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Tese

Efeitos da condição corporal e suplementação materna com fontes de cobalto, ácido fólico e metionina no controle imunometabólico de bezerros neonatos.

Matheus Gomes Lopes

Pelotas, 2021

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Para minha família e amigos, com carinho e gratidão.

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“No matter how right or wrong you have gone, you can always come home”

Alan Jackson

Resumo

LOPES, Matheus. **Efeitos da condição corporal e suplementação materna com fontes de cobalto, ácido fólico e metionina no controle imunometabólico de bezerros neonatos.** 2021. 126f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

O manejo da condição corporal das vacas leiteiras durante o período seco, pode alterar a disponibilidade de nutrientes para o feto nos estágios finais de crescimento no ambiente uterino. Além disso, a exposição materna a diferentes estressores metabólicos no final da gestação, pode contribuir para a desregulação imunológica na prole e influenciar a suscetibilidade a doenças. Por outro lado, a inclusão de doadores de grupamento metil na dieta materna, pode proporcionar respostas positivas no metabolismo energético, inflamação, status antioxidante e desempenho da progênie. O objetivo desta tese foi avaliar os efeitos da condição corporal e da suplementação materna com doadores de grupamento metil durante o final da gestação, sobre parâmetros imunológicos e metabólicos de bezerros neonatos. Para compreender a influência do escore de condição corporal (ECC) materno na função imunológica neonatal, em um primeiro estudo avaliamos a expressão gênica em sangue total de bezerros nascidos de uma coorte maior de vacas e divididos retrospectivamente de acordo com o ECC materno pré-parto (4 semanas) em ECC normal (≤ 3.25; n = 22; NormBCS) ou ECC alto (≥ 3.75; n = 16; HighBCS), por meio de um desafio ex vivo com lipopolissacarídeo (LPS) ao nascimento, 21 e 42 dias de idade. Os genes avaliados por RT-qPCR eram associados à resposta imune, função antioxidante e metabolismo de 1-carbono. A regulação positiva de genes associados com a resposta inflamatória e antioxidante em bezerros gerados por vacas com ECC alto, sugeriu uma maior estimulação imunológica nestes animais. Ainda, a diferença na regulação de genes relacionados ao metabolismo de 1-carbono nos bezerros gerados por vacas com ECC alto, destacou um potencial papel dos doadores de grupamento metil na resposta imune inata dos neonatos. Desta forma, em um segundo estudo, utilizando um desafio ex vivo com LPS e bactérias enteropatogênicas, avaliamos a resposta imune inata no sangue total de bezerros nascidos de vacas suplementadas durante os últimos 30 dias de gestação, com duas fontes diferentes de cobalto [cobalto glucoheptonado (CoPro) ou cobalto pectina (CoPectin)], ácido fólico (FOA) e metionina protegida da degradação ruminal (RPM). Os grupos (n = 12 bezerros/grupo) eram divididos em CoPro, FOA+CoPro, FOA+CoPectin e FOA+CoPectin+RPM. Nossos resultados demonstraram que a suplementação materna modulou alguns biomarcadores do sistema imunológico inato em níveis plasmáticos e moleculares, destacando-se uma menor concentração plasmática de haptoglobina em bezerros nascidos de vacas alimentadas com ácido fólico adicional. Além de uma regulação diferencial de genes relacionados à inflamação e ao reconhecimento de patógenos. A ausência de efeitos na grande maioria dos genes avaliados e na capacidade funcional das células imunes, sugere que o incremento destes nutrientes na dieta materna exerceu pouca influência sobre o controle imunometabólico da prole.

Palavras-chave: Doadores de metil, Estresse metabólico materno, Imunologia neonatal, Nutrigenômica, Programação nutricional.

Abstract

LOPES, Matheus. **Effects of body condition and maternal supplementation with cobalt sources, folic acid, and methionine on immunometabolic control of neonatal calves.**. 2021. 126f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Managing body condition in dairy cows during the dry period can alter the availability of nutrients to the fetus in the final stages of growth in utero. Furthermore, maternal exposure to different metabolic stressors at late pregnancy might contribute to immune dysregulation in offspring and influence disease susceptibility. On the other hand, the inclusion of methyl donors in the maternal diet can provide positive responses in energy metabolism, inflammation, antioxidant status, and offspring performance. The aim of this thesis was to evaluate the effects of body condition and maternal supplementation with methyl donors during late pregnancy on immunological and metabolic parameters of newborn calves. To understand the influence of maternal body condition score (BCS) on neonate immune function, in a first study we evaluated gene expression in whole blood of calves born from a larger cohort of cows and retrospectively divided according to prepartum maternal BCS (4 weeks) in normal BCS (≤ 3.25; n = 22; NormBCS) or high BCS (≥ 3.75; n = 16; HighBCS), through an ex vivo lipopolysaccharide (LPS) challenge at birth, 21, and 42 days of age. The genes evaluated by RT-qPCR were associated with immune response, antioxidant function, and 1-carbon metabolism. Upregulation of genes associated with inflammatory and antioxidant response in calves born from cows with high BCS suggested greater immune stimulation in these animals. Furthermore, the differential regulation of genes related to 1-carbon metabolism in calves born from cows with high BCS underscored the potential role of methyl donors in the innate immune response of neonates. Thus, in a second study, through both an ex vivo LPS and enteropathogenic bacteria challenges, we evaluated the innate immune response in whole blood of calves born from cows supplemented during the last 30 days of pregnancy with two different cobalt sources [cobalt glucoheptonate (CoPro) or cobalt pectin (CoPectin)], folic acid (FOA), and rumen-protected methionine (RPM). Groups were (n = 12 calves/group) CoPro, FOA+CoPro, FOA+CoPectin, and FOA+CoPectin+RPM. Our results underscored that the maternal supplementation modulated few biomarkers of the innate immune system at plasma and molecular levels, e.g. lower plasma concentration of haptoglobin in calves born from cows fed with additional folic acid. These calves also had differential regulation of genes related to inflammation and pathogen recognition. The lack of effects in the most evaluated target genes and on the functional capability of immune cells suggests that the increased maternal supply of these nutrients had little impact on immunometabolic control of the offspring.

Keywords: Maternal metabolic stress, Methyl donors, Neonatal immunology, Nutrigenomics, Nutritional programming.

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Lista de Abreviaturas

APC – *Antigen-Presenting Cells* (Células Apresentadores de Antígenos)

B12 – Vitamina B12

BEM – Balanço Energético Negativo

BHMT – Betaína Homocisteína Metiltransferase

CBS – Cistationina β-Sintase

Co – Cobalto

ECC – Escore de Condição Corporal

FAO – Organização das Nações Unidas para a Alimentação e a Agricultura

GSH – Glutationa

MAT – Metionina Adenosiltransferase

MTR – Metionina Sintase

PAMP – *Pathogen-Associated Molecular Patterns* (Padrões Moleculares Associados a Patógenos)

PC – *Phosphatidylcholine* (Fosfatidilcolina)

PEMT – *Phosphatidylethanolamine Methyltransferase* (Fosfatidiletanolamina Metiltransferase)

PRR – *Pattern Recognition Receptors* (Receptores de Reconhecimento de Padrões)

SAH – S-Adenosil-Homocisteína

SAHH – S-Adenosil-Homocisteína Hidrolase

SAM – S-Adenosil-Metionina

Sumário

1 INTRODUÇÃO GERAL

Com a crescente demanda mundial por alimentos e proteína de alta qualidade, o setor leiteiro representa uma parcela significativa na contribuição para o suprimento destes requisitos (Adesogan & Dahl, 2020). Considerado um alimento completo, o estímulo ao consumo de leite e seus derivados se tornou uma medida importante no combate à desnutrição e subnutrição em nível global (Sipple et al., 2020). Segundo dados da Organização das Nações Unidas para a Alimentação e a Agricultura (FAO), a produção mundial de leite atingiu 906 milhões de toneladas em 2020, um aumento de 2,0% em relação ao ano de 2019 (FAO, 2021). Estes números, foram impulsionados por uma produção crescente em todas as regiões geográficas do planeta, onde o Brasil ocupa a quarta posição no ranking, com uma produção estimada de 36,7 milhões de toneladas em 2020, ou seja, uma representatividade de aproximadamente 4,0% do volume total de leite produzido no mundo (FAO, 2021).

Com o intuito de dar suporte a este cenário positivo, os sistemas de produção desempenham diariamente um papel imprescindível nesta cadeia e precisam estar atentos aos processos fundamentais que compõem a gestão de uma propriedade leiteira (Evink & Endres, 2017). Dentre estes processos, a nutrição do rebanho representa a parcela mais significativa nos centros de custos de uma propriedade e, portanto, medidas que contribuam para incrementar a assertividade nas formulações das dietas em diferentes categorias, são consideradas estratégias essenciais para proporcionar rentabilidade e longevidade aos produtores (VandeHaar & St-Pierre, 2006). Além disso, o adequado manejo dos lotes, considerando as especificidades de cada estágio da vida produtiva dos animais, nos permite a personalização nas tomadas de decisões e, consequentemente, uma maior eficácia das alternativas propostas.

Atualmente, uma visão empresarial dos sistemas produtivos, possibilitou uma visão holística dos sistemas de criação, onde a negligência em quaisquer das categorias de animais, pode refletir em sérias consequências futuras (Van Niekerk et al., 2021). Desta forma, considerar que “a bezerra de hoje, será a vaca do amanhã”, é uma conhecida frase que, de certa forma, já foi atualizada para “o embrião de hoje, será a vaca do amanhã”. Visto que, o avanço das tecnologias laboratoriais, em nível molecular e celular, nos permitiu visualizar que estratégias e situações oferecidas para

estas bezerras ainda durante o desenvolvimento embrionário e fetal, podem contribuir para reflexos na vida pós-natal e por todo o ciclo produtivo (Loor et al., 2013). Por exemplo, a exposição materna a diferentes estressores metabólicos e ao estresse oxidativo durante o final da gestação, pode contribuir para a desregulação imunológica na prole e influenciar a suscetibilidade a doenças durante o período neonatal (Hodyl et al., 2008; Beloosesky et al., 2010; Ling et al., 2018). Por outro lado, o oferecimento de uma nutrição balanceada, com o adequado equilíbrio entre micro e macronutrientes, durante todo o processo gestacional, é um fator chave para o controle imunometabólico do feto e favorece o adequado desenvolvimento da prole após o nascimento (Van Niekerk et al., 2021; Jacometo et al., 2016; Jacometo et al., 2015; Jacometo et al., 2018).

Estudos focados no entendimento de como diferentes estressores metabólicos maternos, por exemplo, inadequada condição corporal pré-parto, podem influenciar na imunidade e metabolismo da progênie; assim como, a identificação de nutrientes funcionais capazes de serem incluídos na dieta materna, com efeitos de longo prazo na fisiologia e imunometabolismo da prole têm sido alvos de substancial atenção dos pesquisadores em todo o mundo ao longo das últimas décadas (Roche et al., 2009; Ling et al., 2018; Coleman et al., 2020; Coleman et al., 2021). Com o aprimoramento dos métodos moleculares, uma importante área de estudo denominada “programação nutricional”, tem permitido um melhor entendimento de estratégias que podem modular as rotas moleculares e metabólicas do bezerro durante a vida uterina (Coleman et al., 2021). Além disso, a pesquisa de alternativas que potencializem o sistema imunológico em bezerros neonatos, ainda durante a vida fetal, faz parte deste campo de estudo e vem proporcionando resultados promissores através de diferentes estratégias de intervenção, como medidas de manejo operacional e nutricional no período pré-parto (Abuelo, 2020; Ling et al., 2018; Coleman et al., 2021; Lopes et al., 2019). Tais abordagens atuam sobre mecanismos epigenéticos, capazes de modular o fenótipo em decorrência de alterações no aporte de determinados nutrientes durante a gestação (Reynolds et al., 2017). Assim, as estratégias de manejo, possibilitam garantir o aporte adequado de nutrientes ao feto e, consequentemente, facilitar as respostas do bezerro aos desafios nos primeiros dias de vida (Ling et al., 2018; Coleman et al., 2021).

O metabolismo dos nutrientes essenciais ácido fólico e metionina é fundamental para a produção de homocisteína e S-adenosilmetionina (SAM), o principal doador de grupamentos metil (Preynat et al., 2010). Os ciclos do ácido fólico e da metionina estão ligados por meio da atividade da enzima metionina sintase (MTR), a qual possui uma necessidade absoluta de vitamina B12 (B12) (Xue e Snoswell, 1985; Lucock, 2000). Esta vitamina hidrossolúvel é sintetizada pelo microbioma ruminal a partir do mineral cobalto (Co) proveniente da dieta (Stemme et al., 2008). Como bezerros recém-nascidos não possuem um rúmen funcional, eles dependem exclusivamente do ácido fólico e B12 adquiridos no ambiente uterino e por meio da ingestão do colostro e leite materno (Duplessis e Girard, 2019). De um ponto de vista fisiológico, o aumento do fornecimento de ácido fólico e Co, com o Co aumentando a síntese ruminal de B12, poderia levar a uma maior síntese de metionina endógena (Xue e Snoswell, 1985). Embora os efeitos isolados da suplementação materna com doadores de grupamento metil durante o início da vida pós-natal já tenham sido demonstrados (Jacometo et al., 2016, Jacometo et al., 2017, Jacometo et al., 2018; Abdelmegeid et al., 2017; Alharthi et al., 2018, Alharthi et al., 2019), resta determinarmos se o aumento do suprimento de ácido fólico e B12, por meio do aumento do suprimento de Co, sozinho ou combinado com metionina geram efeitos semelhantes no controle imunometabólico de bezerros neonatos.

2 REVISÃO BIBLIOGRÁFICA

2.1 Condição corporal e estressores metabólicos maternos

Entre os estágios do ciclo de vida em bovinos leiteiros, as transições da gestação à lactação e do aleitamento ao desmame, são reconhecidas como as mais desafiadoras fisiologicamente (Drackley, 1999; Loor et al., 2013). As vacas leiteiras, inevitavelmente, experienciam diferentes graus de estresse metabólico durante o período de transição (Abuelo, 2020). Neste período, adaptações fisiológicas são necessárias devido ao aumento nas necessidades de nutrientes associados ao crescimento fetal e à produção de leite (Drackley, 1999). Desta forma, as falhas ou demora nestas adaptações, irão resultar em desordens metabólicas com consequente impacto na vaca e sua lactação subsequente, assim como, no bezerro e seu desenvolvimento (Ling et al., 2018).

O estresse metabólico é caracterizado por uma excessiva mobilização de gordura e liberação de ácidos graxos em decorrência do balanço energético negativo (BEN); aumento de metabólitos reativos ao oxigênio e sobrecarga de mecanismos antioxidantes; comprometimento hepático e disfunção inflamatória (Ling et al., 2018; Abuelo et al., 2015; Trevisi & Minuti, 2017), com impactos diretos e indiretos sobre a saúde, função imunológica e produtividade de vacas leiteiras e seus descendentes (Sordillo & Aitken, 2009; Horst et al., 2021). Visto que, estes estressores metabólicos maternos podem iniciar nas semanas que antecedem o parto, os efeitos negativos ao feto dentro do ambiente uterino têm sido alvo de recentes investigações (Abuelo, 2020; Ling et al., 2018).

O escore de condição corporal (ECC) durante o período periparto, é uma ferramenta de manejo aplicável, pois avalia tanto a quantidade de gordura subcutânea quanto o estado nutricional da vaca (Roche et al., 2009). As elevações nas concentrações de ácidos graxos, oriundos do alto ECC pré-parto, predispõem as vacas leiteiras a um maior número de doenças, incluindo um status inflamatório exacerbado e intenso estresse oxidativo (Bernabucci et al., 2005; Akbar et al., 2015), cetose subclínica (Schulz et al., 2014) e disfunções imunológicas (Zhang et al., 2018). As reservas corporais em vacas leiteiras, durante o final da gestação, estão

diretamente relacionadas ao status energético e desempenham um papel fundamental na partição de nutrientes para o crescimento do feto (Loor et al., 2013). Embora garantir um suprimento nutricional materno equilibrado, no final da gestação, seja essencial para o desenvolvimento fetal, estratégias para controlar o adequado ECC são fundamentais (Roche et al., 2009). O ECC ideal ao parto, ainda é mérito de discussões na literatura, porém atualmente, um valor aceitável em uma escala de 5 pontos, está entre 3,0 e 3,25 (Roche et al., 2013).

Estudos em bovinos leiteiros também demonstraram que, a exposição a estressores de ordem ambiental ou nutricional, como por exemplo, ao estresse térmico e a ingestão de energia restrita ou excessiva, durante o final da gestação, afetam a função imunológica e metabólica da prole (Gao et al., 2012; Tao et al., 2012; Tao et al., 2014; Osorio et al., 2013). Está bem estabelecido que vacas leiteiras com alto ECC antes do parto, estão predispostas a um maior número de doenças que podem afetar negativamente suas crias (Roche et al., 2009). Além disso, o estresse oxidativo mais pronunciado enfrentado por vacas que mantêm um alto ECC pré-parto (Bernabucci et al., 2005; Liang et al., 2020), pode levar diretamente a mecanismos de resposta inflamatória e imunológica que impactam o ambiente fetal (Ling et al., 2018). Entretanto, ainda não foi esclarecido até que ponto estas adaptações maternas na condição corporal podem ser transferidas para as células do sistema imunológico do feto e, portanto, nos abrem um vasto caminho de investigações pela frente.

2.2 Doadores de grupamento metil e metabolismo de 1-carbono

Os doadores de grupamento metil, como por exemplo, ácido fólico, betaína, colina, metionina e B12, desempenham papéis funcionais em todo o organismo por meio de suas propriedades metabólicas, epigenéticas e imunomoduladoras (Coleman et al., 2020; Coleman et al., 2021; McFadden et al., 2020). Além disso, compartilham vias bioquímicas em comum, das quais o metabolismo de 1-carbono recebeu substancial atenção durante as últimas décadas (Figura 1). Apesar de os principais aspectos regulatórios neste metabolismo, serem relativamente bem conhecidos em outras espécies, o metabolismo de 1-carbono não é completamente compreendido em ruminantes (Coleman et al., 2021).

Este modelo teórico, abrange um conjunto de vias e reações celulares, em nível citoplasmático, nuclear e mitocondrial, nas quais a função mais bem estudada é

a transferência de grupamentos metil para reações de metilação e síntese de ácido nucleico (Snoswell & Xue, 1987). Até então, os principais impactos conhecidos desta via em vacas leiteiras, derivam do seu papel no desenvolvimento embrionário (Peñagaricano et al., 2013), função placentária (Batistel et al., 2019), crescimento neonatal (Alharthi et al., 2018), metabolismo antioxidante (Liang et al., 2019) e benefícios imunometabólicos na vaca durante o final da gestação e início da lactação (Osorio et al., 2014; Batistel et al., 2018), assim como, na prole durante o período neonatal (Jacometo et al., 2016; Alharthi et al., 2019; Duplessis & Girard, 2019).

Resumidamente, o metabolismo de 1-carbono abrange a transferência de átomos de carbono em uma variedade de reações metabólicas e desempenha um papel fundamental na geração de grupamentos metil lábeis (Vailati-Riboni et al., 2020; Snoswell & Xue, 1987; McFadden et al., 2020). As principais interrelações nesta via incluem a remetilação da homocisteína em metionina, na qual a betaína ou o folato, participam como doadores de grupamento metil através da ação catalizadora das enzimas betaína homocisteína metiltransferase (BHMT) ou MTR, respectivamente (Xue & Snoswell, 1985). A colina também participa da síntese de metionina através da sua oxidação em betaína para apoiar a atividade de BHMT (Coleman et al., 2019). A enzima metionina adenosiltransferase (MAT) converte metionina em SAM, o principal doador celular de grupamentos metil. Esta, em parte, favorece uma reação de transmetilação catalisada pela enzima fosfatidiletanolamina metiltransferase (PEMT) para gerar S-adenosil-homocisteína (SAH) e fosfatidilcolina (PC) (Xue & Snoswell, 1986). Isto é seguido pela conversão de SAH em homocisteína, em uma reação reversível e catalisada pela enzima SAH hidrolase (SAHH). A homocisteína também pode entrar na via da transulfuração, a primeira reação utilizada para sintetizar a cistationina através da ação da enzima cistationina β-sintase (CBS). A cistationina, pode então ser usada para a produção de cisteína, que é utilizada para sintetizar os potentes antioxidantes glutationa (GSH) e taurina (Vailati-Riboni et al., 2019).

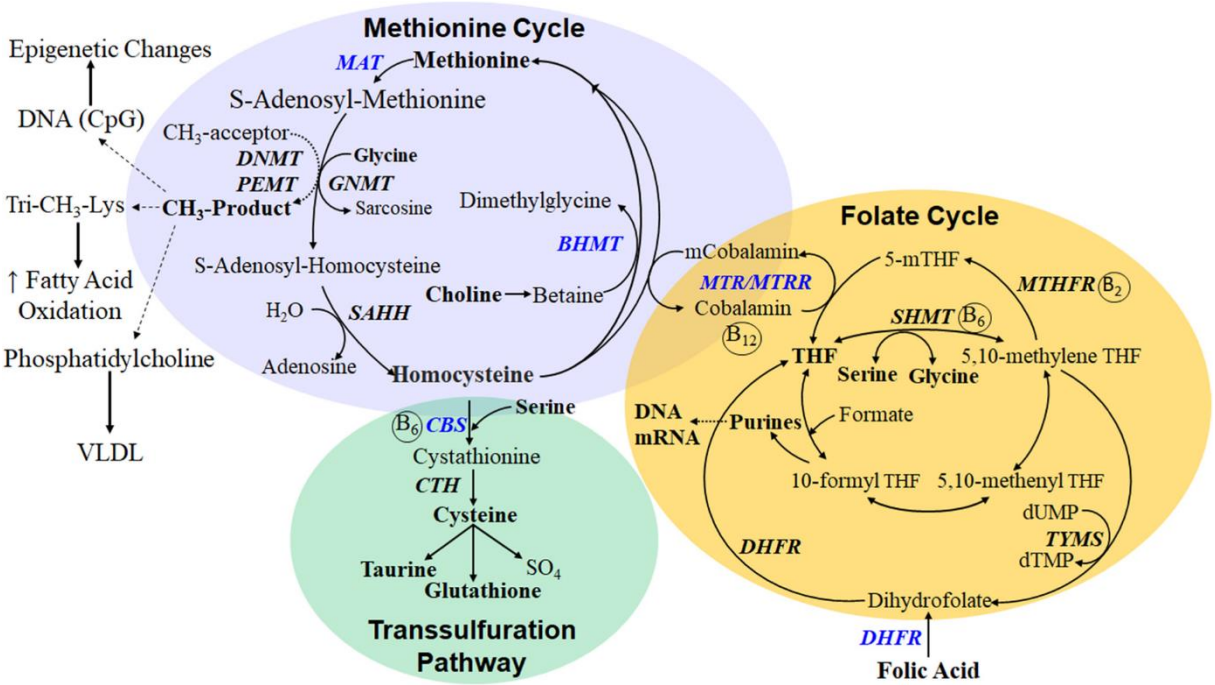

Figura 1. Modelo teórico do metabolismo de 1-carbono: ciclo da metionina, do ácido fólico e via da transulfuração, assim como as interrelações entre os componentes das vias do metabolismo de 1-carbono. 5-mTHF = 5-metil-tetra-hidrofolato; B2 = riboflavina; B6 = piridoxal 5'-fosfato; B12 = cobalamina; BHMT = betaína homocisteína metiltransferase; CBS = cistationina beta sintase; CTH = gama-liase cistationina; DHFR = dihidrofolato redutase; DNMT = DNA metiltransferase; dUMP = monofosfato de desoxiuridina; dTMP = monofosfato de timidina; GNMT = glicina N-metiltransferase; MAT = adenosiltransferase de metionina; MTHFR = metilenotetra-hidrofolato redutase; MTR = 5-metiltetra-hidrofolato-homocisteína metiltransferase; MTRR = 5-metiltetrahidrofolato-homocisteína metiltransferase redutase; PEMT = fosfatidiletanolamina N-metiltransferase; SAHH = S-adenosil-homocisteína hidrolase; SHMT = serina hidroximetiltransferase; THF = tetra-hidrofolato; TYMS = timidilato sintetase; VLDL = lipoproteína de densidade muito baixa. Fonte: Coleman et al., 2021.

Além do período periconcepcional (Peñagaricano et al., 2013), devido às suas funções variadas, o impacto da nutrição com doadores de grupamento metil apresentam múltiplos benefícios durante o final da gestação e início da lactação, ou seja, durante todo o período de transição (Coleman et al., 2021). Desta forma, inúmeras pesquisas foram conduzidas em vacas periparturientes, nas últimas três décadas, explorando essas interações biológicas para identificar os mecanismos por trás dos benefícios imunometabólicos que são evidenciados (Coleman et al., 2020; McFadden et al., 2020; Coleman et al., 2021). Porém, ainda existe a necessidade de ampliarmos nossa compreensão, sobre o papel destes nutrientes no controle imunometabólico de bezerros oriundos de vacas submetidas a um maior aporte de doadores de grupamento metil dietético, durante os diferentes períodos da gestação.

2.3 Imunometabolismo de bezerros neonatos

O período neonatal é um dos mais desafiadores para bezerros (Hulbert & Moisá, 2016), principalmente porque eles nascem sem a presença de imunoglobulinas circulantes, ressaltando a dependência exclusiva da ingestão de colostro nas primeiras horas de vida (Chase et al., 2008). Além disso, as funções celulares do sistema imunológico neonatal requerem um período inicial de maturação por meio da exposição a novos antígenos, e estudos recentes em bezerros sugeriram que o estado nutricional durante os estágios finais da vida fetal é um ponto crítico para o adequado desenvolvimento dos mecanismos de defesa e controle dos processos inflamatórios (Hulbert & Moisá, 2016; Jacometo et al., 2018; Alharthi et al., 2019).

O sistema imunológico do bezerro, começa a se desenvolver no útero aproximadamente aos 42 dias de gestação (Abuelo, 2020) e, desta forma, pode ser afetado por condições do metabolismo materno durante a gestação. Além disso, alguns estudos demonstram que o sistema imunológico possui menor prioridade na partição de nutrientes fetais, quando em comparação com outros sistemas (Wu et al., 2005). Portanto, o sistema imunológico e, consequentemente, a capacidade do animal em enfrentar a invasão de patógenos, é um dos primeiros sistemas afetados quando o fornecimento de nutrientes ao feto é limitado (Abuelo, 2020).

As células imunológicas são dependentes de um suprimento adequado de nutrientes para energia e proliferação (Li et al., 2007). Uma vez ativadas, as células apresentadoras de antígenos (APC) iniciam uma rota de sinalização por meio de interações entre receptores de reconhecimento de padrões (PRR) e padrões moleculares associados a patógenos (PAMP), resultando em uma resposta inflamatória sistêmica (Rosadini & Kagan, 2017). Isso leva à estimulação da transcrição e produção de fatores solúveis (por exemplo, sistema complemento, lisozimas, proteínas de fase aguda e citocinas), porém sob um alto custo em termos de glicose e utilização de aminoácidos (Li et al., 2007; Vailati-Riboni et al., 2017).

A “programação nutricional”, também denominada por alguns autores como “programação fetal”, refere-se ao conceito de que fatores que afetam o crescimento e o desenvolvimento fetal causam mudanças de longo prazo na estrutura e função de células e tecidos (Wu et al., 2005; Abuelo, 2020). Este conceito foi estabelecido pela primeira vez usando estudos epidemiológicos para mostrar uma forte associação

entre características fenotípicas adversas (por exemplo, síndrome metabólica, alterações de crescimento e disfunção imune e reprodutiva) e exposição, dentro do ambiente uterino, a diversos estressores metabólicos maternos (Reynolds et al., 2017). Posteriormente, foi demonstrado em estudos com humanos e animais que todos os sistemas orgânicos e funções metabólicas podem ser afetados pela “programação nutricional” (Reynolds et al., 2019).

A deficiência de micro ou macronutrientes na dieta compromete o adequado funcionamento do sistema imunológico e aumenta a susceptibilidade de doenças infecciosas em animais e seres humanos (Li et al., 2007; Hulbert & Moisá, 2016; Grimble, 2006). Nos últimos anos, mecanismos celulares e moleculares que envolvem estes processos começaram a ser esclarecidos e indicam um papel importante para determinados nutrientes funcionais na resposta imune, regulando a ativação de leucócitos, moléculas antioxidantes, expressão gênica e produção de citocinas e anticorpos (Li et al., 2007). O desenvolvimento da imunologia nutricional teve seu despontamento a partir de casos de desnutrição e infecção, que são os fatores que mais comprometem o sistema vital tanto em animais como em humanos (Grimble, 2006). Este conceito de imunologia nutricional agrega metodologias de pesquisa nutricional e imunológica, com o intuito de esclarecer a função dos nutrientes no organismo (Coleman et al., 2021; Li et al., 2007; Coleman et al., 2020).

O sistema imune está conceitualmente dividido em defesas físicas, exemplificadas por barreiras naturais como a pele, imunidade inata composta pelo sistema complemento, granulócitos, macrófagos, células dendríticas e *natural killers*, e a imunidade específica composta pelos linfócitos T e B, além de imunoglobulinas (Beutler, 2004). Os dois sistemas, inato e específico, são submissos de certa quantidade de nutrientes para a síntese de proteínas e polipeptídeos, manutenção energética dos mecanismos de respostas celulares e, ainda, suporte enzimático às mais diversas reações metabólicas. Neste sentido, estudos têm sido conduzidos na busca de nutrientes funcionais capazes de auxiliar o sistema imune, por desenvolverem funções importantes na ativação e proliferação de leucócitos, redução do estresse oxidativo, expressão gênica e produção de citocinas e imunoglobulinas (Li et al., 2007). No entanto, os mecanismos pelos quais estes nutrientes funcionais podem impactar o sistema e células imunológicas do bezerro, quando adicionados

estrategicamente na dieta materna durante períodos críticos da gestação, ainda são dignos de estudos mais profundos para melhor compreensão.

3 HIPÓTESE E OBJETIVOS

3.1 Hipóteses

Hipótese 1: O sistema imunológico de bezerros neonatos é afetado por diferentes vias de acordo com o escore de condição corporal materno antes do parto.

Hipótese 2: Fontes adicionais de unidades de 1-carbono na dieta materna ao final da gestação, pode estimular a função imune inata da prole durante o início da vida pós-natal.

3.2 Objetivo Geral

Avaliar os efeitos da condição corporal e da suplementação materna com fontes de cobalto, ácido fólico e metionina durante o final da gestação, em parâmetros imunológicos e metabólicos de bezerros neonatos.

3.3 Objetivos Específicos

- Avaliar como o escore de condição corporal materno ao final da gestação, pode afetar a expressão de genes relacionados à resposta imune, função antioxidante e metabolismo de 1-carbono, em leucócitos de bezerros neonatos.
- Avaliar como o escore de condição corporal materno ao final da gestação, pode afetar a concentração plasmática de interleucina 1β em bezerros neonatos.
- Avaliar como o escore de condição corporal materno ao final da gestação, pode afetar o status antioxidante dos bezerros ao nascimento.
- Avaliar os efeitos da suplementação materna com fontes de cobalto, ácido fólico e metionina ao final da gestação, na expressão de genes relacionados à resposta imune, função antioxidante e metabolismo de 1-carbono, em leucócitos de bezerros neonatos.

- Avaliar os efeitos da suplementação materna com fontes de cobalto, ácido fólico e metionina ao final da gestação, na função imunológica de bezerros neonatos.
- Avaliar os efeitos da suplementação materna com fontes de cobalto, ácido fólico e metionina ao final da gestação, no desenvolvimento corporal de bezerros neonatos.
- Avaliar os efeitos da suplementação materna com fontes de cobalto, ácido fólico e metionina no metabolismo energético, inflamação e status antioxidante dos bezerros ao nascimento.

4 CAPÍTULOS

4.1 Artigo 1 - Maternal body condition influences neonatal calf whole blood innate-immune molecular responses to ex vivo lipopolysaccharide challenge

Artigo publicado na revista *Journal of Dairy Science*

Interpretive Summary

Maternal body condition influences neonatal calf whole blood innate-immune molecular responses to ex vivo lipopolysaccharide challenge. ***By Lopes et al.*** Maternal energy reserves in late-pregnancy affect the uterine environment and influence the offspring. The first weeks of life are the most-challenging for calves, largely due to the need for an efficient immune response to overcome stressors. To understand possible effects of maternal body condition (BCS) on neonatal immune function, we challenged whole blood from calves born to cows with normal (NormBCS) or high (HighBCS) BCS with lipopolysaccharide (LPS). Marked upregulation of inflammation-(*IL1B*, *NFKB1*) and antioxidant-responsive (*GSR*) genes in HighBCS calves at birth suggested a heightened immune responsiveness. Marked *MTR* upregulation and *CBS* downregulation in HighBCS calves at birth underscored the role of 1-carbon metabolism during the immune response. Data underscored that managing maternal BCS could alter calf immune response.

RUNNING TITLE: MATERNAL BODY CONDITION AND CALF IMMUNE RESPONSE

Maternal body condition influences neonatal calf whole blood innate-immune molecular responses to ex vivo lipopolysaccharide challenge

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ABSTRACT

Managing body condition in dairy cows during the close-up period could alter the availability of nutrients to the fetus during the final stages of growth in utero. We investigated effects of maternal body condition score (BCS) during late-pregnancy on calf whole blood mRNA abundance and interleukin 1β (IL-1β) concentrations after ex vivo lipopolysaccharide (LPS) challenge. Thirty-eight multiparous Holstein cows and their calves from a larger cohort were retrospectively divided by prepartal BCS into normal BCS ≤3.25 (n = 22; NormBCS) and high BCS ≥3.75 (n = 16; HighBCS) groups. Blood samples from calves collected at birth (before receiving colostrum, d 0), and at 21 and 42 (at weaning) d of age were used for ex vivo whole blood challenge with 3 µg/mL of LPS prior to mRNA isolation. Target genes evaluated by RT-qPCR were associated with immune response, antioxidant function, and 1-carbon metabolism. In addition, plasma IL-1β concentrations were measured. Responses in plasma IL-1β and mRNA abundance from each calf were evaluated as the difference in response between LPS challenged and non-challenged samples (control). Statistical analyzes were performed at birth and also with all time points using a MIXED model in SAS 9.4. Neither birth BW (NormBCS = 43.8 ± 1.01 kg; HighBCS = 43.9 ± 1.2 kg) nor colostrum IgG concentration (NormBCS = 70 ± 5.4 mg/mL; HighBCS = 62 ± 6.5 mg/mL) differ between groups. At birth, whole blood from calves born to HighBCS cows after LPS challenge had greater mRNA abundance of *IL1B, NFKB1*, and *GSR* coupled with lower *GPX1* and *CBS* abundance. The longitudinal analysis of d 0, 21, and 42 data revealed a BCS × age effect for *SOD2* and *NOS2* due to lower mRNA abundance at 42 d of age in the HighBCS calves. Regardless of maternal BCS, there was a decrease in mRNA abundance over time for genes encoding cytokines (*IL1B*, *IL6*, *IL10*, *TNF*), cytokine receptors (*IRAK1*, *CXCR1)*, toll-like receptor pathway (*TLR4*, *NFKB1*), adhesion and migration (*CADM1*, *ICAM1*, *ITGAM*), and antimicrobial function (*MPO*). Concentration of IL-1β after LPS challenge also was markedly

lower at 21 days of age regardless of maternal BCS. Overall, results suggested that maternal BCS in late-prepartum influences the response of the calf immune system to an inflammation challenge after birth. Although few genes among those studied were altered due to maternal BCS, the fact oxidative stress and 1-carbon metabolism related genes responded to LPS challenge in HighBCS calves underscored the potential role of methyl donors (e.g. methionine, choline, and folic acid) on the innate immune response early in life.

Key words: maternal stress, methyl donors, neonatal immunity, nutritional programming

INTRODUCTION

Body reserves of dairy cows in late-pregnancy are directly related to energy status and play a key role in the partition of nutrients to the rapidly-growing fetus (Loor et al., 2013). While ensuring an adequate maternal nutritional supply at the end of pregnancy is essential for adequate fetal development, strategies to control the correct BCS are important (Roche et al., 2013). The optimum calving BCS is still a matter of debate in the literature. Currently, an acceptable value for the ideal calving BCS on a 5-point scale is between 3.0 and 3.25 (Roche et al., 2013). The greater concentrations of NEFA that arise due to high BCS prior to calving predisposes cows to a greater number of disorders including pronounced inflammatory state and oxidative stress (Bernabucci et al., 2005; Akbar et al., 2015), subclinical ketosis (Schulz et al., 2014), and immune dysfunction (Zhang et al., 2018).

Most of the available research on the impacts of BCS has focused on the cow and effects on subsequent lactation (Roche et al., 2009). However, the way in which BCS can also directly affect the fetus during late-pregnancy and the neonatal period (i.e. the first few wk of life) remains to be fully elucidated (Abuelo, 2020). With the improvement of molecular methods, an important area of study referred as nutritional programming has allowed for a better understanding of strategies that could modulate molecular and metabolic routes in the calf during uterine life (Reynolds et al., 2019). These so-called “epigenetic responses” allow the evaluation of phenotypic changes due to alterations in the supply of certain nutrients during gestation (Reynolds et al., 2017). Management strategies make it possible to guarantee an adequate supply of nutrients to the fetus and consequently facilitate responses in the calf to challenges during the first days of life.

These first days of a calf’s life are considered the most challenging period for maintaining adequate growth and development (Hulbert and Moisá, 2016). When leaving the

secure and sterile uterine environment, calves are faced with an external environment full of challenges such as exposure to a wide variety of pathogens. In this sense, the calf immune system faces a daily challenge in an attempt to recognize, present and eliminate antigens, the vast majority of which are seen for the first time (Chase et al., 2008). For the calf to begin developing its defense response, and fundamentally begin to create immune memory against different pathogens, it is essential that the neonatal immune system matures through exposure to new molecules (Barrington and Parish, 2001).

Maternal exposure to different stressors and oxidative stress in late-gestation can contribute to immune deregulation in the offspring, and influence susceptibility to diseases (Hodyl et al., 2008; Beloosesky et al., 2010; Ling et al., 2018). Among these, infectious diseases are the most common in the first's days of a calf's life, e.g. diarrhea and bovine respiratory disease (BRD) (Windeyer et al., 2014; Dubrovsky et al., 2020). The gram-negative bacteria are often involved and induce a potent inflammatory response in the neonate. In this sense, an important pathogen-associated molecular pattern (**PAMP**) is the lipopolysaccharide (**LPS**) present in the gram-negative bacteria cell wall. The LPS is easily recognized by the immune system and can trigger an important systemic inflammatory response. It involves the activation of important molecular pathways as well as the release of potent chemical mediators such as inflammatory cytokines and vasoactive amines (Rosadini and Kagan, 2017). Thus, ex vivo LPS challenges are often used to stimulate the bovine immune system (Vailati-Riboni et al., 2017; Ling et al., 2018).

We hypothesized that the innate immune system of neonatal calves would be affected differently as a function of maternal BCS prior to calving. Thus, we performed an ex vivo whole blood LPS challenge to simulate the immune system of calves during the first weeks of life, and evaluated mRNA abundance and cytokine concentrations. The ex vivo assay

(Vailati-Riboni et al., 2017) allowed us to visualize the dynamics of the immune response as a function of maternal BCS using a non-invasive approach.

MATERIALS AND METHODS

Experimental Design

All procedures were conducted with the approval of the University of Illinois Institutional Animal Care and Use Committee (Protocol #17168). Thirty-eight multiparous Holstein cows from a larger cohort were retrospectively divided according to prepartal BCS -4 wk into normal BCS (NormBCS; n = 22; BCS ≤3.25) and high BCS (HighBCS; n = 16; BCS ≥3.75) groups, through a 5-point scale. During the close-up period, cows were housed in a sand-bedded free-stall barn and fed a total mixed ration (TMR) daily (0600 h) with ad libitum access to the same basal diet (1.37 Mcal/kg of DM, 8.45% RDP and 6.05% RUP), using an individual gate feeding system (American Calan, Northwood, NH, USA). Diet ingredient and nutrient composition are available in Supplemental Table 1. When the expected calving date approached, cows were moved to individual pens bedded with straw.

After calving, total colostrum volume was recorded and total immunoglobulin G (IgG) concentration was immediately estimated using a colostrometer (Bovine Colostrometer catalog no. C10978N, Nasco, Fort Atkinson, WI, USA). Immediately after birth, calves were weighed on a digital scale and removed from their dams to fed fresh colostrum from their respective dams within 6 h. All calves received 3.8 L of first colostrum, if voluntary colostrum intake did not reach 3.8 L the calves were force-fed via esophageal tube to reach the required amount of colostrum. Calves were housed in individual outdoor hutches bedded with straw and fed twice daily (0600 h and 1800 h) with milk replacer (Advance Excelerate, Milk Specialties, Carpentersville, IL, USA; 28.5% CP, 15% Fat) until 35 days of age. From 36 days of age until weaning (42 days of age), calves were switched to once a day feeding at

0600 h. In detail, calves received 4.54 kg/d of milk replacer (0.59 kg of milk replacer in 3.95 L of water) from 1 to 10 d of age, 5.90 kg/d (0.77 kg of milk replacer in 5.13 L of water) from 11 to 20 d of age, 7.26 kg/d (0.94 kg of milk replacer in 6.32 L of water) from 21 to 35 d of age, and 3.63 kg/d (0.47 kg of milk replacer in 3.16 L of water) in a single feeding from 36 to 42 d of age, and had ad libitum access to a starter grain mix (Ampli-Calf Starter 20®; 19.9% crude protein (CP) and 13.5% neutral detergent fiber (NDF), Purina Animal Nutrition, Shoreview, MN, USA).

Whole Blood ex vivo LPS Challenge

The immune response in whole blood samples was assessed as described by Vailati-Riboni et al. (2017) with modifications. Calf blood samples were collected from the jugular vein using 20-gauge needles (BD Vacutainer, Becton Dickinson and Company, Franklin Lakes, NJ, USA) at birth (before colostrum), 21 and 42 (at weaning) d of age. Samples were collected into evacuated tubes containing lithium heparin as an anticoagulant (BD Vacutainer, Becton Dickinson and Company), and immediately transported to the laboratory stored in a thermo-insulated container with 38°C water. The LPS challenge was started no later than 30 min after sampling. Prior to the challenge, lyophilized LPS (*Escherichia coli* O111:B4, Sigma Aldrich, St. Louis, MO, USA) was eluted and diluted in Dulbecco's modified Eagle medium (Sigma Aldrich) in order to obtain a 150 µg of LPS/mL solution. In a laminar airflow cabinet, two aliquots of 980 µL of whole blood were stimulated in duplicates with 20 µL of Dulbecco's modified Eagle medium (non-challenged control samples) or with 20 µL of 150 µg of LPS/mL solution generating a dose of 3 µg of LPS/mL of whole blood (challenged samples). Aliquots were incubated in a water bath for 3.5 hours at 38°C with a horizontal shaking speed of 30 times/min. One of the duplicates was used to remove plasma samples after centrifugation (8,700 × *g* for 16 min at 6°C) and the other kept in whole blood for storage at -80°C.

Whole Blood mRNA Extraction

Whole blood mRNA extraction was performed using Trizol protocol (TRI Reagent® BD, Sigma Aldrich) as recommended by the manufacturer. Possible residue from genomic DNA was removed by purification with on-column DNase digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany), and RNA concentration was measured using the NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE, USA). All samples had an RNA integrity score greater than 8.0 according to the AATI Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA).

cDNA Synthesis and RT-qPCR

Evaluated genes are associated with cytokine production (*IL1B*, *IL6*, *IL10*, *TNF*), cytokine receptors (*IRAK1*, *CXCR1*), toll-like receptor pathway (*TLR4*, *BPI*, *NFKB1*, *STAT3*), adhesion and migration (*CADM1*, *ICAM1*, *ITGAM*, *SELL*), antimicrobial function (*LYZ*, *MPO*, *NOS2*, *SOD2*), antioxidant function (*GPX1*, *GSR*, *GSS*) and 1-carbon metabolism (*CBS*, *MTR*) (Supplemental Table 2). Detailed information on the target genes can be found in our previous manuscripts with calves and cows (Abdelmegeid et al., 2017; Jacometo et al., 2018; Zhou et al., 2018).

The cDNA was synthesized, and RT-qPCR were performed using the same protocol as reported previously by our group (Vailati-Riboni et al., 2019b). Briefly, cDNA synthesis was performed using 100 ng of RNA standardized by dilution in nuclease-free water. Total RNA was mixed using first a mix (Mix 1) containing random primers (Roche Applied Science, Penzberg, Germany) and nuclease-free water. A second mix (Mix 2) consisted of 5x First-Strand Buffer (Thermo Fischer Scientific, Waltham, MA, USA), Oligo dT18 (Integrated DNA Technologies, Coralville, IA, USA), dNTP mix (Invitrogen, Carlsbad, CA, USA), Revert aid (Thermo Fischer Scientific), RNase inhibitor (Thermo Fischer Scientific), and

nuclease-free water. After adding Mix 2 to the RNA+Mix 1 sample, the reaction was performed in a Mastercycler Gradient (Eppendorf, Hamburg, Germany). A 10 µL aliquot of cDNA from all samples was pooled and serially diluted 1:4 with nuclease-free water to generate a 7-point standard curve. RT-qPCR was performed in a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems, Foster City, CA, USA) using diluted cDNA (1:4) and SYBR Green mixture (Applied Biosystems) with 10 µM forward and reverse primers, and nuclease-free water. Each gene was run in triplicate on a single plate with the 7-point standard curve plus the negative control. Data were normalized using the geometric mean of 3 internal control genes: *GAPDH*, *RPS9* and *ACTB* previously validated by our group (Liang et al., 2019; Vailati-Riboni et al., 2019b).

Plasma IL-1β Assay

Plasma samples from the LPS challenge were analyzed for IL-1β concentration through a bovine ELISA according to the manufacturer's recommendations (IL-1β catalog no. ESS0027, Thermo Fischer Scientific). An 8-point standard curve in 1:2 serial dilution was performed with calibrators to interpolate the absorbance values of the target samples. Total IL-1β plasma concentration was calculated in pg/mL, as well as the difference between IL-1β concentration in the non-challenged sample versus the challenged sample (Δ**IL-1β**).

Plasma Antioxidant Status at Birth

Antioxidant status was assessed at birth through various plasma biomarkers. Fatty acids (mmol/L) and β-hydroxybutyrate (mmol/L) were measured using kits from Wako Chemicals GmbH (Neuss, Germany) and Randox Laboratories Ltd. (Crumlin, United Kingdom), respectively, following the procedures described previously (Bionaz et al., 2007; Trevisi et al., 2012). Paraoxonase (U/mL) was measured as described by Bionaz et al. (2007). Total reactive oxygen metabolites (ROMt; mg of H_20_2/100 mL) was determined with a

commercial kit (Diacron International s.r.l., Grosseto, Italy). Ferric reducing ability of plasma (FRAP; µmol/L) was measured using the colorimetric method of Benzie and Strain (1996). Plasma nitric oxide metabolites (NOx; µmol/L), nitrite (NO_2^-; µmol/L), and nitrate (NO_3^-; µmol/L) were measured using the Griess test according to Gilliam et al. (1993) and Bouchard et al. (1999). Retinol (µg/mL), α-tocopherol (µg/mL), and β-carotene (mg/100 mL) were analyzed as described by Trevisi et al. (2013). Zinc (µmol/L) was determined with a commercial kits (Wako Chemicals GmbH) (Bionaz et al., 2007).

Statistical Analysis

The RT-qPCR data were normalized with the geometric mean of the 3 internal control genes and log2 transformed before statistical analysis in order to obtain a normal distribution. The response in mRNA abundance in LPS-challenged versus non-challenged samples was performed in a similar fashion to a previous study with calves (Ling et al., 2018) using delta change values, Δ = LPS challenged - LPS non-challenged samples. Normality of the residuals was tested via Proc Univariate in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). All observations were normally distributed, and observations with absolute value of studentized residual greater than 3 were automatically removed from the analysis. The statistical model used in SAS included BCS, Age, and their interactions as fixed effects. A One-Way ANOVA was used to determine differences at birth with BCS as fixed effect. Total plasma IL1-β concentration data were analyzed including BCS, LPS, Age and their interactions as fixed effects. In addition, analysis of the response in plasma IL1-β (ΔIL-1β) included BCS, Age and their interactions as fixed effects.

RESULTS

Total colostrum IgG concentration did not differ ($P = 0.40$), and averaged 70.00 ± 5.45 mg/mL in NormBCS and 62.69 ± 6.59 mg/mL in HighBCS. Calf BW at birth was

comparable ($P = 0.99$) between groups (NormBCS = 43.85 ± 1.01 kg; HighBCS = 43.86 ± 1.19 kg). All calves were clinically healthy throughout the study.

Whole Blood mRNA Abundance

Cytokines and Cytokines Receptors. At birth, *IL1B* response to the LPS challenge was greater in calves born to HighBCS cows (Δ; $P < 0.01$; Table 1). There was no difference between HighBCS and NormBCS calves at birth for *IL6*, *IL10*, *TNF*, *IRAK1* and *CXCR1*. Analyzing all time points, there was a tendency for a BCS × Age effect on *IL1B* mRNA abundance due to a greater response in HighBCS calves when challenged with LPS at birth (Δ; $P = 0.07$; Figure 1). Regardless of maternal BCS, an Age effect in response to the LPS challenge was detected for all genes measured. As calves grew, there was a decrease in abundance of *IL1B* ($P < 0.01$), *IL6* ($P = 0.04$), *IL10* ($P < 0.01$), *TNF* ($P < 0.001$), *IRAK1* ($P = 0.04$) and *CXCR1* ($P < 0.001$) in response to the LPS challenge (Δ; Figure S1).

Toll-Like Receptor Pathway. At birth, calves from the HighBCS group had greater *NFKB1* abundance in response to the LPS challenge (Δ; $P < 0.01$; Table 1). There was no difference between HighBCS and NormBCS calves at birth for *TLR4*, *BPI* and *STAT3*. Analyzing all time points, regardless of maternal BCS, both *TLR4* and *NFKB1* were downregulated in response to LPS stimulation over time (Δ; $P < 0.0001$; Figure S2).

Adhesion and Migration. There was no difference between HighBCS and NormBCS calves at birth or over time for genes related to adhesion and migration. Analyzing all time points, regardless of maternal BCS, there were interactions over time. The LPS challenge led to downregulation of *ICAM1* ($P = 0.02$) and *ITGAM* ($P < 0.01$) (Δ; Figure S2). Abundance of *CADM1* had a different response to LPS over time, with an upregulation at 21 d of age (Δ; $P = 0.02$; Figure S2).

Antimicrobial Function. At birth, there was no difference between HighBCS and NormBCS calves for genes related to antimicrobial function. Analyzing all time points in response to LPS, calves born to HighBCS cows had a tendency for a decrease in *SOD2* abundance (Δ; $P = 0.09$; Table 2), and a BCS × Age effect was observed where calves from HighBCS cows had lower *SOD2* abundance in response to the LPS challenge at 42 d of age (Δ; $P < 0.01$; Figure 1). Likewise, an effect of BCS × Age was observed for *NOS2* due to downregulation in response to LPS in calves from HighBCS cows at 42 d of age (Δ; $P = 0.05$; Figure 1). In addition, *MPO* abundance decreased over time in response to LPS challenge independent of maternal BCS (Δ; $P < 0.0001$; Figure S2).

Antioxidant Function. At birth, calves born from HighBCS cows had downregulation in *GPX1* mRNA abundance in response to LPS (Δ; $P = 0.02$; Table 1). In contrast, *GSR* mRNA abundance was greater in calves from HighBCS cows, where the downregulation in response to the LPS challenge was attenuated in HighBCS calves (Δ; $P = 0.02$; Table 1). There was no difference between HighBCS and NormBCS calves for *GSS* at birth.

Analyzing all time points in response to the LPS challenge, a tendency for an effect of BCS × Age on *GSR* (Δ; $P = 0.06$) and *GSS* (Δ; $P = 0.10$) was observed. Calves from HighBCS cows had greater *GSR* abundance at birth and decreased over time (Figure 2). Likewise, *GSS* abundance in response to the LPS challenge was lower in the HighBCS group at 42 d of age (Figure 2). Regardless of maternal BCS, there was an Age effect for *GPX1* (Δ; $P < 0.001$) and *GSR* (Δ; $P < 0.05$) abundance. Over time, *GPX1* abundance decreased in response to LPS challenge (Figure S3), whereas *GSR* abundance decreased at 21 d of age (Figure S3).

1-carbon Metabolism. At birth, calves from HighBCS cows had a more pronounced downregulation of *CBS* in response to LPS challenge (Δ; $P = 0.02$; Table 1). In contrast, abundance of *MTR* tended to be greater in calves from HighBCS cows (Δ; $P = 0.09$; Table 1).

Analyzing all time points, a tendency for a BCS × Age effect was observed for *MTR* abundance. Calves from HighBCS cows had greater *MTR* abundance in response to LPS challenge at birth and decreased over time (Figure 2) (Δ; $P = 0.07$; Figure 2). Regardless of maternal BCS, there was an Age effect for *CBS* (Δ; $P < 0.001$) and *MTR* (Δ; $P < 0.05$) abundance. The *CBS* mRNA abundance increased at 21 d of age, while *MTR* abundance has a marked decrease at 21 d of age (Figure S3).

Plasma IL-1β concentration

At birth, there was no difference between HighBCS and NormBCS calves for plasma IL-1β concentration in response to the LPS challenge (ΔIL-1β) ($P > 0.05$; 7838.06 vs. 6959.55 pg/mL; Figure 3). Analyzing all time points, as expected, samples challenged with LPS had a marked increase in plasma IL-1β concentration ($P < 0.0001$; 6551.57 vs. 223.59 pg/mL; Figure 4). In terms of a time effect, calves at birth had a higher IL-1β concentration and decreased substantially at 21 d of age ($P < 0.0001$; Figure 3).

DISCUSSION

Understanding of the relationship between maternal body reserves during late-pregnancy and the immune response of the offspring during the first days of life is still in its infancy (Abuelo, 2020). Through an ex vivo assay with whole blood from calves born to cows categorized into two different prepartal BCS groups (HighBCS vs. NormBCS), we attempted to mimic the main infectious challenges that these calves might be exposed immediately after birth. A similar approach was used recently to study the effect of maternal metabolic stress prior to parturition on the neonatal calf immune response (Ling et al., 2018).

It is well-known that dairy cows with a high BCS prior to calving are predisposed to a greater number of disorders that can negatively affect even the offspring (Roche et al., 2009). The more pronounced oxidative stress experienced by cows that maintain a high BCS prepartum (Bernabucci et al., 2005; Liang et al., 2020) could directly lead to inflammatory and immune response mechanisms that might impact the fetal environment (Ling et al., 2018). In this sense, the extent to which these maternal adaptations in body condition can be transferred to immune cells in the offspring remain to be clarified.

Whole Blood Responses at Birth

We considered the analysis performed at birth (before colostrum) as the closest for assessing the maternal effect of BCS on programming of the newborn immune system. The greater response in *NFKB1* and *IL1B* mRNA abundance in the HighBCS group after LPS challenge is an example of the mechanism of antigen recognition and processing by the innate immune system upon a challenge (Rosadini and Kagan, 2017). Through pattern recognition receptors (PRR) such as toll-like receptor 4 (**TLR4**) polymorphonuclear cells (PMN) in the circulation initiate a signaling cascade culminating in activation of transcription factors (e.g. *NFKB1*) and a consequent stimulation of pro-inflammatory cytokine gene transcription, including *IL1B* (Tapping et al., 2000). Besides pro-inflammatory cytokines, proteins such as LPS-binding protein (LBP) and bactericidal permeability-increasing protein (BPI) are produced to promote cell-antigen interaction through the surface molecule CD14. Together, LBP and CD14 represent the main route by which cells recognize low concentrations of LPS and initiate stimuli to respond to gram-negative bacteria (Ryu et al., 2017), i.e. activation of transcriptional regulators such as nuclear factor kappa B (NFKB) that upregulate genes involved in the inflammatory response (e.g. *IL1B, IL6* and *TNF*) (Hayden and Ghosh, 2011).

Cows with high BCS in late-pregnancy often exhibit high NEFA and BHB levels after calving (Pires et al., 2013), both of which can lead to a chronic inflammation process in addition to over-activating the TLR and NFKB signaling pathway (Zhang et al., 2018). For example, when compared with low BCS cows (≤3.25), our previous work revealed greater NEFA concentrations in high BCS cows (≥3.75) as early as 10 d prepartum (Alharthi et al., 2018). Oxidant status, which is usually greater in cows with high BCS (Bernabucci et al., 2005; Liang et al., 2020), also stimulates inflammation and upregulates *NFKB1* mRNA abundance (Hayden and Ghosh, 2011; Buelna-Chontal and Zazueta, 2013). Thus, the greater *IL1B* mRNA abundance in calves from HighBCS cows suggested these animals might have experienced some degree of stress and/or were more susceptible to stressors. Overall, similar to non-ruminants, data indicate that extracellular stimuli in bovine can contribute to the more pronounced activation of inflammatory transcriptional regulators such as NFKB (Hayden and Ghosh, 2011).

Although there were numerical differences in plasma IL-1β concentrations in response to LPS challenge (ΔIL-1β) in the HighBCS calves at birth, the absence of statistical significance suggested the existence of post-transcriptional regulation of the inflammatory response. It is well-known in non-ruminants that the NFKB pathway is also closely controlled by increases in protein abundance and also post-translational mechanisms including phosphorylation (Hayden and Ghosh, 2011). In addition, the cytosolic inflammasome protein complexes are characterized by promoting IL-1β activation through induction of caspase-1 (CASP1) (Rashidi et al., 2020). Activation of NFKB signaling primes NLR family pyrin domain containing 3 (NLRP3), which is a central component in the inflammasome and CASP1 activation (Shen et al., 2019). Once activated, CASP1 cleaves pro-IL-1β into the mature IL-1β form (Jo et al., 2016). Although we did not measure *CASP1* expression, we speculate that inflammasome signaling might have been the limiting factor precluding the

detection of differences in plasma IL-1β concentrations. Thus, it appears that biological consequences of *IL1B* upregulation would be mitigated due to absence of post-translational over-activation of IL-1β.

Regardless of maternal BCS, this same behavior with the peak in IL-1β concentration at birth was demonstrated after LPS challenge in our previous study with whole blood from calves (Jacometo et al., 2018). In fact, previous and current plasma IL-1β data underscore the high sensitivity to an inflammatory challenge by the newborn calf. In both Jacometo et al. (2018) and the present study LPS challenge at birth compared with later time points resulted in a marked increase in IL-1β concentration (~4,000 to >7,000 pg/mL). Clearly, circulating immune cells in the newborn calf can respond quickly to a stressor such as LPS, even in an environment with high cortisol levels as is often observed in calves at birth (Osorio et al., 2013).

Among antioxidant-related genes, *GPX1* encodes the enzyme glutathione peroxidase 1 (GPx-1), which is responsible for detoxification of hydrogen peroxide and is considered one of the most important antioxidants (Forman et al., 2009). Another key gene in that pathway is *GSR* encoding the enzyme glutathione reductase (GSR), which reduces glutathione disulfide (GSSG) to glutathione (GSH), the most-potent cellular antioxidant used by glutathione peroxidases (GPxs) against cytosolic hydrogen peroxide in PMN (Aquilano et al., 2014). The fact that both GPX1 and GSR work in concert in the GSH pathway, yet their abundance did not follow the same trend after LPS challenge in NormBCS calves was surprising. The marked downregulation of *GSR* after LPS challenge in NormBCS compared with HighBCS calves suggested a quick and dramatic depletion of intracellular reduced GSH. Not all enzymes in the GPxs family use GSH as a reductant; however, it is well-known that GPX1 uses reduced GSH as a mandatory cosubstrate in the reduction of hydrogen peroxide to water (Lubos et al., 2011). Because of the upregulation of *GPX1* in NormBCS vs. HighBCS calves

after LPS challenge, it could be possible that reduced GSH availability was sufficient. Without data on intracellular concentrations of GSH it is challenging to speculate further on the physiologic meaning of such response.

We observed a similar downregulation in *GSR* after LPS challenge in PMN cells isolated from mid-lactation Holstein cows (Vailati-Riboni et al., 2019b). As we reported in that study, the downregulation of key genes in the GSH pathway in response to LPS challenge was unexpected in a condition of intense inflammatory and oxidative stress. Murine models demonstrated that low functionality in PMN was associated with GSR deficiency (Yan et al., 2012), thus, downregulation of *GSR* might be related to a similar condition in these young calves. An in vitro study demonstrated that GSR activity increases markedly immediately after LPS challenge due to high reactive oxygen metabolites (ROM) production, but after 0.5 h GSR activity decreased and also coincided with substantial clearance of ROM (Strauss et al., 1996). Thus, it is also possible that the downregulation of *GSR* after 3.5 h of LPS challenge in the present study reflected a similar behavior.

The *MTR* gene encodes 5-methyltetrahydrofolate-homocysteine methyltransferase, an important catalyst in the final stage of methionine biosynthesis in the 1-carbon metabolism pathway (Steele et al., 2020). Another key enzyme in the pathway is encoded by the *CBS* gene, cystathionine-beta-synthase (CBS), which converts homocysteine to cystathionine in the first step of the transsulfuration pathway (Vailati-Riboni et al., 2019a). In our previous work evaluating the effects of methionine and choline supplementation during the peripartum period on immunometabolic mRNA abundance in Holstein cow PMN, it was observed that both dietary methyl donors downregulated *CBS* abundance (Zhou et al., 2018). Although we are unaware of data measuring activity of 1-carbon metabolism enzyme in immune cells, it could be possible that alterations in mRNA abundance reflect to some extent changes in protein abundance and enzyme activity. For example, activity of CBS in liver tissue together

with intermediate metabolites of the methionine cycle (e.g. methionine, cysteinesulfinate, and hypotaurine) of cows calving at optimal BCS were greater suggesting a greater flow through the transsulfuration pathway (Vailati-Riboni et al., 2020). In this sense, we speculate that upregulation of *MTR* coupled with downregulation of *CBS* abundance in the HighBCS group after LPS challenge might have shifted homocysteine metabolism towards regeneration of methionine rather than synthesis of GSH.

The methionine cycle is a component of 1-carbon metabolism where 1-carbon units from the folate cycle can be used for remethylation of homocysteine to methionine (Zhou et al., 2018). Transfer of methyl groups from betaine to homocysteine is another way to regenerate methionine, hence, besides direct uptake from circulation, cells that possess a functional methionine and folate cycle are capable of generating methionine in different ways. At least in non-ruminants, regulation of epigenetic reprogramming in immune cells is another key function of methionine (Roy et al., 2020). Activated T lymphocytes use exogenous methionine to synthesize S-adenosylmethionine (**SAM**), the main intracellular methyl group donor and essential epigenetic driver (Schvartzman et al., 2018; Roy et al., 2020). In activated T lymphocytes, undersupply of methionine and reduced SAM availability leads to unstable histone methylation, hence, affecting gene transcription. Thus, at least in non-ruminants, methionine and SAM supply are essential for maintenance of histone methylation at active promoters in T cells (Roy et al., 2020). Recent studies in rodents and humans underscored the link between methylation of the *CBS* gene promoter and key physiologic outcomes. Hyper-methylation of *CBS* led to development of hyperhomocysteinemia and prevented folic acid therapy from alleviating this disorder (Behera et al., 2019; Huang et al., 2019). Thus, it could be possible that the downregulation of *CBS* in HighBCS calves was partly due to epigenetic regulation. As such, in HighBCS calves the flow through the transsulfuration pathway might have been curtailed and remethylation of homocysteine to methionine increased.

Whole Blood Responses over Time

The *SOD2* gene encodes the mitochondrial enzyme superoxide dismutase 2 (SOD2) responsible for the destruction of anionic superoxide radicals, normally produced in cells and toxic to the organism (Sordillo and Aitken, 2009). Similar to the present study, it was previously reported that cows with high BCS before calving had lower total circulating SOD activity and greater concentrations of ROM (Bernabucci et al., 2005). Whether these responses prior to parturition have direct impact or can be transferred to the calf in utero is unknown. However, data from Ling et al. (2018) demonstrated that calves born from cows with high oxidative stress index or exposed to high maternal NEFA in utero, characteristics often seen in HighBCS cows, had greater serum concentrations of reactive oxygen and nitrogen species. In addition, those calves had greater basal concentrations of acute-phase proteins and cytokines suggesting a heightened inflammatory status (Ling et al., 2018). Those data along with the greater responsiveness of *IL1B* abundance to LPS in the HighBCS group at birth underscored the potential for the maternal environment, and specifically oxidant status, to affect the calf in the last stages of development in utero.

The upregulation of *NOS2*, which encodes an inducible nitric oxide synthase (NOS) during the oxidative burst (Bogdan, 2015), after LPS challenge underscored the importance of this enzyme as component of the antimicrobial response in the young calf immune system. The response in *NOS2* observed at 42 d of age in the NormBCS group is noteworthy because of the similar response for *SOD2*, *GSR*, and *GSS* abundance all of which participate in the antioxidant response. It is well-known that intracellular killing of pathogens through the production of ROM and nitric oxide (NO) by phagocytes is one of the most important defense mechanisms of the innate immune response (Boulougouris et al., 2019). An in vitro study with murine macrophages indicated that ROM and NO production during the oxidative burst decreased GSR activity (Butzer et al., 1999). Butzer et al. (1999) speculated that this effect on

GSR might be a mechanism to counterbalance the effects of hydrogen peroxide on the GSR enzyme. The interactions detected at d 42 for *GSR*, *GSS*, *SOD2* and *NOS2* further suggest that cellular levels of GSH can decrease in parallel with an increase of anionic superoxide and NO levels. Thus, as cellular levels of GSH decrease in parallel with NO, it is possible that NO regulates its own synthesis through negative feedback mechanisms on the activity of NOS, including the reduction of GSH (Butzer et al., 1999; Vailati-Riboni et al., 2019b).

The greater *MTR* abundance observed in HighBCS calves at birth was not sustained over time, and the longitudinal response assessed by the interaction BCS × Age confirmed the effect was manifested strictly at birth. At least in rodents, data underscore the importance of the methionine cycle on global histone methylation and immune cell function, but also the idea that epigenetic marks respond to environmental changes on a rapid scale, i.e. minutes to hours (Roy et al., 2020). In this context, the possible epigenetic regulation effects in our calves may have been diluted over time due to external environmental factors including changes in nutrition, e.g. colostrum, milk replacer, and starter grain mix.

Regardless of maternal BCS, the longitudinal downregulation of most genes related to the immune response and the decrease in secretion of IL-1β after birth has been previously demonstrated in various studies with neonatal calves (Osorio et al., 2013; Jacometo et al., 2018; Alharthi et al., 2019). Combined, data generated from our laboratory in neonatal calf PMN demonstrate a temporal downregulation of key genes related to the cytokine production (*IL1B*, *IL6*, *IL10*, *TNF*), cytokine receptors (*IRAK1*, *CXCR1*), toll-like receptors (*TLR4*, *NFKB1*), adhesion and migration (*CADM1*, *ICAM1*, *ITGAM*) and antimicrobial function (*MPO*). Our group and others have speculated that these responses might be related to the temporal dynamics in the profile of endocrine factors and immune cell populations (Gao et al., 2012; Jacometo et al., 2018). For instance, during calving, endocrine changes such as high levels of circulating cortisol induce an immunosuppressive state with large changes in

proportions of circulating leukocytes in whole blood of newborn calves (Marcato et al., 2018). At birth, calves have greater proportions of circulating PMN than lymphocytes (ratio 2:1); however, due to the rapid decline in PMN and gradual increase in lymphocyte concentrations, at 1 wk of age proportions are similar and the reverse is observed after the neonatal period (ratio 1:2) (Jones and Allison, 2007; Tornquist and Rigas, 2010). Thus, considering these responses, our data confirm that at birth the immune system is more sensitive to LPS stimulation and underscore the importance of this responsiveness in the context of immune maturation during a time when calves still have not acquired passive immunity.

CONCLUSIONS

Maternal BCS leads to different responses in both immune and antioxidant mechanisms in the calf after an inflammatory challenge with LPS. Responses detected for components of the 1-carbon metabolism pathway after LPS challenge indicated the potential existence of epigenetic regulation. Overall, the data underscored that managing maternal BCS could not only benefit the cow, but the neonatal calf. Long-term effects of maternal exposure to BCS on calf growth and performance remain to be established.

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Table 1. Effects of maternal body condition score during late-pregnancy on the response of calf whole blood mRNA abundance to an ex vivo lipopolysaccharide (LPS) challenge at birth and prior to colostrum feeding. Calves were from cows calving at normal (NormBCS ≤3.25; n = 22) or high (HighBCS ≥3.75; n = 16) body condition. Response calculated as LPS challenged - non-challenged sample for each calf

	Calf group				
Gene symbol	HighBCS	NormBCS	Difference[1]	SEM[2]	*P*-value
Cytokines					
IL1B	3.40	0.16	20	0.75	<0.01
IL6	3.08	3.00	0.03	0.80	0.94
IL10	3.77	2.43	0.55	0.88	0.24
TNF	3.24	2.79	0.16	0.65	0.61
Cytokines receptors					
IRAK1	0.39	-0.13	4.00	0.39	0.30
CXCR1	0.15	0.22	-0.32	0.22	0.81
Toll-like receptors pathway					
TLR4	0.44	0.34	0.29	0.30	0.79
BPI	-0.81	-0.84	0.04	0.29	0.93
NFKB1	1.07	0.28	2.82	0.24	<0.01
STAT3	0.42	0.80	-0.48	0.24	0.22
Adhesion and migration					
CADM1	-0.44	-0.21	-1.10	0.24	0.45
ICAM1	1.49	1.10	0.35	0.43	0.49
ITGAM	0.49	0.30	0.63	0.28	0.61
SELL	-0.43	-0.64	0.33	0.30	0.59
Antimicrobial function					
LYZ	-0.50	-0.11	-3.55	0.38	0.29
MPO	4.06	4.48	-0.09	0.59	0.60
NOS2	4.43	3.52	0.26	1.00	0.49
SOD2	1.05	0.69	0.52	0.41	0.50
Antioxidant function					
GPX1	-0.40	0.33	-2.21	0.23	0.02
GSR	-1.15	-3.55	0.68	0.76	0.02
GSS	-0.89	-1.05	0.15	0.24	0.60
1-carbon metabolism					
CBS	-1.30	-0.22	-4.91	0.35	0.02
MTR	1.98	-0.26	8.62	0.97	0.09

[1]Difference between HighBCS and NormBCS as fold change = (HighBCS - NormBCS)/NormBCS.
[2]Greatest standard error of the mean.

Table 2. Effects of maternal body condition during late-pregnancy on calf whole blood mRNA abundance after ex vivo lipopolysaccharide (LPS) challenge at 0 (at birth and prior to colostrum feeding), 21 and 42 (at weaning) days of age. Calves were from cows calving at normal (NormBCS ≤3.25; n = 22) or high (HighBCS ≥3.75; n = 16) body condition. Response calculated as LPS challenged - non-challenged sample for each calf

		Day of age				*P*-value		
Gene symbol	Calf group	0	21	42	SEM[1]	BCS	Age	BCS × Age
Cytokines								
IL1B	HighBCS	3.41[a]	-0.43	-1.74	1.49	0.21	<0.01	0.07
	NormBCS	0.15[b]	-0.99	-0.97	1.00			
IL6	HighBCS	3.07	3.32	-0.89	1.56	0.45	0.04	0.17
	NormBCS	3.00	2.36	2.14	1.04			
IL10	HighBCS	3.77	1.27	-1.04	1.58	0.84	<0.01	0.18
	NormBCS	2.41	0.95	1.14	1.06			
TNF	HighBCS	2.82	0.47	-1.65	0.93	0.48	<0.01	0.58
	NormBCS	2.76	0.37	-0.49	0.64			
Cytokine receptors								
IRAK1	HighBCS	0.33	-0.50	-0.72	0.64	0.62	0.04	0.41
	NormBCS	-0.10	-1.09	-0.29	0.42			
CXCR1	HighBCS	0.15	-0.03	-1.95	0.41	0.94	<0.01	0.48
	NormBCS	0.22	-0.42	-1.58	0.28			
Toll-like receptors pathway								
TLR4	HighBCS	0.43	0.37	-1.21	0.59	0.18	<0.01	0.28
	NormBCS	0.32	-0.87	-1.45	0.41			
BPI	HighBCS	-0.85	-0.80	-0.47	0.58	0.13	0.88	0.34
	NormBCS	-0.80	-1.11	-1.55	0.39			
NFKB1	HighBCS	1.06[a]	-0.67	-0.63	0.44	0.45	<0.01	0.12
	NormBCS	0.27[b]	-0.54	-0.58	0.31			
STAT3	HighBCS	0.43	1.09	0.22	0.46	0.27	0.07	0.93
	NormBCS	0.80	1.26	0.57	0.31			
Adhesion and migration								
CADM1	HighBCS	-0.44	0.53	-0.57	0.44	0.41	0.03	0.58
	NormBCS	-0.21	0.36	-0.05	0.30			
ICAM1	HighBCS	1.49	0.14	-0.71	0.87	0.44	0.02	0.25
	NormBCS	1.10	0.28	0.66	0.58			
ITGAM	HighBCS	0.49	-0.03	-0.84	0.57	0.80	<0.01	0.77
	NormBCS	0.28	-0.30	-0.59	0.39			
SELL	HighBCS	-0.43	-0.75	-0.47	0.64	0.63	0.74	0.99
	NormBCS	-0.64	-0.94	-0.58	0.43			

Antimicrobial function								
LYZ	HighBCS	-0.54	0.53	-0.65	0.66	0.98	0.65	0.26
	NormBCS	-0.11	-0.42	-0.20	0.46			
MPO	HighBCS	4.07	4.76	1.88	1.44	0.30	<0.01	0.22
	NormBCS	4.51	3.86	-0.01	0.76			
NOS2	HighBCS	4.44	3.47	-0.04[a]	1.81	0.18	0.26	0.05
	NormBCS	3.53	4.01	4.46[b]	1.21			
SOD2	HighBCS	1.04	1.43	-1.56[a]	0.85	0.09	0.17	<0.01
	NormBCS	0.70	1.02	1.70[b]	0.56			
Antioxidant function								
GPX1	HighBCS	-0.42[a]	-0.32	-1.39	0.42	0.11	<0.01	0.35
	NormBCS	0.33[b]	-0.37	-1.02	0.28			
GSR	HighBCS	-1.22[a]	-5.13	-4.58	1.77	0.94	0.05	0.06
	NormBCS	-3.50[b]	-4.86	-2.36	1.26			
GSS	HighBCS	-0.89	-0.77	-2.08[a]	0.47	0.57	0.06	0.10
	NormBCS	-1.05	-1.10	-1.14[b]	0.31			
1-carbon metabolism								
CBS	HighBCS	-1.29[a]	0.98	-1.43	0.63	0.29	<0.01	0.16
	NormBCS	-0.22[b]	0.49	-0.86	0.42			
MTR	HighBCS	2.03	-2.75	-3.77	2.01	0.59	<0.01	0.07
	NormBCS	-0.23	-2.44	-0.12	1.47			

[1]Greatest standard error of the mean.
[a-b]Differences between calf groups within a given time point ($P \leq 0.05$).

Table 3. Effects of maternal body condition score during late-pregnancy on calf plasma concentrations of biomarkers related to energy metabolism and antioxidant status at birth and prior to colostrum feeding. Calves were from cows calving at normal (NormBCS ≤3.25; n = 22) or high (HighBCS ≥3.75; n = 16) body condition

	Calf group			
Item[1]	HighBCS	NormBCS	SEM[2]	*P*-value
Fatty acids, mmol/L	0.94	1.12	0.10	0.16
β-hydroxybutyrate, mmol/L	0.05	0.06	0.01	0.17
Paraoxanase, U/mL	10.6	8.74	2.75	0.61
ROMt, mg of H_20_2/100mL	5.05	5.29	0.34	0.59
FRAP, µmol/L	251	256	11	0.75
NOx, µmol/L	168	183	11	0.34
NO_2^-, µmol/L	3.05	3.70	0.26	0.07
NO_3^-, µmol/L	165	179	11	0.37
Retinol, µg/mL	8.73	8.51	1.02	0.87
α-tocopherol, µg/mL	0.42	0.45	0.06	0.69
β-carotene, mg/100 mL	0.005	0.006	0.001	0.27
Zinc, µmol/L	14.0	11.0	1.7	0.19

[1]NOx = nitric oxide metabolites; NO_2^- = nitrite; NO_3^- = nitrate; FRAP = ferric reducing ability of plasma; ROMt = reactive oxygen metabolites, total.
[2]Greatest standard error of the mean.

Figure 1.

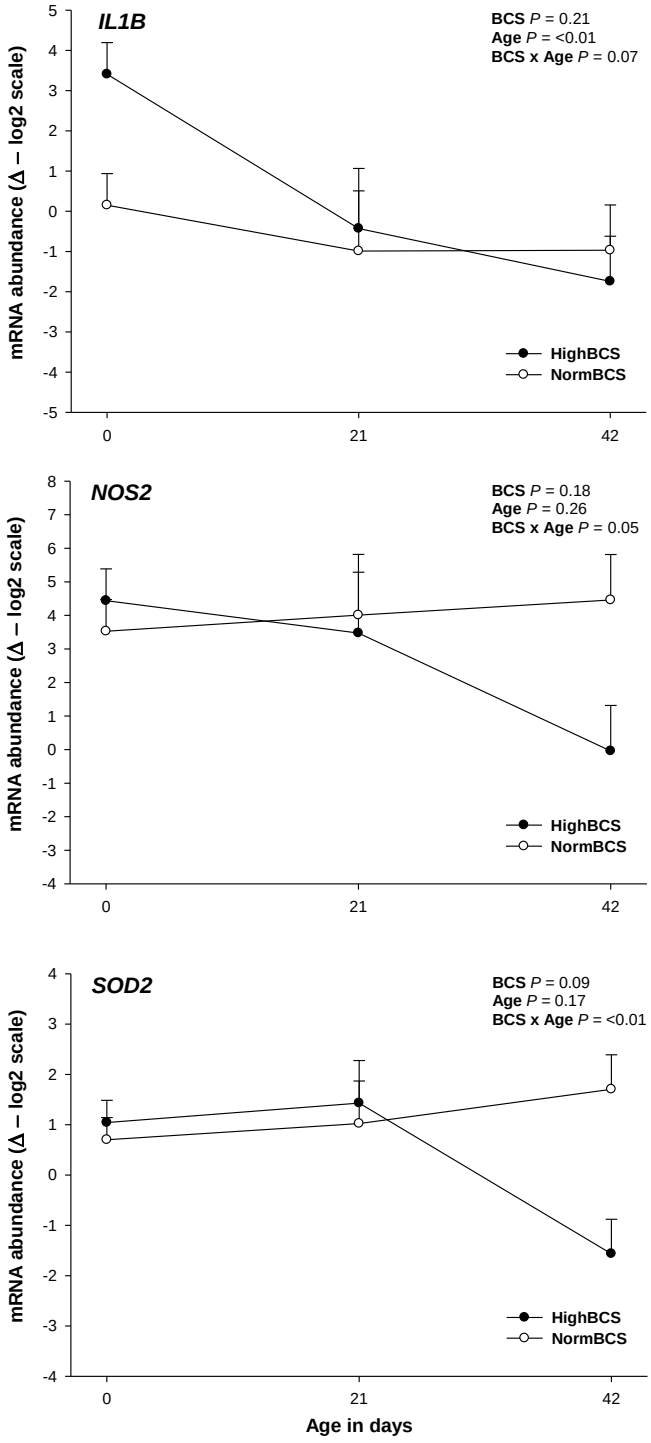

IL1B
BCS P = 0.21
Age P = <0.01
BCS x Age P = 0.07
mRNA abundance (Δ – log2 scale)
HighBCS
NormBCS
NOS2
BCS P = 0.18
Age P = 0.26
BCS x Age P = 0.05
mRNA abundance (Δ – log2 scale)
HighBCS
NormBCS
SOD2
BCS P = 0.09
Age P = 0.17
BCS x Age P = <0.01
mRNA abundance (Δ – log2 scale)
HighBCS
NormBCS
0
21
42
Age in days

Figure 2.

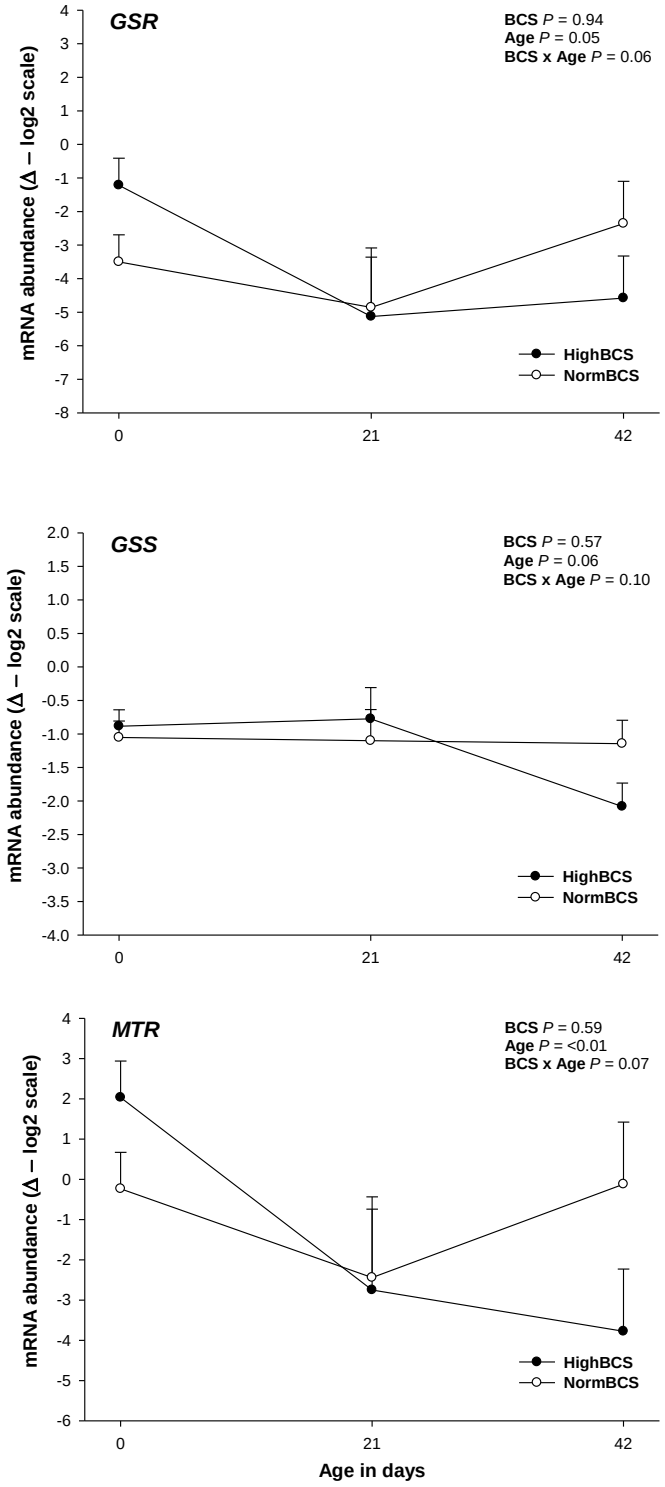

GSR
BCS P = 0.94
Age P = 0.05
BCS x Age P = 0.06
mRNA abundance (Δ – log2 scale)
HighBCS
NormBCS
GSS
BCS P = 0.57
Age P = 0.06
BCS x Age P = 0.10
MTR
BCS P = 0.59
Age P = <0.01
BCS x Age P = 0.07
0
21
42
Age in days

Figure 3.

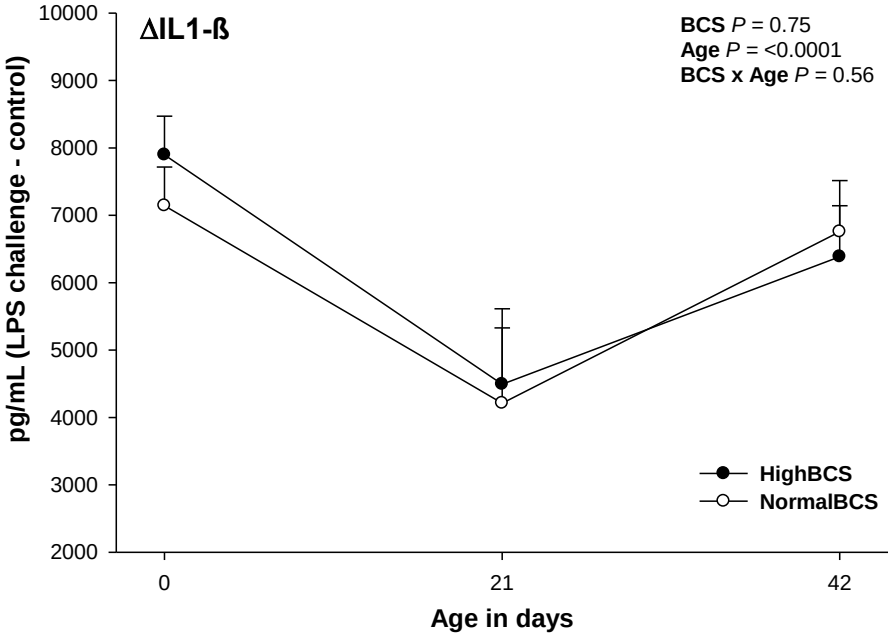

ΔIL1-ß
BCS P = 0.75
Age P = <0.0001
BCS x Age P = 0.56
pg/mL (LPS challenge - control)
10000
9000
8000
7000
6000
5000
4000
3000
2000
0
21
42
Age in days
HighBCS
NormalBCS

Figure 4.

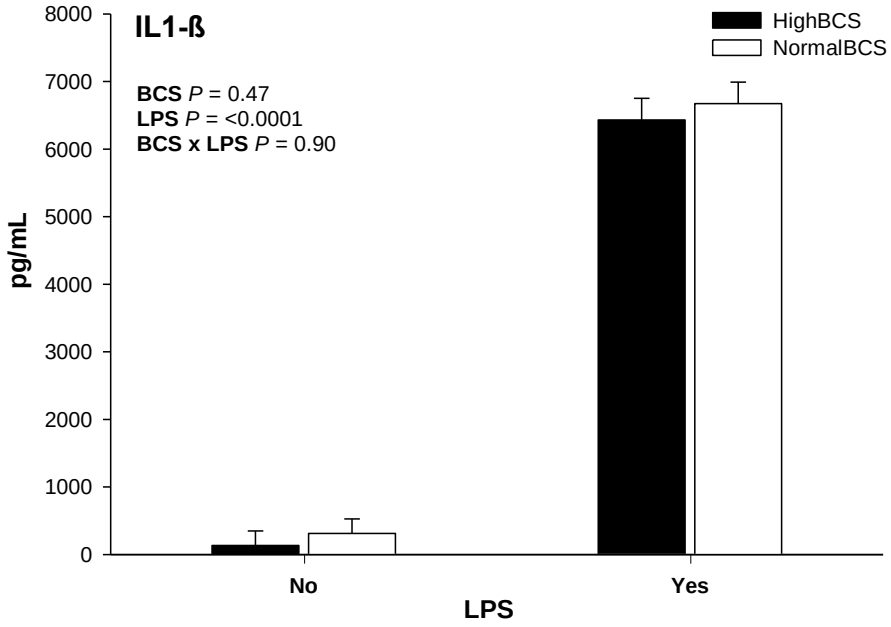

IL1-ß
HighBCS
NormalBCS
BCS P = 0.47
LPS P = <0.0001
BCS x LPS P = 0.90
pg/mL
8000
7000
6000
5000
4000
3000
2000
1000
0
No
Yes
LPS

Figure Legends

Figure 1. Response in mRNA abundance of genes involved in cytokine production (*IL1B*) and antimicrobial function (*NOS2, SOD2*) after ex vivo lipopolysaccharide (LPS) challenge of whole blood at 0 (before colostrum), 21 and 42 days of age. Calves were from cows calving at normal (NormBCS ≤3.25; n = 22) or high (HighBCS ≥3.75; n = 16) body condition. Response calculated as LPS challenged - non-challenged sample for each calf.

Figure 2. Response in mRNA abundance of genes involved in antioxidant function (*GSR, GSS*) and 1-carbon metabolism (*MTR*) after ex vivo lipopolysaccharide (LPS) challenge of whole blood at 0 (before colostrum), 21 and 42 days of age. Calves were from cows calving at normal (NormBCS ≤3.25; n = 22) or high (HighBCS ≥3.75; n = 16) body condition. Response calculated as LPS challenged - non-challenged sample for each calf.

Figure 3. Response in plasma interleukin 1-β concentration (pg/mL) after ex vivo lipopolysaccharide (LPS) challenge of whole blood at 0 (before colostrum), 21 and 42 days of age. Calves were from cows calving at normal (NormBCS ≤3.25; n = 22) or high (HighBCS ≥3.75; n = 16) body condition.

Figure 4. Plasma interleukin 1-β (IL-1β) concentrations (pg/mL) after ex vivo lipopolysaccharide (LPS) challenge. Calves were from cows calving at normal (NormBCS ≤3.25; n = 22) or high (HighBCS ≥3.75; n = 16) body condition.

Supplementary Material

Supplemental Table S1. Ingredient and nutrient composition of diet fed during close-up period (-28 days to calving)

Item	Close-up diet
Ingredient (% of DM)	
Corn silage	37.47
Ground shelled corn	11.60
Wheat straw	21.82
Canola meal	11.67
Soybean meal	6.30
Soychlor[1]	3.37
Corn gluten	2.80
ProvAAL2 AADvantage[2]	0.47
Biotin[3]	0.10
Rumensin[4]	0.19
Calcium sulfate	0.53
Magnesium oxide	0.10
Ca	0.66
P	0.33
Salt	0.10
Na	0.12
Cl	0.78
Mg	0.45
K	1.36
S	0.33
Nutrient composition	
CP, % of DM	14.50
NDF, % of DM	43.30
ADF, % of DM	33.80
aNDFom, % of DM	49.21
NFC, % of DM	28.22
NE_L, Mcal/kg of DM	1.37
RDP, % of DM	8.45
RUP, % of DM	6.05
RDP required, g/d	1,165.00
RDP supplied, g/d	1,152.00
RDP balance, g/d	-18.00
RUP required, g/	158.00
RUP supplied, g/d	821.00
RUP balance, g/d	662.00
MP required, g/d	821.00
MP supplied, g/d	1,360.00
MP balance, g/d	539.00

[1]West Central Soy.
[2]Perdue Agribusiness (Salisbury, MD, USA).
[3]ADM Animal Nutrition (Quincy, IL, USA).
[4]Elanco Animal Health (Greenfield, IN, USA).

Supplemental Table S2. Gene symbol, accession number, and forward and reverse primer sequence of the analyzed genes

Gene[1]	Accession #	Forward sequence	Reverse sequence
GAPDH	NM_001034034.2	TGGAAAGGCCATCACCATCT	CCCACTTGATGTTGGCAG
RPS9	NM_001101152.2	CCTCGACCAAGAGCTGAAG	CCTCCAGACCTCACGTTTGTTC
ACTB	NM_173979.3	ACCAACTGGGACGACATGGA	GTCTCGAACATGATCTGGGTCAT
IL1B	NM_174093.1	TCCACCTCCTCTCACAGGAAA	TACCCAAGGCCACAGGAATCT
IL6	NM_173923	CCAGAGAAAACCGAAGCTCTCAT	CCTTGCTGCTTTCACACTCATC
IL10	NM_174088.1	GAAGGACCAACTGCACAGCTT	AAAACTGGATCATTTCCGACAAG
TNF	NM_173966.3	CCAGAGGGAAGAGCAGTCCC	TCGGCTACAACGTGGGCTAC
IRAK1	NM_001040555.1	CCTCAGCGACTGGACATCCT	GGACGTTGGAACTCTTGACATCT
CXCR1	NM_001105038.1	CGGCACTGGGTCAAGTTCATAT	GTCGGAGTATGGTGGTTGATAGG
TLR4	NM_174198.6	GCTGTTTGACCAGTCTGATTGC	GGGCTGAAGTAACAACAAGAGGAA
BPI	NM_173895.2	ACACCGTCCGCATACACATCT	TTTCTGGTCATGGACTTTTGGA
NFKB1	NM_001076409.1	TTCAACCGGAGATGCCACTAC	ACACACGTAACGGAAACGAAATC
STAT3	NM_001012671.2	CCGGTGTCCAGTTCACAACTAA	CCCCGGAGTCTTTGTCAAT
CADM1	NM_001038558.2	GCAAGGAGCTCAAAGGCAAGT	AACCCCGTCATCCTCCTTGT
ICAM1	NM_174348.2	AGAATTAGCGCTGACCTCTGTTAAG	CGGACACATCTCAGTGACTAAACAA
ITGAM	NM_001039957.1	GGCTTGTCTCTTGCATTTGCT	CCATTTGCATAGGTGTTCTCCTT
SELL	NM_174182.1	CTCTGCTACACAGCTTCTTGTAAACC	CCGTAGTACCCCAAATCACAGTT
LYZ	NM_001077829.1	AAAGCAGTTAACGCCTGTCGTAT	CATGCCACCCATGCTTTAATG
MPO	NM_001113298.2	AGCCATGGTCCAGATCATCAC	ACCGAGTCGTTGTAGGAGCAGTA
NOS2	NM_174182.1	CTGAAGCAGCTGATGGCTACT	ATGATAGCGCTTCTGGTTCTTGAC
SOD2	NM_201527.2	TGTGGGAGCATGCTTATTACCTT	TGCAGTTACATTCTCCCAGTTGA
GPX1	NM_174076.3	AACGCCAAGAACGAGGAGATC	CATTCACCTCGCACTTTTCGA
GSR	NM_001114190.2	CGCTGAGAACCCAGAGACTTG	AAACGGAAAGTGGGAACAGTAAGTA
GSS	NM_001015630.1	CGAGTGATCCAATGCATTTCAG	ATGTCCCACGTGCTTGTTCAT
CBS	NM_001102000.2	GCCACCACCTCTGTCAAATTC	GGACAGAAAGCAGAGTGGTAACTG
MTR	NM_001030298.1	GTTCCACCTGCCACTGTTTTC	CAGCGCTCTCCAATGTTAACAA

[1]*ACTB* = Actin beta; *BPI* = Bactericidal/permeability-increasing protein; *CADM1* = Cell adhesion molecule 1; *CBS* = Cystathionine β-synthase; *CXCR1* = CXC motif chemokine receptor 1; *GAPDH* = Glyceraldehyde-3-phosphate dehydrogenase; *GPX1* = Glutathione peroxidase 1; *GSR* = Glutathione reductase; *GSS* = Glutathione synthetase; *ICAM1* = Intercellular cell adhesion molecule 1; *IL1B* = Interleukin 1β; *IL6* = Interleukin 6; *IL10* = Interleukin 10; IRAK1 = IL1 receptor associated kinase 1; *ITGAM* = Integrin subunit α M; *LYZ* = Lysozyme; *MPO* = Myeloperoxidase; *MTR* = 5-methyltetrahydrofolate-homocysteine methyltransferase; *NFKB1* = Nuclear factor κβ subunit 1; *NOS2* = Nitric oxide synthase 2; *RPS9* = Ribosomal protein S9; *SELL* = Selectin L; *SOD2* = Superoxide dismutase 2, mitochondrial; *STAT3* = Signal transducer and activator of transcription 3; *TLR4* = Toll-like receptor 4; *TNF* = Tumor necrosis factor α.

Supplemental Table S3. RT-qPCR performance among the genes measured in whole blood after lipopolysaccharide (LPS) challenge

Gene symbol	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
IL1B	23.72	1.46	-3.313	0.983	2.004
IL6	26.78	4.52	-3.235	0.988	2.038
IL10	28.08	5.82	-3.131	0.990	2.086
TNF	27.86	5.60	-3.231	0.986	2.039
IRAK1	30.62	8.36	-3.347	0.956	1.990
CXCR1	24.60	2.34	-3.314	0.996	2.003
TLR4	26.12	3.86	-3.222	0.997	2.043
BPI	29.63	7.38	-3.342	0.983	1.992
NFKB1	24.97	2.71	-3.296	0.999	2.011
STAT3	24.15	1.89	-3.353	0.981	1.987
CADM1	28.26	6.00	-3.404	0.998	1.967
ICAM1	25.84	3.58	-3.358	0.995	1.985
ITGAM	28.15	5.89	-3.256	0.966	2.028
SELL	26.53	4.27	-3.151	0.991	2.077
LYZ	24.28	2.02	-3.316	0.996	2.002
MPO	34.87	12.73	-3.198	1.000	2.054
NOS2	27.60	5.34	-3.175	0.995	2.065
SOD2	21.79	-0.47	-3.402	0.997	1.968
GPX1	23.66	1.40	-3.309	0.994	2.005
GSR	29.51	7.36	-3.308	0.981	2.006
GSS	30.19	7.93	-3.500	0.990	1.931
CBS	27.38	5.12	-3.367	0.996	1.982
MTR	31.12	9.05	-3.326	0.992	1.998

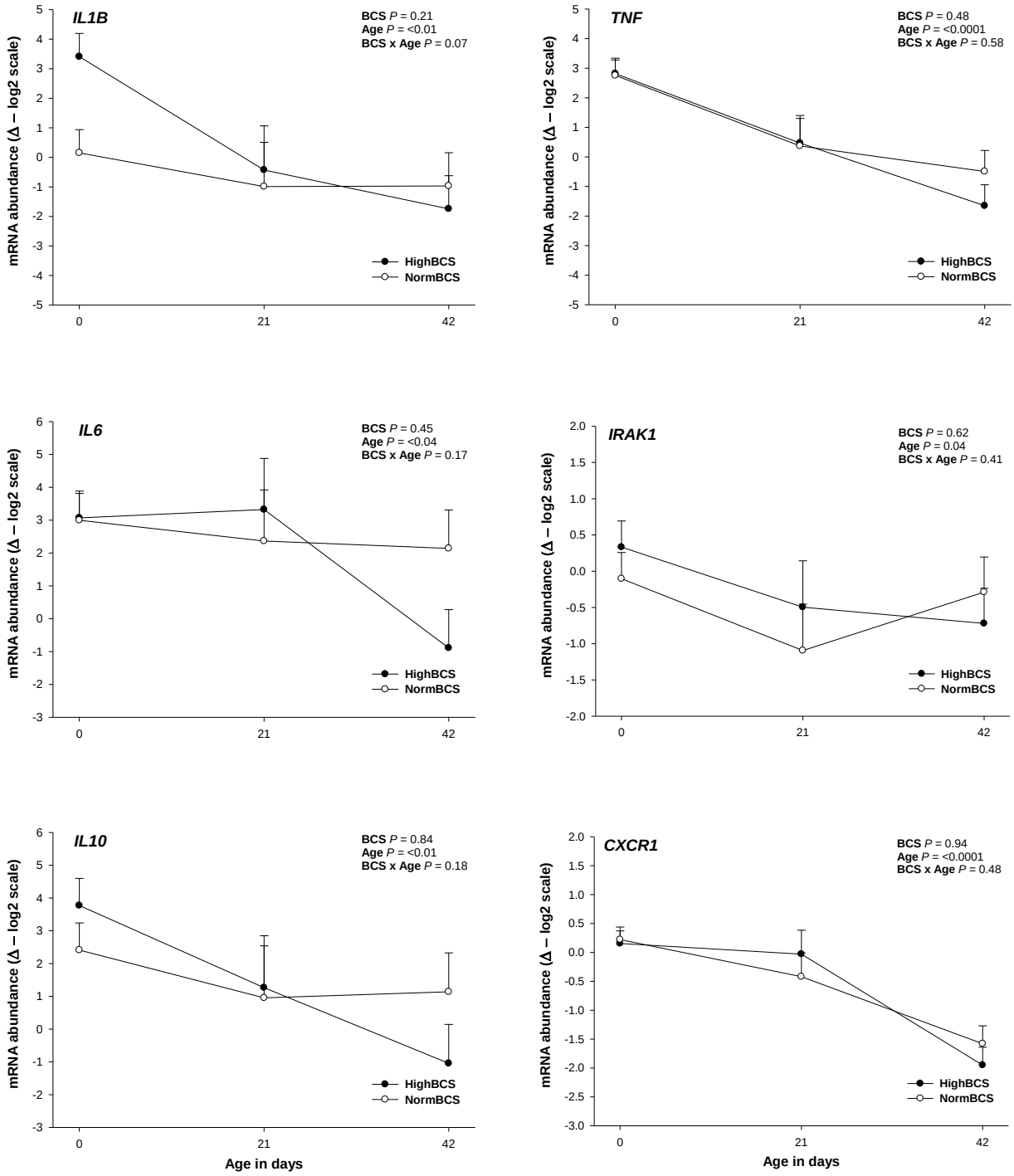
[1]The median is calculated considering all time points and all calves.
[2]The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct internal controls] for all samples.
[3]Slope of the standard curve.
[4]R^2 coefficient of determination of the standard curve.
[5]Efficiency was calculated as [$10^{(-1/Slope)}$].

Supplemental Table S4. Main effect of age on response in mRNA abundance after ex vivo lipopolysaccharide (LPS) challenge of whole blood at 0 (at birth and prior to colostrum feeding), 21 and 42 (at weaning) days of age. Calves were from cows calving at normal (NormBCS ≤3.25; n = 22) or high (HighBCS ≥3.75; n = 16) body condition. Response calculated as LPS challenged - non-challenged sample for each calf. [a,b]Means differ ($P \leq 0.05$)

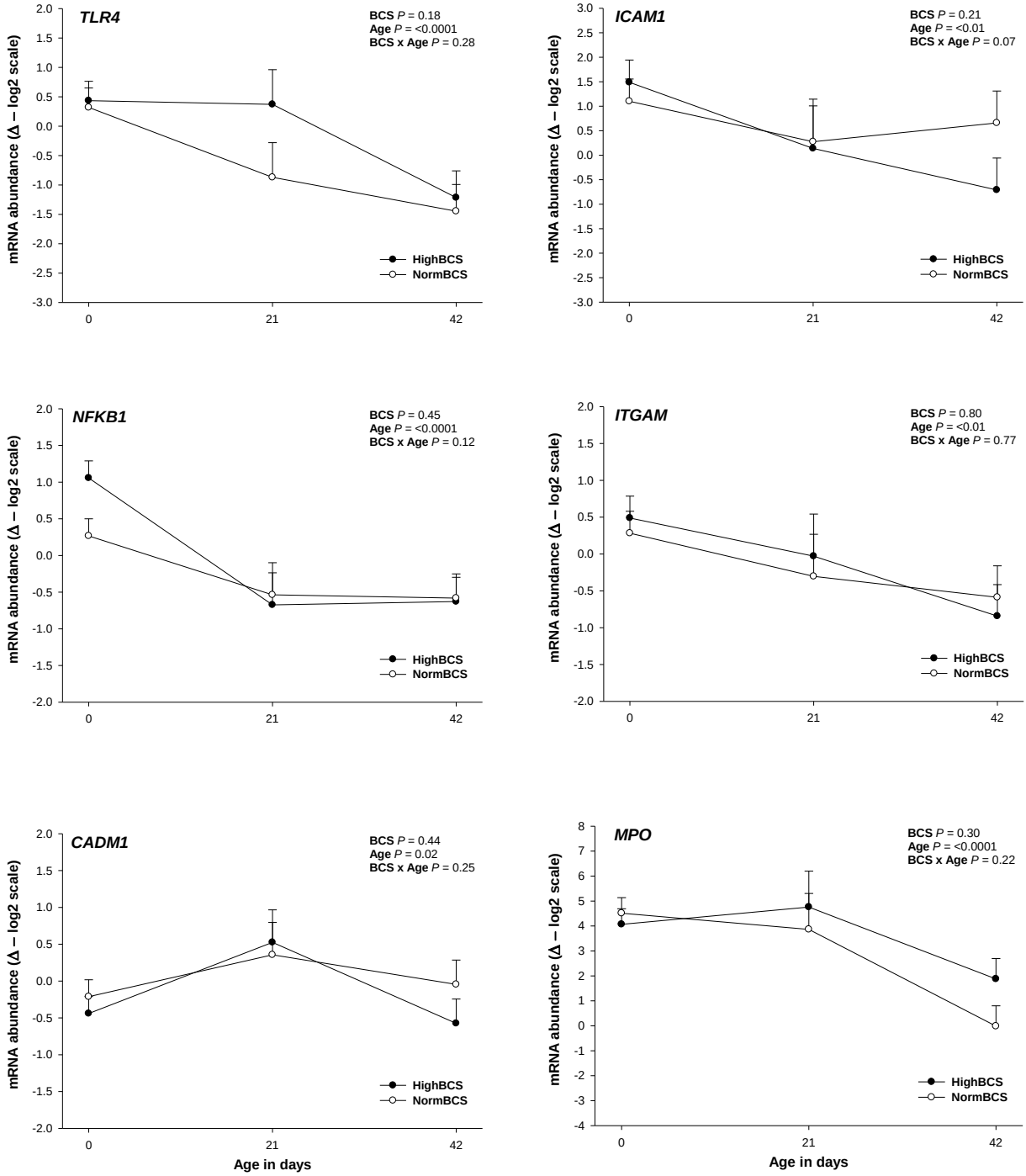
	Age				
Gene symbol	0	21	42	SEM[1]	*P*-value
Cytokines					
IL1B	1.78[a]	-0.71[b]	-1.36[b]	0.84	<0.01
IL6	3.03[a]	2.84[a]	0.63[b]	0.87	0.04
IL10	3.09[a]	1.11[b]	0.05[b]	0.89	<0.01
TNF	2.79[a]	0.42[b]	-1.07[c]	0.53	<0.01
Cytokine receptors					
IRAK1	0.11[a]	-0.80[b]	-0.50[b]	0.36	0.04
CXCR1	0.19[a]	-0.23[a]	-1.77[b]	0.23	<0.01
Toll-like receptor pathway					
TLR4	0.38[a]	-0.25[b]	-1.33[c]	0.33	<0.01
BPI	-0.83[a]	-0.95[a]	-1.01[a]	0.33	0.88
NFKB1	0.66[a]	-0.60[b]	-0.60[b]	0.25	<0.01
STAT3	0.61[ab]	1.18[a]	0.39[b]	0.26	0.07
Adhesion and migration					
CADM1	-0.33[a]	0.44[b]	-0.31[a]	0.25	0.03
ICAM1	1.29[a]	0.21[b]	-0.03[b]	0.49	0.02
ITGAM	0.39[a]	-0.17[ab]	-0.72[b]	0.32	<0.01
SELL	-0.53[a]	-0.84[a]	-0.53[a]	0.36	0.74
Antimicrobial function					
LYZ	-0.27[a]	0.06[a]	-0.42[a]	0.38	0.65
MPO	4.29[a]	4.31[a]	0.93[b]	0.80	<0.01
NOS2	3.98[a]	3.74[a]	2.21[a]	1.02	0.26
SOD2	0.87[a]	1.23[a]	0.07[a]	0.47	0.17
Antioxidant function					
GPX1	-0.04[a]	-0.35[a]	-1.21[b]	0.23	<0.01
GSR	-2.36[a]	-4.99[b]	-3.47[ab]	0.99	0.05
GSS	-0.97[a]	-0.94[a]	-1.61[b]	0.26	0.06
1-carbon metabolism					
CBS	-0.76[a]	0.73[b]	-1.15[a]	0.35	<0.01
MTR	0.90[a]	-2.60[b]	-1.95[b]	1.12	<0.01

[1]Greatest standard error of the mean.
[a-c]Means within a row differ ($P \leq 0.05$).

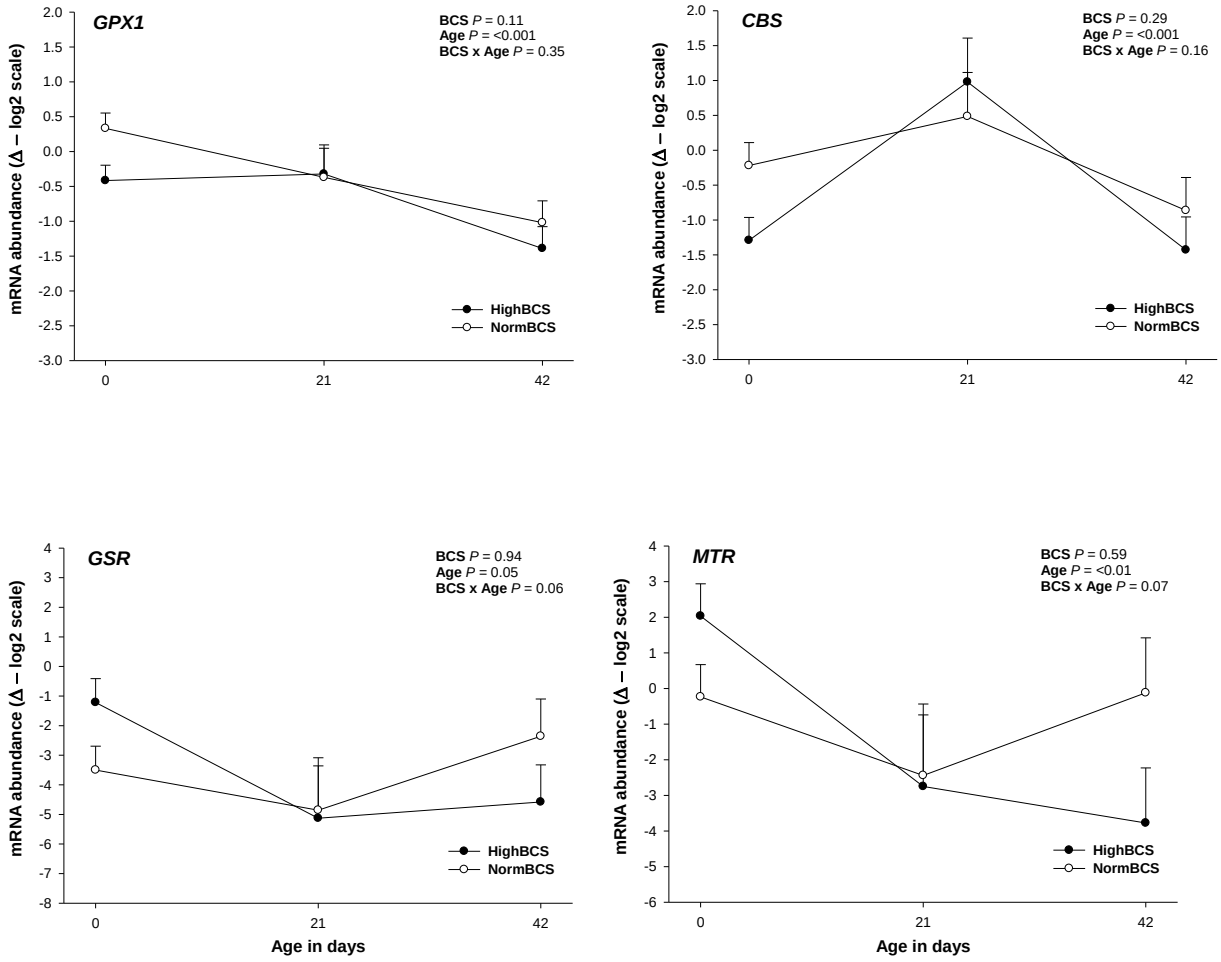
Supplemental Figure S1.



Supplemental Figure S2.



Supplemental Figure S3.



Supplemental Figure Legends

Supplemental Figure S1. Response in mRNA abundance of genes involved in cytokine production (*IL1B*, *IL6*, *IL10*, *TNF*) and cytokine receptors (*IRAK1*, *CXCR1*) after ex vivo lipopolysaccharide (LPS) challenge of whole blood at 0 (before colostrum), 21 and 42 days of age. Calves were from cows calving at normal (NormBCS ≤3.25; n = 22) or high (HighBCS ≥3.75; n = 16) body condition. Response calculated as LPS challenged - non-challenged sample for each calf.

Supplemental Figure S2. Response in mRNA abundance of genes involved in the toll-like receptor pathway (*TLR4*, *NFKB1*), adhesion and migration (*CADM1*, *ICAM1*, *ITGAM*), and antimicrobial function (*MPO*) after ex vivo lipopolysaccharide (LPS) challenge of whole blood at 0 (before colostrum), 21 and 42 days of age. Calves were from cows calving at normal (NormBCS ≤3.25; n = 22) or high (HighBCS ≥3.75; n = 16) body condition. Response calculated as LPS challenged - non-challenged sample for each calf.

Supplemental Figure S3. Response in mRNA abundance of genes involved in antioxidant function (*GPX1*, *GSR*) and 1-carbon metabolism (*CBS*, *MTR*) after ex vivo lipopolysaccharide (LPS) challenge of whole blood at 0 (before colostrum), 21 and 42 days of age. Calves were from cows calving at normal (NormBCS ≤3.25; n = 22) or high (HighBCS ≥3.75; n = 16) body condition. Response calculated as LPS challenged - non-challenged sample for each calf.

4.2 Artigo 2 - Maternal supplementation with cobalt sources, folic acid, and rumen-protected methionine and its effects on molecular and functional correlates of the immune system in neonatal Holstein calves

Artigo publicado na revista *Journal of Dairy Science*

Interpretive Summary

Maternal supplementation with cobalt sources, folic acid, and rumen-protected methionine and its effects on molecular and functional correlates of the immune system in neonatal Holstein calves. ***By Lopes et al.*** The research for alternatives that enhance the immune system in young calves even during fetal life are part of the field of study called "nutritional programming". Through non-invasive methods, we assessed the innate-immune response in whole blood of calves born from cows supplemented during late pregnancy with different cobalt sources, folic acid, and rumen-protected methionine. Data underscored that supplementation with these nutrients modulated few biomarkers of the innate immune system at plasma and molecular levels. Responses were associated with inflammatory biomarkers related to acute-phase response at birth, e.g. lower plasma haptoglobin concentration at birth in calves born from cows fed additional folic acid. Those calves also had differential regulation of genes related to inflammation and pathogen recognition, e.g. greater mRNA abundance in whole blood of genes related to adhesion and migration (*ICAM1*) and toll-like receptor pathway (*BPI*) in response to overstimulation of the immune cells through an ex vivo lipopolysaccharide (LPS) challenge. The lack of effect on most of the evaluated genes, which share similar inflammatory pathways, or immune cell function (phagocytosis, oxidative burst) rendered confirmation of a biological impact of these maternal nutrients challenging.

RUNNING TITLE: MATERNAL METHYL DONORS AND CALF IMMUNE RESPONSE

Maternal supplementation with cobalt sources, folic acid, and rumen-protected methionine and its effects on molecular and functional correlates of the immune system in neonatal Holstein calves

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ABSTRACT

An adequate supply of nutrients during fetal life is an important determinant of immunocompetence in neonates. Calves born to Multiparous Holstein cows fed during the last 30-d of pregnancy two different cobalt (Co) sources [Co glucoheptonate (CoPro) or Co pectin (CoPectin)], folic acid (FOA) and rumen-protected methionine (RPM) were used to study neonatal immune responses after ex vivo lipopolysaccharide (LPS) challenge. Treatment groups were (n = 12 calves/group) CoPro, FOA+CoPro, FOA+CoPectin, and FOA+CoPectin+RPM. After calving, calves were weighed at birth and blood was collected at birth (before colostrum), 21 and 42 (at weaning) d of age. Growth performance was recorded for each calf once a week during the first 6 wk of age. Energy metabolism, inflammation, and antioxidant status were assessed at birth through various plasma biomarkers. Whole blood was immediately transported to the laboratory and challenged with 3 μg/mL of LPS or used for phagocytosis and oxidative burst assays. Target genes evaluated by real-time quantitative PCR in whole blood samples were associated with immune response, antioxidant function, and one-carbon metabolism. The response in mRNA abundance in LPS-challenged versus non-challenged samples was performed using delta change values (Δ = LPS challenged - LPS non-challenged samples). Phagocytosis capacity and oxidative burst activity were measured in neutrophils and monocytes with the data reported as percentages of CD14/CH138A positive cells. Data including all time points were subjected to ANOVA using PROC MIXED in SAS 9.4, with Treatment, Sex, Age, and Treatment × Age as fixed effects. A one-way ANOVA was used to determine differences at birth with Treatment and Sex as fixed effects. Calf birth BW and other growth parameters were comparable between groups and maternal treatment had no effect. At birth, plasma haptoglobin concentration was lower in in FOA+CoPro vs. CoPro calves. There was no effect for other plasma biomarkers or immune function due to maternal treatments at birth. Compared with CoPro calves, in response to LPS challenge,

whole blood from FOA+CoPectin and FOA+CoPectin+RPM calves had greater mRNA abundance of intercellular adhesion molecule 1 (*ICAM1*). There was no effect for other genes. Regardless of maternal treatments, sex-specific responses were observed due to greater plasma concentrations of haptoglobin, paraoxonase, ROMt, NO_2^-, and β-carotene in Female vs. Male calves at birth. In contrast, whole blood from Male calves showed greater mRNA abundance of *IRAK1*, *CADM1*, and *ITGAM* in response to LPS challenge at birth. The longitudinal analysis of day 0, 21, and 42 data revealed greater bactericidal permeability-increasing protein (*BPI*) mRNA abundance in whole blood from FOA+CoPectin vs. FOA+CoPro calves, coupled with greater abundance in FOA+CoPro vs. CoPro calves. Regardless of maternal treatments, most genes related to cytokines and cytokine receptors (*IL1B*, *IL10*, *TNF*, *IRAK1*, *CXCR1*), toll-like receptor pathway (*TLR4*, *NFKB1*), adhesion and migration (*ICAM1*, *ITGAM*), antimicrobial function (*MPO*), and antioxidant function (*GPX1*) were downregulated over time. Phagocytosis capacity and oxidative burst activity in both neutrophils and monocytes did not differ due to maternal treatment. Regardless of maternal treatments, there was an increase in the percentage of neutrophils capable of phagocytosis and oxidative burst activity over time. Overall, these preliminary assessments suggested that maternal supplementation with FOA and Co combined with RPM had effects on a few plasma biomarkers of inflammation at birth, and molecular responses associated with inflammatory mechanisms during the neonatal period. The fact that few differences were detected for most of the plasma and molecular variables seem to agree with the lack of differences in aspects of immune cell functionality, i.e. phagocytosis capacity and oxidative burst activity.

Key words: methyl donors, neonatal immunity, nutritional programming, vitamin B12

INTRODUCTION

Cellular functions of the neonatal immune system require an initial period of maturation through exposure to new antigens, with some data suggesting that nutritional status during late stages of fetal life also is critical (Hulbert and Moisá, 2016; Jacometo et al., 2018; Alharthi et al., 2019b). The role of methyl donors and one-carbon metabolism in dairy cattle has received substantial attention over the past few decades (Girard et al., 1995; Girard and Matte, 2005; Zhou et al., 2017). Benefits of greater methyl donor supply to both dams and offspring in terms of production and health parameters are clear (Zenobi et al., 2018; Coleman et al., 2020). For instance, a number of studies with dietary methyl donors (e.g. methionine, choline, and folic acid) in peripartal dairy cows have confirmed a benefit in performance, health and immune function (Graulet et al., 2007; Osorio et al., 2013a; Vailati-Riboni et al., 2017b; Arshad et al., 2020). Many of these benefits were also linked to positive responses on aspects of energy metabolism, inflammation, antioxidant status and birth body weight in the offspring (Jacometo et al., 2016; Alharthi et al., 2019a; Duplessis and Girard, 2019), underscoring that adequate supply of methyl donors during later stages of fetal life is an important determinant of immunocompetence and growth in the neonatal calf (Alharthi et al., 2018, 2019b; Zenobi et al., 2018).

Metabolism of the essential nutrients folic acid (**FOA**) and methionine (**Met**) is key for production of homocysteine (**Hcy**) and S-adenosylmethionine (**SAM**), the main donor of methyl groups (Preynat et al., 2010). The FOA and Met cycles are linked through the activity of methionine synthase (**MTR**), which has an absolute requirement for vitamin B12 (**B12**) (Xue and Snoswell, 1985a; Lucock, 2000). This water-soluble vitamin is synthesized by the ruminal microbiome from the trace mineral cobalt (**Co**) in the diet (McDowell, 2000). Because newborn calves do not have a functional rumen, they depend on FOA and B12 acquired in the uterine environment and through colostrum and milk intake (Duplessis and

Girard, 2019). From a physiological point of view, increased supply of FOA and Co, the latter by enhancing B12 ruminal synthesis, could lead to greater endogenous Met synthesis (i.e. remethylation) (Xue and Snoswell, 1985b; a). Although isolated effects of maternal supplementation with methyl donors during early postnatal life were already demonstrated (Jacometo et al., 2016, 2017, 2018; Abdelmegeid et al., 2017; Alharthi et al., 2018, 2019a; b), it remains to be determined whether increased supply of FOA and B12, through increased Co supply, alone or combined with Met generate similar effects in neonatal innate-immune responses.

Our hypothesis was that additional sources of one-carbon units from an increase in the supply of dietary methyl donors for the dam enhances offspring innate-immune function during early postnatal life. To test this hypothesis for the first time, calves born to Multiparous Holstein cows fed during the last 30-d of pregnancy an experimental FOA, one of two Co sources [Co glucoheptonate (CoPro) or Co pectin (CoPectin)] and rumen-protected methionine (RPM) were retrospectively selected based on completeness of performance data from birth to 6 wk of age, whole blood mRNA abundance of target genes associated with immune response, antioxidant function, and one-carbon metabolism after ex vivo whole blood lipopolysaccharide (**LPS**) challenge, and peripheral blood neutrophil and monocyte function after ex vivo enteropathogenic bacteria challenge.

MATERIALS AND METHODS

Experimental Design

All procedures were conducted with the approval of the University of Illinois Institutional Animal Care and Use Committee (Protocol #17168). The cow study was performed as a randomized complete blocked design with 72 Holstein cows blocked according to parity, previous lactation milk yield, and expected day of parturition. A power

calculation was performed using an online resource (kindly made available online by Dr. Rollin Brant, University of British Columbia, Vancouver, CA) comparing a mean to a known value. We defined the outcomes of performance and blood of our dataset and its variability. The relevant values for mu0 (known value), mu1 (mean of the population to be sampled), sigma (standard deviation of the sampled population), and sample size were included in the model with α-value = 0.05 (type I error rate). Analyses indicated a power > 0.80. Out of a total of 19, 17, 18, and 18 calves born to cows in the CoPro, FOA+CoPro, FOA+CoPectin, and FOA+CoPectin+RPM groups (described below), respectively, some could not be sampled or complete the entire study due to not meeting the selection criteria below, illness, or missed sampling due to human error. Ultimately, the number of calves (n = 12/group) used to perform all analyses described in the present study was greater than previously reported in studies dealing with the association between maternal nutrition or stress in the prepartal period and neonatal calf growth and development (Jacometo et al., 2015, 2016; Ling et al., 2018).

Despite the removal of calves due to issues outlined above, we believe that retrospective selection of calves with a complete set of performance, molecular, and functional data as the sole criteria strengthens the physiological relevance of the data reported here. According to our hypothesis, supplementation with FOA and Co can increase endogenous Met synthesis via MTR activity, as well as combined Met supplementation can generate additional SAM. Thus, we performed the trial with an approach to investigate two Co sources coupled with an experimental FOA source alone or combined with RPM. Besides evaluating two chemically-distinct Co sources, dividing cows into a CoPro, FOA+CoPro, FOA+CoPectin, and FOA+CoPectin+RPM group allowed us to evaluate the additive effect of FOA or Met, i.e. FOA+CoPectin vs. FOA+CoPro, FOA+CoPro vs. CoPro, and FOA+CoPectin+RPM vs. FOA+CoPectin. Cows in each FOA group received 50 g of FOA

(experimental ruminally-available folic acid source). The Co treatments delivered 1 mg of Co/kg of DM [Co glucoheptonate (CoPro) or Co pectin (CoPectin), Zinpro Corporation, Eden Prairie, MN, USA]. The RPM (Smartamine M, Adisseo NA, Alpharetta, GA, USA) was fed at 0.09% of DM to achieve a ratio of 2.8:1 Lys:Met in the MP. During the close-up period, cows were housed in a sand-bedded free-stall barn and fed a total mixed ration (TMR) daily (0600 h) with ad libitum access to the same basal diet (1.37 Mcal/kg of DM, 14.5% CP), using an individual gate feeding system (American Calan, Northwood, NH, USA). Treatments were top-dressed from -30 to 30-d around parturition. Diet ingredient and nutrient composition during the close-up period are available in Supplemental Table S1. When the expected calving date approached, cows were moved to individual pens bedded with straw.

After calving, total colostrum volume was recorded and total immunoglobulin G (IgG) concentration was immediately estimated at room temperature (22°C) based on specific gravity, with a colostrometer (Bovine Colostrometer catalog no. C10978N, Nasco, Fort Atkinson, WI, USA). Calves (n = 12/group) [CoPro (7 male, 5 female), FOA+CoPro (6 male, 6 female), FOA+CoPectin (5 male, 7 female), and FOA+CoPectin+RPM (7 male, 5 female)] were used in the study if all the following criteria were fulfilled: (1) single calf, (2) calving difficulty score ≤ 3 (5-point scale), (3) colostrum quality assessed through a colostrometer of total IgG ≥ 60 mg/mL, (4) dam produced at least 3.8 L of good quality first colostrum, and (5) calf birth body weight (BW) ≥ 40 kg (Jacometo et al., 2016).

Immediately after birth, calves were weighed using a digital scale and removed from their dams. Cows were milked in the parlor where the volume of colostrum was recorded, and calves were fed with first-milking fresh colostrum from their respective dam using nipple bottles within 6 h after birth. All calves received 3.8 L of first colostrum, if voluntary colostrum intake did not reach 3.8 L calves were force-fed via esophageal tube to reach the required amount of colostrum. Calves were housed in individual outdoor hutches bedded with

straw and fed twice daily (0600 h and 1800 h) with milk replacer (Advance Excelerate, Milk Specialties, Carpentersville, IL, USA; 28.5% CP, 15% Fat) until 35 days of age. From 36 days of age until weaning (42 days of age), calves were switched to once a day feeding at 0600 h. In detail, calves received 4.54 kg/d of milk replacer (0.59 kg of milk replacer in 3.95 L of water) from 1 to 10 d of age, 5.90 kg/d (0.77 kg of milk replacer in 5.13 L of water) from 11 to 20 d of age, 7.26 kg/d (0.94 kg of milk replacer in 6.32 L of water) from 21 to 35 d of age, and 3.63 kg/d (0.47 kg of milk replacer in 3.16 L of water) in a single feeding from 36 to 42 d of age, and had ad libitum access to a starter grain mix (Ampli-Calf Starter 20®; 19.9% crude protein (CP) and 13.5% neutral detergent fiber (NDF), Purina Animal Nutrition, Shoreview, MN, USA). Growth performance including body weight, body length, hip height, wither height and hip width was recorded for each calf once a week during the first 6 wk of age. Health checks including body temperature, respiratory rate, and fecal score (Osorio et al., 2012) were recorded weekly. All calves retained in the study remained clinically healthy and sick animals were excluded to avoid bias in our data due to an exogenous challenge of the immune system.

Energy Metabolism, Inflammation and Antioxidant Status at Birth

Energy metabolism, inflammation, and antioxidant status were assessed only in blood samples at birth and prior to colostrum feeding through several plasma biomarkers. In brief, glucose (mmol/L) and albumin (g/L) were analyzed using the IL Test (catalog no. 0018250840, and 0018250040, respectively) purchased from Instrumentation Laboratory Spa in the ILAB 600 clinical auto-analyzer (Instrumentation Laboratory, Lexington, MA). Fatty acids (mmol/L) and β-hydroxybutyrate (BHBA; mmol/L) were measured using kits from Wako Chemicals GmbH (Neuss, Germany) and Randox Laboratories Ltd. (Crumlin, United Kingdom), respectively (Bionaz et al., 2007; Trevisi et al., 2012). Haptoglobin (g/L) and ceruloplasmin (μmol/L) were analyzed as described by Bertoni et al. (2008).

Myeloperoxidase (U/L) and paraoxonase (U/mL) were determined as previously described by Bradley et al. (1982) and Bionaz et al. (2007), respectively. Total reactive oxygen metabolites (ROMt; mg of H_2O_2/100 mL) was determined with a commercial kit (Diacron International s.r.l., Grosseto, Italy). Ferric reducing ability of plasma (FRAP; µmol/L) was measured using the colorimetric method of Benzie and Strain (1996). Plasma nitric oxide metabolites (NOx; µmol/L), nitrite (NO_2^-; µmol/L), and nitrate (NO_3^-; µmol/L) were measured using the Griess test according to Gilliam et al. (1993) and Bouchard et al. (1999). Retinol (µg/mL), α-tocopherol (µg/mL), and β-carotene (mg/100 mL) were analyzed as described by Trevisi et al. (2013). Zinc (µmol/L) was determined with a commercial kits (Wako Chemicals GmbH) (Bionaz et al., 2007).

Whole Blood ex vivo LPS Challenge

The ex vivo LPS challenge was performed as described in our recent work with whole blood in calves (Lopes et al., 2021). Calf whole blood samples were collected from the jugular vein using 20-gauge needles (BD Vacutainer, Becton Dickinson and Company, Franklin Lakes, NJ, USA) at 0 (at birth and prior to colostrum feeding), 21 and 42 (at weaning) days of age. Samples were collected into evacuated tubes containing lithium heparin as an anticoagulant (BD Vacutainer, Becton Dickinson and Company), and immediately transported to the laboratory in a thermo-insulated container with water (38°C). Whole blood samples were challenged with 3 µg/mL of LPS (*Escherichia coli* O111:B4, Sigma Aldrich, St. Louis, MO, USA) no later than 30 min after sampling. In brief, two aliquots of 980 µL of whole blood were stimulated in duplicate with 20 µL of Dulbecco's modified Eagle medium (non-challenged control samples) or with 20 µL of LPS solution (challenged samples). Aliquots were incubated in a water bath for 3.5 hours at 38°C with a horizontal shaking speed of 30 times/min (Jahan et al., 2015; Vailati-Riboni et al., 2017b). Environmental

contaminations with exogenous endotoxins were prevented by using certified endotoxin-free materials.

Whole Blood mRNA Extraction, cDNA Synthesis and RT-qPCR

After LPS challenge, whole blood mRNA extraction was performed using Trizol protocol (TRI Reagent® BD, Sigma Aldrich) as recommended by the manufacturer. Possible residue from genomic DNA was removed by purification with on-column DNase digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany), and RNA concentration was measured using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). All samples had an RNA integrity score greater than 8.0 according to the AATI Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA).

Target genes evaluated encode proteins associated with immune response, antioxidant function and one-carbon metabolism. Detailed information can be found in the Supplemental Table S2 and in our previous studies with cows and calves (Abdelmegeid et al., 2017; Jacometo et al., 2018; Zhou et al., 2018; Lopes et al., 2021). The cDNA synthesis was performed following the same protocol recently described in our study with calf whole blood (Lopes et al., 2021). Quantitative real-time PCR (RT-qPCR) was performed in a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems, Foster City, CA, USA) using 4 µL diluted 1:4 cDNA and 6 µL SYBR Green mixture (Applied Biosystems) with 0.4 µL 10 µM forward and reverse primers, and 0.2 µL nuclease-free water. Each gene was run in triplicate on a single plate with 7-point standard curve plus the negative control. The RT-qPCR performance and the information required by the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) to ensure quality control can be found in Supplemental Tables S3 and S4. Data were normalized using the geometric mean of 3 internal

control genes: *GAPDH, RPS9* and *ACTB* previously validated by our research group (Liang et al., 2019; Vailati-Riboni et al., 2019; Lopes et al., 2021).

Phagocytosis Capacity and Oxidative Burst Activity

Simultaneous phagocytosis capacity and oxidative burst activity of peripheral blood neutrophils and monocytes were determined through an ex vivo challenge with enteropathogenic bacteria *Escherichia coli* (*E*. coli O118:H8; kindly donated by M. A. Ballou, Texas Tech University, Lubbock) as described by Hulbert et al. (2011) with modifications previously reported by our group (Zhou et al., 2016; Vailati-Riboni et al., 2017b; Batistel et al., 2018). In brief, whole blood was sampled from jugular vein at 0, 21, and 42 d of age into evacuated tubes (BD Vacutainer) containing lithium heparin, and placed on ice until the assay was started. Two hundred microliters of whole blood with 40 μL of 100 μ*M* dihydrorhodamine 123 (Sigma-Aldrich), and 40 μL of propidium iodine-labeled bacteria (10^9 cfu/mL) were incubated at 38.5°C for 10 min. After red blood cells were lysed with ice-cold MilliQ water, cells were resuspended in PBS solution. Subsequently, neutrophils were stained with CH138A primary anti-bovine granulocyte monoclonal antibody (catalog no. BOV2067, Washington State University, Pullman) and phycoerythrin-labeled secondary antibody (catalog no. 1020–09S, Southern Biotech, Birmingham, AL), and monocytes were marked with allophycocyanin-labeled anti-CD14 antibody (catalog no. 301808, Biolegend, San Diego, CA). Lastly, cells were resuspended in 400 μL of PBS solution for flow cytometry analyses (LSR II, Becton Dickinson, San Jose, CA). Neutrophils and monocytes were gated based on their side scatter properties in combination with the phycoerythrin and allophycocyanin signal, respectively. Compared with the negative controls, neutrophils and monocytes from *E*. coli–stimulated samples with greater emissions of propidium iodine were considered positive for phagocytosis. Similarly, compared with negative controls, the *E*. coli–stimulated samples with greater emissions of dihydrorhodamine 123 were considered positive

for oxidative burst. Data are reported as percentages of CD14/CH138A positive cells with phagocytosis and oxidative burst activity.

Statistical Analysis

The RT-qPCR data were normalized with the geometric mean of the 3 internal control genes and log2 transformed before statistical analysis in order to obtain a normal distribution. The response in mRNA abundance in LPS-challenged versus non-challenged samples was performed in a similar fashion to a previous study with calves (Ling et al., 2018) using delta change values, Δ = LPS challenged - LPS non-challenged samples as in our previous study (Lopes et al., 2021). Normality of the residuals was tested via Proc Univariate in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). All observations were normally distributed, and observations with absolute value of studentized residual greater than 3 were automatically removed from the analysis. Statistical analysis was performed with SAS 9.4. Data including all time points were subjected to ANOVA using repeated measures ANOVA with PROC MIXED, including Treatment, Sex, Age, and Treatment × Age as fixed effects. Data including only the time point at birth were subject to a one-way ANOVA, with Treatment and Sex as fixed effects. Means were compared using the PDIFF statement in SAS with Tukey test.

RESULTS

Development, metabolism, and immune function at birth

Growth variables did not differ due to maternal treatment at birth nor during the first 6 wk of age ($P > 0.05$; Table 1). With the exception of haptoglobin, there was no difference in energy metabolism, inflammation, or antioxidant status-related biomarkers according to maternal treatments ($P > 0.05$; Table 2). At birth, haptoglobin was lower in calves born to FOA+CoPro vs. CoPro cows ($P < 0.05$; Table 2). In addition, at birth, phagocytosis capacity

and oxidative burst activity in both neutrophils and monocytes did not differ due to maternal treatment ($P > 0.05$; Table 2).

Regardless of maternal treatments, effects of Sex were observed for some inflammation and antioxidant status-related biomarkers due to greater haptoglobin (0.37 ± 0.02 vs. 0.31 ± 0.02 g/L), paraoxonase (13.19 ± 1.97 vs. 6.28 ± 1.27 U/mL), ROMt (5.66 ± 0.23 vs. 4.49 ± 0.20 mg H_2O_2/100mL), NO_2^- (4.56 ± 0.34 vs. 3.31 ± 0.29 µmol/L), and β-carotene (0.007 ± 0.001 vs. 0.004 ± 0.001 mg/100 mL) concentrations in Female vs. Male calves at birth ($P < 0.05$; Table 2).

Whole Blood Leucocyte mRNA Abundance

Cytokine and Cytokine Receptors. At birth, no difference was observed due to maternal treatments for *IL1B*, *IL6*, *IL10*, *TNF*, *IRAK1* and *CXCR1* mRNA abundance in whole blood leucocytes after LPS challenge ($P > 0.05$; Table 3). In addition, a Sex effect was observed for *IRAK1* mRNA abundance due to upregulation in Male vs. Female calves in response to LPS challenge ($\Delta = 0.71 \pm 0.37$ vs. -0.28 ± 0.31; $P = 0.05$; Table 3). Analyzing all time points, regardless of maternal treatments, an Age effect in response to the LPS challenge was detected for most genes measured (Table 4). Over time, there was an Age effect with a decrease in mRNA abundance of *IL1B* ($P < 0.01$), *IL10* ($P < 0.01$), *TNF* ($P < 0.01$), *IRAK1* ($P < 0.01$) and *CXCR1* ($P < 0.01$) in response to the LPS challenge (Suppl. Figure S1).

Toll-Like Receptor Pathway. At birth, no difference was observed due to maternal treatments for *TLR4*, *BPI*, *NFKB1* and *STAT3* mRNA abundance in whole blood leucocytes after LPS challenge ($P > 0.05$; Table 3). Analyzing all time points in response to LPS challenge, *BPI* mRNA abundance was greater overall in whole blood leucocytes from calves born to FOA+CoPectin vs. FOA+CoPro cows ($P < 0.05$; Table 4), coupled with greater abundance in calves born to FOA+CoPro vs. CoPro cows ($P < 0.05$; Table 4). In addition, a Sex effect was

observed due to greater *BPI* mRNA abundance in Male vs. Female calves in response to LPS challenge (Δ = -0.44 ± 0.23 vs. -1.01 ± 0.16; *P* = 0.04; Table 4). Regardless of maternal treatments, there was an Age effect with downregulation of *TLR4* and *NFKB1* after LPS challenge (*P* < 0.01; Suppl. Figure S2).

Adhesion and Migration. At birth, *ICAM1* response to the LPS challenge was greater in whole blood leucocytes from calves born to FOA+CoPectin and FOA+CoPectin+RPM vs. CoPro cows (*P* = 0.06; Table 3). In addition, a Sex effect was observed in *CADM1* (Δ = 0.05 ± 0.22 vs. -0.56 ± 0.19; *P* = 0.04; Table 3) and *ITGAM* (Δ = 0.99 ± 0.30 vs. 0.04 ± 0.26; *P* = 0.02; Table 3) mRNA abundance due to upregulation in Male vs. Female calves in response to LPS challenge. Analyzing all time points regardless of maternal treatments indicated that *ICAM1* and *ITGAM* were downregulated in response to LPS challenge over time (Age; *P* < 0.05; Suppl. Figure S2). In addition, *CADM1* mRNA abundance had a different response over time, with an upregulation due to LPS challenge at 21 d of age (Age; *P* = 0.03; Suppl. Figure S2).

Antimicrobial Function. At birth, no difference was observed due to maternal treatments for *LYZ*, *MPO*, *NOS2* and *SOD2* mRNA abundance in whole blood leucocytes after LPS challenge (*P* > 0.05; Table 3). Analyzing all time points, a Sex effect was observed in *MPO* mRNA abundance due to upregulation in Male vs. Female calves in response to LPS challenge (Δ = 3.99 ± 0.53 vs. 2.64 ± 0.35; *P* = 0.03; Table 4). In addition, mRNA abundance of *SOD2* was greater in Female vs. Male calves in response to LPS challenge (Δ = 0.19 ± 0.39 vs. 1.39 ± 0.28; *P* = 0.01; Table 4). Regardless of maternal treatments, *MPO* mRNA abundance decreased over time in response to LPS challenge (Age; *P* < 0.01; Suppl. Figure S2).

Antioxidant Function. At birth, no difference was observed due to maternal treatments for *GPX1*, *GSR* and *GSS* mRNA abundance in whole blood leucocytes after LPS challenge ($P > 0.05$; Table 3). Analyzing all time points, regardless of maternal treatments, *GPX1* mRNA abundance decreased over time in response to LPS challenge (Age; $P < 0.01$; Suppl. Figure S3).

One-carbon Metabolism. At birth, no difference was observed due to maternal treatments for *CBS* and *MTR* mRNA abundance in whole blood leucocytes after LPS challenge ($P > 0.05$; Table 3). Analyzing all time points regardless of maternal treatments indicated that *MTR* was overall downregulated in response to LPS challenge over time (Age; $P < 0.05$; Suppl. Figure S3). In addition, *CBS* mRNA abundance had a different response over time, with an upregulation due to LPS challenge at 21 d of age (Age; $P < 0.01$; Suppl. Figure S3).

Whole Blood Phagocytosis and Oxidative Burst

After enteropathogenic bacteria challenge, phagocytosis capacity and oxidative burst activity in both neutrophils and monocytes did not differ due to maternal treatment ($P > 0.05$; Table 5). Regardless of maternal treatments, an Age effect in response to the challenge was detected for phagocytosis capacity and oxidative burst activity in both neutrophils and monocytes. As calves grew, there was an increase in the percentage of neutrophils capable of phagocytosis and oxidative burst activity ($P < 0.01$; Figure 1). However, although the percentage of monocytes capable of oxidative burst increased over time, the percentage of monocytes capable of phagocytosis decreased at 21 d of age coupled with a marked increase at 42 d of age ($P < 0.01$; Figure 1).

DISCUSSION

Work from our group on maternal post-ruminal Met supply has generated data revealing how this essential AA can affect immune function in the neonatal calf (Jacometo et

al., 2015, 2018; Alharthi et al., 2019b). The present study went further than previously to assess for the first time Co, FOA, and Met supply. By design, however, rather than using the entire cohort of calves born to cows on study, we focused on those calves for which there was a complete set of performance, mRNA, and immune function data. Combined, our methods allowed us to visualize the dynamics of the neonate immune system at a molecular and cellular level using a non-invasive approach (Hulbert et al., 2011; Vailati-Riboni et al., 2017a). The analysis performed at birth prior to colostrum feeding allowed the closest assessment of in utero effects. In addition, the longitudinal analysis at 0, 21, and 42 days of age allowed evaluation of carry over effects of maternal exposure to these nutrients.

Acute-Phase Response at Birth

In cattle, studies indicated that the positive acute-phase protein (**APP**) haptoglobin could be a candidate biomarker to predict disease (Gånheim et al., 2007; Huzzey et al., 2009). However, although higher levels of haptoglobin indicate a greater degree of inflammation, the sensitivity of haptoglobin concentrations alone as a diagnostic test for individual calves appears to be low (Murray et al., 2014). By binding and eliminating free hemoglobin from the circulation, this protein plays a key role in dampening oxidative and inflammatory stress (Murata et al., 2004). In the present study, the lower haptoglobin concentrations observed at birth in FOA+CoPro vs. CoPro calves indicated that an additive effect of FOA might have contributed to reducing the inflammatory status. Although data in calves is lacking, a recent study in pregnant women demonstrated that B_{12} in addition to FOA and iron supplementation decreased plasma haptoglobin during the post-intervention period (Zec et al., 2020). Previous work in humans and rodents investigating the immunomodulatory role of FOA, isolated or combined with other methyl donors (e.g. Met, choline, and B_{12}), underscored the effects of FOA on anti-inflammatory responses directly as a result of blocking pro-inflammatory cytokine expression and production, e.g. tumor necrosis factor-α (Chen et al., 2016; Cianciulli

et al., 2016; Samblas et al., 2018). Specific mechanisms for responses detected in the present study still need to be investigated in future, more controlled, studies. Although previous work from our group and others demonstrated lower haptoglobin levels due to increased methyl donor supply in cows during transition (Coleman et al., 2020) and late-gestation periods (Jacometo et al., 2018), there are no studies reporting specifically the role of additional FOA on anti-inflammatory regulation in ruminants.

Among positive APP, ceruloplasmin is a serum ferroxidase that contains most of the blood plasma copper (Hellman and Gitlin, 2002). Its serum concentrations increase considerably during inflammation, infection, and trauma, mainly due to an increase in gene transcription through cytokine-stimulation of liver cells (Gitlin, 1988). The trend observed for lower ceruloplasmin concentration in FOA+CoPectin+RPM vs. FOA+CoPectin calves is an example of the additive effect of this essential AA as a possible regulator of inflammatory mechanisms (Coleman et al., 2020). The fact that effects of additional RPM only were associated with lower ceruloplasmin levels at birth without altering other APP could suggest it had little biological consequence. However, these results along with our previous studies demonstrating that maternal supplementation with RPM during late-pregnancy promoted a lower inflammatory status in the offspring (Jacometo et al., 2016; Alharthi et al., 2019b; Coleman et al., 2021) support data from non-ruminants underscoring the immunomodulatory properties of Met (Wang et al., 2018).

Whole Blood Molecular Responses to LPS Challenge

The *ICAM1* gene encodes the cell surface glycoprotein intercellular adhesion molecule 1 (ICAM1), which acts as a membrane-bound molecule involved in cell-to-cell adhesive interactions on leukocyte adhesion and migration (Zhou et al., 2018). Data underscoring a greater mRNA abundance of *ICAM1* in response to LPS challenge at birth in calves born to

FOA+CoPectin and FOA+CoPectin+RPM vs. CoPro cows are challenging to explain physiologically. An additive effect of FOA could partly help explain the response because some studies in humans reported a relationship between B-vitamins supply (FOA and B_{12}) and regulation of biomarkers related to endothelial function (Alian et al., 2012; Van Dijk et al., 2016). However, the same behavior was not observed in FOA+CoPro calves. In this sense, the absence of transcriptional regulation due to maternal treatment in other important adhesion and migration-related genes (e.g. *CADM1*, *ITGAM*, and *SELL*) also should be taken into account. Thus, it appears that biological consequences of *ICAM1* upregulation in the context of chemotaxis, migration, and adhesion processes would have been a dampening of these events.

Bactericidal permeability-increasing protein (BPI), encoded by *BPI*, plays a central role in immune recognition and binding to LPS (Bülow et al., 2018). At least in non-ruminants, LPS-binding protein (LBP) and BPI interactions with cell surface molecules (e.g. CD14) are the major pathway by which PMN recognize low concentrations of LPS and begin stimuli to respond against gram-negative bacteria (Ryu et al., 2017; Bülow et al., 2018). In non-ruminants, trace amounts of LPS are sufficient to activate NF-κβ via TLR4 and its translocation to the nucleus, stimulating cytokine-related gene expression (e.g. *IL1B*, *IL6*, and *TNF*) (Hayden and Ghosh, 2011). Among toll-like receptor pathway genes evaluated, it is noteworthy that transcriptional regulation due to maternal treatments was observed only for *BPI*. However, at the transcriptional level, this effect did not seem to affect the cell signaling cascade (e.g. *TLR4*, *NFKB1*, and cytokine-related genes). In this sense, with the lack of effects on receptors, transcription factors, and pro-inflammatory cytokines, it is challenging to speculate on the biological significance of greater *BPI* abundance in calves born to FOA+CoPro vs. CoPro and FOA+CoPectin vs. FOA+CoPro cows. In addition, how much of the observed FOA effect was due to an increase in maternal ruminal supply of FOA cannot be

determined, nor whether one of the two different Co sources was able to promote marked effects on immune system activation against LPS.

The *IL10* gene encodes interleukin-10 (IL-10), which is an anti-inflammatory cytokine produced by monocytes, macrophages, dendritic cells, and lymphocytes (Rutz and Ouyang, 2016). Among cytokine-related genes, *IL10* was the sole gene with a possible regulation due to a trend observed in calves born to cows fed additional RPM. Our research group recently demonstrated upregulation of *IL10* mRNA abundance in isolated PMN from lactating Holstein cows and calves (Abdelmegeid et al., 2017) as level of Met supply increased (Lopreiato et al., 2019; Vailati-Riboni et al., 2019). Thus, the trend observed with marked *IL10* upregulation in calves born from RPM cows in response to LPS challenge at birth underscored the importance of this AA as immunomodulator (Coleman et al., 2020, 2021). Overall, combined results suggested that Met might play an important role in the modulation of *IL10* especially during immune challenges.

Whole Blood Cellular Responses to Enteropathogenic Bacteria Challenge

It is noteworthy that the present study was unable to demonstrate the potential for greater maternal supply of methyl donors to impact cellular immune function. Although our group has demonstrated effects of maternal RPM supply on calf phagocytosis capability (Alharthi et al., 2019b), similar responses were not observed in the present study. Furthermore, a recent study evaluating effects of supplementation with FOA and B_{12} on leukocyte function in Holstein cows did not detect differences on phagocytosis and oxidative burst activity (Vanacker et al., 2020). However, the numerically greater percentage (+11.7%) of neutrophils capable of phagocytosis detected in calves from FOA+CoPectin vs. FOA+CoPro cows especially at birth was surprising. Whether numerical effects arose from greater availability in utero of B_{12} synthesized from Co by rumen microbes remains to be

determined. A classic study in humans reported lower metabolic activity in leukocytes from patients with B_{12} deficiency during an in vitro phagocytosis assay (Kaplan and Basford, 1976). In ruminants, however, these types of relationships have not yet been clarified. Thus, our data underscored that despite a numerical difference in neutrophil phagocytosis capacity, these responses did not reflect in an improvement of innate-cell killing capabilities due to the lack of differences observed in oxidative burst activity. Available data from previous and the present study offer support for more in-depth mechanistic studies.

Temporal responses

The downregulation in mRNA abundance after LPS challenge for most genes related to cytokines and cytokine receptors (*IL1B, IL6, IL10, TNF, IRAK1, CXCR1)*, toll-like receptor pathway (*TLR4, NFKB1*), adhesion and migration (*ICAM1*, *ITGAM*), antimicrobial function (*MPO),* and antioxidant function (*GPX1*) was likely due to changes in endocrine and cellular profiles as calves aged (Gao et al., 2012). A limitation in the present study is that we did not perform a differential leukocyte count in whole blood samples. Thus, we cannot determine with certainty if the observed differences were due to changes in function or just a reflection of changes in leukocyte populations. However, it is well-established that in healthy conditions, newborn calves experience a change in proportions of blood leukocyte populations during the neonatal period (Tornquist and Rigas, 2010; Marcato et al., 2018). For instance, at birth, a predominant population of PMN begins to be replaced over time by PBMC, i.e. mainly lymphocytes (Jones and Allison, 2007). As PMN are the main effector cells of the immediate innate response to LPS, the lower abundance of this cell type in calves at 21 and 42 days of age might have been a limiting factor for the responses observed at these time points. Similar molecular responses were also reported in our previous work with whole blood and isolated PMN from Holstein calves during the first few wk of life (Osorio et al., 2013b; Jacometo et al., 2018; Alharthi et al., 2019b). Together, those data indicated that the

calf immune system at birth and prior to colostrum feeding is more sensitive to LPS challenge (Lopes et al., 2021).

Regardless of maternal treatments, besides alterations in cellular profiles, differences in longitudinal immune function and mRNA abundance after LPS challenge can be explained partly through the characteristic of antigens in each type of analysis. As the immune function assay was performed with peripheral blood neutrophils and monocytes stimulated with *E*. coli (Hulbert et al., 2011), a distinct pattern of response as calves grew might have occurred due to maturation of the innate-immune system (e.g. effector cells and complement system) and acquisition of maternal antibodies after colostrum intake (Chase et al., 2008; Batista et al., 2015; Hulbert and Moisá, 2016). Although the assessment of adequate transfer of passive immunity was not undertaken through IgG or serum total protein concentrations at 2 and 7 d of age, an important limitation of this study, we ensured that all calves received the same amount of colostrum from their respective dams. It is well-known that antibodies act as important opsonins to facilitate phagocytosis (Winkelstein, 1973). In this sense, in addition to greater cell maturity of neutrophils and monocytes after the first few wk of life, the presence of maternal antibodies promoting opsonization of enteropathogenic bacteria might have played a role in the overall greater percentage of cells capable of phagocytosis and oxidative burst activity over time (Seto et al., 1976; Menge et al., 1998).

Sex-Specific Responses

Although our group recently reported that increased methyl donor supply during late-pregnancy led to offspring sex-specific divergent changes in metabolic and epigenetic signatures in bovine placenta (Batistel et al., 2019), knowledge about sex-specific gene expression and metabolic control in ruminants is still in its infancy. Previous studies underscored that gene expression with a “sex bias” is predominant in many species, the extent

to which this occurs can vary greatly among tissues or stages of development (Grath and Parsch, 2016). For instance, a recent study across human tissues revealed a repertoire of transcription factors that play important roles in the sex-specific architecture of gene regulatory networks in both health and disease (Lopes-Ramos et al., 2020). The few differences detected in the present study due to sex led us to speculate that Female calves experienced some degree of oxidative stress at birth, e.g. increased paraoxonase, ROMt, NO_2^-, β-carotene, and haptoglobin. In contrast, the upregulation of *IRAK1*, *CADM1*, and *ITGAM* in response to LPS challenge at birth in Male calves suggested a greater capacity for signaling and cell adhesion. Clearly, the present study does not truly allow to ascertain the long-term biological impacts of the sex-specific differences detected. However, we believe that these data are important in terms of follow-up studies because the field of micronutrient supplementation in the overall context of managing the pregnant cow is certain to continue growing (see review by Coleman et al., 2021).

CONCLUSIONS

Despite some inherent limitations in evaluating data from a retrospective selection of calves, the fact that animals used had a complete set of performance, mRNA, and innate immune function data allow for a preliminary evaluation of the role of maternal Co, folic acid, and Met supply. Although a few plasma and molecular biomarkers were significantly affected, at the levels fed in the present study, the data did not provide clear evidence of beneficial effects of nutrients associated with one-carbon metabolism in modulating cellular immune functionality of the neonatal calf. Further studies with greater biological power and different dosages of these nutrients could help ascertain the existence of mechanistic relationships that have an implication on calf growth and development.

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Table 1. Effects of maternal supplementation during the last 30-d of pregnancy with an experimental folic acid source (FOA), two cobalt (Co) sources [Co glucoheptonate (CoPro) or Co pectin (CoPectin)] and rumen-protected methionine (RPM) on calf growth parameters at birth (before colostrum feeding) and during the first 6 wk of age

Item	Maternal treatment[1]				SEM[2]	*P*-value			
	CoPro	FOA+ CoPro	FOA+ CoPectin	FOA+CoPectin +RPM		Trt	Sex	Age	Trt × Age
At birth									
Body weight, kg	44.12	43.75	40.09	44.67	1.76	0.22	0.06		
Body length, cm	110.42	110.39	113.74	110.70	1.48	0.27	0.48		
Hip height, cm	79.00	79.27	77.81	79.18	0.92	0.62	0.35		
Whither height, cm	75.54	76.04	75.40	76.31	0.71	0.76	0.18		
Hip width, cm	15.45	15.35	15.00	15.00	0.29	0.57	0.97		
1 to 6 wk[3]									
Body weight, kg	56.30	55.60	54.35	57.20	1.84	0.73	0.08	<0.01	0.76
Body length, cm	119.90	119.44	122.06	119.23	1.23	0.36	0.94	<0.01	0.57
Hip height, cm	83.27	84.50	82.37	84.08	0.83	0.29	0.72	<0.01	0.45
Whither height, cm	80.15	80.71	78.93	80.54	0.72	0.31	0.12	<0.01	0.28
Hip width, cm	18.26	18.52	18.24	18.26	0.24	0.82	0.22	<0.01	0.80

[1]Maternal treatments were CoPro (n = 12), FOA+CoPro (n = 12), FOA+CoPectin (n = 12), and FOA+CoPectin+RPM (n = 12).
[2]Greatest standard error of the mean.
[3]Least squares means for calf growth parameters including weekly measurements from 1 to 6 wk of age.

Table 2. Effects of maternal supplementation during the last 30-d of pregnancy with an experimental folic acid source (FOA), two cobalt (Co) sources [Co glucoheptonate (CoPro) or Co pectin (CoPectin)] and rumen-protected methionine (RPM) on plasma concentrations of biomarkers related to energy metabolism, inflammation, and antioxidant status, as well as on immune function of calf leukocytes at birth and prior to colostrum feeding

Item[1]	Maternal treatment[2]				SEM[3]	*P*-value	
	CoPro	FOA+ CoPro	FOA+ CoPectin	FOA+CoPectin +RPM		Trt	Sex
Plasma Biomarkers							
Energy metabolism							
Glucose, mmol/L	4.93	4.71	5.08	4.65	0.68	0.95	0.14
Fatty acids, mmol/L	1.18	0.89	0.99	0.94	0.12	0.35	0.91
BHBA, mmol/L	0.05	0.07	0.05	0.04	0.01	0.16	0.09
Inflammation							
Myeloperoxidase, U/L	260	167	232	187.5	30.13	0.12	0.62
Haptoglobin, g/L	0.39[a]	0.27[b]	0.32[ab]	0.34[ab]	0.02	0.02	0.02
Ceruloplasmin, µmol/L	0.13	0.11	0.24	0.09	0.04	0.08	0.45
Albumin, g/L	29.53	28.75	29.75	29.45	0.46	0.45	0.72
Antioxidant status							
Paraoxonase, U/mL	4.25	13.18	7.14	8.57	2.99	0.18	<0.01
ROMt, mg H_2O_2/100mL	5.43	4.44	5.28	4.75	0.36	0.18	<0.01
FRAP, µmol/L	249	255	259	234.6	15.54	0.64	0.39
NOx, µmol/L	204	181	174	184.3	16.69	0.58	0.24
NO_2^-, µmol/L	4.40	3.20	3.53	3.72	0.43	0.26	<0.01
NO_3^-, µmol/L	199	178	171	180.6	16.77	0.62	0.21
Retinol, µg/mL	6.65	7.57	9.27	9.23	0.99	0.12	0.37
α-tocopherol, µg/mL	0.37	0.42	0.36	0.36	0.04	0.74	0.33
β-carotene, mg/100 mL	0.004	0.006	0.005	0.005	0.001	0.80	<0.01
Zinc, µmol/L	8.70	10.94	9.53	11.32	1.53	0.56	0.11
Immune function[4]							
Neutrophil							
Phagocytosis	51.2	50.1	61.8	64.2	5.6	0.19	0.65
Oxidative burst	56.5	55.0	54.5	58.1	4.6	0.94	0.51
Monocyte							
Phagocytosis	18.6	19.4	17.5	18.7	1.2	0.73	0.06
Oxidative burst	18.7	19.8	18.5	18.8	1.5	0.92	0.18

[1]BHBA = β-hydroxybutyrate; NOx = nitric oxide metabolites; NO_2^- = nitrite; NO_3^- = nitrate; FRAP = ferric reducing ability of plasma; ROMt = reactive oxygen metabolites, total.
[2]Maternal treatments were CoPro (n = 12), FOA+CoPro (n = 12), FOA+CoPectin (n = 12), and FOA+CoPectin+RPM (n = 12).
[3]Greatest standard error of the mean.
[4]Data reported as percentages of CD14/CH138A positive cells with phagocytosis and oxidative burst activity.
[a-b]Means within a row differ ($P \leq 0.05$).

Table 3. Effects of maternal supplementation during the last 30-d of pregnancy with an experimental folic acid source (FOA), two cobalt (Co) sources [Co glucoheptonate (CoPro) or Co pectin (CoPectin)] and rumen-protected methionine (RPM) on the response of calf whole blood mRNA abundance to an ex vivo lipopolysaccharide (LPS) challenge at birth and prior to colostrum feeding. Response calculated as LPS challenged - non-challenged sample for each calf

	Maternal treatment[1]					*P*-value	
Gene symbol	CoPro	FOA+ CoPro	FOA+ CoPectin	FOA+CoPectin +RPM	SEM[2]	Trt	Sex
Cytokines							
IL1B	1.68	0.60	2.30	3.62	1.32	0.35	0.23
IL6	2.92	2.12	3.22	4.81	1.10	0.31	0.94
IL10	3.21	1.47	2.89	5.38	1.17	0.09	0.88
TNF	1.67	3.71	2.73	3.85	0.93	0.16	0.94
Cytokines Receptors							
IRAK1	0.16	-0.43	0.32	0.50	0.53	0.52	0.05
CXCR1	0.02	0.16	0.28	0.41	0.31	0.79	0.44
Toll-Like Receptor Pathway							
TLR4	0.27	0.22	0.66	0.45	0.42	0.84	0.67
BPI	-1.49	-0.67	-0.17	-0.84	0.37	0.06	0.06
NFKB1	0.64	0.29	0.76	0.97	0.37	0.52	0.93
STAT3	0.72	0.61	0.57	0.65	0.35	0.98	0.96
Adhesion and Migration							
CADM1	-0.68	-0.28	0.13	-0.34	0.33	0.32	0.04
ICAM1	0.63[a]	1.05[ab]	2.11[b]	2.28[b]	0.55	0.05	0.39
ITGAM	0.28	0.54	0.46	0.01	0.44	0.76	0.02
SELL	-0.39	-0.79	-0.33	-0.67	0.42	0.78	0.34
Antimicrobial Function							
LYZ	0.05	-0.16	0.27	-1.18	0.51	0.20	0.33
MPO	4.44	5.30	2.70	4.65	0.75	0.12	0.16
NOS2	3.34	3.00	3.59	6.64	1.34	0.18	0.49
SOD2	0.90	0.26	2.06	1.25	0.53	0.07	0.41
Antioxidant Function							
GPX1	-0.40	0.18	0.38	-0.07	0.35	0.36	0.38
GSR	-2.12	-2.47	-3.19	-2.55	1.18	0.91	0.50
GSS	-1.08	-1.16	-0.57	-1.00	0.34	0.56	0.13
One-carbon Metabolism							
CBS	-0.92	-0.42	-0.11	-1.35	0.52	0.33	0.57
MTR	1.46	0.45	-0.72	1.44	1.54	0.68	0.40

[1]Maternal treatments were CoPro (n = 12), FOA+CoPro (n = 12), FOA+CoPectin (n = 12), and FOA+CoPectin+RPM (n = 12).
[2]Greatest standard error of the mean.
[a-c]Means within a row differ ($P \leq 0.05$).

Table 4. Effects of maternal supplementation during the last 30-d of pregnancy with an experimental folic acid source (FOA), two cobalt (Co) sources [Co glucoheptonate (CoPro) or Co pectin (CoPectin)] and rumen-protected methionine (RPM) on calf whole blood mRNA abundance after ex vivo lipopolysaccharide (LPS) challenge at 0 (at birth and prior to colostrum feeding), 21 and 42 (at weaning) days of age. Response calculated as LPS challenged - non-challenged sample for each calf

	Maternal treatment[1]					*P*-value			
Gene symbol	CoPro	FOA+ CoPro	FOA+ CoPectin	FOA+CoPectin +RPM	SEM[2]	Trt	Sex	Age	Trt × Age
Cytokines									
IL1B	-0.06	-1.17	0.09	1.15	1.04	0.25	0.12	<0.01	0.91
IL6	2.47	1.42	2.27	2.58	1.06	0.67	0.18	0.09	0.90
IL10	2.13	0.66	0.97	2.69	0.93	0.11	0.58	<0.01	0.90
TNF	0.44	0.86	1.03	1.05	0.62	0.75	0.39	<0.01	0.56
Cytokines Receptors									
IRAK1	-0.27	-0.59	-0.41	-0.23	0.31	0.54	0.06	<0.01	0.11
CXCR1	-0.51	-0.61	-0.59	-0.86	0.24	0.66	0.77	<0.01	0.78
Toll-Like Receptor Pathway									
TLR4	-0.58	-0.52	-0.67	-0.30	0.46	0.94	0.71	<0.01	0.69
BPI	-1.17[a]	-1.00[b]	-0.76[c]	-0.34[c]	0.27	<0.01	0.04	0.98	0.28
NFKB1	-0.06	-0.29	-0.34	0.30	0.28	0.27	0.44	<0.01	0.72
STAT3	0.72	0.84	0.83	0.59	0.32	0.91	0.14	0.08	0.84
Adhesion and Migration									
CADM1	-0.19	0.22	0.01	-0.22	0.27	0.41	0.20	0.03	0.38
ICAM1	0.65	0.27	-0.19	1.18	0.52	0.21	0.12	<0.01	0.39
ITGAM	-0.19	-0.13	-0.14	-0.25	0.36	0.99	0.11	0.02	0.86
SELL	-0.27	-0.85	-0.81	-0.24	0.39	0.31	0.60	0.33	0.56
Antimicrobial Function									
LYZ	0.07	0.01	0.11	-0.84	0.43	0.30	0.31	0.68	0.40
MPO	2.72	3.01	3.93	3.06	0.85	0.50	0.03	<0.01	0.06
NOS2	3.66	2.70	3.49	4.35	1.08	0.48	0.13	0.33	0.92
SOD2	1.23	0.32	1.18	1.20	0.59	0.30	0.01	0.89	0.95
Antioxidant Function									
GPX1	-0.47	-0.36	-0.37	-0.76	0.25	0.56	0.73	<0.01	0.26
GSR	-4.28	-2.28	-3.75	-4.87	1.04	0.31	0.52	0.11	0.68
GSS	-1.07	-1.10	-1.13	-1.19	0.31	0.99	0.93	0.25	0.48
One-carbon Metabolism									
CBS	-0.35	-0.13	-0.20	-1.14	0.36	0.12	0.94	<0.01	0.64
MTR	-0.74	1.36	-2.70	-2.39	1.51	0.06	0.09	0.05	0.18

[1]Maternal treatments were CoPro (n = 12), FOA+CoPro (n = 12), FOA+CoPectin (n = 12), and FOA+CoPectin+RPM (n = 12).
[2]Greatest standard error of the mean.
[a-c]Means within a row differ ($P \leq 0.05$).

Table 5. Effects of maternal supplementation during the last 30-d of pregnancy with an experimental folic acid source (FOA), two cobalt (Co) sources [Co glucoheptonate (CoPro) or Co pectin (CoPectin)] and rumen-protected methionine (RPM) on phagocytosis capacity and oxidative burst activity of calf blood neutrophils and monocytes after enteropathogenic bacteria challenge at 0 (at birth and prior to colostrum feeding), 21 and 42 (at weaning) days of age. Data reported as percentages of CD14/CH138A positive cells with phagocytosis and oxidative burst activity

Leukocyte function (%)	Maternal treatment[1]				SEM[2]	*P*-value			
	CoPro	FOA+ CoPro	FOA+ CoPectin	FOA+CoPectin +RPM		Trt	Sex	Age	Trt × Age
Neutrophil									
Phagocytosis	66.54	66.46	71.55	74.92	2.94	0.13	0.58	<0.01	0.67
Oxidative burst	60.85	61.54	62.15	64.00	2.20	0.77	0.15	<0.01	0.99
Monocyte									
Phagocytosis	17.89	17.94	17.67	19.82	0.74	0.15	0.07	<0.01	0.36
Oxidative burst	21.07	22.86	22.47	22.91	0.90	0.44	0.79	<0.01	0.89

[1]Maternal treatments were CoPro (n = 12), FOA+CoPro (n = 12), FOA+CoPectin (n = 12), and FOA+CoPectin+RPM (n = 12).
[2]Greatest standard error of the mean.

Figure 1

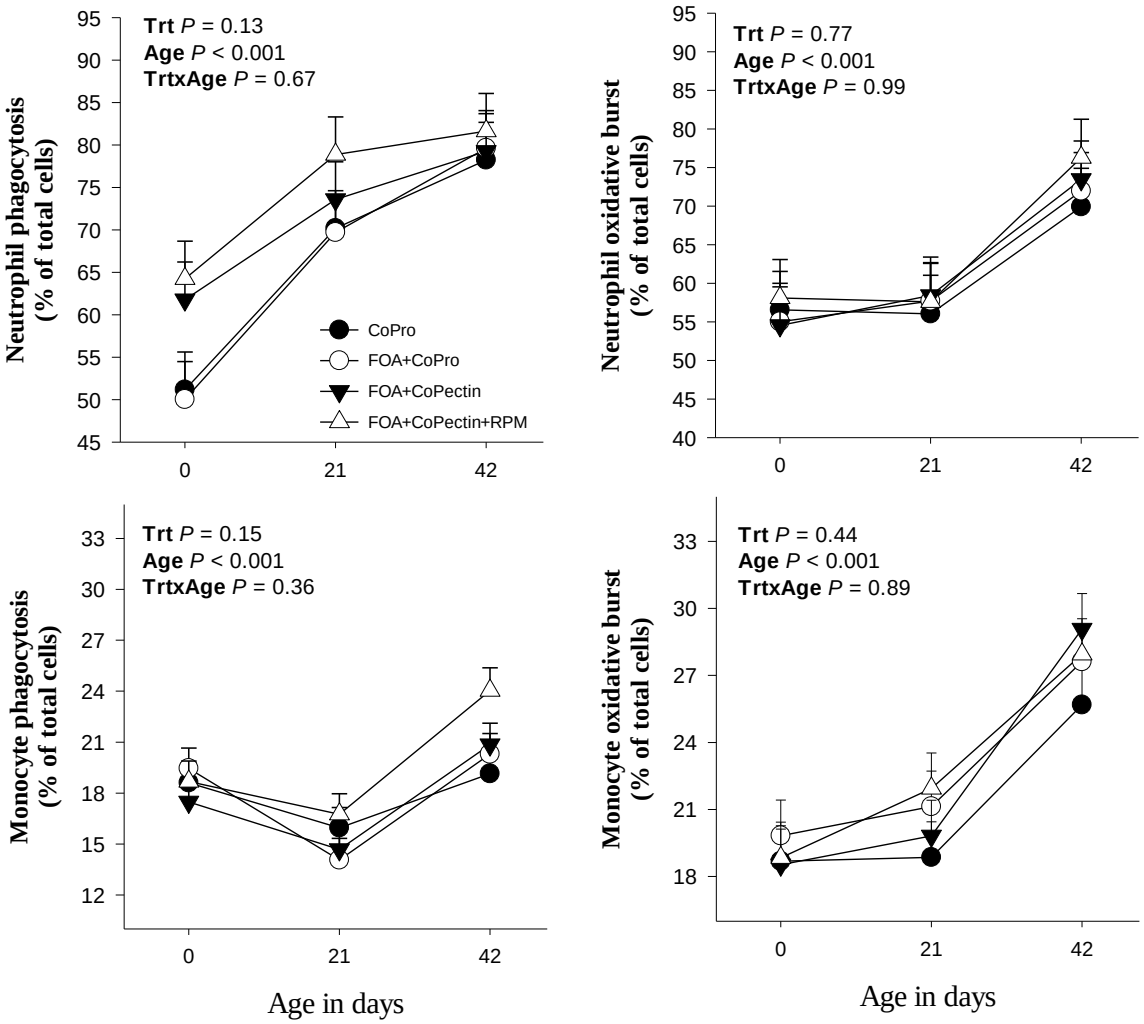

Neutrophil phagocytosis (% of total cells)
Trt P = 0.13
Age P < 0.001
TrtxAge P = 0.67
CoPro
FOA+CoPro
FOA+CoPectin
FOA+CoPectin+RPM
Neutrophil oxidative burst (% of total cells)
Trt P = 0.77
Age P < 0.001
TrtxAge P = 0.99
Monocyte phagocytosis (% of total cells)
Trt P = 0.15
Age P < 0.001
TrtxAge P = 0.36
Monocyte oxidative burst (% of total cells)
Trt P = 0.44
Age P < 0.001
TrtxAge P = 0.89
Age in days
Age in days

Figure Legends

Figure 1. Ex-vivo phagocytosis capacity and oxidative burst activity of blood neutrophils and monocytes from Holstein calves born to cows after enteropathogenic bacteria challenge. Multiparous Holstein cows were fed during the last 30-d of pregnancy an experimental folic acid source (FOA), two cobalt (Co) sources [Co glucoheptonate (CoPro) or Co pectin (CoPectin)] and rumen-protected methionine (RPM). Maternal treatments were CoPro (n = 12), FOA+CoPro (n = 12), FOA+CoPectin (n = 12), and FOA+CoPectin+RPM (n = 12).

Supplementary Material

Supplemental Table S1. Ingredient and nutrient composition of diet fed during close-up period (-30 days to calving)

Item	Close-up diet
Ingredient (% of DM)	
Corn silage	37.47
Ground shelled corn	11.60
Wheat straw	21.82
Canola meal	11.67
Soybean meal	6.30
Soychlor[1]	3.37
Corn gluten	2.80
ProvAAL2 AADvantage[2]	0.47
Biotin[3]	0.10
Rumensin[4]	0.19
Calcium sulfate	0.53
Magnesium oxide	0.10
Ca	0.66
P	0.33
Salt	0.10
Na	0.12
Cl	0.78
Mg	0.45
K	1.36
S	0.33
Nutrient composition	
CP, % of DM	14.50
NDF, % of DM	43.30
ADF, % of DM	33.80
aNDFom, % of DM	49.21
NFC, % of DM	28.22
NE_L, Mcal/kg of DM	1.37
RDP, % of DM	8.45
RUP, % of DM	6.05
RDP required, g/d	1,165.00
RDP supplied, g/d	1,152.00
RDP balance, g/d	-18.00
RUP required, g/	158.00
RUP supplied, g/d	821.00
RUP balance, g/d	662.00
MP required, g/d	821.00
MP supplied, g/d	1,360.00
MP balance, g/d	539.00

[1]West Central Soy.
[2]Perdue Agribusiness (Salisbury, MD, USA).
[3]ADM Animal Nutrition (Quincy, IL, USA).
[4]Elanco Animal Health (Greenfield, IN, USA).

Supplemental Table S2. Gene symbol, accession number, and forward and reverse primer sequence of the analyzed genes

Gene[1]	Accession #	Forward sequence	Reverse sequence
GAPDH	NM_001034034.2	TGGAAAGGCCATCACCATCT	CCCACTTGATGTTGGCAG
RPS9	NM_001101152.2	CCTCGACCAAGAGCTGAAG	CCTCCAGACCTCACGTTTGTTC
ACTB	NM_173979.3	ACCAACTGGGACGACATGGA	GTCTCGAACATGATCTGGGTCAT
IL1B	NM_174093.1	TCCACCTCCTCTCACAGGAAA	TACCCAAGGCCACAGGAATCT
IL6	NM_173923	CCAGAGAAAACCGAAGCTCTCAT	CCTTGCTGCTTTCACACTCATC
IL10	NM_174088.1	GAAGGACCAACTGCACAGCTT	AAAACTGGATCATTTCCGACAAG
TNF	NM_173966.3	CCAGAGGGAAGAGCAGTCCC	TCGGCTACAACGTGGGCTAC
IRAK1	NM_001040555.1	CCTCAGCGACTGGACATCCT	GGACGTTGGAACTCTTGACATCT
CXCR1	NM_001105038.1	CGGCACTGGGTCAAGTTCATAT	GTCGGAGTATGGTGGTTGATAGG
TLR4	NM_174198.6	GCTGTTTGACCAGTCTGATTGC	GGGCTGAAGTAACAACAAGAGGAA
BPI	NM_173895.2	ACACCGTCCGCATACACATCT	TTTCTGGTCATGGACTTTTGGA
NFKB1	NM_001076409.1	TTCAACCGGAGATGCCACTAC	ACACACGTAACGGAAACGAAATC
STAT3	NM_001012671.2	CCGGTGTCCAGTTCACAACTAA	CCCCGGAGTCTTTGTCAAT
CADM1	NM_001038558.2	GCAAGGAGCTCAAAGGCAAGT	AACCCCGTCATCCTCCTTGT
ICAM1	NM_174348.2	AGAATTAGCGCTGACCTCTGTTAAG	CGGACACATCTCAGTGACTAAACAA
ITGAM	NM_001039957.1	GGCTTGTCTCTTGCATTTGCT	CCATTTGCATAGGTGTTCTCCTT
SELL	NM_174182.1	CTCTGCTACACAGCTTCTTGTAAACC	CCGTAGTACCCCAAATCACAGTT
LYZ	NM_001077829.1	AAAGCAGTTAACGCCTGTCGTAT	CATGCCACCCATGCTTTAATG
MPO	NM_001113298.2	AGCCATGGTCCAGATCATCAC	ACCGAGTCGTTGTAGGAGCAGTA
NOS2	NM_174182.1	CTGAAGCAGCTGATGGCTACT	ATGATAGCGCTTCTGGTTCTTGAC
SOD2	NM_201527.2	TGTGGGAGCATGCTTATTACCTT	TGCAGTTACATTCTCCCAGTTGA
GPX1	NM_174076.3	AACGCCAAGAACGAGGAGATC	CATTCACCTCGCACTTTTCGA
GSR	NM_001114190.2	CGCTGAGAACCCAGAGACTTG	AAACGGAAAGTGGGAACAGTAAGTA
GSS	NM_001015630.1	CGAGTGATCCAATGCATTTCAG	ATGTCCCACGTGCTTGTTCAT
CBS	NM_001102000.2	GCCACCACCTCTGTCAAATTC	GGACAGAAAGCAGAGTGGTAACTG
MTR	NM_001030298.1	GTTCCACCTGCCACTGTTTTC	CAGCGCTCTCCAATGTTAACAA

[1]*ACTB* = Actin beta; *BPI* = Bactericidal/permeability-increasing protein; *CADM1* = Cell adhesion molecule 1; *CBS* = Cystathionine β-synthase; *CXCR1* = CXC motif chemokine receptor 1; *GAPDH* = Glyceraldehyde-3-phosphate dehydrogenase; *GPX1* = Glutathione peroxidase 1; *GSR* = Glutathione reductase; *GSS* = Glutathione synthetase; *ICAM1* = Intercellular cell adhesion molecule 1; *IL1B* = Interleukin 1β; *IL6* = Interleukin 6; *IL10* = Interleukin 10; IRAK1 = IL1 receptor associated kinase 1; *ITGAM* = Integrin subunit α M; *LYZ* = Lysozyme; *MPO* = Myeloperoxidase; *MTR* = 5-methyltetrahydrofolate-homocysteine methyltransferase; *NFKB1* = Nuclear factor κβ subunit 1; *NOS2* = Nitric oxide synthase 2; *RPS9* = Ribosomal protein S9; *SELL* = Selectin L; *SOD2* = Superoxide dismutase 2, mitochondrial; *STAT3* = Signal transducer and activator of transcription 3; *TLR4* = Toll-like receptor 4; *TNF* = Tumor necrosis factor α.

Supplemental Table S3. RT-qPCR performance among the genes measured in whole blood after lipopolysaccharide (LPS) challenge

Gene symbol	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]
IL1B	23.73	1.47	-3.313	0.983	2.004
IL6	26.80	4.55	-3.235	0.988	2.038
IL10	28.12	5.86	-3.131	0.990	2.086
TNF	27.86	5.60	-3.231	0.986	2.039
IRAK1	30.64	8.38	-3.347	0.956	1.990
CXCR1	24.60	2.34	-3.314	0.996	2.003
TLR4	26.13	3.88	-3.222	0.997	2.043
BPI	29.71	7.45	-3.342	0.983	1.992
NFKB1	24.98	2.72	-3.296	0.999	2.011
STAT3	24.15	1.89	-3.353	0.981	1.987
CADM1	28.27	6.01	-3.404	0.998	1.967
ICAM1	25.84	3.58	-3.358	0.995	1.985
ITGAM	28.17	5.91	-3.256	0.966	2.028
SELL	26.51	4.25	-3.151	0.991	2.077
LYZ	24.33	2.07	-3.316	0.996	2.002
MPO	34.88	12.75	-3.198	1.000	2.054
NOS2	27.65	5.39	-3.175	0.995	2.065
SOD2	21.81	-0.45	-3.402	0.997	1.968
GPX1	23.68	1.42	-3.309	0.994	2.005
GSR	29.55	7.40	-3.308	0.981	2.006
GSS	30.20	7.94	-3.500	0.990	1.931
CBS	27.37	5.11	-3.367	0.996	1.982
MTR	31.12	9.05	-3.326	0.992	1.998

[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 samples.
[3]Slope of the standard curve.
[4]R^2 stands for the coefficient of determination of the standard curve.
[5]Efficiency is calculated as [$10^{(-1/Slope)}$].

Supplemental Table S4. Information required by the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) to ensure quality control

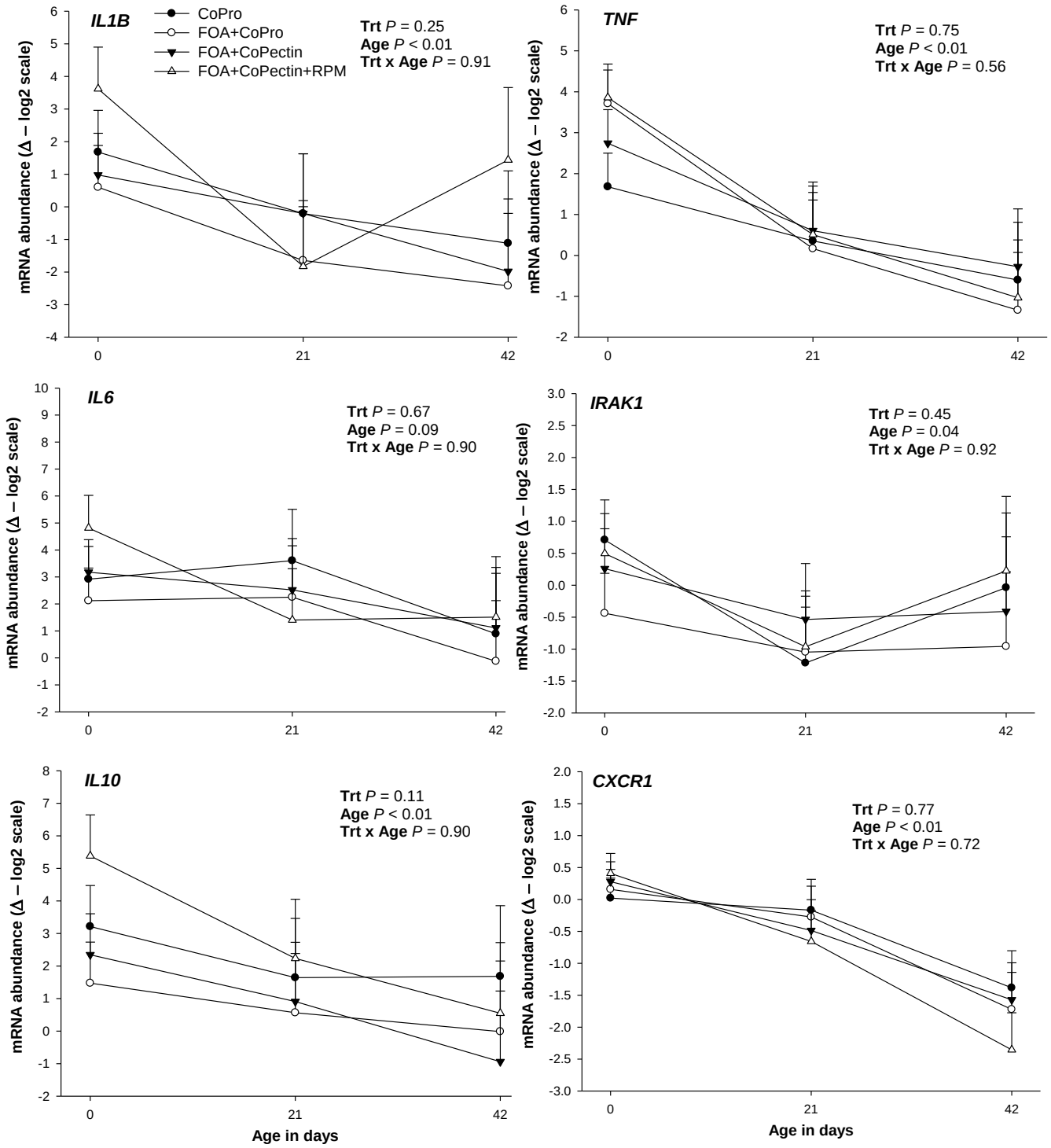
Item to check	Importance*	
		Nucleic acid extraction
Source of additional reagents used	D	TRI Reagent® BD (Sigma Aldrich)
Contamination assessment (DNA or RNA)	E	Reverse transcription controls (without enzyme) were performed in order to assess the absence of DNA in the RNA sample. For that purpose, RNA was processed as a normal sample in the RT step, except that no reverse transcriptase was added to the reaction mixture (see "complete reaction conditions" in Reverse Transcription).
Nucleic acid quantification	E	RNA concentration was determined by measuring the absorbance at 260 nm UV light
Instrument and method	E	NanoDrop ND-1000 (NanoDrop Technologies)
Purity (A260/A280)	D	RNA purity was determined by measuring the absorbance ratio 260/280
RNA integrity: method/instrument	E	AATI Fragment Analyzer (Agilent Technologies)
RIN/RQI or Cq of 3' and 5' transcripts	E	All samples had an RNA integrity score greater than 8.0
Electrophoresis traces	D	
Inhibition testing (Cq dilutions, spike, or other)	E	The standard curve has been considered sufficient to rule out the presence of inhibitors of reverse-transcription activity or PCR
		Reverse transcription
Complete reaction conditions	E	RNA isolated from whole blood was used for RT-qPCR analysis. The cDNA synthesis was performed using 100 ng of RNA standardized by dilution in nuclease-free water. The total RNA was mixed using a first mix (Mix 1) containing 1 µL random primers (Roche Applied) and 9 µL nuclease-free water. The RNA+Mix 1 was incubated at 65°C for 5 min and kept on ice for 3 min. A second mix (Mix 2) consisted of 4 µL 5x First-Strand Buffer (Thermo Fischer Scientific), 1 µL Oligo dT18 (Integrated DNA Technologies), 2 µL 10 mmol/L dNTP mix (Invitrogen), 0.25 µL 200 IU/µL of Revert aid (Thermo Fischer Scientific), 0.125 µL 20 U/µL of RNase inhibitor (Thermo Fischer Scientific), and 1.625 µL nuclease-free water. After adding Mix 2 to the RNA+Mix 1 sample, the reaction was performed in an Mastercycler Gradient (Eppendorf) using the following temperature program: 25 °C for 5 min, 42 °C for 60 min and 70 °C for 5 min. cDNA was then diluted 1:4 with DNase/RNase free water.
Amount of RNA and reaction volume	E	Amount of RNA: 100 ng; Reaction volume: 20 µL
Priming oligonucleotide (if using GSP) and concentration	E	Not applicable
Reverse transcriptase and concentration	E	Revert aid (Thermo Fischer): 2.5 IU/µL
Temperature and time	D	Specified in "Complete reaction conditions-Reverse transcription"
Manufacturer of reagents	D	Specified in "Complete reaction conditions-Reverse transcription"
Cqs with and without reverse transcription	D	
Storage conditions of cDNA	D	-20°C

		qPCR protocol
Complete reaction conditions	E	Quantitative PCR was performed in a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems) using 4 µL diluted 1:4 cDNA and 6 µL SYBR Green mixture (Applied Biosystems) with 0.4 µL 10 µM forward and reverse primers, and 0.2 µL nuclease-free water. Each gene was run in triplicate on a single plate with 7-point standard curve plus the negative control.
Reaction volume and amount of cDNA/DNA	E	Reaction volume: 10 µL; amount of cDNA: 1:4 dilution.
Additives	E	Specified in "Complete reaction conditions-qPCR protocol"
Manufacturer of plates/tubes	D	Specified in "Complete reaction conditions-qPCR protocol"
Complete thermocycling parameters	E	2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15 s plus 65 °C for 15 s.
Reaction setup (manual/robotic)	D	Robotic
Manufacturer of qPCR instrument	D	Specified in "Complete reaction conditions-qPCR protocol"
		qPCR validation
Evidence of optimization (from gradients)	D	
Specificity (gel, sequence, melt, or digest)	E	Melting curve analysis, ramping from 55°C to 95°C, where fluorescence data are measured continuously (measured melting temperature values are provided as supplementary data). Gene-specific amplification was confirmed by a single band in 2% agarose gel electrophoresis stained with ethidium bromide. No template controls (no cDNA in PCR) were run for each gene to detect unspecific amplification and primer dimerization.
For SYBR Green, Cq of the NTC	E	The signal of the amplification plot never crossed the threshold (Cq > 40) and therefore there was a high Cq value difference between the negative control and all the cDNA sample.
Calibration curves with slope and y intercept	E	The slopes are provided in Supplemental Table S2
PCR efficiency calculated from slope	E	Provided in Supplemental Table S3
Cis for PCR efficiency or SE	D	
r2 of calibration curve	E	Provided in Supplemental Table S3
		Data analysis
qPCR analysis program	E	Specified in "Complete reaction conditions-qPCR protocol"
Method of Cq determination	E	The threshold is determined using the Amplification-based Threshold method. The threshold is used to specify Cq values of samples
Outlier identification and disposition	E	None of the Cq values were discarded
Results for NTCs	E	The signal of the amplification plot never crossed the threshold (Cq > 40) and therefore there was a high Cq value difference between the negative control and all the cDNA sample.
Justification of number and choice of reference genes	E	The use of *GAPDH*, *RPS9*, and *ACTB* as reference genes for whole blood leucocytes gene expression has been successfully used by Lopes et al. (2020, J. Dairy Sci. 104:2266-2279), Vailati-Riboni et al. (2019, J. Dairy Sci. 102:8343-8351) and Lopreiato et al. (2019, J. Dairy Sci. 102:10395-10410)

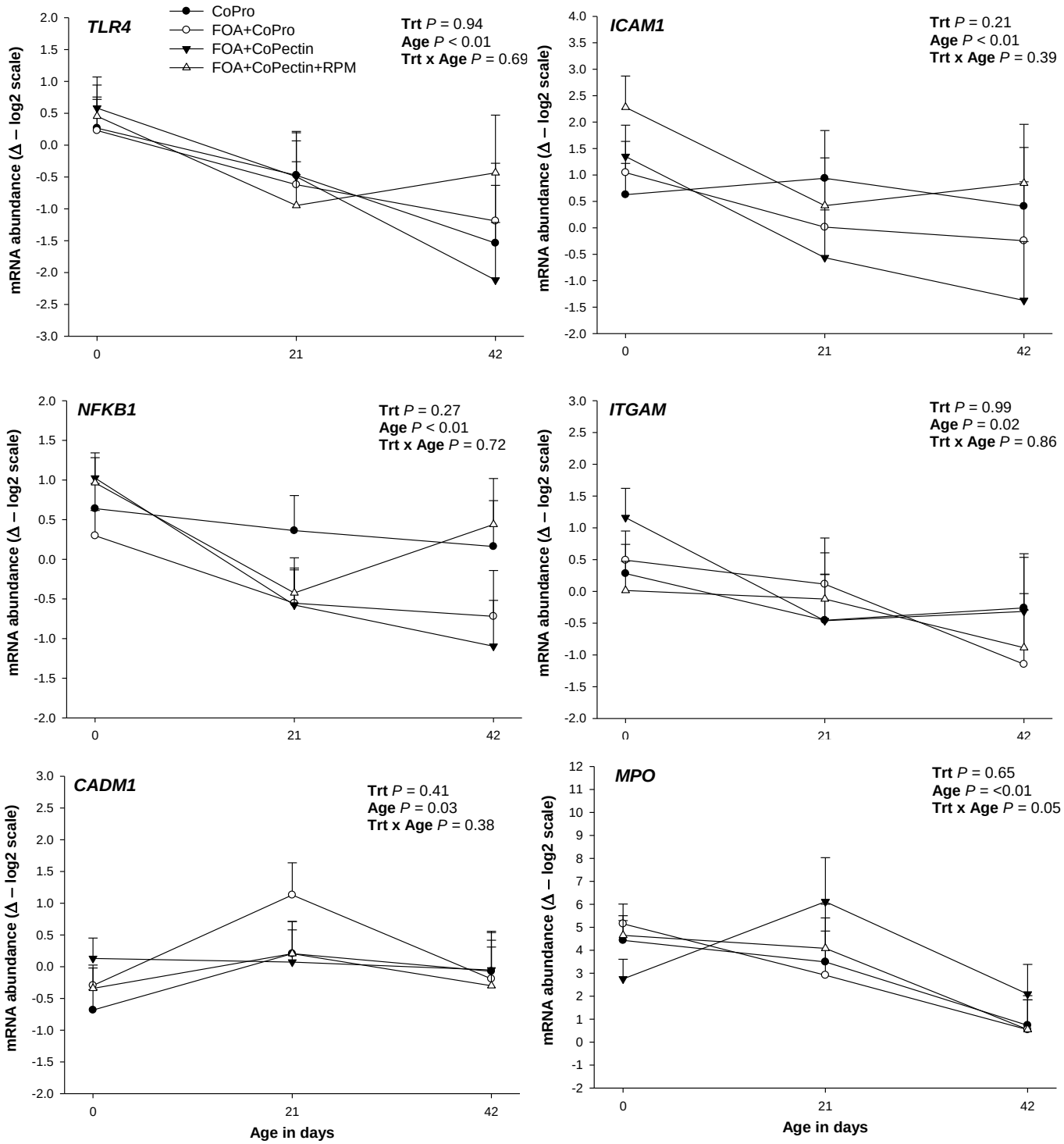
Description of normalization method	E	Described in main body
Number and concordance of biological replicates	D	
Number and stage (RT or qPCR) of technical replicates	E	qPCR reactions were performed in triplicate
Repeatability (intraassay variation)	E	Mean coefficient of variation of triplicates was lower than 5%
Reproducibility (interassay variation, CV)	D	
Power analysis	D	
Statistical methods for results significance	E	Described in main body
Software (source, version)	E	Described in main body
Cq or raw data submission with RDML	D	
		qPCR target information
Gene symbol	E	Provided in Supplemental Table S2
Sequence accession number	E	Provided in Supplemental Table S2
Location of amplicon	D	
Amplicon length	E	Reported in Zhou et al. (2018, J. Dairy Sci. 101:10374-10382)
In silico specificity screen (BLAST, and so on)	E	Reported in Zhou et al. (2018, J. Dairy Sci. 101:10374-10382)
Pseudogenes, retropseudogenes, or other homologs?	D	
Sequence alignment	D	
Secondary structure analysis of amplicon	D	
		qPCR oligonucleotides
Primer sequences	E	Provided in Supplemental Table S2
RTPrimerDB identification number	D	
Probe sequences	D	
Location and identity of any modifications	E	No modifications were done
Manufacturer of oligonucleotides	D	IDT Oligo
Purification method	D	Desalted

*E = Essential information; D = Desirable information.

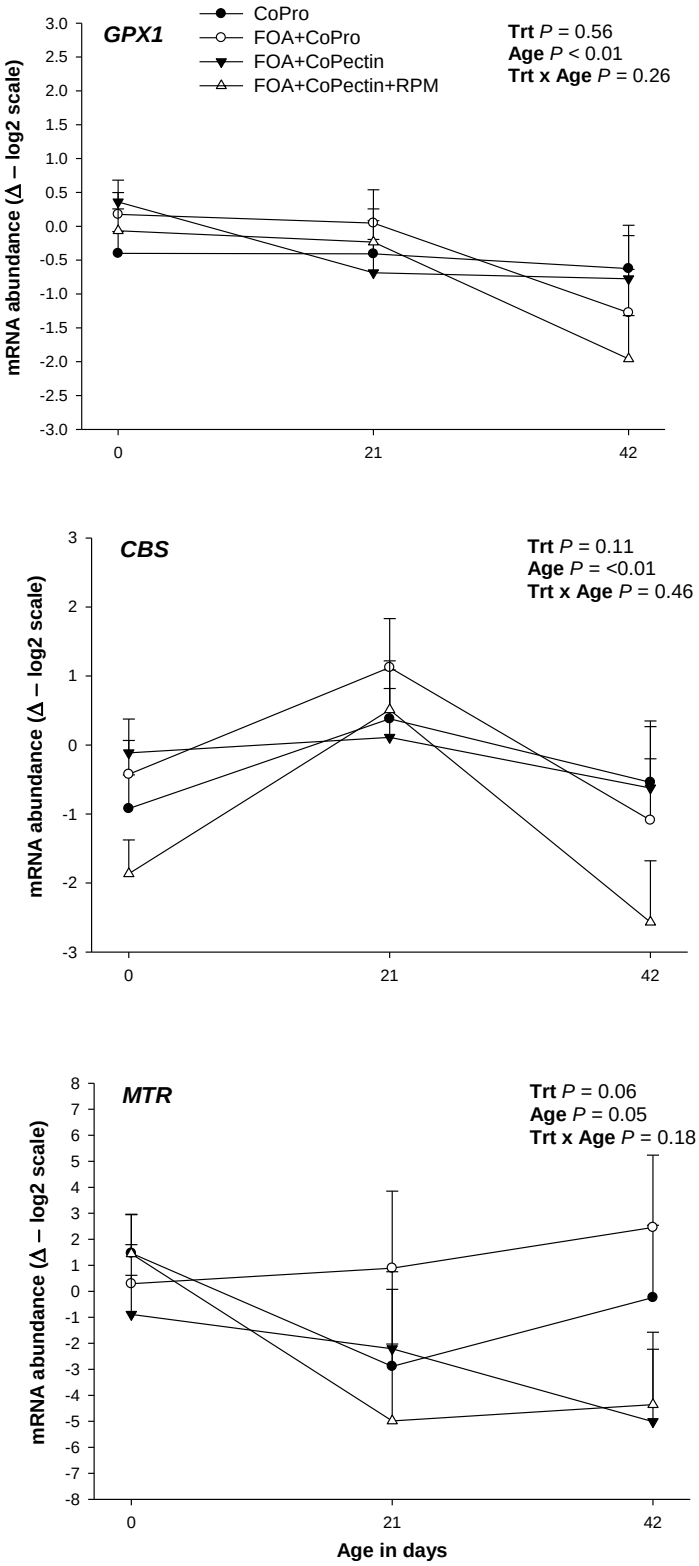
Supplemental Figure S1.



Supplemental Figure S2.



Supplemental Figure S3.



Supplemental Figure Legends

Supplemental Figure S1. Effects of maternal supplementation with CoPro, CoPectin, folic acid (FOA) and rumen-protected methionine (RPM) during late-pregnancy on calf whole blood mRNA abundance of genes involved in cytokine production (*IL1B*, *IL6*, *IL10*, *TNF*) and cytokine receptors (*IRAK1*, *CXCR1*) after ex vivo lipopolysaccharide (LPS) challenge including the time points at 0 (at birth and prior to colostrum feeding), 21 and 42 (at weaning) days of age. Response calculated as LPS challenged - non-challenged sample for each calf.

Supplemental Figure S2. Effects of maternal supplementation with CoPro, CoPectin, folic acid (FOA) and rumen-protected methionine (RPM) during late-pregnancy on calf whole blood mRNA abundance of genes involved in the toll-like receptor pathway (*TLR4*, *NFKB1*), adhesion and migration (*CADM1*, *ICAM1*, *ITGAM*), and antimicrobial function (*MPO*) after ex vivo lipopolysaccharide (LPS) challenge of whole blood at 0 (before colostrum), 21 and 42 days of age. Response calculated as LPS challenged - non-challenged sample for each calf.

Supplemental Figure S3. Effects of maternal supplementation with CoPro, CoPectin, folic acid (FOA) and rumen-protected methionine (RPM) during late-pregnancy on calf whole blood mRNA abundance of genes involved in antioxidant function (*GPX1*) and one-carbon metabolism (*CBS*, *MTR*) after ex vivo lipopolysaccharide (LPS) challenge of whole blood at 0 (before colostrum), 21 and 42 days of age. Response calculated as LPS challenged - non-challenged sample for each calf.

5 CONCLUSÃO GERAL

No geral, nosso primeiro estudo ressaltou que a condição corporal materna promoveu diferentes respostas, em nível molecular, nos mecanismos imunológicos e antioxidantes de bezerros neonatos após um desafio inflamatório com LPS. Além disso, os dados ressaltaram que o manejo do ECC materno, não só pode beneficiar a vaca, mas também os bezerros neonatos. Os efeitos de longo prazo dos diferentes níveis de ECC materno, no crescimento e desempenho dos bezerros ainda precisam ser estabelecidos.

Em nosso segundo estudo, apesar de algumas limitações inerentes da avaliação de dados de uma seleção retrospectiva de bezerros, o fato de que os animais utilizados tinham um conjunto completo de dados de desempenho, expressão gênica e função imunológica, permitiu uma avaliação preliminar do papel da suplementação materna com fontes de cobalto, ácido fólico e metionina na imunidade e metabolismo dos neonatos. Embora alguns biomarcadores plasmáticos e moleculares tenham sido significativamente afetados, nos níveis fornecidos no presente estudo, os resultados não forneceram evidências concretas dos benefícios de nutrientes relacionados ao metabolismo de 1-carbono, na modulação da capacidade e função imune de células neonatais. Novos estudos, com maior poder biológico e diferentes dosagens desses nutrientes, podem auxiliar a verificar a existência de relações diretas e indiretas, que tenham implicações no crescimento e desenvolvimento, assim como, no controle imunometabólico de bezerros neonatos.

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