inglês *acetycholine*). Fonte Jiang et al. (2014).

No mesmo sentido, o feto obtém os ácidos graxos da circulação materna que atravessam a placenta (Figura 4) (Haggarty, 2010; Herrera et al., 2006). Estudos demostraram que a suplementação materna de PUFA ômega-3, que possui propriedade antioxidante e anti-inflamatórias, exerce efeitos benéficos, incluindo crescimento fetal e redução do stress oxidativo (Jones et al., 2013). Niculescu et al. (2013) observaram que a disponibilidade materna de ALA durante a gestação e a lactação induziu alterações epigenéticas no fígado materno e do recém nascido. Os mesmos autores também observaram que a disponibilidade de ácidos graxos ômega-3 durante a gestação e lactação foi capaz de induzir alterações na expressão de das enzimas alongasses e desnaturasses, estas que regulam paralelamente as vias metabólicas dos AG.

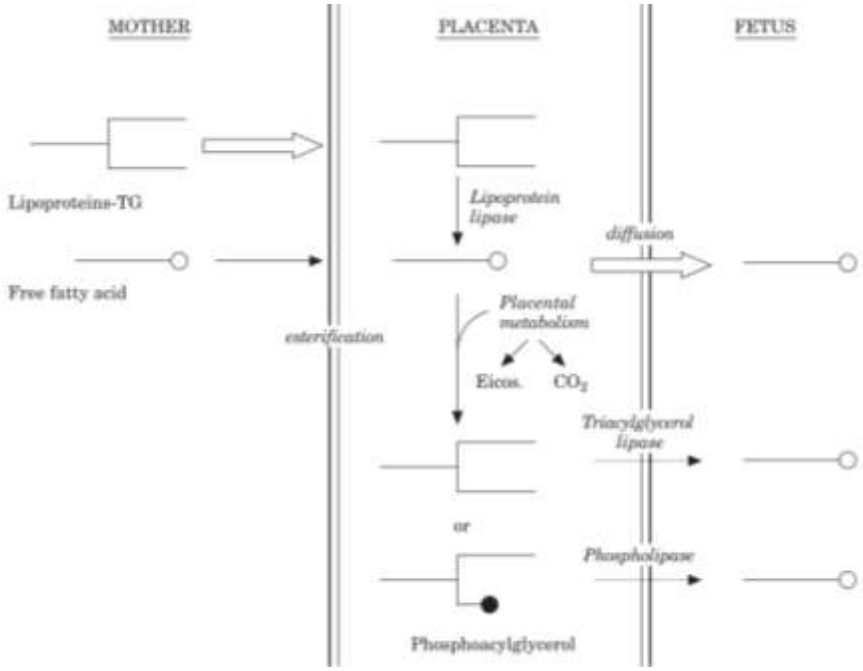

Figura 4: Representação esquemática da transferência placentária de ácidos graxos para o feto. O tecido placentário de diferentes espécies possue a lipoproteína lipase (na figura em inglês - *lipoprotein lipase*), a fosfolipase A2 e a lipase intracelular. Através deste mecanismo, os triglicerídeos plasmáticos (na figura em inglês -*lipoprotein -TG*) maternos são hidrolisados e absorvidos pela placenta, na qual a reesterificação e a hidrólise intracelular facilitam a difusão dos ácidos graxos livres (na figura em inglês - *free faty acids*) para o feto (na figura em inglês -0*fetus*) e seu transporte subsequente ao fígado fetal. Fonte Herrera (2002).

Portanto, elucidar melhor os mecanismos destes ingredientes imunonutricionais na prole é de extrema importância para esclarecer os seus efeitos nos fetos.

3 HIPÓTESE E OBJETIVOS

3.1 Hipótese

A suplementação com doadores do grupo metil ou ácidos graxos poli-insaturados é capaz de modular a expressão gênica gerando benefícios durante o periparto e uma melhor resposta inume na prole de mães suplementadas, reduzindo os efeitos provocados pela imunossupressão.

3.2 Objetivo Geral

Avaliar os efeitos da suplementação com doadores do grupo metil ou ácidos graxos poli-insaturados na expressão gênica de células polimorfonucleadas e do tecido hepático.

3.3 Objetivos Específicos

- Avaliar a funcionalidade hepática durante o periparto de bovinos de leite e sua relação com as proteínas de fase aguda;
- Avaliar a suplementação de colina e metionina durante o periparto em genes relacionados a rota inflamatória em neutrófilos de vacas leiteiras;
- Avaliar os efeitos da nutrição materna durante o periparto com doadores do grupo metil, nos biomarcadores sanguíneos relacionados à rota inflamatória e estresse oxidativo e na expressão de genes no metabolismo da colina e metionina;
- Avaliar, no tecido hepático, por meio da expressão de genes envolvidos na rota do GPR120, os efeitos da alimentação com diferentes fontes de AGNE ao longo de três gerações.

4 CAPÍTULOS

4.1 Artigo 1 – Reduction of liver function delays resumption of postpartum ovarian activity and alters the synthesis of acute phase proteins in dairy cows.

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Reduction of liver function delays resumption of postpartum ovarian activity and alters the synthesis of acute phase proteins in dairy cows

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Abstract

The aim of this study was to evaluate the concentration of acute phase proteins, milk production, and resumption of postpartum ovarian activity of clinically healthy dairy cows in a semi-extensive system with different Liver Functionality Index (LFI) values. The animals were divided into two groups: Low LFI (LLFI: -7 to -12; n: 10) and High LFI (HLFI: -7 to -4; n: 10). Animals with LLFI had lower paraoxonase activity and lower albumin concentration in the pre- and postpartum periods ($P<0.05$), higher non-esterified fatty acids prepartum ($P<0.005$), and higher haptoglobin concentration postpartum ($P<0.01$). The LLFI group showed lower resumption of ovarian activity until 44 days postpartum (29%; $P<0.05$) than HLFI (86%). Milk production did not differ between groups. Therefore, this study suggests that the LFI is an important biomarker of synthesis of acute phase proteins and the first ovulation interval, and it can be used to improve the production and reproductive performance.

Keywords: Liver functionality index; ovarian activity; haptoglobin; paraoxonase; albumin.

Introduction

The transition period from three weeks prepartum to three weeks after calving (Mulligan and Doherty, 2008) is a critical phase for dairy cows, since it is characterized by a high incidence of metabolic and infectious diseases, and reproductive problems (Goff and Horst, 1997). It is also marked by inflammatory conditions due to an increase in inflammatory cytokine synthesis as result of adipose tissue mobilization (Contreras and Sordillo, 2011).

The main effects of cytokines, during inflammation, are in the partitioning of nutrients (Elsasser et al., 2000), and a decrease in the dry matter intake (DMI) (Johnson and Finck 2001) and reproductive activity (Butler, 2000). In the liver, cytokines are primarily responsible for promoting an inflammatory response and inducing an increase in positive acute phase proteins (APP), such as haptoglobin (HP), and for decreasing the production of negative APP, such as paraoxonase (PON) and albumin (ALB) (Fleck, 1989, Bionaz et al. 2007).

The search for accurate markers to define immunity, inflammatory, and metabolic conditions in dairy cows is a constant concern. One way to characterize the extent and severity of inflammation on protein synthesis is using the Liver Functionality Index (LFI), which takes into account changes in ALB synthesis, cholesterol (CHOL), and bilirubin (BIL) in the first month of lactation (Bertoni and Trevisi 2013). In intensive systems, cows with low LFI show lower DMI, lower milk production, and higher incidence of disease (Trevisi et al., 2012). However, these results are limited to intensive systems, there is no evidence if cows in semi-extensive systems show the same performance, since animal challenges are different, such as heat stress, and walking to milking and grazing. Thus, the aim of this study was to evaluate changes in APP concentration, milk production, and resumption of ovarian activity in clinically healthy dairy cows in a semi-extensive system with diffent LFI.

Materials and Methods

Experimental design

Thirty-seven pluriparous dairy Holstein cows from a commercial farm in southern Brazil (32° 16' S, 52° 32'L) were used in the trial. This study was approved by the Ethics and Animal Experimentation Committee from the Federal University of Pelotas, under the registration number 5273. Animals were kept in a semi-extensive system, had three calves or more, 7891 ± 1184 kg/305 days of milk production average, and they were kept in the same nutritional conditions (Table 1) from 21 days prepartum to day 30 postpartum. Cows were monitored daily (rectal temperature, heart rate and respiration rate) during the experiment and were excluded in cases of disease (mastitis, hypocalcemia, retained placenta). Twenty cows that did not have a history of illness during the experiment were used for the laboratory analysis.

Cows were milked twice a day (3:00 AM and 3:00 PM) and milk yield (kg/cow) was recorded daily by software (ALPRO Tetra Laval Group®, Sweden). Every five days (16 days to 60 days in milk) has generated a mean of the total milk produced. Body weight was measured weekly using a weighing platform (EziWeigh5, TRU Test®, Farm Tech Group Ljutomer, Slovenia). Body condition score (BCS) was determined weekly by three evaluators on a scale from 1 to 5 according to Wildman et al. (1982).

The animals were divided into two groups based on the LFI (Trevisi et al, 2012; Bertoni and Trevisi 2013), where animals with Low LFI (-7 to -12; n = 10, LLFI) and animals with High LFI (-4 to -7; n = 10, HLFI). The LFI includes concentrations of albumin, lipoproteins (indirectly measured as total cholesterol), and bilirubin (as indirect measure of the enzymes synthesized by the liver, which also coordinate bilirubin clearance). LFI measures the relevant changes in concentrations between 3 and 28 DIM.

Blood sampling and analyses

Blood was were collected on days -21, -14, -7 and -3 prepartum, 0, 3, 6, 9, 23 and 30 postpartum, after milking through the coccygeal complex in vacuum tubes containing potassium fluoride (13 x 75 mm, 3 mL, Vacuplast®, Zhejiang, China), no anticoagulant (16 x 100 mm, 1 mL, Vacuplast®, Shandong, China), or EDTA (13 x 75 mm, 4 ml, BD Vacutainer™, Franklin Lakes, USA).

PON activity was determined by an enzymatic technique using a commercial kit (ZeptoMetrix Corporation®, Buffalo, NY, USA). The HP concentration was analyzed by a colorimetric method described by Jones and Mould (1984) and adapted by Schneider et al. (2013). Absorbance was obtained using a plate reader (Thermo Plate® TP-Reader, Sao Paulo, Brazil). Plasma non-esterified fatty acids (NEFA) concentration was obtained using a commercial kit (Wako NEFA-HR, WakoChemicals®, Richmond, USA), performed in accordance with the micro-method as described by Ballou at al (2009) using a plate reader (Thermo Plate® TP-Reader, São Paulo, Brazil). The insulin concentration was determined by a commercial ELISA kit (Ins-Easia®, DiaSource, Louvain-La-Neuve, Belgium), which presents 100% cross-reactivity in cattle (Beitinger at al., 2012) and a minimum detection limit of 1.13 μlU/mL. Albumin (ALB), aspartate aminotransferase (AST) gamma-glutaryl transferase (GGT), glucose (GLU), bilirubin (BIL), and cholesterol (CHOL) concentrations were measured in plasma using a light-visible spectrophotometer (Biospectro®, SP-220, Curitiba PR Brazil) using commercial kits (LabTest Diagnostica®, Lagoa Santa, MG, Brazil). All these analyses was realized in the days -21, -14, -7 and -3 prepartum, 0, 3, 6, 9, 23 and 30 postpartum.

Serum progesterone (P4) was analyzed on days 16, 23, 30, 37, and 44 postpartum using a commercial radioimmunoassay kit (Coat-A Count®, Diagnostic Products Corporation, Los Angeles, USA) previously described by Burke et al. (2003). Cows that had P4 concentration

higher than 1 ng/mL in two consecutive samples were considered ovulatory, and cows that did not resume ovarian activity (no increase in P4 above 1 ng/ml during same period) were considered anovulatory (Stevenson and Britt, 1979).

The intra- and inter-assay coefficients of variation (CV) for NEFA, PON, HP, INS, ALB, AST, GGT, PG, and GLU were lower than 12%.

Statistical analysis

All statistical analyses were performed using SAS 9.0 software (SAS® Institute Inc., Cary, NC, USA, 2004). Metabolite concentrations (NEFA, PON, HP, INS, ALB, AST, GGT, CHOL, BIL, and GLU), body weight, and BCS were evaluated through analysis of variance (ANOVA) with a MIXED procedure to assess the main effect of group, time (in days), and their interaction (Littell et al., 1998). The statistical model CATMOD (Categorical Data Analysis Procedures) from SAS was used for analysis of categorical data ovulation. $P<0.05$ was considered significant, and data analyses were done separately for pre- and postpartum periods.

Results

In the first 7 week of lactation a lower proportion of 29% (3/10) in the LLFI group had normal ovarian activity resumption in comparison to 86% (9/10; $P <0.05$) of HLFI.

The HP concentration prepartum did not differ between groups ($P>0.05$). The LLFI group had higher HP concentrations in the postpartum period ($P<0.01$; Figure 1A). In LLFI group, PON activity showed a trend towards a decrease ($P = 0.07$) in the prepartum period, in postpartum period both groups showed a decrease in PON levels. However, in LLFI group this decrease was more significant (86.38 ± 3.84 KU/l, $P <0.001$) compared to group HLFI (113.91 ± 3.87 KU/L; Figure 1B) and showed a delayed increase compared to group HLFI. The levels

of ALB were lower in LLFI pre- (P<0.02) and postpartum (P<0.001; Figure 1C) periods than HLLFI. NEFA concentration was higher in the LLFI group during the prepartum period (P<0.005; Figure 2A) than HLFI, but no difference was observed in the postpartum period (P>0.05). The INS shown a trend towards reduction (P=0.08) in the LLFI treatment in the prepartum period (9.93 ± 1.52 U/mL LLFI vs. 13.76 ± 1.58 U/mL HLFI), but there was no effect in the postpartum period (P=0.05; Figure 2B). Blood levels of liver enzymes did not differ between the groups during the prepartum period, but postpartum animals with LLFI had higher levels of GGT (P<0.03) and tended to have higher levels of AST (P<0.07).

Bilirubin values analyzed in the days 3 and 28 to compose the LFI calculus, the results showed a difference (P> 0.05) in the interaction group*time in both moments. The LLIF groups (7.49 ± 0.8mg/dL) showed lower values compared to HLFI (9.51 ± 0.8mg/dL) on the day 3 postpartum; but on the 28 day postpartum the LLFI the group (8.87 ± 0.8mg/dL) had higher values compared to HLFI (6.91 ± 0.8mg/dL, P> 0.05). Cholesterol values also analyzed on 3 and 28 showed differences between groups (P> 0.05) and a tendency in group*time (P<0.07). The LLFI group (day 3, 1.75 ± 00.15 mg/dL and day 28, 2.22 ± 0.15 mg/dL) showed lower values in both times compared to HLFI group (day 3, 1.90 ± 00.15 mg/dL and day 28, 2.92 ± 0.15 mg/dL).

The BCS value (prepartum 3.52 ± 0.12 LLFI vs. 3.32 ± 0.10 HLFI, and postpartum 2.78 ± 0.07 LLFI vs. 2.82 ± 0.05 HLFI), body weight (prepartum 697.20 ± 12.17 kg LLFI vs. 632.67 ± 16.04 kg HLFI; postpartum 580.54 ± 12 kg LLFI vs. 572.80 ± 22 kg HLFI), milk production (26.04 ± 0.9 kg/day LLFI vs. 27.44 ± 0.8 kg/day HLFI) and blood concentrations of GLU (prepartum 70.20 ± 3.19 mg/dL LLFI vs. 65.07 ± 3.36 mg/dL HLFI; postpartum 76.22 ± 3.82 mg/dL LLFI vs. 79.73 ± 3.87 mg/dL HLFI) were not different (P>0.05) between the groups at any time.

Discussion

The results indicate that animals with low LFI had an increase in adipose tissue mobilization in the prepartum period, as indicated by the higher concentration of NEFA during this period, also these animals lose more BCS and BW compared with the animal from the group HLFI. As reported by Wathes et al. (2009), an increase in NEFA levels is detrimental to immune function in the peripartum period. Furthermore, the NEFA and INS are strongly associated with the anovulatory postpartum interval (Butler, 2003; Wiltbank, et al., 2006). These results are similar to our study, in which animals with LLFI and higher concentrations of NEFA had lower concentrations of INS. Consequently, had an impaired reproductive function, as suggested by the low number of animals that have ovulated before 44 days of postpartum (3/10).

Another factor associated with ovarian activity is the lower concentration of ALB and PON in the LLFI group. ALB, which was lower in pre- and postpartum cows with LLFI, may be indicative of impaired liver function (Bertoni et al., 2008), higher incidence of fatty liver (Bobe et al., 2004), and energy deficiency (Bell et al., 2000). Moreover, low plasma ALB is associated with reproductive disorders, studies have observed in prepartum lower concentrations of ALB in cows with metritis (Schneider, Correa, and Butler, 2013) and in anovulatory cows (Krause et al., 2014), agreeing with the lower ALB levels observed in the LLFI group. PON is correlated with reproduction due to its antioxidant activity in ovarian follicles (Soran, Younis, Charlton-Menys, and Durrington, 2009), and because it is linked to HDL, the main energy source of the follicles (Browne et al., 2008). Confirming this effect, the LLFI group had delayed return to cyclicity and lower PON activity.

The LFI values of the animals in this study were lower (-12 to -4) than those found by Trevisi et al. (2012), which were from -4.9 to +2.5. Despite the different value in LFI, the

responses were similar. It is possible to say that the classification used was adequate for the animals in our study. The lower LFI in this study compared to Trevisi et al. (2012) may be related to the ALB values, which were maintained within low level in both groups (2.8 g/L), compare with the result obtain by Trevisi et al. (3.6 g/L). Low ALB concentrations may indicate a reduction in liver function, protein synthesis deviation (Bertoni et al., 2008), and increased albumin catabolism due to an energy deficit (Bell et al., 2000), which may have occurred in this production system. Other values no usual, and maybe be because by the system is the glucose levels. Glucose concentrations were not different between groups, but cow present lower levels of glucose in the pre-partum compared with postpartum.

The other markers (cholesterol and bilirubin) required to compose the calculation of LFI also showed different between groups. The values obtained for bilirubin on the day 3 and day 28 showed that the LLFI group had a impairment of liver functionality, because the values did not show a reduction on day 28. This increase in bilirubin in LLFI group on day 28 may be an indicative of reduced in the synthesis of liver enzyme (Trevisi et al., 2012). The Cholesterol values also show lower in LF group compared to HLFI. Serum cholesterol concentration in dairy cows are gradually reduced during the prepartum period, but begins to increase gradually after calving (Quiroz-Rocha et al., 2009). Sepúlveda-Varas (et al., 2015) observed low concentrations of cholesterol are associated with postpartum health disorders, especially severe metritis. Burle et al. (1995) reported lower serum cholesterol concentration in anestrous than in cycling cows, this is in agreement with the results found in this experiment, suggestion a possible minor substrate for the steroidogenesis hormones synthesis in the cow from the group LLFI.

According to Bionaz et al. (2007), a decrease in the liver functionality is not related to lower hepatic activity or tissue injury, but with the reduction in protein synthesis that occurs

during inflammation, noting that the liver increases the synthesis of positive APP and decreases negative APP synthesis in this situation. This change is clearly observed in the LLFI group, with the reduction of PON and ALB and an increase in HP. This demonstrates that the LFI is a useful index also for use in animals raised in a semi-extensive system, as observed by Trevisi et al. (2012), using lower absolute values for LFI, indicating that there may be a difference in animal classification in different systems.

Milk production did not differ between the LLFI and HLFI groups. Trevisi et al. (2012) observed differences between groups, but maintained sick animals in the experiment. Our result for milk production is similar to that of Bertoni et al. (2008) who evaluated the hepatic activity (Liver Activity Index, LAI) and did not observe difference in the first three months of lactation in milk production between groups with different values of LAI. Lower LFI changed the metabolites and the resumed ovarian activity, but did not have an effect on milk production, possibly because only clinically healthy animals remained in the experiment and due to lower milk production in the semi-extensive system when compared to the intensive system.

Conclusion

Dairy cows in a semi-extensive system with less liver function postpartum showed changes in the synthesis of acute phase proteins and delayed return to ovarian cyclicity due to the energy deficit in the postpartum. It is recommended the determination of LFI in cows fed with different sources of energy in the diet to prevent or to reduce adverse effects in the liver and in the performance.

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Table 1 Ingredient and nutrient composition of prepartum and postpartum diets.

	Prepartum (kg/day)	Postpartum (kg/day)
Ingredients		
Native pasture	*Ad libitum*	-
Forage Sorghum	-	*Ad libitum*
Pre-dried	-	15
Wheat bran	0.5	1.5
Soybean meal	1.0	2.4
Rice bran	0.68	2.88
ground corn	1.05	3.0
Sorghum grain	1.05	2.13
Bicarbonate of soda	0.4	0.11
Urea	-	0.09
Mineral Supplement	-	0.19
Calcitic limestone	0.12	0.19
Salt	-	0.002
Protected fat	0.2	-

Nutrient composition (dry matter basis)

	Prepartum *(%)*		Postpartum *(%)*		
	Forage	Concentrate	Forage	Pre-dried	Concentrate
NDF	67.65	47.42	64.32	63.46	32.57
ADF	51.37	13.56	41.74	45.75	13.14
Crude protein	9.16	15.61	9.84	8.88	14.92
Fat	1.73	3.57	2.02	2.00	4.01
Minerals	9.23	8.9	9.99	8.84	9.02

*Estimated based on diet analyses in National Research Council (NRC) software (2001).

Neutral detergent fiber (NDF) and acid detergent fiber (ADF).

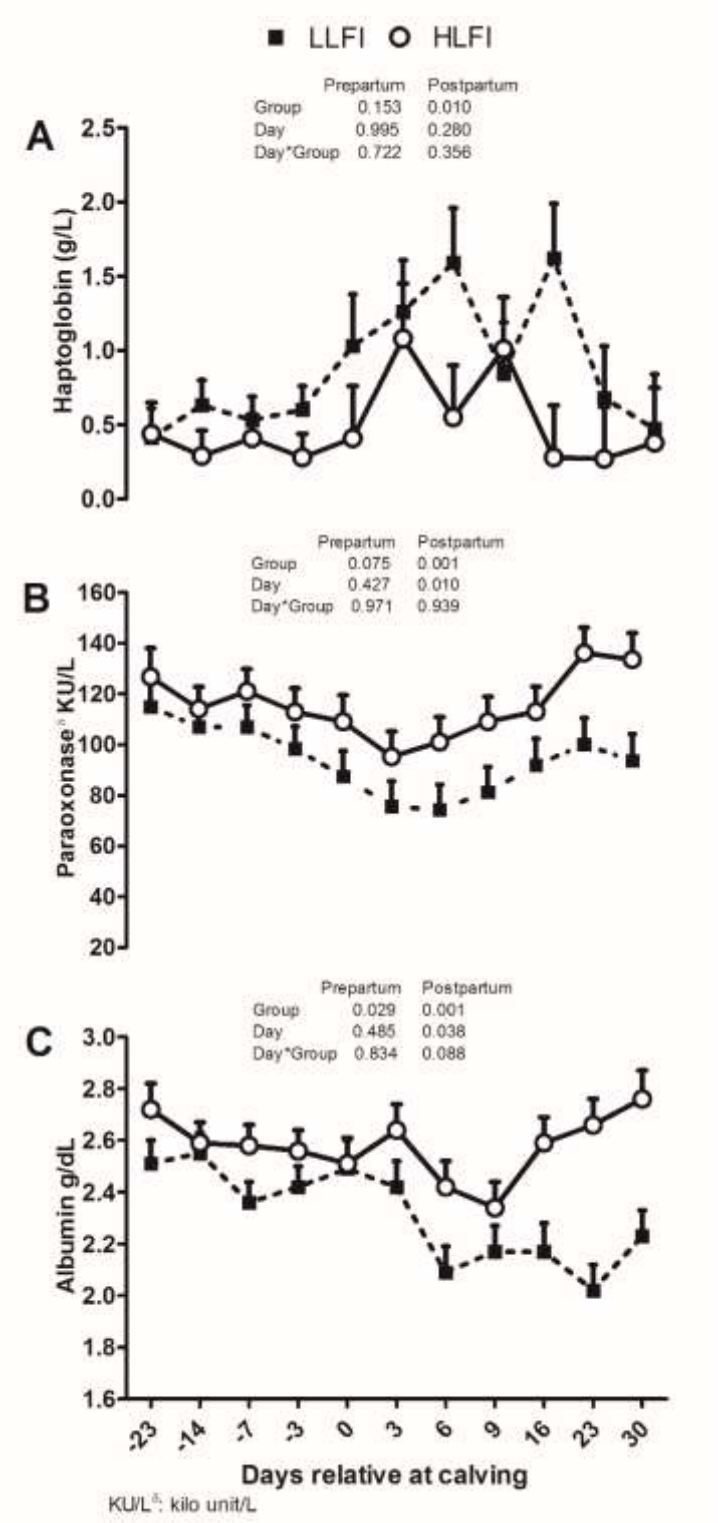

Figure1. Concentrations of haptoglobin (g/dL) (A), albumin (g/dL) (B) and paraoxonase (KU/L) (C) during peripartum in the group LLFI (■) and in the group HLFI (○).

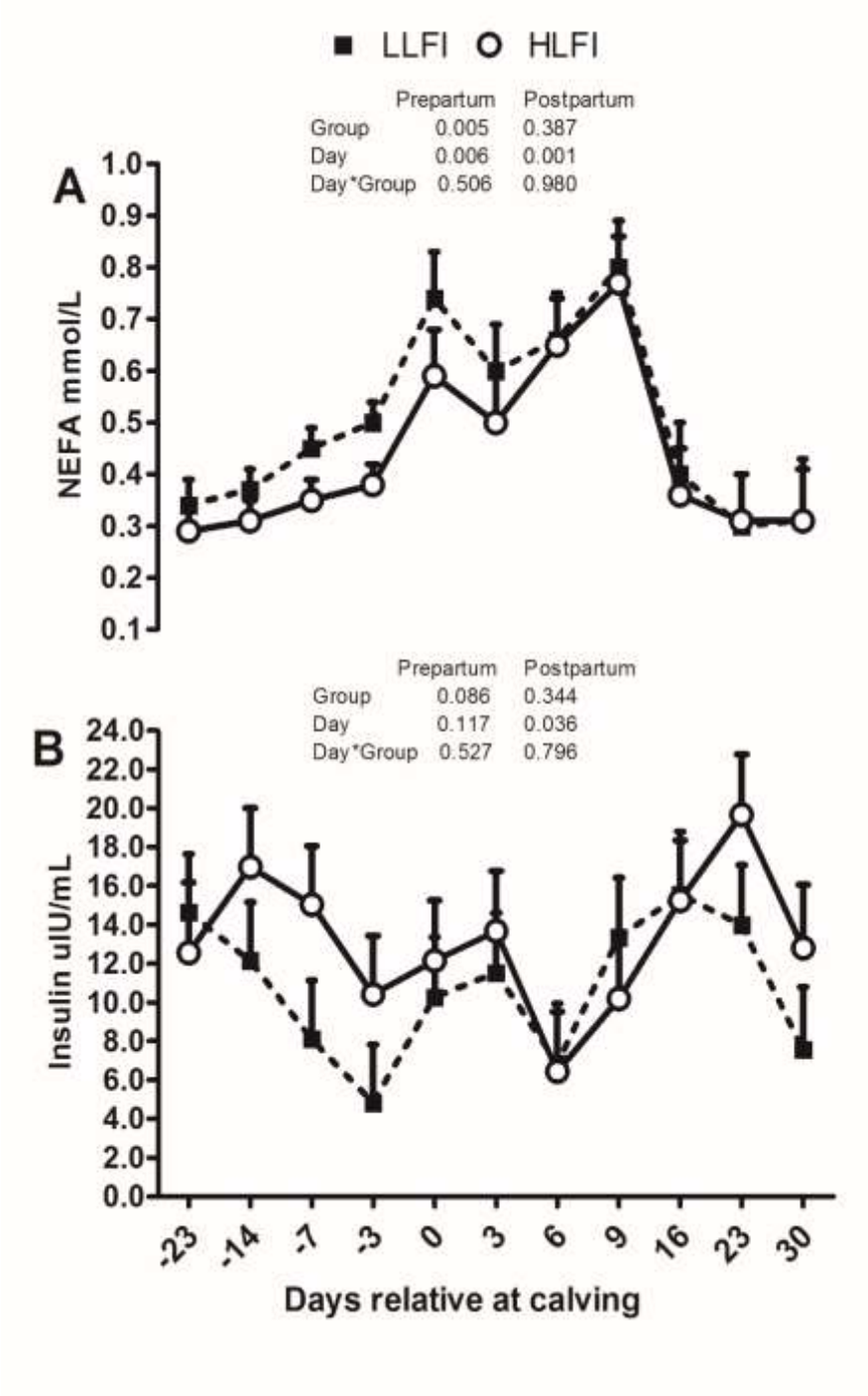

Figure 2. Concentrations of plasma non-esterified fatty acids (NEFA, mmol/L) (A), insulin (g/dL) (B) during peripartum in the group LLFI (■) and in the group HLFI (○).

4.2 Artigo 2 – Immunometabolic gene expression in blood neutrophils are altered in response to peripartum supplementation of methionine or choline

Artigo apresentado nas normas da revista *Journal of Dairy Science*, na qual o será submetido

Running Title: RUMEN-PROTECTED METHYL DONORS AND THE PMN EXPRESSION

Immunometabolic gene expression in blood neutrophils are altered in response to peripartum supplementation of Methionine (Smartamine M) or Choline (Reashure)

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ABSTRACT

The objectives of this study were to measure the gene expression and function of PMN and the concentrations of biomarkers in plasma of cows supplemented with rumen-protected methionine (MET) or choline (CHOL). Forty multiparous Holstein cows were used in a randomized complete block design with 2×2 factorial arrangement of MET (Smartamine M, Adisseo NA) and CHOL (ReaShure, Balchem Inc.) level (with or without). Treatments (10 cows each) were control (CON), no MET or CHOL; CON+MET (SMA); CON+CHOL (REA); and CON+MET+CHOL (MIX). From −50 d to −21 d before expected calving, all cows received the same diet (1.40 Mcal NEL/kg DM) with no MET or CHOL. From −21 d to calving, cows received the same close-24 up diet (1.52 Mcal NEL/kg DM) and were assigned randomly to each treatment. From calving to 30 d, cows were on the same postpartal diet (1.71 Mcal NEL/kg DM) and continued to receive the same treatments until 30 d. MET supplementation was adjusted daily at 0.08% DM of diet and CHOL was supplemented at 60 g/cow/d. Blood was sampled for biomarker analysis on -10, 8 and 30 d relative to calving date. Neutrophil phagocytosis and oxidative burst were assessed at d 1, 7, and 28 d. Blood PMN isolated at −10, 7, and 28d relative to calving was used to evaluate gene expression. The MET group had lower ROM compared with CON group. The blood metabolic had great concentration of total and reduced glutathione in cows fed MET compared with CON. Higher concentration of BHBA was observe in the MET group. Supplemental CHOL led to greater blood glucose and insulin concentrations. Blood PMNL phagocytosis capacity and oxidative burst activity was greater in MET supplemented cows. Changes in PMN gene expression regardless of treatments were observer to proinflammatory genes (TNFα and BPI) with highest expression on 8 d, suggesting more pronounced inflammatory status compared with -10 d and 29 d relative to parturition. In the other way, ITGA4, ICAM1, IL6, INSR, NOS2 and CBS were lower ($P<0.05$) on day 8

compared with other times. The expression of IRAK1, AKT1, GSK3, ITGB2 and MPO ($P<0.05$) increased after parturition (8 and 29d) compared with -10d. The main effect of CHOL ($P<0.05$) towards greater expression were observed to CADM1, IL10, AKT1, CREB1, INSR. Interactions of CHOL×day in PMN gene expression was associated with gradual increases from 8 to 28 d in genes that facilitate migration to inflammatory sites and the activation and development of PMN (SELL, CXCR2, BPI, IL10, IL10R, TLR2, RXRA). Different response was observed in MET supplementation group, it had higher concentration of SOD2 ($P=0.02$) is associated with reactive oxygen production in PMN and lower expression of ITGAB2 and IRAK1, suggesting less activated status of PMN. Myeloperoxidase gene expression (MPO) was higher in the both supplementation groups (CHOL and MET) suggestion a better immune response. Results from the present study indicate that peripartal supplementation of rumen-protected MET and CHOL has positive effects in PMN expression indicating a better immune status.

Key Words: methionine, choline, inflammation, oxidative stress, transition cow

INTRODUCTION

Methionine (MET) and choline (CHOL) are ingredients that have beneficial influence in animal metabolism by effect direct on hepatic metabolism reducing the lipid accumulation through lipotrofic action (Osorio et al., 2013) increase the phosphatidylcholine (Auboiron et al., 1995), also increase hepatic triglycerides, besides the increase milk production and protein level in the milk (Armentano et al., 1997). However, few studies highlight the MET and CHOL effect on the inflammatory response in dairy cows.

The inflammatory response is challenged around the time of parturition, and it is associated with higher incidence of diseases (Sordillo et al., 2009; Goff and Horst, 1997). Neutrophils are the major pathogen-fighting immune cells, being first line of immune defense in dairy cows (Paape et al., 2003). Upon maturation, neutrophils are released into circulation and play key roles for immune surveillance and defense against pathogens (Burton and Erskine, 2003). The changes in the inflammatory responses during the peripartum period can consist of a hyporesponsive state, described by impaired neutrophil functions, including impaired of superoxide production, myeloperoxidase activity, phagocytic capabilities, expression of cell surface adhesion molecules, and capacity for migration (Kehrli et al., 1989; Burton et al., 1995 Rinaldi et al., 2008)

Recent studies demonstrated that genomic expression in neutrophils (PMN) can be influenced by body condition score (Lange et al., 2016), prepartal energy intake (Moyes et al., 2014; Zhou et al., 2015), level of energetic in the diet (Li et al., 2016a) and mineral supplementation (Osorio et al., 2016), however research concerning supplementation of other aminoacids on bovine PMN response is scarce (Garcia et al., 2016), and currently seeking to elucidate the supplementation effects AA on the mediators of inflammation, especially MET that eh the first limiting amino acid.

Recent research has suggested that AA play an important role in immune modulation by regulating immune cell activation, proliferation, antibody production, and cytokine production, whereas dietary AA deficiency directly impairs immune function of animals (Cruzat et al., 2014). In human, Sipka et al. (2014) observed that 14 of the 21 supplemented AA, reduced oxidative response, whereas only supplemental Arg increased oxidative response of human PMN. The supplementation with methionine is highlighted because can influence in the oxidative stress status, because serves as substrate for glutathione synthesis, most abundant natural antioxidant (Martinov et al., 2010). However, little research has been conducted to determine effects of MET a CHOL on immune function in dairy cows. Therefore, our hypothesis is that MET and CHOL supplementation during the peripartal period have a positive effect on PMN gene expression, which are ultimately reflected in an impaired immune response during the peripartum period.

MATERIALS AND METHODS

Experiment design and Dietary treatment

All the procedures for this study were conducted in accordance with a protocol (#13023) approved by the Institutional Animal Care and Use Committee of the University of Illinois. Complete details of the experimental design have been reported previously (Zhou et al., 2016).

Briefly, a subset of cows from a group of 40 with complete set of PMN samples (d −10, 7, and 28) were selected and the experiment was conducted as a randomized complete bloc design 2 × 2 factorial arrange of MET (Smartamine M, Adisseo NA, Alpharetta, GA) and CHOL (ReaShure, Balchem Inc) level (with or without). Cows were blocked according to parity, previous lactation milk yield, and expected calving date. The cows randomly assigned to receive a basal control (CON) diet (n = 10) with no rumen-protected MET or CHOL supplementation; CON plus MET (SMA, n = 10) at a rate of 0.08% of DM; CON+CHOL (REA,

n = 10) at 60 g/d; or CON+MET+CHOL (MIX, n = 10). Dosage of MET was based on Osorio et al. (2013), whereas CHOL was supplied following manufacturer's recommendations.

All cows received the same far-off diet (1.40 Mcal/kg of DM, 10.2% RDP, and 4.1% RUP) from -50 to -22 d before expected calving, close-up diet (1.52 Mcal/kg of DM, 9.1% RDP, and 5.4% RUP) from -21d to expected calving, and lactation diet from calving (1.71 Mcal/kg of DM, 9.7% RDP, and 7.5% RUP) through 30 DIM. The TMR for the close-up and lactation diets was measured weekly for estimation of daily TMR DM offered. Feed offered was adjusted to achieve ~10% refusals. Ingredient and chemical composition of the diets is in the Supplemental Table S1.

The MET and CHOL supplement was top-dressed once daily at the morning feeding using approximately 50 g of ground corn as carrier for all treatments. Supplementation of MET (SMA; 0.08% DM of TMR offered) was calculated daily for each cow. Smartamine M was supplied as small beads containing a minimum of 75% DL-Met, physically protected by a pH sensitive coating, which is considered to have a Met bioavailability of 80% (Graulet et al., 2005); therefore, per 10 g of Smartamine, the cows received 6 g of metabolizable MET. The ReaShure supplement is reported to contain 28.8% choline chloride and is protected by microencapsulation. The product is considered to have CHOL bioavailability of 72% (Benoit, 2009), therefore, per 60 g of ReaShure, the cows received 12.4 g of metabolizable choline chloride.

Animal Management and Diet

During the dry period, cows were housed in a ventilated, sand-bedded freestall 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 at 0630 h using an individual gate feeding system (American Calan, Northwood, NH) and 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 housed in a tie-stall barn and were fed a common lactation diet once daily and milked 3 times daily (0400, 1200, or 2000 h).

Blood Sample Collection

Blood samples for biomarker analyses were collected from the coccygeal vein using 20-gauge BD Vacutainer needles (Becton Dickinson, Franklin Lakes, NJ) on −10, 8, and 30 relatives to parturition. At each time point, a total of 40 mL of blood were collected in vacutainer tubes (10 mL, BD Vacutainer, Becton Dickinson) containing serum clot activator or lithium heparin, for serum and plasma, respectively. After blood collection, tubes with lithium heparin were placed on ice and tubes with clot activator were kept at room temperature (21 °C) until centrifugation (~30 min). Serum and plasma were obtained by centrifugation of clot activator and lithium heparin tubes, at 1,900 × *g* for 15 min at 4°C. Serum and plasma were aliquoted and stored at −80°C until further analysis.

An additional tube was collected on 1, 7, 28 d relative to calving for neutrophil phagocytosis and oxidative burst analyses.

Blood Metabolites and Biomarkers of Liver

Measurements of non-esterified fatty acids (NEFA), beta- hydroxybutyrate (BHBA), glucose, myeloperoxidase (MPO), ferric-reducing antioxidant power (FRAP), reactive oxygen metabolites (ROM), were did using commercial kits in a clinical auto-analyzer 120 (ILAB 600, Instrumentation Laboratory) at the University of Illinois Veterinary Diagnostic Laboratory (Urbana), following the procedures previously described in (Jacometo et al., 2015). Bovine IL-1β (Cat. No. ESS0027; Thermo Scientific, Rockford, IL) and IL-6 (Cat. No. ESS0029; Thermo Scientific, Rockford, IL) plasma concentration were performed using commercial kits. Plasma phosphatidylcholine (Cat. No.10009926; Cayman chemical, Ann Arbor, MI) and free choline

(Cat. No. K615-100; Biovision Inc., Milpitas, CA) concentration was quantified using commercial kits. Insulin concentration was analyzed using a commercial bovine insulin ELISA kit (catalog no. 10120101; Mercodia AB, Uppsala, Sweden).

Glutathione (GSH) in liver tissue were measured at using a commercial kit (Cat. 136 No. NWH-GSH01; Northwest Life Science Specialties LLC, Vancouver, WA). Reduced GSH was calculated as reduced GSH = total GSH – oxidized GSH. Liver puncture biopsy was describing at Zhou et al. (2016).

Whole Blood Phagocytosis and Oxidative Burst

The phagocytic capacity and oxidative burst activity of peripheral neutrophil was determined was determined upon challenge with enteropathogenic bacteria (*Escherichia coli* 0118:H8) as described by Hulbert et al. (2011) with modifications. Briefly, 200 μL of whole blood with 40 μL 100 μ*M* dihydrorhodamine (Sigma-Aldrich, St. Louis, MO), and 40 μL of propidium iodine labeled bacteria (10^9 cfu/mL) at 38.5°C in a water bath for 10 min. To eliminate the fluorescence of nonphagocytosed bacteria, 100 μL of quenching solution was added. After red blood cells were lysed with ice cold MilliQ water, and resuspended in PBS solution. Subsequently neutrophils were stained with CH138A primary anti-bovine granulocyte monoclonal antibody (Cat. No. BOV2067, Washington State University, WA) and PE-labeled secondary antibody (Cat. No. 1020-09S, Southern Biotech, AL). Lastey, the cells were re-suspended in 100 μL of DNA-staining solution, and light-protected in an ice bath until analyzed by flow cytometry (LSR II, Becton Dickinson, San Jose, CA). After red blood cells were lysed with ice cold MilliQ water, and resuspended in PBS solution. Subsequently neutrophils were stained with CH138A primary anti-bovine granulocyte monoclonal antibody (Cat. No. BOV2067, Washington State University, WA) and PE-labeled secondary antibody (Cat. No. 1020-09S, Southern Biotech, AL). Lastly, the cells were re-suspended in PBS solution for flow

cytometry analyses (LSR II; Becton Dickinson, San Jose, CA). Data are reported as percentages of CD14/CH138A positive cells with phagocytosis and oxidative burst capability.

Neutrophil Isolation

Neutrophils were isolated based on procedures described by Moyes et al. (2014) with modifications. Briefly, blood (~100 mL) was sampled in ACD Vacutainer tubes and mixed well by inversion and placed on ice until 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 were discarded. Twenty-five milliliters of deionized water at 4 °C was added to lyse red blood cells, followed by addition of 5 mL of 5 × PBS at 4 °C to restore an isosmotic environment. Samples were centrifuged at 200 × g for 10 min at 4 °C and the supernatants were decanted. The pellet was washed with 10 mL of 1 × PBS and centrifuged for 5 min (200 × g at 4 °C) and supernatants were decanted. Eight milliliters of deionized water at 4 °C was added, followed by addition of 2 mL of 5 × PBS at 4 °C. Samples were centrifuged at 500 × g for 5 min at 4 °C and supernatant was decanted. Two subsequent washings using 10 mL of 1 × PBS at 4 °C were performed with samples centrifuged at 500 × g for 5 min at 4 °C and supernatant was decanted. Neutrophils were immediately homogenized in 2 mL of Trizol Reagent (Invitrogen, Carlsbad, CA) with 1 µL of liner acrylamide (Ambion Inc., Austin, TX) using a Polytron power homogenizer at maximum speed. The suspension was transferred equally into 2 RNA-free microcentrifuge tubes (2 mL; Fisher Scientific) and stored at −80 °C until further analysis.

RNA Isolation and cDNA Synthesis and Quantitative Real-Time PCR

For PMN total RNA extraction, the miRNeasy kit® (Cat. #217004, Qiagen, Hilden, Germany) was used following the manufacturer's protocols (supplemental file). Samples were treated column with DNaseI (Qiagen), concentration was measured using the NanoDrop ND-

1000 (NanoDrop Technologies, Rockland, DE), and RNA quality- (RNA integrity number - RIN) was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). Samples used in the analysis had a mean RIN number of 6.5±0.2. Specific details of RNA extraction are presented in the Supplementary File.

The protocols used for cDNA synthesis and quantitative real-time PCR were exactly the same as those reported by (Jacometo et al., 2015). Based on relevant biological functions in PMN, 34 target genes selected in this study are involved in migration, cell adhesion, chemotaxis, inflammation (TLR pathway and cytokines), oxidative stress, methionine cycle, nuclear receptors and factors of transcription. The official symbol, name, and a short summary description of these genes are presented in Additional Table 2. Primers were designed via Primer Express 3.0.1 software (Applied Biosystems). For this study, *GAPDH*, ubiquitously expressed prefoldin-like chaperone (*UXT*), and Ribosomal Protein S9 (RPS9) were used as internal control gene, and their geometric mean was used to normalize the expression data. These were previously confirmed as stably expressed for PMN gene expression (Moyes et al., 2014). All evaluated qPCR performance and primer information are reported in the supplemental material (Supplemental Tables).

Statistical Analysis

Data were analyzed using Proc MIXED of SAS (SAS Institute Inc., Cary, NC) according to the following model:

$$yijklm=\mu+Bi+Mj+Ck+MCjk+Tl+TMjl+TCkl+TMCjkl+Pm+PMjm+PCkm$$
$$+PMCjkm+PTlm+PTMjlm+PTCklm+PTMCjklm+An+\varepsilon ijklm \quad 142$$

where $Yijklm$ is the dependent, continuous variable; μ is the overall mean; Bi is the random effect of the i the block; Mj is the fixed effect of MET (j = with or without); Ck is the fixed effect of CHOL (k = with or without); Tl is the fixed effect of time (day or week) of the

experiment; Pm is the fixed effect of the m the parity of the experiment (m = 2 or 3); An is the random effect of the n the animal (cow); $\varepsilon ijklmn$ is the residual error. Parity effect and interactions were removed from the model when not significant ($P \geq 0.05$). Blood metabolites, liver composition and PMN gene expression were analyzed at various time points that were not equally spaced. Therefore, an exponential correlation covariance structure SP (POW) was used for repeated measures. Least square means separation between time points was performed using the PDIFF statement. Statistical differences were declared significant at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

The effect of prepartum MET or CHOL supplementation in PMN gene expression during peripartum period is reported in the Table 1. Genes that differed at specific time point (day: -10, 7 and 30) between MET and W.MET and CHOL and W.CHOL are shown in Figure 1 and Figure 2. The effects of supplementing on blood biomarker was reposted in the Table 2 and the blood neutrophil phagocytosis and oxidative burst was reported in the Table 3.

Neutrophil expression

Cell adhesion, chemotaxis and migration gene expression

The expression of ICAM1 showed an interaction in MET×T ($P < 0.05$) in day 30, where animals that received MET had an upregulation of this gene compared with animal that didn't received MET. The CHOL supplementation showed a tendency to upregulated ($P < 0.09$) in CHOL×T of ICAM1 in the day -10. Besides the diet the ICAM1 have a time effect ($P < 0.004$) with lower expression in the day 7 (1.74±0.13) compared with day -10 (1.74±0.23) and day 30 (1.62± 0.13).

The expression of SELL ($P < 0.07$), CXCR2 ($P < 0.01$) showed an interaction of CHOL×T. These genes had an upregulate of expression compared with animals that didn't receive CHOL at day 30 postpartum. In the CHOL supplementation, also was observed a main effect to CADM1($P < 0.01$) animals that received CHOL had an upregulate of gene expression of CADM1 compared that animal W.CHOL.

The expression of BPI ($P < 0.004$) showed an interaction of CHOL×T at day -10 and day 7, in the prepartum the CHOL have a lower, but at day 7 there had an upregulated of the expression in this compared with W.CHOL (FIG 1 – BPI). An effect of time ($P < 0.01$), beside the diet also was observed to BPI, with higher expression at day 7 (0.83± 0.09) compared with day -10 (0.49±0.08) and day 30 (0.50± 0.08).

In the expression of genes related with integrins cell-surface, was observed a main effect to ITGB2 ($P < 0.02$) with a downregulation MET. The expression of ITGA4 ($P < 0.001$) was regulated regardless of diet, were in the day 7 showed the lower expression (1.36± 0.17) compared with day -10 (2.00±0.18) and day 30 (2.10± 0.18). The other integrins (ITGA4 and ITGAM) not showed a main effect, interactions, and time effect to MET and CHOL supplementation.

TLR pathway and cytokines

The expression of IL10 showed a main effect of CHOL supplementation ($P < 0.06$) with an upregulation of the gene expression in CHOL group compared with W.CHOL, also an interaction of CHOL×T ($P < 0.02$) in the day 30 was observed with the same response, bigger expression in CHOL group. Associate to IL10, your receptors IL10R show similar result to interaction CHOL×T ($P < 0.01$), with an upregulate in the day 30 (CHOL: 1.89±0.17 and W.CHOL: 0.84±0.19) These genes did not show effects to MET supplementation. The other cytokines (IL1β and IL6) evaluated in the study didn't show differences to MET or CHOL, only

IL6 show an effect of Time ($P < 0.01$), with lower concentration in the day 7 (1.22±0.14) compared with day -10 (1.78±0.13) and day 30 (1.78± 0.14).

The gene expression of TLR2 ($P < 0.01$) had effect in interaction of CHOL×T, and the same effect did not observe to MET×T. These genes had an upregulation in the expression at day 30 in CHOL group (Figure 2). The TLR4 did not show differences in the gene expression from CHOL and MET supplementation and time effect.

The main effect ($P < 0.01$) of MET supplementation was observed to IRAK1, MET group had a downregulation of these gene compared with W.MET. Also there was an effect of time ($P < 0.01$), besides of the diet, day 30 (1.52±0.07) had a higher compared with day -10(1.29±0.06), day 7 (1.45±0.07) had not difference to day -7 and day 30

The TNFα had an interaction effect of CHOL×T ($P < 0.01$) in the day 7 with an upregulation of the group W.CHOL compared with CHOL supplementation. Also there was an effect of time, regardless of the diet, with higher expression in the day 7 (1.01±0.05) compared with day -10 (0.84±0.05). Day 30 (0.94±0.05) did not have different from other days.

The gene IFNγ, FKB1, STAT3 and S100A8 did not have effect of CHOL and MET supplementation, interactions (CHOL×T and MET×T), and time.

Oxidative stress and Methionine cycle and glutathione metabolism

The expression of MPO showed an effect to MET ($P < 0.04$) and CHOL ($P < 0.03$) supplementation and also interaction to MET×T ($P < 0.001$) and CHOL×T ($P < 0.001$). Animals that receive MET or CHOL have an upregulation of MPO expression, especial at day 30 compared with animal without supplementation. Regardless of the diet, MPO have a time effect, with an upregulate at day 30(2.14±0.28), compared with day -10(1.09±0.23) and day 7(1.09±0.24).

The biologic mediator NOS2 showed an interaction of MET×T ($P < 0.02$), these gene was downregulated at day -10 in MET group. Also a time effect ($P < 0.0001$) was observed at day -10, with a higher expression (3.01±0.35) compared with day 7 (1.37±0.33) and day 30 (1.63± 0.34)

The expression of SOD2, show a main effect ($P < 0.02$) to MET supplementation and did not show the same effect to CHOL supplementation. Animals that receive MET had an upregulate of SOD2. The expression of SOD1 did not show effect of MET and CHOL, and also the interactions.

The expression of CBS showed a repost to time ($P < 0.02$) beside the diet, with lower expression in the day 7 (1.23±0.13) compared with day -10 (1.61±0.13) and 30 (1.74±0.13). A tendency was observed to MET×T ($P <0.12$), between MET (1.82±0.19) and W.MET (1.4±0.20) at day 30, and the same effect was not see to CHOL supplementation. The other genes involved in the methionine cycle (CTH and SAHH) and glutathione metabolisms (GSS, GSR and GPX1) no differences were observed for CHOL and MET supplementation, interaction between CHOL×T and MET×T, and time.

Nuclear receptors and factors of transcription

The expression of RXRA have an effect in the interaction CHOL×T ($P < 0.04$), were we observed an upregulation of the gene expression in the day 30 in the CHOL group (Figure 2).

A main effect was observed in CHOL supplementation of INSR ($P < 0.07$), AKT1($P < 0.02$) and CREB1($P < 0.03$), these genes had an upregulation in CHOL group. The INSR had also effect in interaction in CHOL×T ($P < 0.07$), and MET×T ($P < 0.004$) with an upregulation in the expression of INSR at day 30, compared with animals that not received the supplementation.

The expressions of GSK3 showed an effect of time, with higher expression at day 30 (1.75 ±0.14) compared with day -10 (1.28±0.14) and (1.37±0.14).

Blood Metabolites and Biomarkers Analyses

Inflammation

A significant time effect with greater concetracion in postpartum was detect to IL-6 (*P* = 0.001). In the other way IL-1β showed a lower concentration in the postpartum period (*P* = 0.08).

The MPO concentration showed a time effect with higher concentration at day 7 (479.04±14.81) compared with day -10 (409.64±14.10) and day 30 (420.51±14.58)

Oxidative stress

A main effect (P =0.03) was detected for plasma ROM in MET supplementation, animal in MET group had lower concetracion of ROM (Table 2), also an interaction MET×time (P = 0.03) was observed at day 30, with lower concentration of ROM in MET group (13.58±0.49) compared with W. MET (16.06±0.53). A time effect (P <0.001) was in observed regardless of diet, with higher levels in the postpartum period compared with prepartum.

The liver total and reduced GSH concentrations showed a time effect with lower concentration at day 7 compared with day -10 and day 30. The MET supplemented group showed higher concetracion of reduced (*P* = 0.02) and total GSH (*P* = 0.02) compared with W.MET

Choline metabolism and Energetics Metabolists

Phosphatidil choline (PC) showed a time effect (P <.0001) with higher concentration at day 30 compared with day 7 and -10. In the other why, choline, showed a time effect with lower concetracion at day 30. Also a tendency (P = 0.08) in the main effect was observed to CHOL supplementation, with higher concentration in CHOL group compared with W.CHOL.

Glucose (P = 0.05) and insulin (P = 0.02) had a main effect to CHOL supplementation, which were greater in CHOL group compared with W.CHOL. Also a tendency (P = 0.08) in CHOL supplementation was detected to glucose:insulin in CHOL cows (Table 2). An interaction for MET×time was detected for insulin (P = 0.01) and glucose:insulin (p = 0.07), which was mainly associated with higher insulin at -10 day in MET group. The time effect observed to this metabolists was related with the lower concentrations in the postpartum period compared with the prepartum. In the other way, NEFA and BHBA had a time with higher concentration of this metabolists especial at day 7. A main effect in to BHBA (P = <.0001) in MET supplemented cow compared with W.MET.

Blood Phagocytosis and Oxidative Burst

A increase in neutrophil phagocytosis capability was detected in cows supplemented with MET (P = 0.001) and not in CHOL (P = 0.73). Concomitant, MET supplementation also resulted in greater blood neutrophil oxidative burst (P < 0.001) compared with CON (Table 3).

In CHOL group was observed an interaction CHOL×time at day 30, with higher oxidative burst activity. No significant temporal changes were detected for blood neutrophil phagocytosis and oxidative burst activity in MET supplementation.

DISCUSSION

Gene Expression

Cell adhesion, chemotaxis and migration gene expression

A large number of cell surface receptors are express in neutrophils to recognize microbial invasion (Futosi et al., 2013). Activation of those receptors, leads to complex cellular activation of processes like as phagocytosis, exocytosis, production of reactive oxygen species, as well migration and cytokine release (Robinson, 2009). The initial PMN-endothelial

interaction, called rolling, is mediated by members of the selectins, especial SELL following by integrins (ITGB2) and immunoglobulins (ICAM-1) that are important for firm adhesion and cell diapedesis (van de Vijver et al., 2012).

A prospective clinical study in humans, showed that Selectin-L expression is regulated by TNFγ, the stimulation with TNFγ leads to a decrease SELL expression on PMN (Mommsen et al., 2011), our results showed an upregulation of SELL in CHOL in the day 30, this results can be link with no change in TNFγ expression of CHOL group, since in W.CHOL at day 7, showed an upregulation of TNFγ and a possible relation with no increase the expression of SELL in the pos-partum period, leading to a compromise in the activation of rolling in animals W.CHOL In the other way, MET did not showed effect in the SELL expression, although Li et al. (2016) showed a beneficial effect of MET in dairy cow fed with a prepartum higher-energy diet in the upregulate of SELL expression and enhanced the ability for cell adhesion.

Lee and Kehrli (1998) observed that SELL had a lower expression after parturition, suggested that the ability for cell adhesion in PMN have been reduced after partum, and it is related with elevated concentrations of cortisol (Weber et al., 2001), however this time effect not was observed.

The upregulation of ICAM-1, an epithelial adhesive ligand for migrating, in animals that receive CHOL and MET supplementation at day 30 compared with no supplementation could be in response to endotoxin, how demonstrated by Corl et al., (2008) in bovine primary mammary endothelial, resulting in a better immune response compared with animal without supplementation.

The receptor for endothelial ICAM-1, the integrin molecular ITGB2 showed a diet effect of downregulation in MET group. The upregulation of ITGB2 is related with higher-energy diet in prepartum of dairy cows (Zhou et al., 2015), after intermammary administration

of LPS (Diez-Fraile et al., 2003) and with activation of nitric oxide biosynthetic and NF-kB (Leite et al., 2009), genes that did not change in this paper.

The Bactericidal/Permeability Increasing protein (BPI) plays an important role in host-defense in dairy cows (Wheeler et al., 2007). The upregulation of BPI protein observed at day 7 in animal that receive CHOL, can be associated with response to activation of Toll-like receptor (TLR) in order to attenuating the TLR response by acts in permeability the plasma membrane of Gram negative bacteria (Elsbach et al., 1994), suggest an activation of the immune response in the CHOL cows compared with control.

Increased expression of adhesion molecules is important for directing peripheral blood leukocytes to the underlying infected (Lee and Kehrli, 1998), the postpartal upregulation of most of these genes (BPI, ICAM1, ITGA4, ITGB2) regardless of prepartal dietary energy is suggestive of additional factors, like change in hormonal environment, in the postpartum period, stimulating the coordinated production of intracellular signaling molecules that help maintain some degree of inflammation.

TLR pathway and cytokines

The upregulation of IL10 (main effect and interaction in the day 30) observed in the animal CHOL, an anti-inflammatory cytokine (Couper et al., 2008) and also the upregulation of your receptor (IL-10R) at day 30, can be linked with the activation of the immune system in CHOL, because one effect of IL10 is induces the NF-Kb activation. The CREB, can activate the IL10 production (Brenner et al., 2003) and in CHOL group is higher expressed compared with W.CHOL.

The higher expression of IL6 in the week after partum supports the observation that cows experience a degree of inflammation around parturition (Bossaert et al., 2012).

The TNFα represents a primary proinflammatory cytokine which response to tissue damage, pathogen invasion and other mechanisms (Schroder et al., 2004). The lower mRNA expression of TNFα in CHOL group indicates that the positive effect supplementation. Numerous studies showed that TNF-α was linked with the mastitis during the periparturient period when dairy cattle have oxidative stress, suggestion an alteration in the animal that not receive CHOL (Locksley et al., 2001). Wherever the ROM effect only was observed in the cow that not receive MET.

Oxidative stress and Methionine cycle and glutathione metabolism

Superoxide dismutase 1 and 2 are enzymes that converts superoxide anion to H_2O_2 in the mitochondria, by this action is considered an efficient antioxidant enzyme. Hu et al., (2005) observed that the inhibition of SOD2 promoted the accumulation of ROM, and the higher expression of SOD2 in MET group maybe act direct in the catalyze of ROM, because when elevated, leads to loss of cell function, necrosis and apoptosis (Nordberg and Arnér, 2001) and how observed, ROM is lower in MET group.

Concomitant with SOD2, the marked increase of MPO postpartum suggests a major contribution of this enzyme in H_2O_2 catabolism (Olsson et al., 2011). This enzyme catalyzes the formation of hypochlorous acid, a major weapon used by phagocytes to kill ingested microorganisms (Lau et al., 2005). Metzler et al., (2011) observed in patients with complete deficient in MPO activity suffer from a more severe immunodeficiency than those with only a partial deficiency. Myeloperoxidase is involved in PMN respiratory burst and has been associated with neutrophil extracellular traps-mediated microbial killing (Parker and Winterbourn, 2012), and how observed in the MET group, the oxidative burst was higher in this group, and could indicate a better response in MET group of MPO compared with CHOL, that sowed higher expression of MPO, but no difference in the oxidative burst.

Associated with MPO and SOD2, the lower concentration of NOS2 at day -10 in MET group and along the period, indicated a control of the transcription factor of inflammation, because NOS2 expression modulated by inflammatory mediators. Li et al., (2010) observed that NO, synthetize by NOS2, concentration was higher blood and uterine secretions in animals with subclinical and clinical endometritis when compared with control cows, indicated that animals in MET group probably have a better control to response to inflammation mediators.

Nuclear receptors and factors of transcription

Crookenden et al., (2014) and Osorio et al., (2013) observe a decrease in the 1 week poscalving in the expression of RXRA, in the animals of this study this time effect not was observed. The upregulation of RXR observe to CHOL group at day 30 could be related with lower neutrophil differentiation and impaired proliferation how demonstrated by Taschner et al., (2007), and the down-regulation of RXRA is required for neutrophil generation from primary human progenitor cells.

Cyclic AMP-response element-binding protein (CREB) is known to regulate a number of immune-regulatory genes such as IL-10, this action can be modulating because CREB interacting with NFkB activation (Brenner et al., 2003). Wen et al., (2013) observed in transgenic mice were CREB was overexpressed, that animals had abnormal chemokine production in macrophages and increased NADPH oxidase activity in neutrophils and suggest that CREB overexpressing neutrophils may be primed or are hypersensitive to stimuli such as bacteria, resulting in increased abscess formation. This observation related with our dates suggest that the higher concentration in CHOL group could indicate changes in the inflammatory response, with negative effects.

Blood Phagocytosis and Oxidative Burst

Phagocytosis is a fundamental function of PMN, which are involved in host defense (Zhou et al., 2015), and when this activity reduces, it is an indirect indicator of the impaired of the immune response. The greater neutrophil phagocyte and oxidative burst detect in MET cow provide evidences that MET supply could urge the immune system, possible by the action of antioxidants taurine and glutathione, this last higher in MET cows. The increase in *SOD2* and *MPO* expression in this group could have been linked with an increase in oxidative stress status and explain the better response PMN phagocytosis observed. Moreover, the higher concentration of ketone body no impaired phagocytic capacity of PMN in MET group, opposing Suriyasathaporn et al. (1999), that observed the impaired of the activity when high-BHB levels was tested. The inhibition of negative effects ketone body in this groups could be related with the higher antioxidants concentration (GSH) in this groups, that reduces the effect of BHBA (Li et al., 2016b).

The levels of glucose and insulin observed in the CHOL group could compromised the activity of the PMN, as observed in studies with diabetic rats a decreased of phagocytosis capacity (Panneerselvam and Govindasamy, 2003), in the other way the reduction of blood glucose levels by insulin treatment of diabetic patients (Alba-Loureiro et al., 2006) was significantly correlated with improvement of neutrophil phagocytosis capacity.

CONCLUSION

The temporal adaptations in the PMN transcriptome associated with chemotaxis and migration (CADM1 and SELL) in MET and CHOL supplemented cows indicates better capacity for a faster response to pathogens during the transition period. The overall lower mRNA expression of IRAK1 and ITGB2 over time in response to MET supplementation

indicates that PMN were less active. In contrast, greater BPI and TLR2 together with greater PMN adhesion related genes (CADM1, CXCR2, SELL) seems to indicate that the PMN in CHOL cows were activated to some extent. The lack of an overall MET or CHOL effect on CTH, CBS, SAHH, GSR, and GSS expression indicates that PMN may not be very active in terms of utilizing MET and/or CHOL for intracellular synthesis of glutathione or taurine. Additional studies to examine Methionine and Choline mechanisms of action on PMN function and activity around parturition appear warranted.

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Table 1: Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected Met, rumen-protected choline, or both (MIX) on relative mRNA expression of polymorphonuclear leukocyte (PMNL).

	MET			CHOL			*P*-value				
Genes	With	Without	SEM[1]	With	Without	SEM	MET[2]	CHOL[3]	TIME	M×T[4]	C×T[4]
Cell adhesion, chemotaxis and migration											
BPI	0.69	0.53	0.07	0.65	0.56	0.07	0.11	0.36	0.01	0.25	0.004
CADM1	3.20	2.74	0.33	3.56[A]	2.39[B]	0.32	0.32	0.01	0.28	0.63	0.12
CXCR2	1.88	2.06	0.21	1.89	2.05	0.21	0.28	0.63	0.27	0.76	0.01
ICAM1	2.00	1.86	0.19	1.82	2.03	0.19	0.60	0.43	0.004	0.05	0.09
ITGA4	1.94	1.70	0.17	1.84	1.79	0.17	0.31	0.83	0.001	0.31	0.12
ITGAM	1.60	1.81	0.11	1.72	1.69	0.11	0.17	0.86	0.45	0.26	0.62
ITGB2	2.06[b]	2.32[a]	0.08	2.22	2.16	0.08	0.02	0.62	0.06	0.38	0.16
SELL	1.41	1.20	0.12	1.39	1.22	0.12	0.28	0.63	0.89	0.93	0.07
TLR pathway and cytokines											
IFNγ	2.01	1.96	0.27	1.80	2.16	0.28	0.89	0.37	0.18	0.58	0.50
IL-1β	2.18	1.71	0.25	2.13	1.76	0.25	0.19	0.30	0.15	0.51	0.13
IL-6	1.66	1.54	0.11	1.57	1.63	0.11	0.44	0.71	0.01	0.25	0.38
IL-10	1.66	1.40	0.15	1.74[A]	1.33[B]	0.15	0.23	0.06	0.79	0.15	0.02
IL-10R	1.36	1.18	0.10	1.39	1.15	0.10	0.23	0.11	0.18	0.62	0.001
IRAK1	1.33[b]	1.51[a]	0.04	1.41	1.43	0.05	0.01	0.67	0.05	0.31	0.84
NFKB1	1.85	1.81	0.11	1.90	1.76	0.11	0.82	0.36	0.77	0.85	0.59
STAT3	1.71	1.67	0.18	1.79	1.59	0.18	0.90	0.45	0.31	1.00	0.81
S100A8	1.41	1.20	0.12	1.39	1.22	0.12	0.23	0.31	0.12	0.97	0.60
TLR2	1.59	1.56	0.07	1.63	1.52	0.07	0.75	0.26	0.70	0.42	0.06
TLR4	2.05	1.78	0.18	2.05	1.78	0.18	0.28	0.29	0.48	0.76	0.21
TNFα	0.94	0.93	0.05	0.89	0.98	0.05	0.97	0.30	0.04	0.17	0.03

[a,b] Mean values with different superscripts differ in the MET effect ($P < 0.05$); [A, B] Mean values with different superscripts differ in the CHOL effect ($P < 0.05$);
[1]Greatest SEM. [2]Overall effect of methionine supplementation; [3]Overall effect of choline supplementation; [4]Interaction of MET × time; [5]Interaction of CHOL × time.

Table 1 Continuation: Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected Met, rumen-protected choline, or both (MIX) on relative mRNA expression of polymorphonuclear leukocyte (PMNL).

	MET			CHOL			*P*-value				
Genes	With	Without	SEM[1]	With	Without	SEM	MET[2]	CHOL[3]	TIME	M×T[4]	C×T[4]
Oxidative stress and Methionine cycle											
CBS	1.55	1.51	0.12	1.49	1.57	0.12	0.82	0.67	0.02	0.12	0.23
CTH	1.75	1.94	0.16	1.99	1.70	0.16	0.43	0.22	0.90	0.50	0.30
GPX1	2.24	2.22	0.14	2.15	2.30	0.14	0.53	0.58	0.52	0.68	0.32
GSR	1.85	1.95	0.21	1.82	1.98	0.21	0.93	0.45	0.24	0.34	0.16
GSS	1.88	2.06	0.21	1.89	2.05	0.21	0.74	0.59	0.24	0.98	0.61
MPO	1.95[a]	1.16[b]	0.26	1.97[A]	1.14[B]	0.27	0.04	0.03	0.01	<.0001	0.001
NOS2	1.58	2.43	0.40	1.70	2.30	0.40	0.14	0.29	<.0001	0.02	0.73
SAHH	1.66	1.61	0.13	1.70	1.57	0.13	0.81	0.47	0.15	0.72	0.65
SOD1	0.95	1.00	0.06	1.00	0.94	0.06	0.54	0.50	0.22	0.93	0.46
SOD2	2.33[a]	1.75[b]	0.18	2.19	1.89	0.18	0.02	0.20	0.93	0.65	0.76
Nuclear receptors and factors of transcription											
AKT1	1.16	1.02	0.07	1.22[A]	0.95[B]	0.08	0.20	0.02	<.0001	0.70	0.19
CREB1	1.50	1.46	0.04	1.54[A]	1.41[B]	0.04	0.49	0.03	0.12	0.28	0.72
GSK3	1.48	1.46	0.13	1.57	1.36	0.13	0.93	0.26	0.07	0.31	0.25
INSR	1.52	1.48	0.12	1.65[A]	1.35[B]	0.12	0.81	0.07	<.0001	0.004	0.05
RXRA	1.73	1.65	0.15	1.77	1.60	0.15	0.69	0.43	0.15	0.30	0.04

[a,b] Mean values with different superscripts differ in the MET effect ($P < 0.05$); [A, B] Mean values with different superscripts differ in the CHOL effect ($P < 0.05$);
[1]Greatest SEM. [2]Overall effect of methionine supplementation; [3]Overall effect of choline supplementation; [4]Interaction of MET × time; [5]Interaction of CHOL × time.

Table 2: Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected Met, rumen-protected CHO, or both (MIX) on blood biomarker

	MET		SEM[1]	CHOL		SEM	*P*-value				
Metabolits	With	Without		With	Without		MET[2]	CHOL[3]	TIME	M×T[4]	C×T[4]
Inflammation											
IL-6 (pg/mL)	1028.00	1053.98	149.93	998.85	1083.12	149.73	0.90	0.69	0.004	0.64	0.65
IL-1β (pg/mL)	5.17	6.83	0.83	6.08	5.92	0.85	0.16	0.89	0.08	0.46	0.50
MPO (U/L)	425.63	447.16	15.11	437.64	435.15	15.02	0.31	0.91	0.0007	0.69	0.81
Oxidative stress											
Frap (µmol/L)	132.84	128.23	3.09	128.17	132.90	3.11	0.29	0.28	<.0001	0.45	0.05
ROM (mg H_2O_2/100mL)	13.01[a]	14.17[b]	0.38	13.93	13.24	0.38	0.03	0.19	<.0001	0.03	0.41
Reduced glutathione (µmol/g protein)	411.14[a]	142.04[b]	75.99	203.96	349.22	77.89	0.02	0.18	0.04	0.29	0.42
Glutathione (µmol/g protein)	375.34[a]	127.91[b]	70.23	208.45	294.80	71.34	0.02	0.38	0.02	0.11	0.54
Choline metabolism											
Plasma choline (mg/dL)	3.74	3.56	0.11	3.80[A]	3.51[B]	0.12	0.26	0.08	<.0001	0.93	0.37
Plasma PC (mg/mL)	122.86	115.49	5.70	118.07	120.28	5.87	0.36	0.78	<.0001	0.09	0.58
Energetics Metabolites											
Glucose (mmol/L)	3.91	3.92	0.06	4.00[A]	3.83[B]	0.06	0.95	0.05	<.0001	0.39	0.60
Insulin (µg/L)	0.61	0.50	0.06	0.65[A]	0.46[B]	0.06	0.15	0.02	0.005	0.01	0.93
Glucose:insulin	9.67	10.19	0.85	8.91[B]	10.95[A]	0.83	0.65	0.08	0.07	0.02	0.27
BHBA	0.74[a]	0.61[b]	0.02	0.69	0.66	0.02	<.0001	0.24	0.001	0.27	0.38
NEFA	0.56	0.49	0.06	0.56	0.50	0.06	0.39	0.52	<.0001	0.67	0.40

[a,b] Mean values with different superscripts differ in the MET effect ($P < 0.05$); [A, B] Mean values with different superscripts differ in the CHOL effect ($P < 0.05$);
[1]Greatest SEM. [2]Overall effect of methionine supplementation; [3]Overall effect of choline supplementation; [4]Interaction of MET × time; [5]Interaction of CHOL × time

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Table 3. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected Met, rumen-protected CHOL on blood neutrophil and monocyte phagocytosis and oxidative burst.

	MET		SEM[1]	CHOL		SEM	*P*-value				
Cell activity	With	Without		With	Without		MET[2]	CHOL[3]	TIME	M×T[4]	C×T[4]
Neutrophils phagocytosis	58.98[a]	48.33[b]	2.32	53.13	54.18	2.22	0.001	0.73	0.85	0.86	0.69
Neutrophils oxidative burst	59.22[a]	42.65[b]	1.80	52.10	49.78	1.67	<.0001	0.31	0.82	0.91	0.01

[a,b] Mean values with different superscripts differ in the MET effect ($P < 0.05$); [A, B] Mean values with different superscripts differ in the CHOL effect ($P < 0.05$); [1]Greatest SEM. [2]Overall effect of methionine supplementation; [3]Overall effect of choline supplementation; [4]Interaction of MET × time; [5]Interaction of CHOL × time

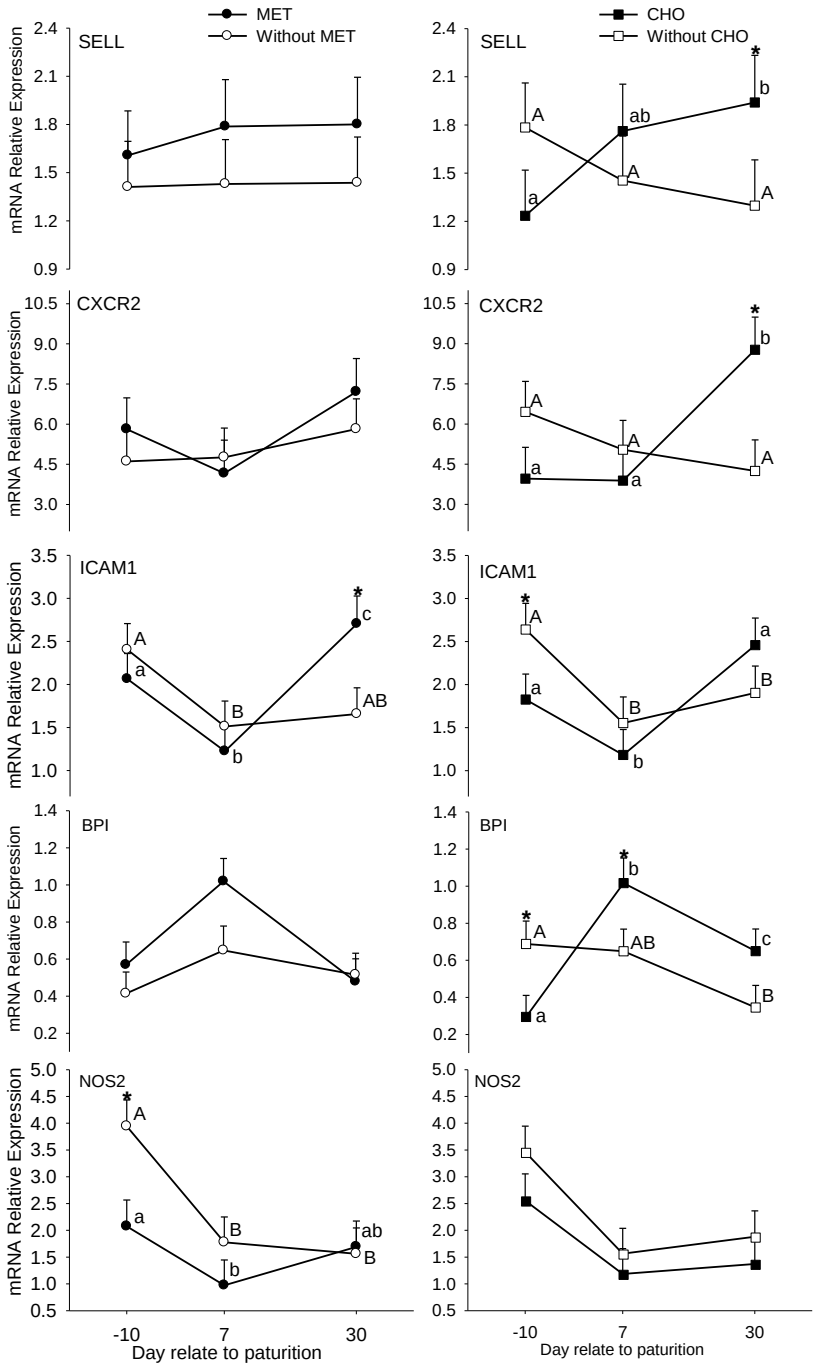

Figure 1: Expression of genes related to cell adhesion (ICAM1), chemotaxis (BPI) and oxidative stress (MPO and NOS2) in polymorphonuclear leukocytes of cows supplement during the peripartal period with rumen-protected Met, rumen-protected choline, or both (MIX). [a, b] and [A, B] Diet × day effects ($P < 0.05$) at a specific time point, *diet × day effects ($P < 0.05$) within diet and across days. Error bars indicate SEM.

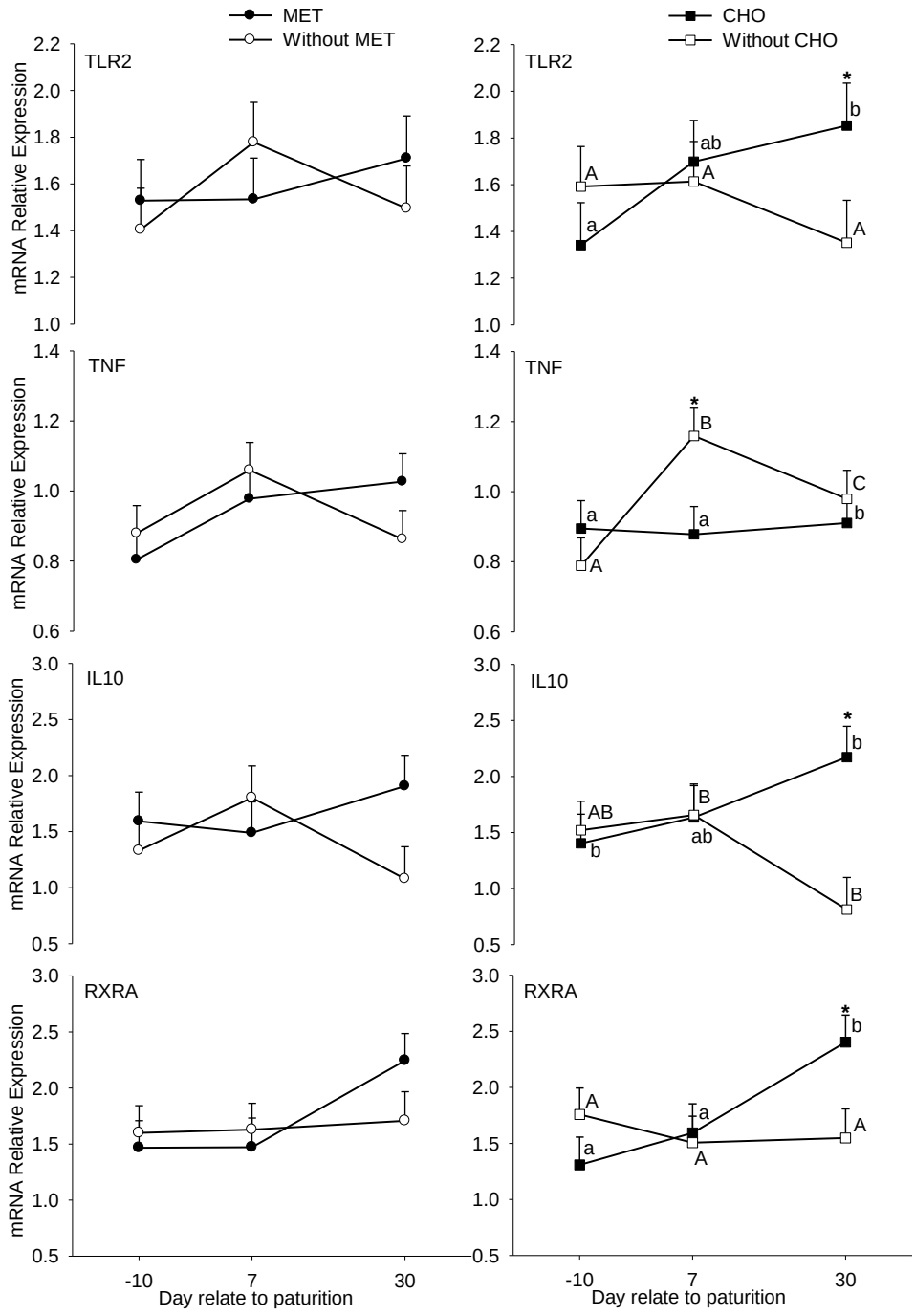

Figure 2: Expression of genes related to TLR pathway (TLR2 and TNF) and cytokines (IL1β and IL10) in polymorphonuclear leukocytes of cows supplement during the peripartal period with rumen-protected Met, rumen-protected choline, or both (MIX). [a, b] and [A, B] Diet × day effects ($P < 0.05$) at a specific time point, *diet × day effects ($P < 0.05$) within diet and across days. Error bars indicate SEM

Supplemental file

RNA extraction, Quality Assessment, and cDNA synthesis

Total RNA was extracted from blood PMN using TRIZol regent combination with miRNeasy® Mini Kit (Cat. #217004, Qiagen). Isolated neutrophils were completely homogenized in 1 mL TRIZol reagent (Invitrogen, Carlsbad, CA) using Beadbeater twice for each 30 sec. Each tube was added into 200 µL Chloroform and put in room temperature 3 min after shaking vigorously for 15 sec. The upper phase was transferred into a new collection tube without disturbing the mid and lower phase after 12,000 g at 4℃ for 15 min centrifuge. Ethanol 100% of 750 µL was added and mixed well. All amount of liquid supernatants were pipetted into a miRNeasy mini spin column in a 2 mL collection tube, then followed manufacturer's instructions of miRNeasy® Mini Kit. DNase I digestion mix (Cat. #79254, Qiagen) of 80 µL was added to each column to remove genomic DNA. Finally, 50 µL RNase free water were added to elute RNA and total RNA were obtained. The RNA concentration was measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The purity of RNA was assessed by ratio of optical density OD260/280, which were above 1.80 for all samples. The RNA integrity (RIN) was evaluated via electrophoretic analysis of 28S and 18S rRNA subunits using a 2100 Bioanalyzer (Agilent Technologies), and values were above 5.50 for all samples.

A portion of RNA was diluted to 100 ng/µL by adding DNase-RNase free water prior to cDNA synthesis. Complementary DNA was synthesized using 1 µL of 100 ng total RNA, 1 µL of Random Primers (Cat. #11034731001, Roche), and 9 µL of DNase/RNase-free water. The mixture was incubated at 65℃ for 5 min and kept on ice for 3 min. A total of 9 µL of master mix composed of 4 µL of 5X First-Strand Buffer (Cat. #18064-022, Invitrogen), 1 µL of Oligo dT18 (Operon Biotechnologies, Huntsville, AL), 2 µL of 10 mM dNTP mix (Cat. #18427-088, Invitrogen), 1.625

μL of DNase/RNase-free water, 0.25 μL (200U/μL) of Revert Aid Reverse Transcriptase (Cat. #EP0442, Thermo Scientific), and 0.125 μL (40U/μL) of RiboLock RNase Inhibitor (Cat. #EO0382, Thermo Scientific) were added. The reaction was performed in an Eppendorf Mastercycler Gradient following such temperature program: 25℃ for 5 min, 50℃ for 60 min, and 70℃ for 15 min. The cDNA was then diluted 1:4 with DNase/RNase-free water.

Primer Design and Evaluation

Primers were designed and evaluated as previously described (Bionaz and Loor, 2008). Briefly, primers were designed using Primer Express 3.0.1 (Applied Biosystems) with minimum amplicon size of 80 bp (amplicons of 100-120 bp were of superiority, if possible) and limited percentage of 3' G + C. Major part of primer sets were designed to fall across exon-exon junctions. Then, primers were aligned against publicly available databases using BLASTN at NCBI and UCSC's Cow (*Bos taurus*) Genome Browser Gateway to determine the compatibility of primers with already annotated sequence of the corresponding gene. Prior to qPCR, a 20 μL PCR reaction comprised of 8 μL dilute cDNA, 10 μL Power SYBR Green PCR Master Mix (Cat. #4367659, Applied Biosystems), 1 μL forward primer and 1 μL reverse primer was established to verify the primers. Of these, a universal reference cDNA amplified from all samples was utilized to ensure identification of desired genes. PCR product of 5 μL was run in a 2% agarose gel stained with SYBR Safe DNA Gel Stain (Cat. #S33102, Invitrogen), and the remaining 15 μL were cleaned with a QIAquick PCR Purification Kit (Cat. #28104, Qiagen) and sequenced at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana. The sequencing product was confirmed through BLASTN at NCBI database. Only primers

that did not present primer-dimer, 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 pair also was evaluated by the presence of a unique peak during the dissociation step at the end of qPCR. The biological functions of all target genes are presented in Supplemental Table 2. Supplemental Table 3 shows all designed primers in this study as well as all sequence information confirmed by BLASTN. Sequencing results for all genes are reported in Table 4.

Quantitative PCR (qPCR)

The qPCR was conducted in triplicate as described previously (Graugnard et al, 2009). Briefly, four microliters of diluted DNA (dilution 1:4) combined with 6 μL of mixture composed of 5 μL 1 × SYBR Green master mix (Cat. #4309155, Applied Biosystems), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL of DNase/RNase-free water were added in a MicroAmp™ Optical 384-Well Reaction Plate (Cat. #4309849, Applied Biosystems). A 6-point standard curve plus the nontemplate control (NTC) together with three replicates of each sample were run to detect the relative expression level. The reactions were conducted in ABI Prism 7900 HT SDS instrument (Applied Biosystems) following the conditions below: 2 min at 50℃, 10 min at 95℃, 40 cycles of 15 s at 95℃ (denaturation), and 1 min at 60℃ (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95℃ for 15 s, then 65℃ for 15 s. The threshold cycle (Ct) data were analyzed and transformed using the standard curve with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems). The final data were normalized with the geometric mean of the 3 ICGs, as reported previously (Moyes et al, 2010).

Relative mRNA Abundance of Genes within PMN

Efficiency of qPCR amplification for each gene was calculated using the standard curve method (Efficiency = $10^{(-1/slope)}$). Relative mRNA abundance among measured genes was calculated as previously reported (Bionaz and Loor, 2008), using the inverse of PCR efficiency raised to ΔCt (gene abundance = $1/E^{\Delta Ct}$, where ΔCt = Ct of tested gene – geometric mean Ct of 3 ICGs). Overall mRNA abundance for each gene among all samples of the same PMN was calculated using the median ΔCt, and overall percentage of relative mRNA abundance was computed from the equation: 100 × mRNA abundance of each individual gene / sum of mRNA abundance of all the genes investigated. Supplemental Table 6 shows the qPCR performance among the genes measured in PMN

Suppl. Table 1. Ingredient composition of diets fed to periparturient cows that were unsupplemented or were supplemented with Smartamine M or Reashure

		Close-up				Lactation			
Ingredients (g·kg^{-1} DM)	Far off	CON[1]	SMA	REA	MIX	CON	SMA	REA	MIX
Alfalfa silage	120	83.4	83.4	83.4	83.4	50.7	50.7	50.7	50.7
Alfalfa hay	-	42.9	42.9	42.9	42.9	29.8	29.8	29.8	29.8
Corn silage	330	364	364	364	364	334	334	334	334
Wheat straw	360	156	156	156	156	29.8	29.8	29.8	29.8
Cottonseed	-	-	-	-	-	35.8	35.8	35.8	35.8
Wet brewers grains	-	42.9	42.9	42.9	42.9	90.9	90.9	90.9	90.9
Ground shelled corn	40	129	129	129	129	239	239	239	239
Soy hulls	20	42.9	42.9	42.9	42.9	41.8	41.8	41.8	41.8
Soybean meal, 48% CP	79.2	25.7	25.7	25.7	25.7	23.9	23.9	23.9	23.9
Expeller soybean meal[2]	-	25.7	25.7	25.7	25.7	59.7	59.7	59.7	59.7
Soychlor[3]	1.5	38.6	38.6	38.6	38.6	-	-	-	-
Blood meal, 85% CP	10	-	-	-	-	-	-	-	-
ProVAAl AADvantage[4]	-	8.6	8.6	8.6	8.6	15.0	15.0	15.0	15.0
Urea	4.5	3.0	3.0	3.0	3.0	1.8	1.8	1.8	1.8
Rumen-inert fat[5]	-	-	-	-	-	10.2	10.2	10.2	10.2
Limestone	13	12.9	12.9	12.9	12.9	13.1	13.1	13.1	13.1
Salt	3.2	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Dicalcium phosphate	1.2	1.8	1.8	1.8	1.8	3.0	3.0	3.0	3.0
Magnesium oxide	2.1	0.8	0.8	0.8	0.8	1.2	1.2	1.2	1.2
Magnesium sulfate	9.1	9.9	9.9	9.9	9.9	-	-	-	-
Sodium bicarbonate	-	-	-	-	-	7.9	7.9	7.9	7.9
Potassium carbonate	-	-	-	-	-	3.0	3.0	3.0	3.0
Calcium sulfate	-	-	-	-	-	1.2	1.2	1.2	1.2

Suppl. Table 1 Continuation. Ingredient composition of diets fed to periparturient cows that were unsupplemented or were supplemented with Smartamine M or Reashure

Ingredients (g·kg^{-1} DM)		Close-up				Lactation			
	Far off	CON[1]	SMA	REA	MIX	CON	SMA	REA	MIX
Mineral vitamin mix[6]	2	1.7	1.7	1.7	1.7	1.8	1.8	1.8	1.8
Vitamin A[7]	0.15	-	-	-	-	-	-	-	-
Vitamin D[8]	0.25	-	-	-	-	-	-	-	-
Vitamin E[9]	3.8	3.9	3.9	3.9	3.9	-	-	-	-
Biotin	-	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
SMA[10]	-	-	0.8	-	0.8	-	0.8	-	0.8
REA[11]	-	-	-	60 g	60 g	-	-	60 g	60 g

[1]CON = control; SMA = rumen-protected methionine; REA = rumen-protected choline; MIX = SMA+REA; MET = SMA+MIX; CHOL = REA+MIX.
[2]SoyPLUS (West Central Soy, Ralston, IA).
[3]By West Central Soy.
[4]Blood meal-based product (Perdue AgriBusiness,Salisbury, MD).
[5]Energy Booster 100 (Milk Specialties Global, Eden Prairie, MN).
[6]Composition in g·kg-1 DM: Mg 50, S 100, K 75, Fe 20, Zn 30, Mn 30, Cu 5, I 0.25, Co 0.04, Se 0.15. Vitamins in mg·kg-1 DM: retinol 660, cholecaciferol 16.5, alpha-tocopherol 5159.
[7]Contained 9 g·kg-1 retinol.
[8]Contained 0.125 g·kg-1 cholecaciferol.
[9]Contained 29.5g·kg-1 alpha-tocopherol.
[10]Rumen protected methionine (Smartamine, Adisseo NA) was supplemented on top of the diet given to the animals.
[11]Rumen protected choline (Reashure, Balchem Inc. NH) was supplemented to cows at 60 g·cow-1d -1 to each cow

1

Suppl. Table 2: Gene symbol, gene name, and description of the main biological function and biological process of the targets analyzed in PMN.

Symbol	Name	Summary description from NCBI
CELL ADHESION, CHEMOTAXIS AND MIGRATION		
BPI	Bactericidal/permeability-increasing protein	This gene encodes a lipopolysaccharide binding protein. It is associated with human neutrophil granules and has antimicrobial activity against gram-negative organisms.
CADM1	Cell adhesion molecule 1	This gene encodes for a protein that acts as a cell adhesion molecule essential for several functions of neutrophils.
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.
ICAM1	Intercellular adhesion molecule	This gene encodes a cell surface glycoprotein which is typically expressed on endothelial cells and cells ofthe immune system.
ITGA4	Integrin, Alpha 4	The gene encodes a member of the integrin alpha chain family of proteins. The encoded preproprotein is proteolytically processed to generate light and heavy chains that comprise the alpha 4 subunit. This subuni associates with a beta 1 or beta 7 subunit to form an integrin that may play a role in cell motility and migration.
ITGAM	Integrin, Alpha M	This gene encodes the integrin alpha M chain. Integrins are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain. This I-domain containing alpha integrin combines with the beta 2 chain (ITGB2) to form a leukocyte-specific integrin referred to as macrophage receptor 1 ('Mac-1'), or inactivated-C3b (iC3b) receptor 3 ('CR3').
ITGB2	Integrin, Beta 2	This gene encodes an integrin beta chain, which combines with multiple different alpha chains to form different integrin heterodimers. Integrins are integral cell-surface proteins that participate in cell adhesion as well as cell-surface. The encoded protein plays an important role in immune response and defects in this gene cause leukocyte adhesion deficiency.
SELL	Selectin L	This gene encodes a cell surface adhesion molecule that belongs to a family of adhesion/homing receptors. The encodedprotein contains a C-type lectin-like domain, a calcium-binding epidermal growth factor-like domain, and two short complement-like repeats
TLR PATHWAY and CYTOCINES		
IFNγ	Interferon, Gamma	The encoded protein is secreted by cells of both the innate and adaptive immune systems. The active protein is a homodimer that binds to the interferon gamma receptor which triggers a cellular response to viral and microbial infections
IL1β	Interleukin 1, beta	The protein encoded by this gene is a member of the interleukin 1 cytokine This cytokine is produced by activated macrophages as a proprotein, which is proteolyticallyprocessed 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

Suppl. Table 2 Continuation: Gene symbol, gene name, and description of the main biological function and biological process of the targets analyzed in PMN.

Symbol	Name	Summary description from NCBI
TLR PATHWAY and CYTOCINES		
IL6	Interleukin 6	Cytokine with a wide variety of biological functions. It is a potent inducer of the acute phase response.
IL10	Interleukin 10	The protein encoded by this gene is a cytokine produced primarily by monocytes and to a lesser extent by lymphocytes. This cytokine has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th1 cytokines, MHC class II Ags, and costimulatory molecule
IL10R	Interleukin 10 receptor	The protein encoded by this gene is a receptor for interleukin 10. It has been shown to mediate the immunosuppressive signal of interleukin 10, and thus inhibits the synthesis of proinflammatory cytokines. This receptor is reported to promote survival of progenitor myeloid cells through the insulin receptor substrate-2/PI 3-kinase/AKT pathway. Activation of this receptor leads to tyrosine phosphorylation of JAK1
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 IL0 upon stimulation. This gene is partially responsible for IL1-induced upregulation of the transcription factor NF-kappa B.
NFKB1	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer In B-Cells 1	NF-kappa-B is a pleiotropic transcription factor present in almost all cell types and is the endpoint of a series of signal transduction events that are initiated by a vast array of stimuli related to many biological processes such as inflammation, immunity, differentiation, cell growth tumorigenis and apoptpsis.
STAT3	Signal Transducer and Activator Of Transcription 3	Signal transducer and transcription activator that mediates cellular responses to interleukins
S100A8	S100 Calcium Binding Protein A8	The protein encoded by this gene is a member of the S100 family of proteins containing 2 EF-hand calcium-binding motifs. S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation
TLR2	Toll-like receptor 2	The protein encoded by this gene is a member of the Toll-like receptor (TLR) family which plays a 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. This gene is expressed most abundantly in peripheral blood leukocytes, and mediates host response to Gram-positive bacteria and yeast via stimulation of NFKB

Suppl. Table 2 Continuation: Gene symbol, gene name, and description of the main biological function and biological process of the targets analyzed in PMN.

Symbol	Name	Summary description from NCBI
TLR PATHWAY and CYTOCINES		
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 infectiousagents, and mediate the production of cytokines necessary for the development ofeffective immunity. This receptor has been implicated in signal transduction event induced by lipopolysaccharide (LPS) found in most G -bacteria.
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.
OXIDATIVE STRESS AND METHIONINE CYCLE		
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 . .
CTH	Cystathionine gamma-lyase	Cystathionine gamma-lyase (CSE) (or cystathionase) is an enzyme which breaks down cystathionine into cysteine and α-ketobutyrate.
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
GSR	Glutathione reductase	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 antioxidante.
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.
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.
SAHH	Adenosylhomocysteinase (AHCY)	S-adenosylhomocysteine hydrolase 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.

Suppl. Table 2 Continuation: Gene symbol, gene name, and description of the main biological function and biological process of the targets analyzed in PMN.

Symbol	Name	Summary description from NCBI
OXIDATIVE STRESS AND METHIONINE CYCLE		
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	Destroys superoxide anion radicals which are normally produced within the cells and which are toxic to biological systems
NUCLEAR RECEPTORS AND FACTORS OF TRANSCRIPTION		
AKT1	SERINE/THREONINE KINASE 1	AKT is responsible of the regulation of glucose uptake by mediating insulin-induced translocation of the SLC2A4/GLUT4 glucose transporter to the cell surface.
CREB1	cAMP responsive element binding protein 1	The protein is phosphorylated by several protein kinases, and induces transcription of genes in response to hormonal stimulation of the cAMP pathway.
GSK3B	Glycogen Synthase Kinase 3 Beta	The protein encoded by this gene is a serine-threonine kinase belongin to the glycogen synthase kinase subfamily. It is involved in energy metabolism, neuronal cell development, and body pattern formation. Constitutively active protein kinase that acts as a negative regulator in the hormonal control of glucose homeostasis,
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.
RXRA	Retinoid X receptor, alpha	Receptor for retinoic acid. Retinoic acid receptors bind as heterodimers to their target response elements in response to their ligands, all-trans or 9-cis retinoic acid, and regulate gene expression in various biological processes.
CONTROL GENES		
UXT	Ubiquitously Expressed Prefoldin Like Chaperone	The protein encoded by this gene functions as a cofactor that modulates androgen receptor-dependent transcription and also plays a critical role in tumor necrosis factor-induced apoptosis.
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	The product of this gene catalyzes an important energy-yielding step in carbohydrate metabolism, the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide (NAD).
RPS9	Ribosomal Protein S9	This gene encodes a ribosomal protein that is a component of the 40S subunit

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Suppl. Table 3: GeneBank accession number, hybridization position, sequence and amplicon size of primers used to analyze gene expression by qPCR

Gene ID	Accession #	Gene	Primers[1]	Primers (5'-3')	bp[2]
280734	*NM_173895.2*	*BPI*	F.502 R.601	ACACCGTCCGCATACACATCT TTTCTGGTCATGGACTTTTGGA	100
529873	*NM_001038558.2*	*CADM1*	F:578 R:670	GCAAGGAGCTCAAAGGCAAGT AACCCCGTCATCCTCCTTGT	90
281863	NM_174360.2	*CXCR2*	F. 518 R. 619	GGCACTGGGTCAAGTTCATATGT CGGAGTACGGTGGTTGATAGG	102
281839	NM_174348.2	*ICAM1*	F.2961 R.3060	AGAATTAGCGCTGACCTCTGTTAAG CGGACACATCTCAGTGACTAAACAA	100
282882	NM_174748.1	*ITGA4*	F.2149 R.2250	ACAGACAGCTCTGGCATAGTGAGA AGCTGGCATCCAGAAGAAAGC	102
407124	NM_001039957.1	*ITGAM*	F.268 R.362	GGCTTGTCTCTTGCATTTGCT CCATTTGCATAGGTGTTCTCCTT	95
281877	NM_175781.1	*ITGB2*	F. 1231 R. 1338	GACACCCTGAAAGTCACCTACGA GAAGGTGATCGGGACGTTGAT	108
281485	NM_174182.1	*SELL*	F.588 R.691	CTCTGCTACACAGCTTCTTGTAAACC CCGTAGTACCCCAAATCACAGTT	104
281237	NM_174086.1	*IFNγ*	F.68 R.167	TCCGGCCTAACTCTCTCCTAA CCCTGGCCATAAGAACCAGAA	100
281251	NM_174093.1	*IL1β*	F. 30 R. 129	ATTCTCTCCAGCCAACCTTCATT TTCTCGTCACTGTAGTAAGCCATCA	100
280826	NM_173923.2|	*IL6*	F. 190 R. 289	CCAGAGAAAACCGAAGCTCTCAT CCTTGCTGCTTTCACACTCATC	100
281246	NM_174088.1	*IL10*	F. 171 R. 268	GAAGGACCAACTGCACAGCTT AAAACTGGATCATTTCCGACAAG	98
513478	NM_001205757.1	*IL10R*	F. 238 R. 397	GTATCGCAGCAATGGTTAC CCGTCAGAGTCACTTCAT	110
533953	NM_001040555.1	*IRAK1*	F. 950 R. 1052	CCTCAGCGACTGGACATCCT GGACGTTGGAACTCTTGACATCT	103
616115	NM_001076409.1	*NFKB1*	F.172 R.266	TTCAACCGGAGATGCCACTAC ACACACGTAACGGAAACGAAATC	95
508541	NM_001012671.2	*STAT3*	F.3804 R.3913	CCGGTGTCCAGTTCACAACTAA CCCCGGAGTCTTTGTCAAT	110
616818	NM_001113725.2	*S100A8*	F. 53 R. 152	ACACCATGCTGACGGATCTG TCCCTATAGACGGCGTGGTAA	100
281534	NM_174197.2	*TLR2*	F. 2238 R. 2340	CTGGCAAGTGGATTATCGACAA TACTTGCACCACTCGCTCTTCA	102
281536	NM_174198.6	*TLR4*	F.555 R.664	TGCGTACAGGTTGTTCCTAACATT TAGTTAAAGCTCAGGTCCAGCATCT	110
280943	NM_173966.3	*TNF*	F. 174 R. 287	CCAGAGGGAAGAGCAGTCCC TCGGCTACAACGTGGGCTAC	114
514525	NM_001102000.2	*CBS*	F. 2349 R. 2448	GCCACCACCTCTGTCAAATTC GGACAGAAAGCAGAGTGGTAACTG	100
539159	BC151523.1	*CTH*	F. 926 R. 1025	AAGTCCGCATGGAGAAGCATT GAAGGCAGCCCAGGATAAATAA	100

Suppl. Table 3 Continuation: GeneBank accession number, hybridization position, sequence and amplicon size of primers used to analyze gene expression by qPCR

Gene ID	Accession #	Gene	Primers[1]	Primers (5'-3')	bp[2]
281209	NM_174076.3	*GPX1*	F.325 R. 430	CCCCTGCAACCAGTTTGG GAGCATAAAGTTGGGCTCGAA	106
506406	NM_001114190.2	*GSR*	F. 1319 R. 1418	CGCTGAGAACCCAGAGACTTG AAACGGAAAGTGGGAACAGTAAGTA	100
525059	BC109713.1	*GSS*	F. 1334 R. 1423	CGAGTGATCCAATGCATTTCAG ATGTCCCACGTGCTTGTTCAT	90
511206	NM_001113298.2	*MPO*	F.1311 R.1415	AGCCATGGTCCAGATCATCAC ACCGAGTCGTTGTAGGAGCAGTA	105
281485	NM_174182.1	*NOS2*	F. 3283 R. 3372	CTGAAGCAGCTGATGGCTACT ATGATAGCGCTTCTGGTTCTTGAC	89
508158	NM_001034315.1	*SAHH*	F.887 R.995	TGTCAGGAGGGCAACATCTTT AGTGCCCAATGTTACACACAATG	109
281495	NM_174615.2	*SOD1*	F. 268 R. 368	GGCTGTACCAGTGCAGGTCC GCTGTCACATTGCCCAGGT	100
281496	NM_201527.2	*SOD2*	F. 620 R.714	TGTGGGAGCATGCTTATTACCTT TGCAGTTACATTCTCCCAGTTGA	95
280991	NM_173986.2	*AKT1*	F. 864 R. 963	GGATTACCTGCACTCGGAAAAG TCCGAAGTCGGTGATCTTGAT	100
281713	NM_174285.1	*CREB1*	F. 85 R. 225	TTCAAGCCCAGCCACAGATT GGCCGCCTGAATAACTCCAT	140
536561	NM_001102192.1	*GSK3A*	F. 83 R. 168	CACAAGCTTTAAGTGAGGCTCAGA GCGCCCTCAGGAAGAGTTG	86
408017	XM_005208817.2	*INSR*	F. 245 R. 328	CCCTTCGAGAAAGTGGTGAACA AGCCTGAAGCTCGATGCGATAG	84
507554	NM_001304343.1	*RXRA*	F. 224 R. 356	TGTCCCCGATGAGCTTGAAG GAGGCGTACTGCAAACACAAGT	133

[1] Primer direction (F – forward; R – reverse) and hybridization position on the sequence.

[2] Amplicon size in base pair (bp).

Suppl. Table 4: qPCR performance among the genes measured in PMN.

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]	Relative mRNA abundance[6]
BPI	26.98	1.33	-2.91	0.99	2.20	0.35
CADM1	22.65	1.11	-2.69	0.99	2.36	0.39
CXCR2	20.44	0.99	-2.44	0.98	2.57	0.39
ICAM1	27.59	1.33	-3.00	0.99	2.16	0.36
ITGA4	21.33	1.05	-2.99	1.00	2.16	0.44
ITGAM	21.88	1.08	-3.36	1.00	1.98	0.48
ITGB2	20.47	1.01	-2.85	0.99	2.24	0.44
SELL	19.01	0.92	-3.13	1.00	2.09	0.51
IFNγ	29.75	1.49	-2.34	0.91	2.68	0.23
IL1B	18.27	0.90	-3.03	0.99	2.14	0.51
IL6	28.81	1.42	-2.57	1.00	2.45	0.28
IL10	28.92	1.43	-2.63	0.94	2.40	0.29
IL10R	23.20	1.16	-3.20	0.98	2.05	0.43
IKAK1	23.40	1.15	-2.81	0.99	2.27	0.39
NFKB1	21.14	1.06	-2.91	0.99	2.21	0.43
STAT3	21.63	1.07	-3.07	1.00	2.12	0.45
S1000A8	17.82	0.87	-3.14	1.00	2.08	0.53
TLR2	23.28	1.15	-2.90	0.99	2.21	0.40
TLR4	23.32	1.14	-3.14	0.99	2.08	0.43
TNF	25.49	1.26	-2.88	1.00	2.23	0.37
CBS	30.89	1.52	-2.60	0.99	2.42	0.26
CTH	25.01	1.24	-2.60	0.96	2.43	0.33
GPX1	17.21	0.84	-2.51	0.99	2.50	0.46
GSR	21.25	1.04	-2.91	0.99	2.21	0.44
GSS	26.26	1.29	-2.94	1.00	2.19	0.36
MPO	29.73	1.44	-2.02	0.83	3.13	0.19
NOS2	28.82	1.40	-2.93	0.99	2.19	0.33
SAHH	20.91	1.03	-3.01	1.00	2.15	0.45
SOD1	23.98	1.18	-3.07	0.99	2.12	0.41
SOD2	19.29	0.96	-2.96	1.00	2.18	0.48
AKT1	23.09	1.16	-3.08	0.98	2.11	0.42
CREB1	22.68	1.14	-3.06	0.98	2.12	0.42
GSK3B	22.34	1.12	-3.15	0.99	2.08	0.44
INSR	22.61	1.13	-2.74	0.98	2.32	0.39
RXRA	21.36	1.05	-3.05	0.99	2.13	0.45

[1] The median is calculated considering all time points and all cows.

[2] The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each cow.

[3] Slope of the standard curve.

[4] R^2 stands for the coefficient of determination of the standard curve.

[5] Efficiency is calculated as $[10^{(-1 / Slope)}]$.

[6] Relative mRNA abundance is calculates as $[1/ \text{Efficiency}^{\text{Median } \Delta Ct}]$, then [(relative mRNA abundance/∑relative mRNA abundance)*100]. mRNAs = 100% and microRNAs = 100%

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4.3 Artigo 3 – Methyl-donors methionine (smartamine m) and choline (reashure) during late pregnancy its association with hepatic gene expression and blood biomarker in neonatal holstein calves.

Artigo apresentado nas normas da revista *Journal of Dairy Science*, na qual o será submetido

Running Title: MATERNAL NUTRION WITH RUMEN-PROTECTED METHYL DONORS

METHYL-DONORS METHIONINE (SMARTAMINE M) AND CHOLINE (REASHURE) DURING LATE PREGNANCY ITS ASSOCIATED WITH HEPATIC GENE EXPRESSION AND BLOOD BIOMARKER IN NEONATAL HOLSTEIN CALVES.

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ABSTRACT

The maternal nutrition has a direct bearing on the availability of nutrients to the fetus and an effect on the neonates metabolism in general. The aim of this study was to assess the effect of maternal methionine (MET) or choline (CHOL) supplementation on neonatal calf blood biomarkers and liver expression of genes related to methyl pathways, one-carbon pathway and glutathione cycle. Holstein cows were assigned in a randomized complete block design with 2×2 factorial arrangement of MET and CHOL level (with or without). Treatments were control (CON); CON+MET (MET); CON+CHOL (CHOL); and CON+MET+CHOL (MIX). Liver biopsies were harvested (n = 8/ group) in the calves at 4 and 50 (~1 wk post-weaning) days of age. No maternal diet effect (P>0.05) was observed on ALDH7, MAT1A, CDO1, GCLC, SCL2A2, KAT2B, FGF21, PC, PCK1. The GSR and PPARA expression was lower at day 50 (P<0.02) in MET calves, CHOL supplementation had no effect (P>0.05), might indicate a lower requirement for the antioxidant activity of glutathione in MET maternal supplemented calves. Day 4, BHMT and CSAD (P>0.05) had a higher expression in CHOL supplementation, and PEMT had a lower expression (P>0.001). At day 50, was observed a tendency to lower expression in CHOL supplementation to CBS (P>0.07) CHDH (P>0.09) and NR3C1 (P>0.09). SAHH expression (P<0.02) was lower in the CHOL and MET supplementation in the day 4 compared with no supplementation. The expression of MAT2A, at day 4, showed different responses to supplementation with CHOL and MET, in the first, had a higher expression and in MET cows a lower expression, compared with the control. In the day 50, not had deference to MAT2A. Methionine adenosyltransferases (MATs) are responsible for the conversion of methionine to S-adenosylmethionine (SAM), an essential methyl donor, especial MAT2A is induced on liver during active growth and cell differentiation, suggested a

different hepatic adaptation in animal supplemented with CHOL. Our study suggests that maternal gestational methyl donor diet affected hepatic genes expression involved in cell cycle and glutathione cycle.

Key Words: fetal programming, amino acids, maternal nutrition.

INTRODUTION

Many studies have been examined the direct contribution of the nutrient, such as glucose or amino acids, to fetal mass (Jahan-Mihan et al., 2015; Riskin-Mashiah et al., 2009). However, some amino acids, for example methionine, not only contribute to the fetal (protein) mass, but also, may impact fetal growth. The metabolism of methionine, along with folate, (one carbon pool) influences a large number of processes that directly or indirectly affect cell proliferation and gene expression, particularly in the growing organism (Kalhan and Marczewski, 2012). Methionine may also impact nutrient transport by its effect (via homocysteine) on the uteroplacental vasculature.

Methionine is an essential amino acid for dairy cows (Nutrient Requirements of Dairy Cattle, 2001) and also the immediate source of the methyl (one carbon) groups required for the methylation of nucleic acids, proteins, biogenic amines, and phospholipids (Brosnan and Brosnan, 2006). Methionine and folate are the key constituents of one carbon transfer, providing the one carbon units for the numerous methyl transferase reactions. Since the methionine and folate cycles are ubiquitously present in every cell in the body and participate in key metabolic reactions, in DNA synthesis and by methylation of DNA in gene expression, perturbation in their metabolism either by nutrient deficiency, or by nutrient, hormonal and environmental interactions can have profound impact on the cell function, metabolism, growth and proliferation.

Choline is another nutrient that have a critical purpose, it is a precursor for the phospholipid phosphatidylcholine, which is a major constituent of membranes, lipoproteins, bile, and surfactants, also, it is a precursor for betaine (Zeisel et al., 2003). Choline is especially important for fetal development (Zeisel, 2013) In rodent models, maternal intake of choline during pregnancy

influenced the development of the hippocampus in the fetus and memory function in the offspring (Craciunescu et al., 2003).

Though the nutritional physiology of the neonatal calf has been well-studied (Blum, 2006), the extent to which prenatal maternal supplementation with methionine and choline affects the profiles of stress and inflammatory markers, metabolites, and gene expression in the liver of neonatal calves are yet to be defined. This way, our hypothesis was that rumen-protected choline and methionine maternal supplementation, would benefit calf liver metabolism during the neonatal period. The specific objectives were to identify the residual effects of choline and methionine supplementation during late-pregnancy on calves' blood immunometabolic markers and liver expression of genes related 1 carbon metabolism genes 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 2x2 factorial 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", 1.40 megacalories/kg dry matter) 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”, 1.52 megacalories/kg dry matter), 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 treatments representing the 4 combinations of **MET** (n = 10, 0.08% of the dry matter of the diet/d methionine, Smartamine M® Adisseo, Alpharetta, GA, USA, ration 2.9:1 Lys:Met), **CHOL** (n =10, supplementation at 60 g/d, ReaShure®, Balchem Inc., NY, USA) ;included **CON** (n = 10; no MET or CHOL supplementation, ration 3.6:1 Lys:Met)); and **MIX** (n= 10 both MET e CHOL supplementation with Smartamine M® and ReaShure®). The dosage of choline was supplemented by following the manufacturer’s recommendations, whereas MET was based on a previous study (Zhou et al., 2016).

The MET and CHOL were supplemented to each cow by placing them on top of the mixed diet from last 24 d of gestation, hence, any observed treatment effects are attributed to maternal nutrition during this period.

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). 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, USA) before receiving colostrum (baseline - day 0), 24 h after receiving colostrum (day 1), 14, 28 and 50 days (n = 10/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.

Blood metabolites, APP and oxidative stress biomarkers

Blood samples were analyzed to albumin, cholesterol, total bilirubin, creatinine, urea, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), glucose, beta-hydroxybutyrate (BHBA), haptoglobin (HAP), ceruloplasmin, antioxidant potential (FRAP), paraoxonase, myeloperoxidase, reactive oxygen metabolites (ROMt) The kits and procedure are described in the Jacometo et al (2015).

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 (Osorio et al., 2013) using a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA).

mRNA isolation, cDNA synthesis and qPCR

The liver was sampled via puncture biopsy (Dann et al., 2006) from calves under local anesthesia previously to the afternoon feeding, at approximately 3 PM, on days 4, and 50 of age (n = 8/group). Tissue were stored in liquid Nitrogen until the RNA extraction.

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).

The protocols used for cDNA synthesis and quantitative real-time PCR was performed as describe previously by Jacometo et al. (2015). Primer design and testing also was performed exactly as described by Jacomento et al. (2015).

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 analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC), apropriede to a 2×2 fatorial desing, according to the following model:

$$y_{ijklm} = \mu + b_i + M_j + C_k + MC_{jk} + T_l + TM_{jl} + TC_{kl} + TMC_{jk}l + A_{m:ijk} + \varepsilon_{ijklm},$$

where y_{ijklm} is the dependent, continuous variable; μ is the overall mean; b_i is the random effect of the ith block; M_j is the fixed effect of MET (j = with or without); C_k is the fixed effect of CHOL (k = with or without); T_l is the fixed effect of time (day or week) of the experiment; $A_{m:ijk}$ is the random effect of the m the animal (calve) nested within block × MET × CHOL; and ε_{ijklm} is the residual error. The covariate for parity (second vs. third lactation and above) was maintained in the model for all variables when significant ($P < 0.05$). Blood metabolites and liver composition were

analyzed at various time points that were not equally spaced. Therefore, an exponential correlation covariance structure SP (POW) was used for repeated measures. Least squares means separation between time points was performed using the PDIFF statement. Statistical differences were declared significant at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

Gene expression

The effect of maternal supplementation of MET or CHOL liver gene expression is reported in the Table 1 and in the Figure 1 and 2. The genes MAT1, GCLC, PC, PCK1, NR3C1, SL2A2, KAT2, FGF21 did not show effect ($P > 0.05$) to maternal supplementation with choline or methionine in the day 4 and day 50.

In the day 4, the genes SAHH ($P < 0.02$) and MAT2 ($P < 0.05$) were lower expression in the MET group compared with animals Without MET (W.MET). Also in the same day, the genes BHMT ($P < 0.001$), MTR ($P < 0.07$), MAT2 ($P < 0.02$), and CSAD ($P < 0.05$) show an effect to maternal supplementation to Choline, in the CHOL group these genes were higher expression than Without CHOL (W. CHOL). In the other way, the gene SAAH ($P < 0.02$) was lower expression in the CHOL group compared with W. CHOL.

In the day 50, GSR ($P < 0.01$) and PARA ($P < 0.02$) show an effect to maternal supplementation with Methionine, in the MET group these genes had a lower expression compared with calves of W. MET group. Also in the day 50, the genes CBS ($P < 0.07$), PEMT ($P < 0.001$) show a lower expression in CHO group than W. CHOL. The gene NR3C1 ($P < 0.09$) and CHCH

($P < 0.09$), show a tendency to lower expression in the CHOL group compared with W. CHOL group. In the other way the PC ($P < 0.10$) had a higher expression in CHOL group than W.CHOL.

Metabolists

The effects of supplementing on blood biomarker was reposted in the Table 2. The metabolites with differed at specific time point (day: 0,1 14, 28, 50 day of age) between MET and W. MET or CHOL and W. CHOL are shown in Figure 3 and Figure 4.

For all the blood biomarkers was observed a time effect ($P < 0.01$). The animals had a marked increase ($P < 0.001$) in glucose, AST, GGT and total bilirubin concentration from birth to 24h after colostrum intake (day 1), and decreases posteriorly in the collections of days 14, 28 and 50 day of age. Only NEFA and Creatinine had higher concentration in the moment of the birth (Day 0), with a considerable reduction in day 1 ($P < 0.001$). The others blood biomarkers (BHBA, PON, HAP, ceruloplasmina, tocoferol, urea, cholesterol, retinol, myeloperoxidade) increased over the days of life, with the peak at day 50 ($P < 0.001$).

In the MET group was observed higher total bilirubin ($P < 0.01$), and a tendency to higher concentration of NEFA ($P < 0.09$) and HAP ($P < 0.06$) compared with W. MET. The concentration of GGT ($P < 0.02$) and Retinol ($P < 0.02$) was lower in MET group compared with W. MET. An interaction MET ×TIME was observed to GGT ($P < 0.001$) with lower concentration in the day 1 in MET group than W. MET, total bilirubin ($P < 0.001$) and creatinine ($P < 0.05$), dad a higher concentration in the MET group than W. MET, in the day 0 and 1 respectively (Figure 4). A tendency was observed to ROMt ($P < 0.08$) with lower concentration in MET group than W. MET at day 14 and 28.

In the CHOL group, was observed lower concentration of to GGT ($P < 0.001$) and NEFA ($P < 0.001$) compared with W. CHOL. In the other way FRAP ($P < 0.06$) had a higher concertation in CHOL group than W. CHOL. The interaction CHOL× TIME was observed to creatinine ($P < 0.001$), NEFA ($P < .0001$), with lower concertation in CHOL group at day 0 compared with W. CHOL. The interaction at day 0 was observed to GGT ($P < .001$) with lower concentration in CHOL group and to tocopherol ($P < 0.01$), with higher concentration compared with W. CHOL. Also myeloperoxidase ($P < 0.06$). show a tendency to lower concertation in the day 28 in CHOL group compared to W. CHOL

DISCUSSION

As a nutritionally important methyl donor, rumen-protected MET supplementation during the transition period is expected to promote overall production in and health of dairy cows not only by serving as a limiting AA for milk synthesis, but also by promoting the synthesis of important methylated compounds, including phosphatidylcholine (Pinotti et al., 2002). Also this effects is extend to calves (Kalhan and Marczewski, 2012).

Considering the fact that MET can be resynthesized *in vivo* from choline-derived methyl groups and homocysteine, similar benefits during the transition period would be expected if a comparable amount of MET is generated from CHOL. Choline could promote MET synthesis in vivo by supplying methyl groups for homocysteine remethylation. Although remethylation of homocysteine to MET can be achieved through either BHMT in the MET cycle or MTR from the tetrahydrofolate cycle, betaine and methyltetrahydrofolate, rather than CHOL, are the direct methyl donors for these reactions. The maternal supplementation with CHOL show a higer expression of

BHMT and MTR at day 4, suggestion that calves supplemented with CHOL indicate that might have been greater betaine generated in response to choline supplementation, and probably synthesis more MET.

For instance, it is possible that hepatic MTR and BHMT activity are not limiting, but rather, the availability of homocysteine and their respective methyl donors control the flux of homocysteine to MET (Pellanda et al., 2012). In fact, the greater MAT2A in calves supplemented with choline could be an indication of increased Met synthesis from choline, because high hepatic Met concentrations lead to increased hepatic SAM concentrations. However in the day 4 the lower SAHH in calves maternal supplemented with CHO indicated the lower potential for synthesis of homocysteine. Also this result agree with the lower CDHD in the same time in the CHO group, CHDH mediates the first and committed step in which choline is first oxidized to betaine aldehyde This may have led to the lower levels of CBS at day 50 in the choline group.

In fact, the lack of change in MTR expression support the idea of decreased Met synthesis in the calves of the MET groups (Ganz et al., 2016). It is noteworthy that CHOL relies on homocysteine to provide the sulfur required for MET synthesis. Because of its key role in the remethylation and transsulfuration pathway, homocysteine could have been a limiting substrate for MET synthesis, especially considering the greater demands after parturition for intracellular sulfur-containing antioxidants, such as glutathione (GSH) and taurine.

However increased flux via the transsulfuration pathway for antioxidant synthesis could be responsible for the similar hepatic MET concentrations observed in treatments. Similarly, the decrease in hepatic total bilirubin concentration in response to MET supplementation could have been the result of increased liver export, although enzyme of GGT and HAP can serve as an

indicator of liver capacity (Smallwood et al., 2016). In fact, because a saturated concentration of substrate was used during incubations in the present study, it is reasonable to assume that the changes in enzyme activities observed reflect corresponding changes in enzyme protein content plus potential posttranslational changes, such as phosphorylation, acetylation, and methylation.

CONCLUSION

In conclusion, our findings provide evidence that MET and CHOL supplementation during maternal live can affect the hepatic mRNA expression and enzyme activity of enzymes controlling flux between MET and CHOL, as well as the transsulfuration pathway. Especial calves supplemented with CHOL, had a higher expression of BHMT and MTR, indicated that CHOL supplementation probably increase MET synthesis as postulated.

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Table 1: Relative mRNA expression of liver in calves born to dams fed with rumen-protected Met, rumen-protected choline, or both (MIX) during the last 30 d prepartum

		MET			CHOL			*P*-value	
Gene	Day	With	Without	SEM[1]	With	Without	SEM	MET[2]	CHOL[3]
GLUCONEOGENESIS									
PC	4	1.20	1.28	0.07	1.26	1.22	0.07	0.42	0.70
	50	0.79	0.89	0.06	0.90 ^{A}	0.77 ^{B}	0.05	0.18	0.10
PCK1	4	0.98	0.97	0.23	1.07	0.88	0.23	0.94	0.29
	50	1.22	1.53	0.14	1.24	1.51	0.13	0.11	0.16
FATTY ACID OXIDATION AND GLUCOSE SIGNALING PATHWAY									
ALDH7	4	0.81	0.83	0.05	0.82	0.83	0.05	0.78	0.82
	50	1.04	0.94	0.05	0.96	1.03	0.05	0.14	0.24
FGF21	4	0.95	0.68	0.20	0.84	0.79	0.20	0.35	0.84
	50	0.72	0.89	0.27	0.91	0.69	0.27	0.65	0.56
PPARA	4	0.97	1.04	0.07	1.07	0.93	0.07	0.50	0.18
	50	0.91^{b}	1.07^{a}	0.05	0.96	1.02	0.05	0.02	0.40
SLC2A	4	1.27	1.36	0.15	1.43	1.21	0.15	0.68	0.30
2	50	1.02	1.09	0.06	1.06	1.05	0.06	0.46	0.93
GLUTATHIONE METABOLISM									
GCLC	4	0.80	0.91	0.21	0.78	0.92	0.21	0.42	0.33
	50	1.39	1.33	0.11	1.30	1.42	0.12	0.67	0.44
GSR	4	0.86	0.96	0.09	0.94	0.88	0.09	0.41	0.62
	50	1.19^{b}	1.45^{a}	0.07	1.25	1.39	0.07	0.01	0.14
MET AND CHOL METABOLISM									
BHMT	4	0.99	0.87	0.08	1.08^{A}	0.79^{B}	0.08	0.13	0.001
	50	1.03	1.00	0.09	1.06	0.98	0.09	0.80	0.51
CBS	4	0.93	0.92	0.04	0.95	0.90	0.04	0.71	0.26
	50	1.15	1.20	0.05	1.11^{B}	1.25^{A}	0.05	0.52	0.07
CDO1	4	0.68	0.63	0.13	0.72	0.59	0.13	0.62	0.19
	50	0.85	0.91	0.06	0.87	0.90	0.06	0.44	0.74
CHDH	4	0.20	0.26	0.07	0.22	0.24	0.07	0.17	0.58
	50	0.38	0.35	0.07	0.32^{B}	0.41^{A}	0.07	0.52	0.09
CSAD	4	0.27	0.25	0.04	0.29^{A}	0.23^{B}	0.03	0.32	0.05
	50	1.11	1.63	0.25	1.11	1.63	0.25	0.14	0.15
MAT1	4	0.28	0.28	0.06	0.30	0.26	0.06	0.86	0.34
	50	0.55	0.45	0.06	0.49	0.51	0.06	0.24	0.77
MAT2	4	0.80^{b}	0.95^{a}	0.05	0.96^{A}	0.78^{B}	0.05	0.05	0.02
	50	1.34	1.37	0.15	1.40	1.31	0.15	0.81	0.40
MTR	4	1.26	1.11	0.24	1.35^{A}	1.02 ^{B}	0.24	0.40	0.07
	50	1.50	1.51	0.17	1.48	1.53	0.17	0.91	0.70
PENT	4	0.31	0.26	0.08	0.24	0.33	0.08	0.47	0.17
	50	0.90	0.89	0.18	0.69^{B}	1.10^{A}	0.18	0.95	0.001
SAHH	4	0.13^{b}	0.25^{a}	0.06	0.18^{B}	0.22^{A}	0.06	0.02	0.02
	50	0.20	0.21	0.04	0.19	0.23	0.04	0.92	0.47
GLUCOCORTICOID RECEPTOR									
NR3C1	4	0.98	0.94	0.03	0.98	0.95	0.03	0.35	0.47
	50	1.14	1.20	0.03	1.13^{B}	1.20^{A}	0.03	0.18	0.09
HISTONE ENZIME									
KAT2B	4	1.06	0.98	0.09	1.02	1.03	0.09	0.26	0.86
	50	1.32	1.37	0.11	1.31	1.37	0.11	0.60	0.51

^{a,b} Mean values with different superscripts differ in the MET effect ($P < 0.05$).

$^{A, B}$ Mean values with different superscripts differ in the CHOL effect ($P < 0.05$).

[1]Greatest SEM. [2]Overall effect of methionine supplementation.

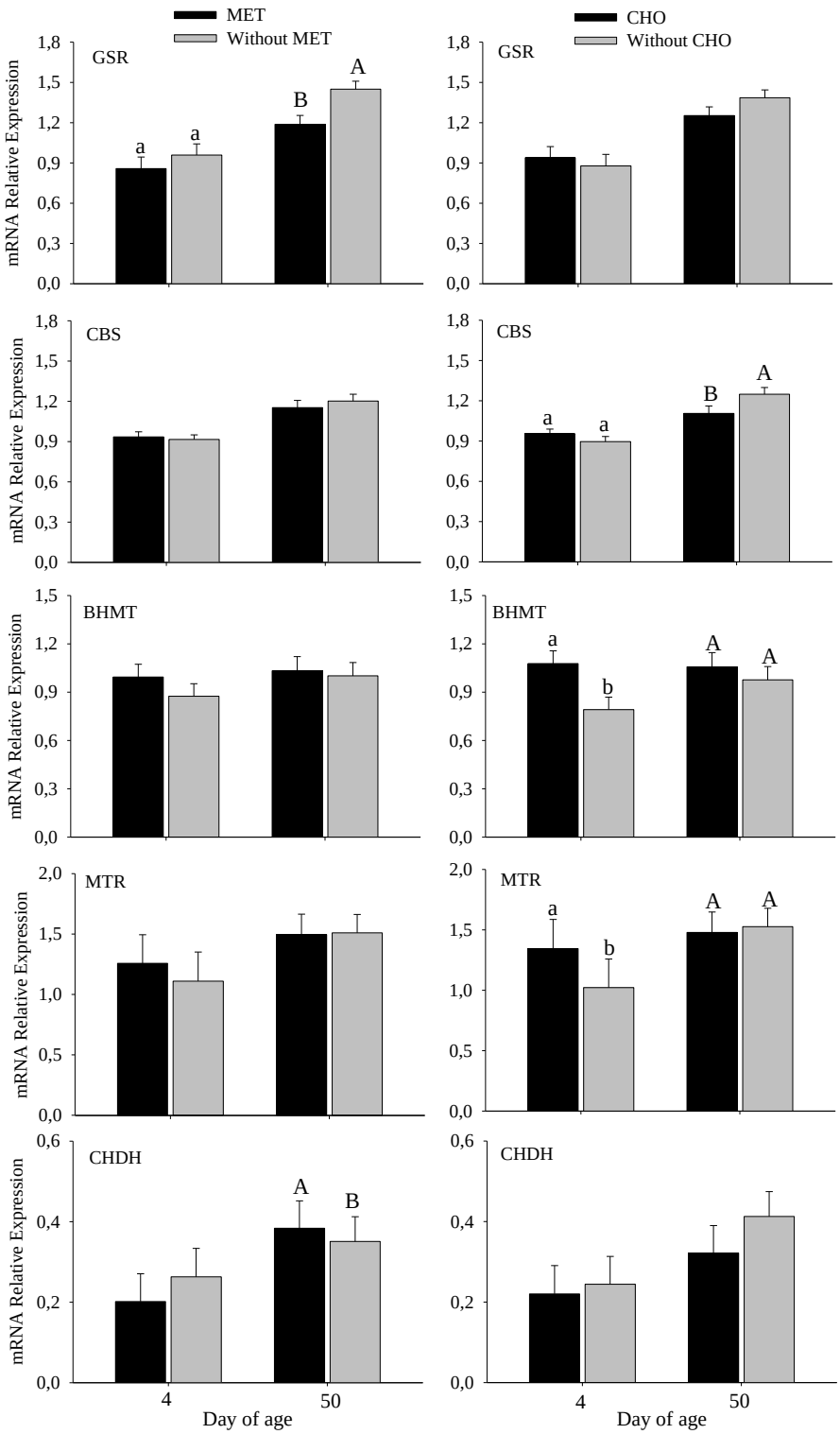

Figure 1: Expression of genes related to choline and methionine metabolism in maternal supplemented calves. [a, b] and [A, B] Diet effects ($P < 0.05$) at a specific time point.

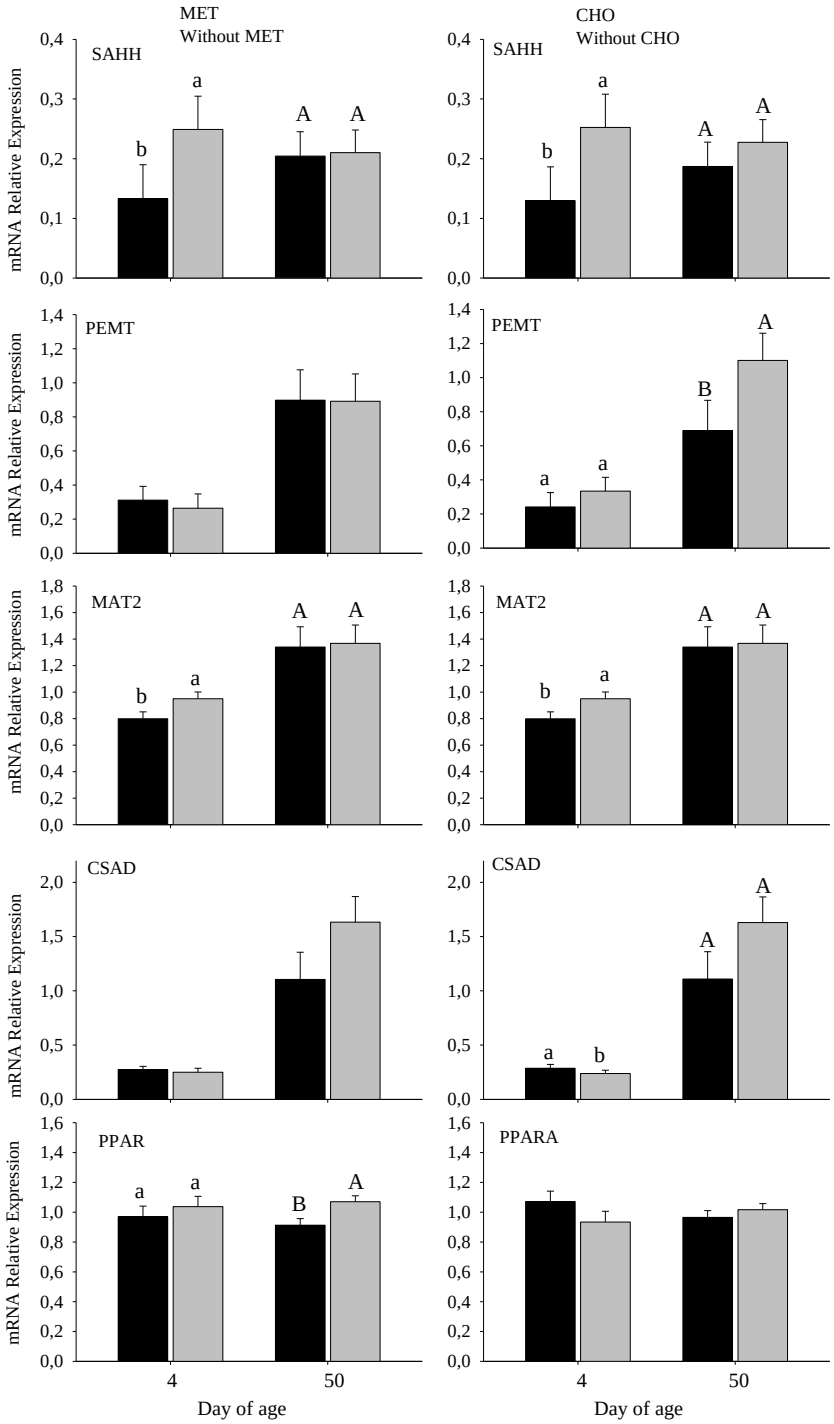

Figure 2: Expression of genes related to choline and methionine metabolism in maternal supplemented calves. [a, b] and [A, B] Diet effects ($P < 0.05$) at a specific time point.

Table 2: Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected Met, rumen-protected choline, or both (MIX) on relative mRNA expression of polymorphonuclear leukocyte (PMNL).

	MET			CHOL			*P*-value				
Metabolits	**With**	**Without**	**SEM[1]**	**With**	**Without**	**SEM**	**MET[2]**	**CHOL[3]**	**TIME**	**M×T[4]**	**C×T[4]**
Glucose	6.14	6.31	0.13	6.09	6.36	0.13	0.33	0.14	<.0001	0.67	0.02
Cholesterol	1.49	1.58	0.06	1.54	1.53	0.06	0.35	0.97	<.0001	0.98	0.81
Urea	4.55	4.42	0.13	4.62	4.36	0.14	0.50	0.16	<.0001	0.11	0.16
AST/GOT	60.85	61.90	2.04	61.19	61.56	2.23	0.72	0.90	<.0001	0.10	0.18
GGT	402.46^{B}	555.00^{A}	42.98	364.16^{b}	593.30^{a}	45.66	0.02	0.001	<.0001	0.001	<.0001
Total bilirubin	9.97^{A}	7.15^{B}	0.69	9.31	7.82	0.77	0.01	0.13	<.0001	0.001	0.21
Creatinine	122.18	115.60	3.87	115.71	122.07	4.17	0.24	0.25	<.0001	0.05	0.001
NEFA	0.43^{A}	0.36^{B}	0.03	0.33^{b}	0.47^{a}	0.03	0.09	0.001	<.0001	0.24	<.0001
BHBA	0.13	0.12	0.02	0.14	0.11	0.02	0.52	0.29	<.0001	0.22	0.10
Ceruloplasmin	2.00	2.19	0.09	2.16	2.03	0.09	0.12	0.28	<.0001	0.60	0.61
Haptoglobin	0.35^{A}	0.26^{B}	0.03	0.32	0.28	0.04	0.06	0.31	0.0001	0.50	0.81
Paraoxonase	29.33	31.66	1.32	30.09	30.91	1.35	0.21	0.66	<.0001	0.79	0.49
Retinolo	21.10^{B}	24.54^{A}	1.12	22.36	23.28	1.10	0.03	0.56	<.0001	0.66	0.42
Albumin	30.72	30.69	0.22	30.68	30.73	0.22	0.93	0.86	<.0001	0.87	0.83
Tocoferolo	1.93	1.85	0.13	1.87	1.91	0.13	0.67	0.82	<.0001	1.00	0.01
FRAP	151.69	155.31	4.12	158.86^{a}	148.14^{b}	4.04	0.52	0.06	<.0001	0.48	0.43
ROMt	11.40	12.08	0.52	11.70	11.78	0.56	0.36	0.90	<.0001	0.08	0.26
Myeloperoxidase	435.09	465.26	27.19	482.37	417.97	29.43	0.42	0.11	0.01	0.61	0.06

[a,b] Mean values with different superscripts differ in the MET effect ($P < 0.05$); [A, B] Mean values with different superscripts differ in the CHOL effect ($P < 0.05$); [1]Greatest SEM. [2]Overall effect of methionine supplementation; [3]Overall effect of choline supplementation; [4]Interaction of methionine × time; [5]Interaction of choline × time.

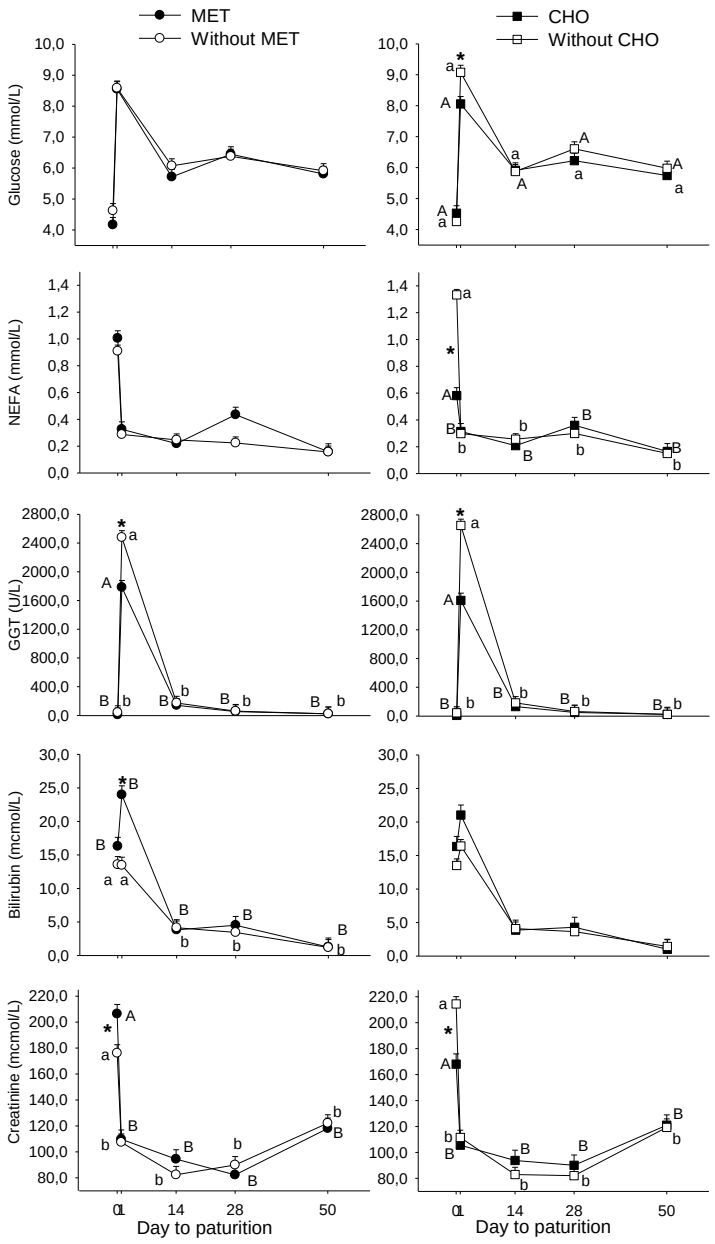

Figure 3: Expression of genes related to inflammation in the calves that received maternal supplement with rumen-protected Met, rumen-protected choline, or both (MIX). [a, b] and [A, B] Diet × day effects ($P < 0.05$) at a specific time point, *diet × day effects ($P < 0.05$) within diet and across days. Error bars indicate SEM

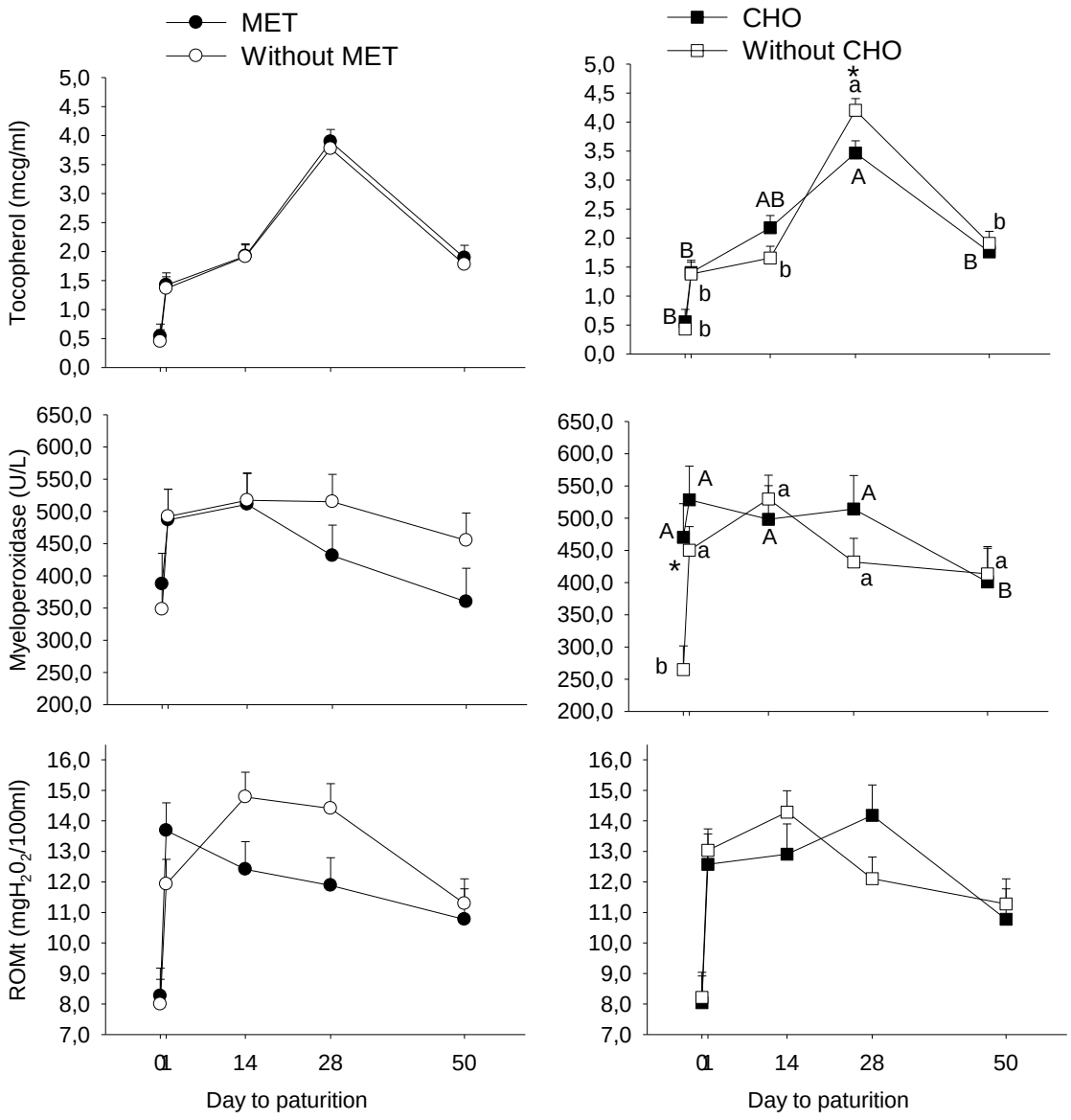

Figure 4: Expression of genes related to inflammation in the calves that received maternal supplement with rumen-protected Met, rumen-protected choline, or both (MIX). [a, b] and [A, B] Diet × day effects ($P < 0.05$) at a specific time point, *diet × day effects ($P < 0.05$) within diet and across days. Error bars indicate SEM

Supplemental file

Suppl. Table 1. Ingredient composition of diets fed during far-off (-50 to -21 d relative to calving) and close-up (-21 d to calving) periods offer to mother of the calves.

	Diets	
Ingredient (% of DM)	Far-off	Close-up
Alfalfa silage	12.00	8.34
Alfalfa hay	-	4.29
Corn silage	33.00	36.40
Wheat straw	36.00	15.63
Cottonseed	-	-
Wet brewers grains	-	4.29
Ground shelled corn	4.00	12.86
Soy hulls	2.00	4.29
Soybean meal, 48% CP	7.92	2.57
Expeller soybean meal[1]	-	2.57
Soychlor[2]	0.15	3.86
Blood meal, 85% CP	1.00	-
ProVAAl AADvantage[3]	-	0.86
Urea	0.45	0.30
Limestone	1.30	1.29
Salt	0.32	0.30
Dicalcium phosphate	0.12	0.18
Magnesium oxide	0.21	0.08
Magnesium sulfate	0.91	0.99
Mineral vitamin mix[4]	0.20	0.17
Vitamin A[5]	0.015	-
Vitamin D[6]	0.025	-
Vitamin E[7]	0.38	0.39
Biotin	-	0.35

[1]SoyPLUS (West Central Soy, Ralston, IA)
[2]By West Central Soy
[3]Perdue AgSolutions LLC (Ansonia, OH)
[4]Contained a minimum of 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5000 mg of Cu/kg, 250 mg of I/kg, 40 mg of Co/kg, 150 mg of Se/kg, 2200 kIU of vitamin A/kg, 660 kIU of vitamin D3/kg, and 7,700 IU of vitamin E/kg.
[5]Contained 30,000 kIU/kg
[6]Contained 5,009 kIU/kg
[7]Contained 44,000 kIU/kg

RNA extraction, Quality Assessment, and cDNA synthesis

Total RNA was extracted from the liver (40mg)using TRIZol regent combination with miRNeasy® Mini Kit (Cat. #217004, Qiagen). The liver tissue were completely homogenized in 1 mL TRIZol reagent (Invitrogen, Carlsbad, CA) using Beadbeater twice for each 30 sec. Each tube was added into 200 µL Chloroform and put in room temperature 3 min after shaking vigorously for 15 sec. The upper phase was transferred into a new collection tube without disturbing the mid and lower phase after 12,000 g at 4℃ for 15 min centrifuge. Ethanol 100% of 750 µL was added and mixed well. All amount of liquid supernatants were pipetted into a miRNeasy mini spin column in a 2 mL collection tube, then followed manufacturer's instructions of miRNeasy® Mini Kit. DNase I digestion mix (Cat. #79254, Qiagen) of 80 µL was added to each column to remove genomic DNA. Finally, 50 µL RNase free water were added to elute RNA and total RNA were obtained. The RNA concentration was measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The purity of RNA was assessed by ratio of optical density OD260/280, which were above 1.80 for all samples. The RNA integrity (RIN) was evaluated via electrophoretic analysis of 28S and 18S rRNA subunits using a 2100 Bioanalyzer (Agilent Technologies), and values were above 5.50 for all samples.

A portion of RNA was diluted to 100 ng/µL by adding DNase-RNase free water prior to cDNA synthesis. Complementary DNA was synthesized using 1 µL of 100 ng total RNA, 1 µL of Random Primers (Cat. #11034731001, Roche), and 9 µL of DNase/RNase-free water. The mixture was incubated at 65℃ for 5 min and kept on ice for 3 min. A total of 9 µL of master mix composed of 4 µL of 5X First-Strand Buffer (Cat. #18064-022, Invitrogen), 1 µL of Oligo dT18 (Operon Biotechnologies, Huntsville, AL), 2 µL of 10 mM dNTP mix (Cat. #18427-088, Invitrogen), 1.625

μL of DNase/RNase-free water, 0.25 μL (200U/μL) of Revert Aid Reverse Transcriptase (Cat. #EP0442, Thermo Scientific), and 0.125 μL (40U/μL) of RiboLock RNase Inhibitor (Cat. #EO0382, Thermo Scientific) were added. The reaction was performed in an Eppendorf Mastercycler Gradient following such temperature program: 25℃ for 5 min, 50℃ for 60 min, and 70℃ for 15 min. The cDNA was then diluted 1:4 with DNase/RNase-free water.

Primer Design and Evaluation

Primers were designed and evaluated as previously described (Bionaz and Loor, 2008). Briefly, primers were designed using Primer Express 3.0.1 (Applied Biosystems) with minimum amplicon size of 80 bp (amplicons of 100-120 bp were of superiority, if possible) and limited percentage of 3' G + C. Major part of primer sets were designed to fall across exon-exon junctions. Then, primers were aligned against publicly available databases using BLASTN at NCBI and UCSC's Cow (*Bos taurus*) Genome Browser Gateway to determine the compatibility of primers with already annotated sequence of the corresponding gene. Prior to qPCR, a 20 μL PCR reaction comprised of 8 μL dilute cDNA, 10 μL Power SYBR Green PCR Master Mix (Cat. #4367659, Applied Biosystems), 1 μL forward primer and 1 μL reverse primer was established to verify the primers. Of these, a universal reference cDNA amplified from all samples was utilized to ensure identification of desired genes. PCR product of 5 μL was run in a 2% agarose gel stained with SYBR Safe DNA Gel Stain (Cat. #S33102, Invitrogen), and the remaining 15 μL were cleaned with a QIAquick PCR Purification Kit (Cat. #28104, Qiagen) and sequenced at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana. The sequencing product was confirmed through BLASTN at NCBI database. Only primers

that did not present primer-dimer, 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 pair also was evaluated by the presence of a unique peak during the dissociation step at the end of qPCR. The biological functions of all target genes are presented in Supplemental Table 2. Supplemental Table 3 shows all designed primers in this study as well as all sequence information confirmed by BLASTN. Sequencing results for all genes are reported in Table 4.

Quantitative PCR (qPCR)

The qPCR was conducted in triplicate as described previously (Graugnard et al, 2009). Briefly, four microliters of diluted DNA (dilution 1:4) combined with 6 μL of mixture composed of 5 μL 1 × SYBR Green master mix (Cat. #4309155, Applied Biosystems), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL of DNase/RNase-free water were added in a MicroAmp™ Optical 384-Well Reaction Plate (Cat. #4309849, Applied Biosystems). A 6-point standard curve plus the nontemplate control (NTC) together with three replicates of each sample were run to detect the relative expression level. The reactions were conducted in ABI Prism 7900 HT SDS instrument (Applied Biosystems) following the conditions below: 2 min at 50℃, 10 min at 95℃, 40 cycles of 15 s at 95℃ (denaturation), and 1 min at 60℃ (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95℃ for 15 s, then 65℃ for 15 s. The threshold cycle (Ct) data were analyzed and transformed using the standard curve with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems). The final data were normalized with the geometric mean of the 3 ICGs, as reported previously (Moyes et al, 2010).

Relative mRNA Abundance of Genes within liver tissue

Efficiency of qPCR amplification for each gene was calculated using the standard curve method (Efficiency = $10^{(-1/slope)}$). Relative mRNA abundance among measured genes was calculated as previously reported (Bionaz and Loor, 2008), using the inverse of PCR efficiency raised to ΔCt (gene abundance = $1/E^{\Delta Ct}$, where ΔCt = Ct of tested gene – geometric mean Ct of 3 ICGs). Overall mRNA abundance for each gene among all samples of the same sample was calculated using the median ΔCt, and overall percentage of relative mRNA abundance was computed from the equation: 100 × mRNA abundance of each individual gene / sum of mRNA abundance of all the genes investigated. Supplemental Table 6 shows the qPCR performance among the genes measured in liver tissue.

Suppl. Table 2: Gene symbol, gene name, and description of the main biological function and biological process of the targets analyzed in calves' liver.

Symbol	Name	Summary description from NCBI
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
FATTY ACID OXIDATION AND GLUCOSE SIGNALING PATHWAY		
ALDH7	Aldehyde Dehydrogenase 7 Family	The protein encoded by this gene is a member of subfamily 7 in the aldehyde dehydrogenase gene family. These enzymes are thought to play a major role in the detoxification of aldehydes generated by alcohol metabolism and lipid peroxidation.
FGF21	Fibroblast Growth Factor 21	Theis gene encodes a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities and are involved in a variety of biological processes. This protein is a secreted endocrine factor that functions as a major metabolic regulator. The encoded protein stimulates the uptake of glucose in adipose tissue.
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 specificreceptors,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.
SLC2A2	Solute carrier family 2 (facilitated glucose transporter), member 2	This gene encodes an integral plasma membrane glycoprotein of the liver, is let beta cells, intestine, and kidney epithelium. The encoded protein mediates facilitated bidirectional glucose transport. It is the principal transporterfor transfer of glucose between liverand blood, and has a role in renal glucose reabsorption.
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.
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.

Suppl. Table 2 Continuation: Gene symbol, gene name, and description of the main biological function and biological process of the targets analyzed in calves' liver.

Symbol	Name	Summary description from NCBI
MET AND CHOL METABOLISM		
BHMT	betaine-homocysteine S-methyltransferase	This gene encodes a cytosolic enzyme that catalyzes the conversion of betaine and homocysteine to dimethylglycine and methionine, respectively.
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.
CDO1	Cysteine dioxygenase 1, cytosolic	It catalyzes the conversion from cysteine to cysteine sulfinic acid.
CHDH	Choline Dehydrogenase	The protein encoded by this gene is a choline dehydrogenase that localizes to the mitochondrion.
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.
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.
MAT2A	Methionine adenosyltransferase 2A	The protein encoded by this gene catalyzes the production of S-adenosylmethionine (AdoMet) from methionine and ATP. AdoMet is the key methyl donor in cellular processes.
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.
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.
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.
GLUCOCORTICOID RECEPTOR		
NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1	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
HISTONE ENZIME		
KAT2B	Lysine Acetyltransferase 2B	It has histone acetyl transferase activity with core histones and nucleosome core particles, indicating that this protein plays a direct role in transcriptional regulation.

Suppl. Table 2 Continuation: Gene symbol, gene name, and description of the main biological function and biological process of the targets analyzed in calves' liver.

Symbol	Name	Summary description from NCBI
CONTROL GENES		
UXT	Ubiquitously Expressed Prefoldin Like Chaperone	The protein encoded by this gene functions as a cofactor that modulates androgen receptor-dependent transcription and also plays a critical role in tumor necrosis factor-induced apoptosis.
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	The product of this gene catalyzes an important energy-yielding step in carbohydrate metabolism, the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide (NAD).
RPS9	Ribosomal Protein S9	This gene encodes a ribosomal protein that is a component of the 40S subunit.

Suppl. Table 3: GeneBank accession number, hybridization position, sequence and amplicon size of primers used to analyze gene expression by qPCR

Gene ID	Accession #	Gene	Primers[1]	Primers (5'-3')	bp[2]
338471	NM_177946.4	***PC***	F. 3577 R. 3700	GCAAGGTCCACGTGACTAAGG GGCAGCACAGTGTCCTGAAG	124
282855	NM_174737.2	***PCK1***	F:601 R:720	AAGATTGGCATCGAGCTGACA GTGGAGGCACTTGACGAACTC	120
537497	L36128.1	***ALDH7***	F.68 R.167	TCCGGCCTAACTCTCTCCTAA CCCTGGCCATAAGAACCAGAA	100
983000257	XM_005195429.3	***FGF21***	F. 223 R. 238	CAGAGCCCCGAAAGTCTCTTG AAAGTGCAGCGATCCGTACAG	106
281992	NM_001034036.1	***PPARA***	F.729 R.8300	CATAACGCGATTCGTTTTGGA CGCGGTTTCGGAATCTTCT	102
282357	NM_001103222.1	***SLC2A2***	F. 1359 R. 145	TTCAGCAACTGGACAGGCAAT AAGACCACACCAGCAAAAGGA	100
512468	NM_001083674.1	***GCLC***	F. 223 R. 328	TACGATCAGTTGGCTACC CCGAGTTCTATCATCTACAGA	106
506406	NM_001114190.2	***GSR***	F. 1319 R. 1418	CGCTGAGAACCCAGAGACTTG AAACGGAAAGTGGGAACAGTAAGTA	100
60687507	NM_001011679.1	***BHMT***	F. 002 R. 102	GCTCTCCTCGTCCATCCTCAT CCGTTCTAGGATGCCCTTCTT	100
514525	NM_001102000.2	***CBS***	F. 2349 R. 2448	GCCACCACCTCTGTCAAATTC GGACAGAAAGCAGAGTGGTAACTG	100
103472132	NM_001034465	***CDO1***	F. 319 R. 420	ATGGAAGCCTATGAGAGCAA TTCAGAAAGCAGTGGGAGTC	120
329663300	NM_001205564.1	***CHDH***	F. 1305 R.1307	AAACTGAGAAGTGCCAAC ACGGAAGTCTTTAATGTCA	103
982924854	XM_005206240.3	***CSAD***	F. 1169 R. 1259	GACACCTGCAACGTCCTCAAG CTGTGTCCAGAGCCACATCGT	91
114052193	NM_001046497.1	***MAT1A***	F.509 R..609	CAAGGGCTTTGACTTTAA CCGACATCCTCTTCATTT	100
155371988	NM_001101131.1	***MAT2A***	F. 825 R. 929	AATCTATCATCTACAGCCAAGTG CCAACGAGCAGCATAAGC	103
71892429	NM_001030298.1|	***MTR***	F. 809 R. 908	GTGCAGCTGAAATGAGACCTTTTA TCACCGAAGGTATTGGGAAGA	100
402693004	NM_182989.3	***PEMT***	F. 765 R. 867	TGTTCGTCCTTTCCAGTTTCCT CTGAACGGGAACATGGTCACT	103
77735582	NM_001034315.1	***SAHH***	F.887 R.995	TGTCAGGAGGGCAACATCTTT AGTGCCCAATGTTACACACAATG	109
331284121	NM_001206634.1	***NR3C1***	F. 395 R. 494	AAGCACCCCCAGTAGAGAAGAA CACAGTAGCTCCTCCCCTTAGG	100
982975327	XM_015461953.1	***KAT2B***	F.576 R.685	TCGAAGTCATTCATGGTCACCTT GACGACCCCATGGACTACAAGT	100

[1] Primer direction (F – forward; R – reverse) and hybridization position on the sequence.

[2] Amplicon size in base pair (bp).

Suppl. Table 6: qPCR performance among the genes measured in liver.

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]	Relative mRNA abundance[6]
PC	22.99	1.07	-3.69	1.00	1.87	0.51
PCK1	20.66	0.96	-3.36	1.00	1.98	0.52
ALDH7	22.80	1.06	-3.65	0.98	1.88	0.51
FGF21	29.23	1.36	-3.40	0.99	1.97	0.40
PPARA	20.97	0.98	-3.12	0.99	2.09	0.48
SLC2A2	20.98	0.98	-3.15	0.99	2.08	0.49
GCLC	22.03	1.03	-3.29	0.99	2.01	0.49
GSR	23.79	1.10	-3.35	0.99	1.99	0.47
BHMT	19.20	0.89	-3.37	0.98	1.98	0.54
CBS	23.46	1.09	-3.49	0.99	1.93	0.49
CDO1	20.75	0.97	-3.60	0.99	1.90	0.54
CHDH	27.09	1.26	-3.28	0.99	2.02	0.41
CSAD	25.12	1.18	-3.00	0.99	2.16	0.40
MAT2A	23.38	1.09	-3.34	0.99	1.99	0.47
MAT1A	22.96	1.07	-3.25	0.98	2.03	0.47
MTR	25.96	1.21	-3.10	0.99	2.10	0.41
PEMT	25.96	1.21	-2.87	0.99	2.23	0.38
SAHH	24.85	1.15	-3.33	0.98	2.00	0.45
NR3C1	24.00	1.12	-3.30	0.99	2.01	0.46
KAT2B	23.91	1.11	-3.20	1.00	2.05	0.45

[1] The median is calculated considering all time points and all cows.

[2] The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each cow.

[3] Slope of the standard curve.

[4] R^2 stands for the coefficient of determination of the standard curve.

[5] Efficiency is calculated as [$10^{(-1 / Slope)}$].

[6] Relative mRNA abundance is calculates as [1/ Efficiency $^{Median\ \Delta Ct}$], then [(relative mRNA abundance/∑relative mRNA abundance)*100]. mRNAs = 100% and microRNAs = 100%.

4.4 Artigo 4 – Dietary regulation of hepatic expression of genes related to inflammation: consumption of polyunsaturated fatty acids in successive generations of rats

O Artigo será apresentado no layout (model) disponibilizado pela revista *Nutrients,* para a qual será submetido

Article

Dietary regulation of hepatic expression of genes related to inflammation: consumption of polyunsaturated fatty acids in successive generations of rats

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Abstract: The consume during pregnancy of essential fatty acids, omega-3 and omega- 6, can benefit maternal and offspring health. They can in the liver mediate the inflammation pathway by the G-protein-coupled receptors (GPCRs). The present study aimed to investigate the effects of diets with different omega-3/omega-6 ratio (group OM3 and group OM6) consumed over three generations (G0, F1, and F2) on hepatic expression of genes related to inflammation. During three consecutive generations adult *Wistar* rats were evaluated in the prepartum (19 days) and postpartum period (21 days after parturition). The OM3 group had higher expression of Arb2, Tab1, Slc2a4. Also was observed a generation effect in the OM3 group with the decrease of NF-κB expression through generations, with low expression in F2 generation. Our results indicated that the polyunsaturated fatty acids (PUFA) effect on the modulation of genes related to GPR120 – Arrestin pathway through the generation.

Keywords: omega-3; Arb2; liver expression; PUFAs; maternal nutrition

PACS: J0101

1. Introduction

The nutrients crossing the placenta impact the fetal metabolism, and consequently fetal growth, thus the fetal development depends on the mother's metabolism and mother adaptation to different nutrients [1]. One of the most important and essential class of nutrient that the fetus obtains from maternal circulation by transfer across the placenta are the fatty acids (FAs), especially the essential fatty acids (EFAs); linoleic acid (LA, 18:2 n-6) and linolenic acid (ALA, 18:3 n-3), both have impacts on the fetal and postnatal development [2,3].

The modulation of the inflammatory response can be altered by FAs, which acts as endogenous ligands, but not all FAs work in the same way. Saturated FAs (SFAs) are proinflammatory, unsaturated FAs are weakly proinflammatory or neutral, and n-3 FAs can as anti-inflammatory molecules [4,5]. It was reported that omega-3 FAs reduce blood triglyceride levels [6] and it is prescribed for hypertriglyceridemia and the prevention of myocardial infarction [7]. Regular intake may reduce the

risk of a secondary or primary heart attack [8]. Also, it was already reported the benefits of polyunsaturated fatty acids consumption on hepatic fat metabolism [9] and tissue composition [10]. However, the anti-inflammatory effects of omega-3 FAs have not been clearly explained at the molecular level, several molecular targets have been suggested to explain the anti-inflammatory effects of omega-3 fatty acids, such as: PPARγ (peroxisome proliferator-activated receptor gamma, resolvins and their receptors, and G-protein-coupled receptors (GPCRs).

Recently, five GPCRs (GPR40, GPR43, GPR41, GPR84, and GPR120) were reported to recognize FAs [11,12]. GPR40 recognizes medium-long chain fatty acids like palmitic acid and linoleic acid [13]. GPR43 and GPR41 recognize short-chain fatty acids like acetate and butyrate [14] GPR84 recognizes the medium-chain fatty acid like lauric acid [15]. GPR120 has attracted much attention because it recognizes long-chain fatty acids, especially n-3 FAs and n-6 FAs [16,17]. However, several questions remain to be answered, especially the relationship between the activation of B-arrestin by GRP120, and pathways for activation of TAK1 and though TBA1 [18].

Activation of Toll-like receptors, such as, TLR-4, which are important in the activation of the innate immune system, can be mediated by omega-3 FA (n-3), which competes with lipopolysaccharide and saturated fatty acids. This ligation with n-3 FA will result in the suppression of NF-κB activation [19]. Furthermore, it has been suggested that omega-3 fatty acids could inhibit NF-κB in a PPARγ-independent manner under certain anti-inflammatory conditions [20].

Some investigations have evaluated the effect of prenatal nutrition with FAs on metabolic pathways and gene regulation from hepatic metabolism but with focus on the lipid metabolism and whether alterations could be transmitted between generations [21,22]. Apart from previously established evidence that dietary FAs have an effect on the regulation gene expression related to inflammation, the multi-generational effect of the n-3 and n-6 ratio during pregnancy on the offspring metabolism has not yet been established. Therefore, our objective are to evaluate the effect of diets with the different n-3/n-6 ratio in successive generations on the hepatic expression level of some target genes involved in inflammatory response

2. Materials and Methods

Experimental design

Male and female Wistar rats, 8 weeks old, were obtained from Central Vivarium/UFPel. Animals were housed individually in a temperature (21–23°C), humidity controlled (60–70%), 12:12 h light–dark cycling (lights from 6 a.m. to 6 p.m.) and free access to a pelleted diet and water. The diets were elaborated in accordance with AIN-93G recommendations (AIN-93G for growth, pregnancy and lactation – Table 1)[23] and the food intake was recorded daily. Every week a portion of the diet was randomly sampled for chemical analyses and fatty acid profile. The fat was isolated by the Bligh and Dyer method (Bligh and Dyer 1959) and the fatty acid profile analyzed by gas chromatography.

The founder generation (G0) was composed of 36 females that were randomly assigned to one of two groups: (1) rats fed with a diet elaborated with flaxseed oil, with a high n-3/ n -6 ratio (2.44/1) (OM3 Group, n = 18), and (2) rats fed with a diet rich in n-6 elaborated with soybean oil, with lower n -3/n-6 ratio (0.07/1) (OM6 Group, n = 18). During the experiment, the males received only the OM6 diet.

The animals were acclimatized to housing and diets for 30 days. Shortly thereafter, the females were mated in a male:female ratio of 1:3 for 3 days. Dam weight was recorded weekly. The number and weight of offspring were recorded at birth, and offspring development was assessed by weighing the group of pups weekly. From the G0 offspring, female progeny were sorted at weaning (21 days) to compose the F1 generation. Sixteen males were selected from the CON group. The animals were fed the diets for 60 days. From the F1 offspring, female progeny were selected at weaning (21 days) to compose the F2 generation.

A schematic design of the experimental groups can be found in Fig. 1. All females were evaluated for pregnancy rate, number of pups per litter and average weight at birth, the results are drescribed in Jacometo et al [9].

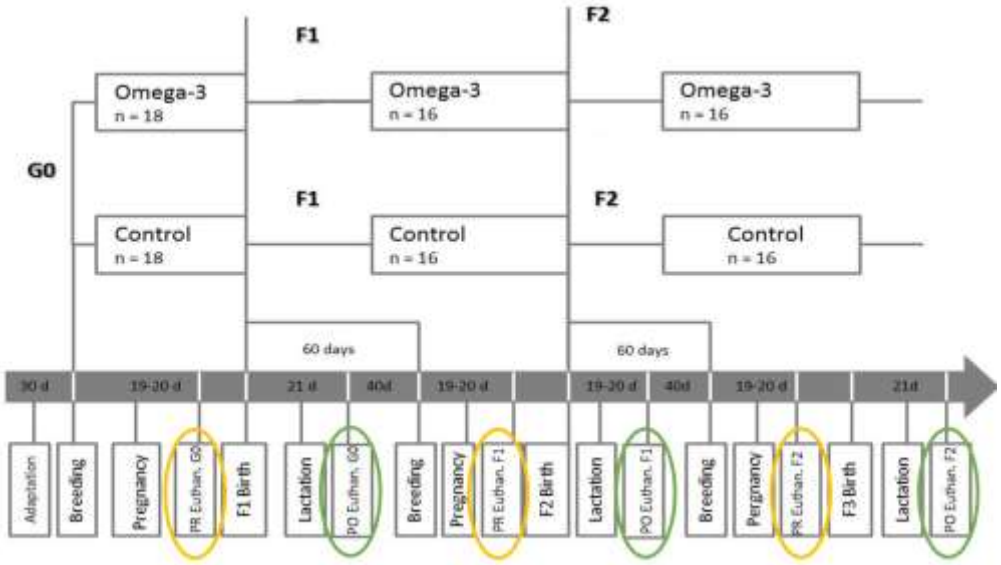

Figure 1. Schematic design of the groups, generation and all the experimental procedures. Yellow circle showed the pre-partum euthanasian and green circle the post-partum euthanasian

Tissue collection, RNA isolation and qRT-PCR analyses

Five female per group, with 19 days of gestation and 21 days postpartum were euthanized (according to the protocol approved by the University Animal Care and Use Committee) in each of the three generations (G0, F1 and F2). Immediately after euthanasia the liver was collected, stored in cryotubes and snap-frozen by submerging in liquid nitrogen. Total RNA was isolated from the liver samples using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was purified using RNeasy columns and on-column RNase-free DNase treatment (Qiagen, Germany), following the manufacturer's protocol.

The RNA concentration was measured using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The purity of RNA (A_{260}/A_{280}) for all samples was above 1.81. The quality of RNA was evaluated using the Agilent Bioanalyzer. system (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). The average RNA integrity number for samples was 7.0 ± 0.4.

For cDNA was synthesized using 100 ng RNA, 1 μg dT18 (Operon Biotechnologies, Huntsville, AL, USA), 1 μL 10 mmol/L dNTP mix (Invitrogen Corp., CA, USA), 1 μL random primer $p(dN)_6$ (Roche®, Roche Diagnostics GmbH, Mannheim, Germany), and 10 μL DNase/RNase free water. qPCR was performed using 4 μL diluted cDNA (dilution 1:4) combined with 6 μL of a mixture composed of 5 μL 1 × SYBR Green master mix (Applied Biosystems, CA, USA), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp®, Optical 384-Well Reaction Plate (Applied Biosystems, CA, USA). The gene primers selected were listeed in the Table 2. Actin beta (Actb) and Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were used as internal controls and the mean calculated and used to normalize data.

Statistical analyses

All the statistical analyses were performed PROC MIXED procedure of SAS 9.4 using SAS (SAS Institute Inc, Cary, NC, USA). The mRNA abundance considering the effects of diet, physiologic moment (pre-partum and post partum) generation, and its interactions. The gene expression results were log2-scale transformed if needed to comply with normal distribution of residuals. Least squares means separation was performed using the PDIFF statement. Statistical significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

Table 1. Ingredient composition of the experimental diets (g/kg diet).

Ingredient	AIN-93G (g/kg diet)	
	OM3	OM6
Cornstarch	407.15	397.48
Casein	200.00	200.00
Dextrinized cornstarch	132.00	132.00
Sucrose	100.00	100.00
Soybean oil	0.00	70.00
Flaxseed oil	60.33	0.00
Fiber	50.00	50.00
Mineral mix	35.00	35.00
Vitamin mix	10.00	10.00
L-Cystine	3.00	3.00
Choline bitartrate	2.50	2.50
Tert-butylhydroquinone	0.014	0.014

Table 1. Primers sequence used for qRT-PCR, source and accession number at NCBI site

Gene	Forward and Reverse primera (5′-3′)		#Acession
Arb2	CCACGTCACCAACAATTCTG	TTGGTGTCTTCGTGCTTGAG	NM_012911.1
Crp	GCAGTAGGTGGGCCTGAAAT	CCCGTCAAGCCAAAGCTCTA	NM_017096.3
Gpr120	CTGGCCGTCCCTTTTCTTCT	TGTTCCT CCACTCGCTCCTG	NM_001047088.1
Icam1	TGCTATATGGTCCTCACCTG	GATCATGGTACAGCACTGTCA	NM_012967.1
Igf1	GACCCGGGACGTACCAAAAT	GAACTGAAGAGCGTCCACCA	NM_001082477.2
Il1b	ATCCCAAACAATACCCAAAG	GAACTGTGCAGACTCAAACTCC	NM_017183.1
Il10	AGCCAGACCCACATGCTCCGA	ACAGGGGAGAAATCGATGACAG	NM_012854.2
Jnk1	CTTTTCCAGCACATCACCGC	AAGCATCAGTACTCGGCAGG	NM_00100738.1
NF-κB	AGTGACAGCGACAGTGACAA	TCATCAGGAAGAGGTTTGGCTGC	NM_001276711.1
Scl2a4	GGGCTGTGAGTGAGTGCTTTC	CAGCGAGGCAAGGCTAG	NM_012751.1
Scos1	CAGTGGCTAGAAAAGCAAG	AGAGCAGGTCATGGAAGCGGAT	NM_001173374.1
Stat1	GGGAAGTACTACTCCAGGCC	GGAGACATGGGAAGAGGTT	NM_032612.3
Stat3	CAAAGAAAACATGGCCGGCA	GGGGGCTTTGTGCTTAGGAT	NM_012747.2
Tab1	GTCAGGCTCTGACTTGGACC	TGATGGGAGGGCTCAGGTAA	NM_001009976.2
Tfg	GCAGGTCAGATTGAAGGTCAG	TGGTTGTTGGCCATATCCCT	NM_001012144.1
Tlr2	GCCGTCTTCAATCTGACTAAT	ACACTGACCACCGATACACT	NM_198769.2
Tlr4	CCCAATCTGTGTCCTTCTAACT	CACTACTTCAGCGTCTCGTGT	NM_019178.1
Tnfα	AACCCACAGATCCAGCACA	TACCCCAGAATCAGCACCGA	NM_012675.3
INTERNAL CONTROL GENES			
Gpdha	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	NM_017008.B
Actβ	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA	NM_031144.3

3. Results

The gene expression of physiologic moments is showed in the Table 3. The gene expression of Gpr120, Cpr, Igf-1, Il1β, Scos1, Tfg, Tlr4 was higher in the pre-partum moment ($P < 0.05$), in the other way only TLR2 had higher expression ($P = 0.0001$) in the post-partum moment.

The group effect is showed in the Table 4. All genes with significate difference ($P < 0.05$), had a higher expression in the OM3 group (Arrb2, Icam1, Igf1, Il1b, Jnk1, Scl2a4, Tab1).

Also in the Table 4 are show the interaction in the group and generation. At the generation GO, the gene expression of Arb2, Jnk1, Sco1, Stat1 was higher in the OM3 group ($P < 0.05$). In the generation F1, the gene expression of Arrb2, Scos1, Tlr4 was higher in the OM3 group ($P < 0.05$). In the other way, the generation F2, the was observe a higher expression in OM6 group to Sco1 and Tlr4 ($P < 0.05$).

The generational effect was observed in the OM3 group to Arb2, NF-κB, Stat1, these genes had a lower expression in the last generation analyzed (F2) compared with the other (F1 and G0) ($P < 0.05$). In the OM6 group the Grp120 increase between the generation, with higher expression in the F2 ($P < 0.05$), in contrat, Scos1 had lower expression in the F2 ($P < 0.05$). The TLR4 gene expression of in the OM6 was at its minimum level expression in the generation F1 ($P < 0.05$).

The figures 2, 3, 4 and 5 show the interaction between group, generation, and physiologic moment. In the pre-partum moment, the Arb2, Tab1, Scos1, NF-κB 1 and Cpr was higher in OM3 group in the generation GO ($P < 0.05$). At the generation F1 only was observed an effect to NF-κB, with higher expression in OM6 group ($P < 0.05$). At the F2, was observed a higher expression of Tab1, Tnf, Il1β, and Scl2 in OM3 group ($P < 0.05$).

The post-partum moment showed the differences in GO to Scos1 and Icam1, both with higher expression in OM3 group ($P < 0.05$). At generation F1, the expression of Arb2, NF-κB , Scl2a4, Il1β, Scos1, Gpr, Igf1 was higher in OM3 group ($P < 0.05$), only TLR2 was higher in OM6 ($P < 0.05$). At generation F3, the expression of NF-κB, Tnf, Slc2a4, Stat3 and Soc1 was higher in the OM6 group ($P < 0.05$).

Table 3. mRNA expression (R. U.) in the pre-partum and post-partum moment

	MOMENT			P value
GENES	PRE-PARTUM	POST-PARTUM	SEM[1]	MOMENT
Arb2	1.301	1.349	0.0703	0.8952
Cpr	1.476^{A}	1.120^{B}	0.109	0.0058
Gpr120	1.897^{A}	1.001^{B}	0.0603	<.0001
Icam1	0.843	0.992	0,063	0.0985
Igf1	1.689^{A}	1.282^{B}	0.072	<.0001
Il1β	2.876^{A}	1.662^{A}	0.192	<.0001
Il10	3.392	3.744	0.416	0.5820
Jnk1	1.364	1.400	0,044	0.7099
Nf-κb 1	1.483	1.339	0.111	0.2499
Scl2a4	3.037	3.059	0.169	0.9416
Soc1	1.293^{A}	1.147^{B}	0.039	0.0022
Stat1	1.223	1 .079	0.064	0.0890
Stat3	1.527^{A}	1.169^{B}	0.082	0.0014
Tab1	1.958	1.841	0.108	0.2725
Tfg	2.424^{A}	1.556^{B}	0.130	<.0001
Tlr2	0.530^{B}	1.500^{A}	0.154	0.0001
Tlr4	1.867^{B}	1.200^{B}	0.103	<.0001
Tnfα	1.773	1.719	0.133	0.6969

1Greatest SEM: Stander error of means; A and B means values with different superscripts differ in MOMENT effect $P < 0.05$.

Table 4. mRNA expression (R. U.) of OM6 and OM3 groups, and the mRNA expression during the generations

	GROUP			GO		F1		F2			P value			
GENES	OM6	OM3	SEM[1]	OM6	OM3	OM6	OM3	OM6	OM3	SEM	GROUP	GROUP *GEN	GROUP *MOMENT	GROUP* MOM*GEN
Arb2	1.212^{A}	1.437^{B}	0.0691	1.095^{b}	1.429^{aYW}	1.217^{b}	1.658^{aY}	1.325	1.226^{W}	0.122	0.0168	0.0767	0.9682	0.0664
Crp	1.300	1.296	0.107	1.284	1.635	1.324	1.471	1.665	1.344	0.104	0.9368	0.3115	0.4944	0.1857
Gpr120	1.424	1.483	0.0593	1.111^{Y}	1.408	1.247^{YW}	1.038	1.542^{W}	1.444	0.189	0.5398	0.0009	0.8328	0.0132
Icam1	0.836^{B}	0.999^{A}	0.062	0.857	1.266	0.918	1.035	0.732	0.695	0.108	0.0387	0.3642	0.5339	0.0008
Igf1	1.334^{B}	1.637^{A}	0.070	1.202	1.551	1.391	1.595	1.408	1.765	0.123	0.0026	0.9163	0.9619	0.0688
Il1β	2.127^{B}	2.411^{A}	0.194	2.206	2.325	2.131	2.487	2.044	2.422	0.358	0.0330	0.3657	0.2390	0.0012
Il10	3.350	3.786	0.416	3.247	3.397	2.874	4.539	3.929	3.422	0.739	0.2781	0.3428	0.0412	0.1645
Jnk1	1.307^{B}	1.457^{A}	0.044	1.268^{b}	1.588^{a}	1.335	1.532	1.318	1.250	0.079	0.0144	0.0447	0.9329	0.2201
Nf-κb 1	1.412	1.409	0.112	1.223	1.496^{W}	1.408	1.562^{W}	1.604	1.169^{Y}	0.207	0.7803	0.0647	0.1213	0.0006
Scl2a4	2.813^{B}	3.283^{A}	0.164	2.552	3.446	2.993	3.508	2.894	2.896	0.290	0.0128	0.7974	0.3838	<.0001
Socs1	1.183	1.257	0.037	1.050^{bW}	1.427^{a}	1.166^{bYW}	1.320^{a}	1.331^{bY}	1.025^{a}	0.070	0.0891	<.0001	0.5440	<.0001
Stat1	1.124	1.179	0.067	0.909^{b}	1.318^{bW}	1.144	1.239^{WY}	1.319	0.979^{Y}	0.116	0.3442	0.0064	0.7632	0.9679
Stat3	1.400	1.296	0.081	1.194	1.264	1.469	1.341	1.538	1.283	0.142	0.5473	0.3328	0.4939	0.0253
Tab1	1.717^{B}	2.082^{A}	0.106	1.542	2.155	1.884	1.871	1.726	2.221	0.186	0.0072	0.3026	0.8540	0.0313
Tfg	2.008	1.972	0.127	2.275	2.125	1.924	1.812	1.825	1.979	0.224	0.4223	0.4886	0.9554	0.2257
Tlr2	1.210	0.820	0.154	1.162	1.182	1.809	0.523	0.659	0.756	0.266	0.1288	0.0993	0.0454	0.0499
Tlr4	1.542	1.525	0.102	1.543^{YW}	1.659	1.355^{bY}	1.668^{a}	1.728^{aW}	1.249^{b}	0.179	0.8087	0.0237	0.1932	0.1888
Tnfα	1.667	1.825	0.135	1.214	1.779	1.673	1.679	2.115	2.018	0.249	0.5830	0.1831	0.4608	0.0001

1 Greatest SEM: Stander error of means; A and B means values with differec in diet effect. a b means values with different of the diet in the generation. Y, W means with diference in the same group throuth the generations.

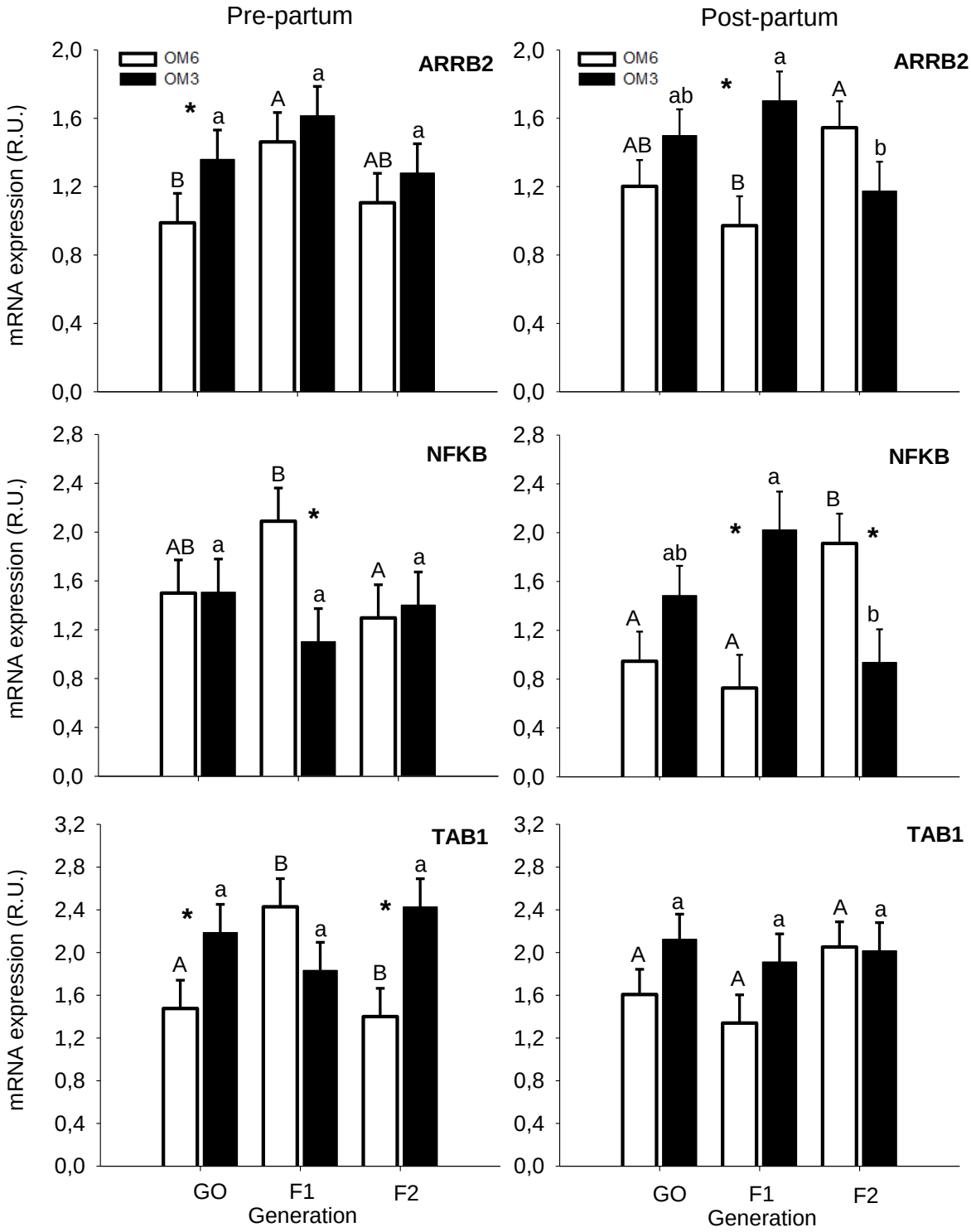

Figure 2. mRNA expression (R. U.) of OM6 and OM3 groups, in G0, F1 and F2 for ARRB2, NF-KB , TAB1. Capital letters indicate differences between generations in the OM6 group ($P < 0.05$). Lowercase letters indicate differences in the OM3 group ($P < 0.05$). The symbol * indicate differences between the groups in the same generation ($P < 0.05$).

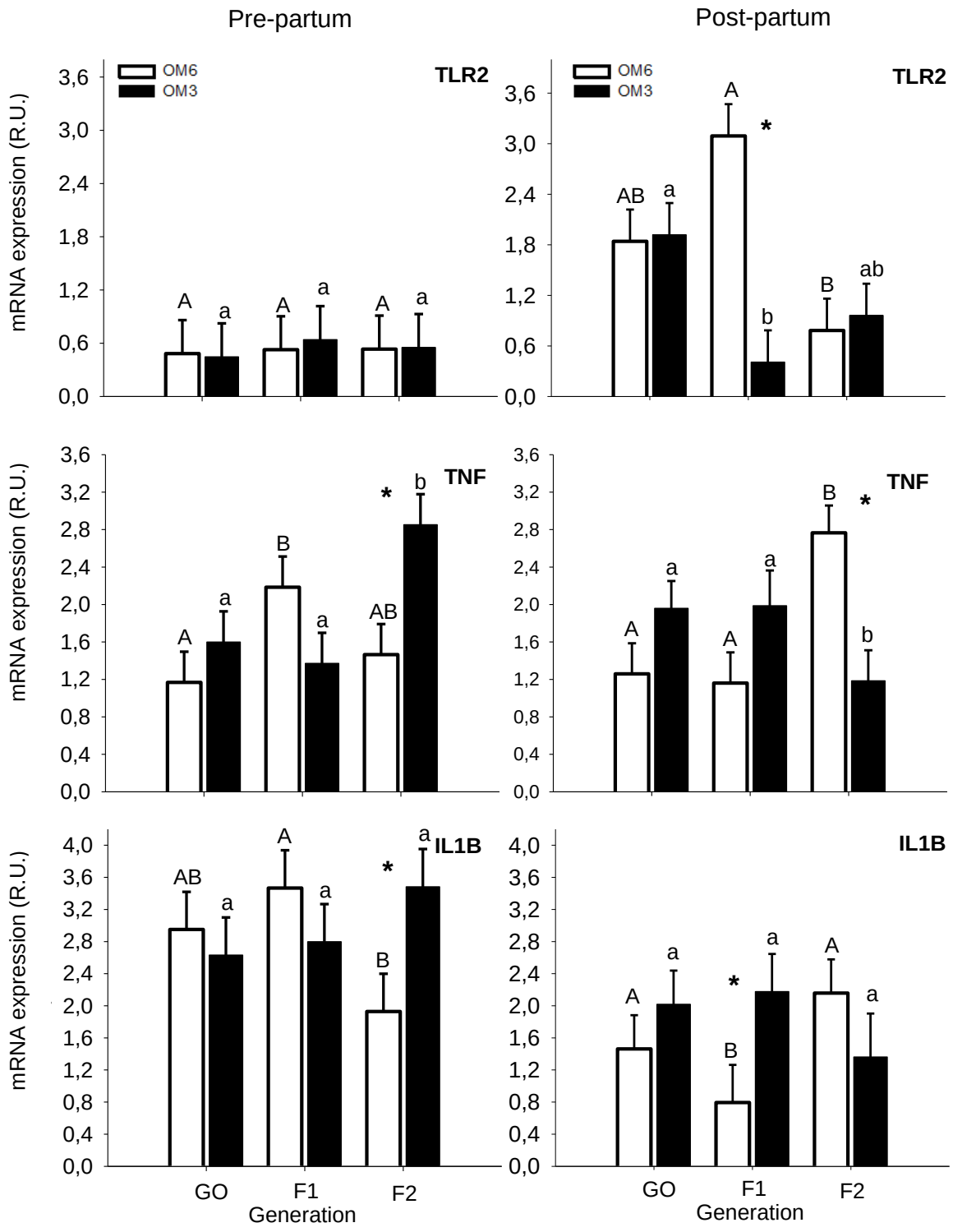

Figure 3. mRNA expression (R. U.) of OM6 and OM3 groups, in G0, F1 and F2 for TLR2, TNF, IL1B. Capital letters indicate differences between generations in the OM6 group ($P < 0.05$). Lowercase letters indicate differences in the OM3 group ($P < 0.05$). The symbol * indicate differences between the groups in the same generation ($P < 0.05$).

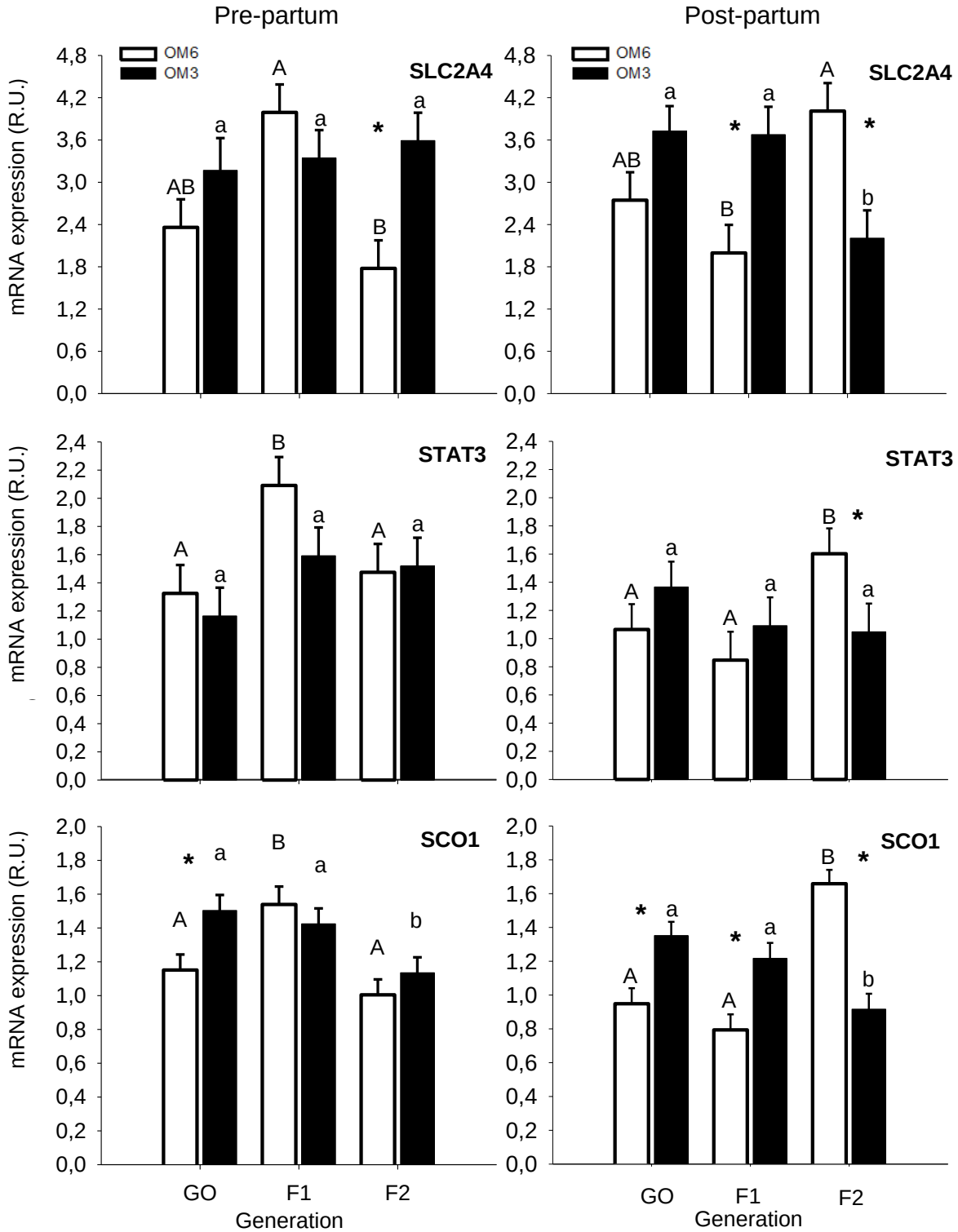

Figure 4. mRNA expression (R. U.) of OM6 and OM3 groups, in G0, F1 and F2 for SCL2A4, STAT3, SCO1. Capital letters indicate differences between generations in the OM6 group ($P < 0.05$). Lowercase letters indicate differences in the OM3 group ($P < 0.05$). The symbol * indicate differences between the groups in the same generation ($P < 0.05$).

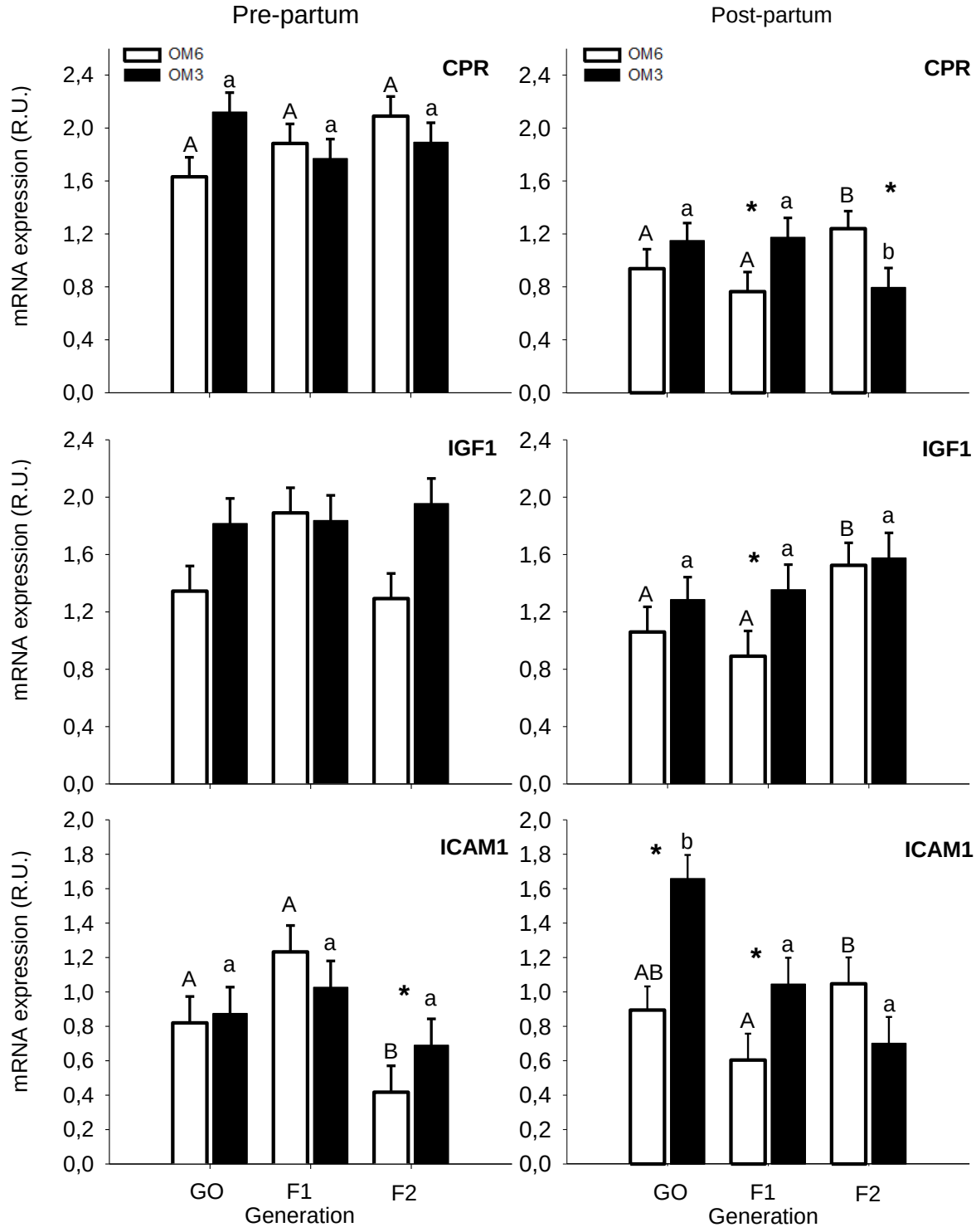

Figure 5. mRNA expression (R. U.) of OM6 and OM3 groups, in G0, F1 and F2 for CRP, IGF-1, ICMA1. Capital letters indicate differences between generations in the OM6 group ($P < 0.05$). Lowercase letters indicate differences in the OM3 group ($P < 0.05$). The symbol * indicate differences between the groups in the same generation ($P < 0.05$).

4. Discussion

Omega-3 (n-3) polyunsaturated fatty acids are known to modulate hepatic gene expression resulting in increased β-oxidation and decreased lipogenesis, effects mediated through Ppar-α and its target genes [24], also can control expression of genes involved in glucose and lipid metabolism [25]. In this paper, we evaluated the expression of inflammatory genes modulated by the PUFA, and we detected a direct effect o n-3 PUFA in this genes, especial in the expression of Arb2. High intakes of n-3 FAs during the life showed a higher expression in the generation G0 and F1 compared with OM6 group. β-Arrestins are important regulators of G-protein-coupled receptors (GPCRs)[26]. In a series of gene silencing experiments, OH and colleagues [16,27] demonstrate that β-Arrestins is essential for the anti-inflammatory effects of n-3 fatty acids in macrophage cells. Also, Arb2 inhibits both the Jnk1 and Nf-κb1pathways by sequestering the Tak1 binding protein Tab1. The inhibition of Tab1 prevents phosphorylation and thus activation of Nf-κb1.

Our results agree with the OH findings to the gene of the TAB1 in OM3 group, its expression was higher in this group, especially in the GO and F2 generation at the prepartum time. However, the gene expression of Jnk1 and Nf-κb1demonstrated that there was not a complete inhibition of the inflammatory pathway since Jnk1 had greater expression in OM3. Nf-κb1 did not present general group effect, however, the expression decreased over the generations in OM3, and in the F2 generation the expression was lower than OM6. These results demonstrate that Nf-κb1 has been controlled by n3-PUFA diet over the generations. This anti-inflammatory effect of n 3 PUFAs has been reported elsewhere, when n-3 PUFAs antagonize the NF-B signaling pathway, and inhibit the expression of inflammatory genes downstream of Nf-κb1[28].

The Nf-κb1 also has a relation with the gene expression of Icam-1. Inflammation-induced Icam-1 expression has been linked to activation of the transcription factor, nuclear factor kappa B (NF-κB1), by various inflammatory stimuli (ie, lipopolysaccharide [LPS], tumor necrosis factor-alpha [TNFα], and interleukin -1 beta [IL-1β]) [29]. The highest expression in the OM3 group of the Icam-1 gene is due to the high levels of expression in the GO on the postpartum moment. This result was not expected, once that the omega-3 supplementation decreases Icam-1 expression wholly or partially through suppression of Nf-κb1activation [30].

The greater expression of Icam-1 in the OM3 group in the F2 generation, at the pre-partum time, is directly linked to the greater expression of the proinflammatory cytokines IL1b and Tnfα [29], in the OM3 group at this same time. However we did not find any factors Which might have led to this change in the expression of cytokines in the OM3 group since a decrease in them was expected as observed by other authors [31].

The effect of supplementation with OM3 increasing Igf-1 expression is equal to the result found by Dirandeh et al. [32], that dairy cows supplemented with OM3- showed higher expression of Igf-1, and Ghr1A, demonstration of coupling of somatic axis was maintained in this animals

Insulin resistance during the peripartum is considered an inflammatory condition [33] Epidemiological studies have reported a low prevalence of impaired glucose tolerance in populations consuming large amounts of n-3 PUFA such as the Greenland Inuit and Alaskan natives [34]. Our results suggest this because of increased expression of Slc2a4 (Glut 4). These cytokines and chemokines induce activation of transcription factors such as Nf-κb1causing both the down-regulation and decreased activation of insulin signaling proteins (Glut4 and Irs-1), which blocks insulin action and causes a state of insulin resistance [35]. The diets enriched with n-3 PUFA was shown to maintain Glut4 content, insulin receptor, IRS-1 tyrosine phosphorylation, and have a lower pro-inflammatory condition[36].

The expression of Gpr120 did not show any difference between the groups, contrasting several studies that show that diets rich in Gpr120 increased expression of Gpr120[16,18,37,38]. Increased expression of Gpr120 in the OM6 group, across generations, being higher in the last generation (F2), may suggest a compensatory effect, or other mechanisms that have influenced the expression of Gpr120.

5. Conclusions

Supplementation with n-3 omega in the generation of rats been associated with favorable gene changes involved in inflammatory pathways as Arb2 and Tab1, suggested an intrauterine programming of gene expression. More study involving DNA methylation needs to be elaborated to elucidate the effects of diets rich in omega 3 in this pathway and the effect omega3 in Jnk1- pathway

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5 CONCLUSÃO GERAL

- O Indicie de funcionalidade hepática é uma fermenta útil no sistema semi-extensivo, em que animais com baixo LFI apresentaram atraso na retomada da atividade ovariana, bem como mudanças mais intensas nas proteínas de fase aguda.
- Animais que receberam MET ou COL apresentaram uma adaptação mais rápida as mudanças observadas durante o periparto. Não foi observado efeito da suplementação com metionina ou colina no metabolismo da glutationa em PMN.
- A suplementação materna com metionina e colina modificou a expressão hepática, dando destaque para a genes MAT2, MTR e BHMT principalmente no dia 4.
- A suplementação com ácidos graxos de cadeia longa demostrou uma modulação dos genes envolvidos na rota do GPR120. Especialmente, a suplementação com ômega 3, proporcionou maior expressão do gene Arb2 em duas gerações consecutivas, e os animais que receberam ômega 6 apresentaram maior expressão do GPR120 na terceira geração.

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