UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

EFEITO DA SUPLEMENTAÇÃO COM FONTES NATURAIS DE ANTIOXIDANTES EM VACAS LEITEIRAS RECEBENDO DIETAS RICAS EM ÁCIDOS GRAXOS POLI-INSATURADOS

Autor: Luciano Soares de Lima
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MARINGÁ
Estado do Paraná
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Tese apresentada, como parte das exigências para obtenção do título de DOUTOR EM ZOOTECNIA, no Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá – Área de Concentração Produção Animal

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TITULAÇÃO: Doutor em Zootecnia – Área de Concentração - Produção Animal

APROVADA em 27 de maio de 2013.

Prof. Dr. Antonio Ferriani Branco

Profª Drª Claudete Regina Alcalde

Profª Drª Daniele Cristina da Silva Kazama

Profª Drª Maria Laura Astigarraga Fernandez

Prof. Dr. Geraldo Tadeu dos Santos
(Orientador)

"Procure obter sabedoria e entendimento; não se esqueça das minhas palavras nem delas se afaste.
Não abandone a sabedoria e ela o protegerá; ame-a, e ela cuidará de você.
O conselho da sabedoria é: procure obter sabedoria; use tudo o que você possui para adquirir entendimento.
Dedique alta estima à sabedoria e ela o exaltará; abrace-a, e ela o honrará".

Provérbios 4:5-8

Aos meus pais, Lourival e Inês de Lima, que dignamente me apresentaram o caminho da honestidade e persistência, o qual serviu de base para minhas conquistas.

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DEDICO

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BIOGRAFIA

Luciano Soares de Lima, filho de Lourival Soares de Lima e Inês Maria Ortega de Lima, nasceu na cidade de Santa Isabel do Ivaí, no dia 25 de agosto de 1984.

Em fevereiro de 2008, foi graduado em Zootecnia pela Universidade Estadual de Maringá.

Em fevereiro de 2008, ingressou no mestrado e em março de 2010, foi titulado Mestre em Produção Animal pelo Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá.

Em março de 2010, ingressou no doutorado em Produção Animal do Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá.

De janeiro de 2012 a novembro de 2012, foi contemplado com bolsa de doutorado-sanduíche pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico para a realização do estágio no Dairy and Swine Research Centre – Agriculture and Agri-Food Canada, na cidade de Sherbrooke, Estado de Quebec, Canadá.

No dia 27 de maio de 2013, submeteu-se à banca de defesa da Tese e foi aprovado para receber a titulação de Doutor em Produção Animal pelo Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá.

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RESUMO

Foram conduzidos três estudos para avaliar os efeitos da suplementação com fontes naturais de antioxidantes em vacas leiteiras recebendo dietas ricas em ácidos graxos poli-insaturados. O primeiro estudo foi realizado para avaliar os efeitos da suplementação de dois produtos, óleo de soja (OS) ou óleo de soja + polpa cítrica (OS+PC) em dois locais, rúmen ou abomaso. Para isto, quatro vacas da raça Holandês, fistuladas no rúmen, foram distribuídas em um quadrado Latino 4 × 4 com arranjo fatorial 2 × 2 dos tratamentos: 1) OS (0,2 kg/d) fornecido no rúmen; 2) OS (0,2 kg/d) perfundido no abomaso; 3) OS+PC (0,2+1,0 kg/d) fornecido no rúmen e 4) OS+PC (0,2+1,0 kg/d) perfundido no abomaso. A ingestão total de MS tendeu a reduzir com a perfusão abomasal de OS e OS+PC. Não houve efeito de produto ou local sobre a produção e composição do leite, no entanto, as vacas que foram perfundidas no abomaso apresentaram maiores proporções de ácidos graxos poli-insaturados no leite resultando em aumento no índice promotor de saúde (IPS) do mesmo. A perfusão abomasal aumentou a produção de dienos conjugados no leite. O pH e o N amoniacal no líquido ruminal foram similares entre os tratamentos, mas a produção de ácidos graxos voláteis foi reduzida quando OS foi fornecido no rúmen. Em conclusão, não foram observadas vantagens no fornecimento de OS e PC no rúmen ou no abomaso em relação às propriedades antioxidantes no leite, embora o fornecimento de OS *bypass* tenha aumentado a proporção de ácidos graxos poli-insaturados e o IPS no leite. O segundo e o terceiro estudos foram gerados a partir de um único experimento no qual foram utilizadas oito vacas da raça Holandês, fistuladas no rúmen, distribuídas em duplo quadrado Latino 4 × 4 com arranjo fatorial 2 × 2 dos tratamentos: 1) ração controle (sem farelo de linhaça-FL) sem perfusão abomasal de óleo de linhaça (OL); 2)

ração contendo 124 g FL/kg (com base na MS); 3) ração controle + perfusão abomasal de 0,25 kg/d de OL; 4) ração contendo 124 g de FL/kg (com base na MS) + perfusão abomasal de 0,25 kg/d de OL. O segundo estudo foi realizado para avaliar os efeitos de antioxidantes provenientes do farelo de linhaça (FL) sobre o desempenho e o *status* oxidativo de vacas leiteiras perfundidas com óleo de linhaça (OL) no abomaso. A ingestão de MS foi maior para vacas que receberam FL na ração e menor nas vacas perfundidas com OL no abomaso. A gordura do leite de vacas alimentadas com FL apresentou maiores proporções de ácidos graxos monoinsaturados e ômega-6. A perfusão abomasal de OL aumentou as proporções de ácidos graxos poli-insaturados no leite melhorando o seu IPS. O fornecimento de FL não alterou a concentração de TBARS no plasma e no leite, a duração da *lag phase* e a taxa de oxidação de dienos conjugados (DC) no plasma. No entanto, a perfusão abomasal de OL aumentou a concentração de TBARS no plasma e no leite e, reduziu a duração da *lag phase* para formação de DC no plasma. A capacidade antioxidante total no plasma não foi alterada pelos tratamentos. De forma geral, os resultados sugerem que a suplementação com FL para vacas recebendo fontes *bypass* de ácidos graxos poli-insaturados não promove benefícios para a proteção do organismo animal, bem como do leite, contra a lipoperoxidação. O terceiro estudo foi realizado para avaliar o efeito da suplementação com FL sobre a atividade das enzimas catalase, glutationa peroxidase e superóxido dismutase no sangue e tecido mamário, bem como a expressão de genes relacionados à defesa antioxidante e à lipogênese no tecido mamário de vacas perfundidas com OL no abomaso. A atividade da catalase tendeu a ser maior com suplementação de FL. A perfusão abomasal de OL não teve efeito sobre a atividade ou sobre a expressão gênica das enzimas antioxidantes bem como dos genes relacionados à lipogênese na glândula mamária, exceto para o gene GPX1 cuja expressão foi aumentada pelo FL, mas somente quando o OL não foi perfundido no abomaso. Os resultados sugerem que a suplementação de vacas leiteiras com 124 g FL/kg e a perfusão abomasal de 0,25 kg/d de OL não induzem mudanças significativas na atividade das enzimas antioxidantes no sangue e no tecido mamário, bem como na expressão dos genes que codificam para enzimas antioxidantes e dos genes relacionados à lipogênese no tecido mamário.

Palavras-chave: farelo de linhaça, lipoperoxidação, perfusão abomasal**,** polpa cítrica

ABSTRACT

Three studies were conducted to evaluate the effects of natural antioxidant sources on performance, milk quality and plasma and milk lipoperoxidation of dairy cows given high polyunsaturated diets. The first study was performed to investigate the effects of supplementing two products, SBO (0.2 kg/d) or SBO+CPP (0.2+1.0 kg/d), at two different sites, rumen or abomasum, on the transfer of antioxidant properties to milk, milk FA profile, milk production, and ruminal fermentation. Four ruminally fistulated lactating Holstein cows were assigned to a 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments: 1) SBO administered in the rumen; 2) SBO infused in the abomasum; 3) SBO+CPP administered in the rumen; and 4) SBO+CPP infused in the abomasum. Total DM input tended to be decreased with abomasal infusion of SBO and SBO+CPP. Product and site of supplementation had no effect on milk production and composition. However, cows infused in the abomasum compared to those administered in the rumen showed higher proportions of polyunsaturated which resulted in enhanced health-promoting index (HPI) of milk. Ruminal pH and ammonia N concentration were similar among treatments, but total volatile FA production was reduced when SBO was administered in the rumen. There was no advantage to supplement SBO and CPP in the rumen or the abomasum on milk antioxidant properties although rumen bypass of SBO increased the proportion of polyunsaturated FA in milk fat and enhanced milk HPI. The second and the third studies were from the same experiment in which eight ruminally fistulated lactating Holstein cows were assigned to a double 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments: 1) no FM in the diet and no abomasal infusion of FO; 2) diet containing 124 g FM/kg (dry matter (DM) basis) and no abomasal infusion

of FO; 3) no FM meal in the diet + abomasal infusion of 0.25 kg/d FO; 4) diet containing 124 g /kg FM (DM basis) + abomasal infusion of 0.25 kg/d FO. The second study was conducted to evaluate the effects of dietary antioxidants from FM on performance and antioxidant status in dairy cows infused with FO in the abomasum. Intake of DM was increased for cows fed FM and reduced for cows infused with FO. Milk production and milk composition did not differ among treatments except for lactose concentration that was increased by FO. Milk fat from cows fed FM had higher monounsaturated and lower omega-6 FA proportions. Abomasal infusion of FO increased proportions of polyunsaturated FA and improved the HPI. Feeding FM did not change plasma and milk TBARS concentration and the lag phase duration of formation and oxidation rate of conjugated dienes in plasma as well. Neither FM nor FO affected total antioxidant capacity in plasma. Overall, the results suggest that FM supplementation to dairy cows receiving a source of polyunsaturated FA that bypasses the rumen does not provide any benefits for protecting cows and milk against lipoperoxidation. The third study was performed to evaluate the effects of antioxidants from FM and abomasal infusion of FO on the activity of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase (GPX)) in blood and mammary tissue and the mRNA abundance of antioxidant and lipogenic-related genes in mammary tissue of dairy cows were determined. Catalase activity in erythrocytes tended to increase when cows were fed FM. Abomasal infusion of FO had no effect on activity and gene expression of antioxidant enzymes and gene expression of lipogenic-genes in mammary tissue, except for an increase in GPX1 expression in the absence of FM. The results suggest that feeding 124 g FM/kg FM and infusing 0.25 kg/d FO in the abomasum of dairy cows does not induce significant changes in the activity of antioxidant enzymes in blood and mammary tissue, and expression of antioxidant and lipogenic-genes in mammary tissue. However, more studies are required to determine any beneficial effects of natural antioxidants such as FM on the oxidative status of cows supplemented with polyunsaturated fatty acids, which could lead to feeding strategies to prevent diseases affecting the health status of dairy cattle.

Keywords: abomasal infusion, citrus pulp, flax meal, lipoperoxidation

I - INTRODUÇÃO

A gordura do leite bovino é importante fonte de nutrientes e energia na alimentação humana. No entanto, seu consumo é, muitas vezes, associado a aumentos no risco de doenças cardiovasculares em humanos, por causa de sua alta proporção de ácidos graxos saturados, aproximadamente 72%, e baixa proporção de ácidos graxos mono e poli-insaturados, aproximadamente 24% e 4%, respectivamente (Bu et al., 2007).

Por este motivo, ao longo dos anos muitas pesquisas são realizadas na busca por novos ingredientes e novas tecnologias que melhorem a composição da gordura do leite. Para isto, uma das ferramentas da ciência animal é a utilização de perfusão abomasal de óleos ricos em ácidos graxos poli-insaturados. Embora, esta prática não seja viável em fazendas comerciais, em nível de pesquisa é de grande utilidade e permitiu inúmeras inferências, não somente sobre a utilização de ingredientes da dieta animal, mas também sobre os efeitos do fornecimento de gordura *bypass* e sobre o metabolismo lipídico em ruminantes

Estudos com perfusão abomasal de óleos vegetais ricos em ácidos graxos poli-insaturados têm demonstrado que o fornecimento de fontes lipídicas *bypass* é capaz de melhorar a composição da gordura do leite com aumentos significativos na concentração dos ácidos graxos ômega-6 (Litherland et al., 2005) e ômega-3 (Kazama et al., 2010; Côrtes et al., 2011).

Diversos estudos em humanos demonstraram que o consumo de ácidos graxos poli-insaturados proporciona diversos benefícios à saúde (Cicero et al., 2012). A ingestão de ácidos graxos ômega-3, por exemplo, tem sido associada à prevenção de doenças cardiovasculares, câncer de próstata, colón e mama (Simopoulos, 2002; Simopoulos, 2003; Abeywardena & Patten, 2011; Nicholson et al., 2013). Desta forma,

o consumo de leite com maior proporção destes ácidos graxos poderia ser interessante à saúde humana caso sua estabilidade no produto final seja preservada.

No entanto, a mudança na quantidade e na composição dos lipídios na dieta pode afetar o metabolismo celular e diversos sistemas fisiológicos. Estudos realizados tendo ratos como modelo demonstraram que ácidos graxos poli-insaturados, especialmente os ácidos graxos ômega-3, desempenham funções de regulação negativa sobre genes relacionados à lipogênese e regulação positiva da extensa rede de genes que codificam para enzimas envolvidas com a oxidação de ácidos graxos (Takahashi et al., 2002; Lapillonne et al., 2004).

Além disso, a ingestão de dietas ricas em ácidos graxos poli-insaturados pode predispor os tecidos do organismo animal, bem como o leite, à lipoperoxidação mediada por radicais livres (Shiota et al., 1999; Chen et al., 2004; Gobert et al., 2009). Tal condição pode ser agravada diante da suposta regulação negativa de genes que codificam para enzimas relacionadas à eliminação de radicais livres do organismo conforme previamente demonstrado em ratos (Sreekumar et al., 2002). Estes primeiros registros são corroborados pelos resultados de estudo recente (Côrtes et al., 2012a), no qual a expressão dos genes das enzimas catalase, glutationa peroxidase e superóxido dismutase foram regulados negativamente pela perfusão abomasal de óleo de linhaça em vacas leiteiras. No caso de vacas leiteiras de alta produção, esta situação pode ser agravada, tendo em vista que estas são naturalmente mais suscetíveis ao estresse oxidativo (Bernabucci et al., 2005; Castillo et al., 2005) por causa das diversas reações oxidativas realizadas de forma mais intensa para aproveitamento dos nutrientes necessários à produção de leite.

Em mamíferos, a fosforilação oxidativa converge todas as etapas oxidativas iniciais do catabolismo de carboidratos, aminoácidos e lipídios para gerar a energia a necessária à síntese de ATP. No entanto, o metabolismo oxidativo que embora seja de vital importância para a sobrevivência celular, traz consigo um efeito colateral que consiste na síntese de radicais livres. Estes são átomos, moléculas ou íons que possuem um ou mais elétrons desemparelhados nos orbitais atômicos ou moleculares (Carocho & Ferreira, 2013) e, podem promover transferência de elétrons através de reações de oxidação e redução (Halliwell & Gutteridge, 2007). Os principais radicais livres em organismos vivos são as espécies reativas de oxigênio (ROS) geradas em diversas reações realizadas na presença de oxigênio molecular (O_2). Entretanto, ROS é um termo

usado por cientistas para descrever não somente os radicais livres hidroxila ($OH^{\bullet}$), superóxidos ($O_2^{\bullet}$), óxido nítrico ($NO^{\bullet}$) e peroxila ($ROO^{\bullet}$), mas também não radicais como peróxido de hidrogênio (H_2O_2) e ozônio (O_3)(Aruoma, 1994).

De acordo com Wojcik et al. (2010), ROS podem ser formados de quatro formas diferentes: 1) na mitocôndria, na qual a fosforilação oxidativa ocorre, resultando na formação de ROS como subprodutos das reações de transferência de elétrons; 2) nas células que realizam fagocitose, tais como neutrófilos, eosinófilos e macrófagos; 3) nos peroxissomos que produzem unicamente H_2O_2 sob condições fisiológicas; e 4) nas enzimas citocromo P450 que oxidam ácidos graxos insaturados e reduzem o oxigênio molecular ao ânion superóxido e/ou H_2O_2. A superprodução de ROS promove um desequilíbrio entre espécies oxidantes e redutoras no meio intracelular, com predominância das primeiras. Isto resulta em modificações químicas nas macromoléculas biológicas como DNA celular, proteínas e lipídios (Lindahl, 1993; Ridnour et al., 2004) podendo promover efeitos deletérios aos sistemas biológicos.

A ocorrência de peroxidação em lipídios depende da composição e concentração de ácidos graxos, temperatura, presença de mecanismos de iniciação e catálise, pressão de oxigênio e, especialmente, da disponibilidade de hidrogênio captável dos lipídios e outras fontes (Schaich, 2005). Em essência, este processo consiste na incorporação de oxigênio molecular a um ácido graxo para produzir um hidroperóxido lipídico (LOOH). Nos sistemas biológicos a peroxidação de lipídios pode ocorrer por múltiplas vias, entre as quais se destacam a via enzimática envolvendo as ciclo-oxigenases e lipoxigenases na oxidação dos ácidos graxos e a peroxidação não enzimática, que envolve a participação de ROS, metais de transição e outros radicais livres (Al-Mehdi et al., 1993; Porter et al., 1995).

O radical hidroxila é um dos principais radicais envolvidos no processo de lipoperoxidação produzidos em sistemas biológicos (Carocho & Ferreira, 2013). Outro radical livre formado a partir do oxigênio que atua sobre ácidos graxos é o radical peroxila ($ROO^{\bullet}$), cuja forma mais simples é a hidroperoxila ($HOO^{\bullet}$), que consiste na configuração protonada (ácido conjugado) do superóxido ($O_2^{\bullet}$) (Valko et al., 2007). Radicais hidroperoxila iniciam a oxidação de ácidos graxos e são documentados em estudos como iniciadores da lipoperoxidação por meio da seguinte equação: $LH + HOO^{\bullet} = L^{\bullet} + H_2O_2$ (Carocho & Ferreira, 2013). Peróxidos lipídicos podem também ser formados a partir da ação do oxigênio singlete ($^1O_2$) sobre ácidos graxos poli-

insaturados. No entanto, esta via não é considerada de iniciação porque o oxigênio reage com o ácido graxo ao invés de abstrair um átomo de hidrogênio como ocorre com os radicais hidroxila, por exemplo (Carocho & Ferreira, 2013).

De forma geral, a oxidação de lipídios pode ser dividida em três fases, iniciação, propagação e terminação (Figura 1). Na fase de iniciação, o ácido graxo sofre o ataque de uma espécie que é suficientemente reativa para abstrair um átomo de hidrogênio a partir de um grupo metileno (-CH_2-), formando um radical lipídico ($L^{\bullet}$). Quanto maior o número de duplas ligações presentes em um ácido graxo, mais fácil é a remoção de átomos de hidrogênio e, consequentemente, mais fácil é a formação de um radical. Desta forma, os ácidos graxos saturados e monoinsaturados são mais resistentes aos radicais livres que ácidos graxos poli-insaturados.

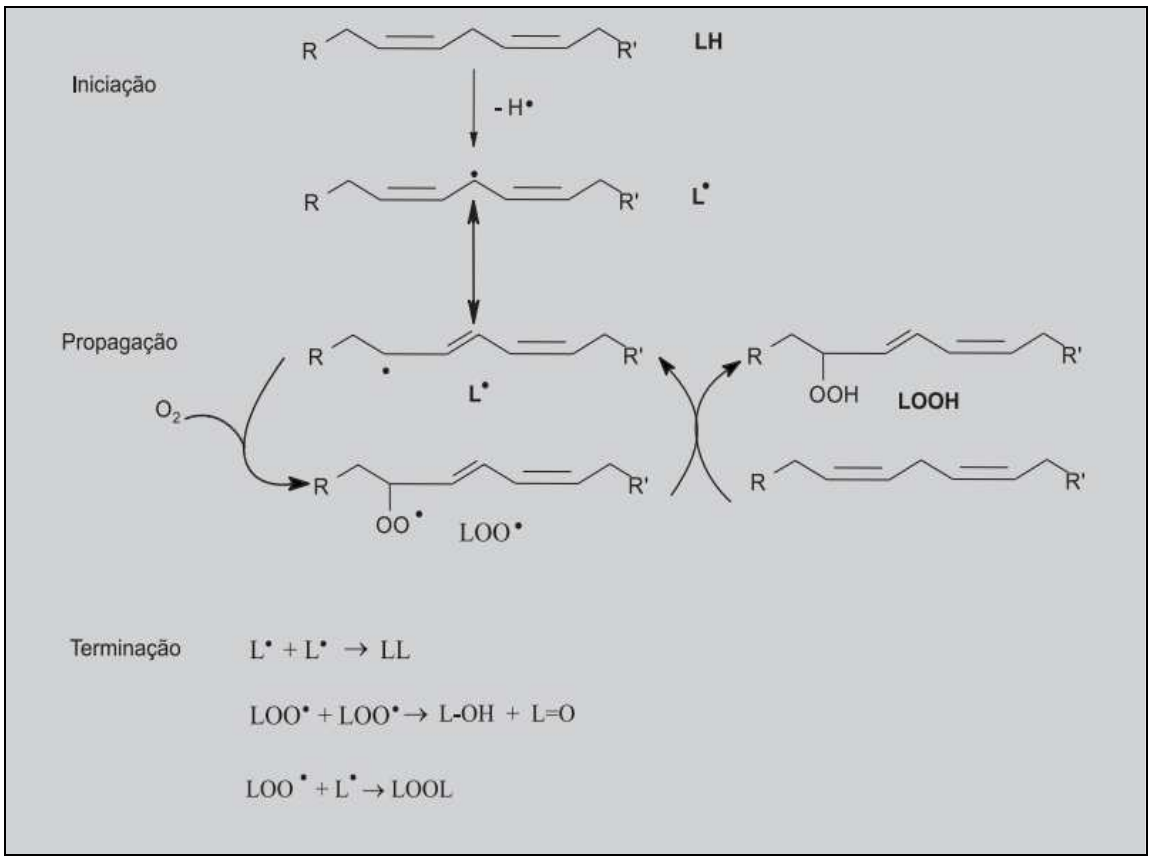

Figura 1. Esquema das principais reações ocorridas durante o processo de peroxidação lipídica
Fonte: Lima & Abdalla (2001).

Após a remoção de átomos de hidrogênio, o radical lipídico é estabilizado por um rearranjo molecular para formar um dieno conjugado, ou seja, duas duplas ligações intercaladas por uma ligação simples (Halliwell & Gutteridge, 2007). Em meio

aeróbico, o radical lipídico formado anteriormente combina com o oxigênio formando um radical peroxila lipídico ($LOO^{\bullet}$). Os $LOO^{\bullet}$ são moléculas altamente reativas que podem propagar reações em cadeia de peroxidação lipídica, ou seja, podem abstrair átomos de hidrogênio de moléculas circunvizinhas, como outro ácido graxo poli-insaturado, formando um novo radical $L^{\bullet}$ e um hidroperóxido lipídico (LOOH), promovendo assim a fase de propagação. Os LOOH produzidos são chamados de produtos primários da oxidação.

A fase de terminação se dá pela eliminação dos radicais formados originando produtos não radicais (Schaich, 2005). Nesta fase, dois radicais formados nas duas fases iniciais reagem e formam os chamados produtos secundários da oxidação, tais como aldeídos, cetonas e ácidos. Os produtos secundários da lipoperoxidação são considerados potencialmente tóxicos e responsáveis pelo surgimento de odores e sabores indesejáveis e reduzem a qualidade e segurança alimentar (Moure et al., 2001). Entre os aldeídos produzidos na fase de terminação estão o malonaldeído (MDA) e o 4-hidroxi-2-nonenal (HNE). O MDA é documentado em estudos como agente mutagênico para as células em mamíferos (Valko et al., 2007). O HNE, por sua vez, não possui ação mutagênica tão intensa como o MDA, mas é apresentado como o maior produto tóxico da peroxidação lipídica (Valko et al., 2007).

Tendo em vista que a produção de radicais livres em organismos vivos é intensa e constante, existe a necessidade do desenvolvimento de sistemas de defesa antioxidante capazes de inibir a oxidação das moléculas biológicas. Os antioxidantes interrompem as reações em cadeia eliminando os radicais livres intermediários e inibindo outras reações de oxidação (Halliwell & Gutteridge, 2007). Isto é conseguido através da sua própria oxidação, pelo que os antioxidantes são frequentemente agentes de redução (Sies, 1997). Os tipos de antioxidantes são bastante diversos, podendo ser sintetizados *in vivo* ou derivados da dieta e podem ser encontrados em todas as células (Sordillo & Aitken, 2009).

Entre os antioxidantes que podem ser sintetizados pelo organismo animal estão as enzimas catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPX). Estas enzimas constituem o sistema de defesa antioxidante primário (Carocho & Ferreira, 2013), sendo, portanto, de crucial importância aos organismos vivos (Alia et al., 2003). Entre os antioxidantes, as enzimas são os mais eficientes podendo catalisar diretamente a redução de diferentes tipos de ROS (Sordillo & Aitken, 2009). Por

exemplo, a CAT está envolvida na detoxificação do peróxido de hidrogênio (Zámocký & Koller, 1999; Chelikani et al., 2004). A GPX cataliza a redução de hidroperóxidos lipídicos e peróxidos de hidrogênio pela glutationa reduzida (Forstrom et al., 1978; Ursini et al., 1985). A superoxido dismutase é uma família de metaloenzimas que catalizam a dismutação do $O_2^{\bullet}$ para oxigênio molecular e peróxido de hidrogênio (Bannister et al., 1987; Zelko et al., 2002).

Além dos antioxidantes enzimáticos existem os não enzimáticos, os quais podem ser obtidos a partir da dieta. Entre estes, encontram-se o ácido ascorbico (vitamina C), tocoferol (vitamina E), glutationa, carotenoides, polifenois (Valko et al., 2007; Wojcik et al., 2010) e também antioxidantes sintéticos, os quais quando adicionados aos alimentos permitem que estes resistam a vários tratamentos e condições, bem como tenham a vida de prateleira prolongada (Carocho & Ferreira, 2013).

Entre os antioxidantes naturais estão os polifenóis que são um grupo amplo e heterogêneo de moléculas formadas como produtos secundários do metabolismo de vegetais superiores (Manach et al., 2004) e desta forma, são encontrados em diversos alimentos de origem vegetal constituintes da dieta animal. Estruturalmente, polifenol é o termo usado para nominar moléculas com uma ou mais hidroxilas ligadas ao anel aromático bezenoide (Wojcik et al., 2010). Assim, são diversos os tipos de polifenóis existentes e estes são qualificados de acordo com sua estrutura. Por exemplo, os flavonoides possuem dois anéis benzenoides (A e B), ligados por meio de três carbonos (estrutura difenilpropano) enquanto as lignanas são formadas por dois anéis benzenoides ligados por duas unidades difenilpropano (Figura 2). A atividade antioxidante dos polifenóis se dá pela eliminação de radicais livres com especial impacto sobre os radicais hidroxila e peroxila (Carocho & Ferreira, 2013).

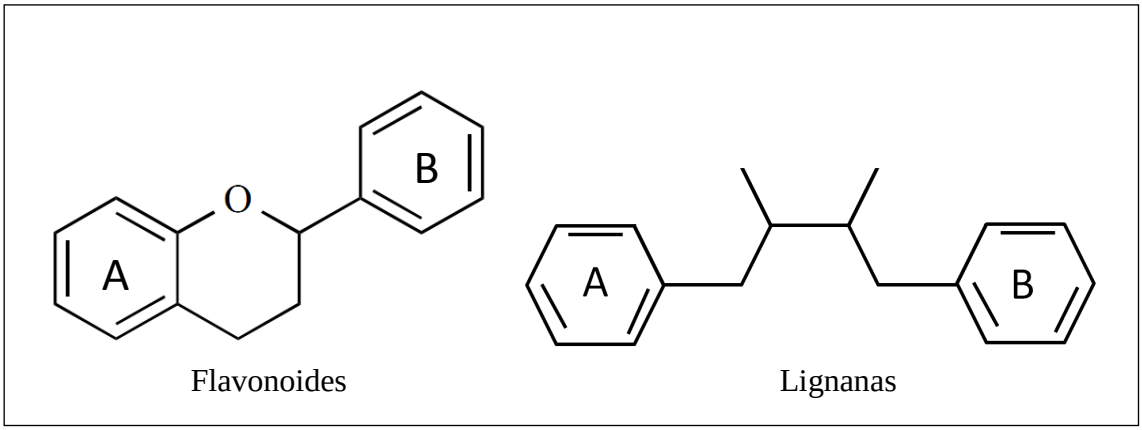

Figura 2. Estrutura química de flavonoides e lignanas
Fonte: Wojcik et al. (2010) e Landete (2012).

Muitas pesquisas na ciência animal sãoo focadas na busca por proteção dos tecidos animais e da gordura do leite contra a lipoperoxidação. De fato, estudos demonstraram que a inclusão de antioxidantes na dieta melhora os efeitos negativos da oxidação dos lipídios pelo sequestro de peróxidos (Frankel, 2005), previne a oxidação e o desenvolvimento de odores e sabores indesejáveis em leites enriquecidos com ácidos graxos poli-insaturados (Barrefors et al., 1995) e, aumenta o desempenho e o *status* oxidativo de vacas leiteiras (Vazquez-Anon et al., 2008).

A suplementação de antioxidantes sintéticos demonstra ser uma ferramenta eficaz, como pode ser constatado no estudo realizado por Wang et al. (2010), no qual foi observado que o *status* oxidativo de vacas leiteiras suplementadas com diferentes fontes *bypass* de ácidos graxos é melhorado com a suplementação de uma mistura de etoxiquina e butil-hidroquinona terciária.

No entanto, mesmo com a comprovada eficácia de antioxidantes sintéticos, existe a tendência a busca pela utilização de moléculas antioxidantes de ocorrência natural por causa de questões relacionadas à segurança e toxidade de aditivos alimentares. Gobert et al. (2009) observaram que a suplementação de vitamina E associada aos polifenóis de origem vegetal reduz a lipoperoxidação plasmática em vacas leiteiras recebendo dietas ricas em ácidos graxos ômega-3. Conclusões similares foram obtidas a partir do estudo realizado por Gladine et al. (2007), no qual quatro diferentes tipos de polifenóis de origem vegetal se mostraram eficientes em limitar a lipoperoxidação plasmática em ovelhas recebendo dietas ricas em ácidos graxos ômega-3. Adicionalmente, os antioxidantes são também documentados como reguladores positivos da expressão de genes que codificam para enzimas envolvidas na eliminação de radicais livres do organismo (McCord, 1994; Abarikwu et al., 2013).

Tais resultados sugerem que a suplementação com ingredientes ricos em antioxidantes naturais poderia ser uma forma de preservar o *status* oxidativo de vacas leiteiras quando estas se encontram em condições promotoras da síntese de radicais livres no organismo, tais como o período de transição ou a suplementação com ácidos graxos poli-insaturados. Neste sentido, muitos alimentos ou produtos de origem vegetal são estudados como fontes naturais de moléculas antioxidantes, tais como os flavonoides e as lignanas, que têm apresentado com fortes propriedades antioxidantes (Hagerman et al., 1998; Kitts et al., 1999).

Frutas cítricas são consideradas ricas fontes de moléculas antioxidantes e dependendo da espécie podem conter flavonoides como naringina, esperidina e tangeritina (Bampidis & Robinson, 2006; Tsai et al., 2007) e beta-criptoxantina (Manthey & Grohmann, 2001; Tanaka et al., 2010). A laranja (*Citrus sinensis*), por exemplo é a fruta cítrica cuja casca é rica em flavonoides como a esperidina (Kanaze et al., 2008), que é documentada como pontente antioxidante (Wilmsen et al., 2005; Al-Ashaal & El-Sheltawy, 2011). O processamento industrial da laranja gera grandes quantidades de coprodutos no mundo todo. No Brasil, por exemplo, este fruto é abundamente utilizado para a produção de suco e, paralelamente a este processo, existe também a produção de óleo essencial, que consiste em compostos voláteis, basicamente hidrocarbonetos (aproximadamente 99% m/m) como limoneno e mirceno (Verzera et al., 2004), utilizado no mercado interno e externo para diversas aplicações, as quais incluem fabricação de produtos químicos, solventes, aromas, fragrâncias, tintas e cosméticos.

Após o processamento do suco, as cascas, sementes, polpas e demais resíduos, que equivalem a aproximadamente 50% do peso de cada fruta (Widmer et al., 2010), são prensados para remover a umidade. A torta obtida, também chamada de polpa cítrica, pode ser submetida à secagem e comercializada como suplemento para a alimentação animal, por exemplo. No caso de vacas leiteiras, a polpa cítrica desidratada se destaca como importante alimento e pode substituir grãos com carboidratos altamente fermentáveis (Santos et al., 2001; Assis et al., 2004). Devendo principalmente ao fato da polpa cítrica ser boa fonte de pectina, cerca de 223 g/kg de MS (Bampidis & Robinson, 2006), cuja fermentação produz maior quantidade de ácido galacturônico ao invés de ácido láctico, como ocorre com alimentos ricos em amido. A partir do ácido galacturônico são produzidos ácidos graxos voláteis comuns à fermentação ruminal e, desta forma, o fornecimento de polpa cítrica auxilia na prevenção de acidose ruminal (Arthington et al., 2002) e, pode melhorar a eficiência de utilização dos alimentos para a produção leiteira (Miron et al., 2002).

Tendo em vista que a polpa cítrica é composta por grande proporção de casca e que esta, geralmente, não sofre tratamento com solventes durante a extração do óleo essencial, é possível que esta seja também fonte em potencial de antioxidantes na alimentação animal. Todavia, são poucos os trabalhos que avaliam os efeitos da utilização de polpa cítrica como fonte de antioxidantes na dieta de vacas leiteiras. Em

um estudo realizado por Tanaka et al. (2010) foi observado aumento na concentração de um composto com antioxidantes (beta-criptoxantina) no leite e no plasma de vacas recebendo silagem de polpa cítrica (*Citrus unshiu* Marc.) na ração total misturada. Tais resultados sugerem que a polpa cítrica poderia ser também utilizada como fonte de antioxidantes para vacas leiteiras.

Outro alimento amplamente conhecido por ser uma fonte de moléculas antioxidantes é o grão de linhaça. Este é rico em lignanas vegetais que, em humanos ou animais monogástricos, são metabolizadas em duas lignanas mamíferas, enterodiol (ED) e enterolactona (EL), sob a ação da microflora do cólon (Setchell et al., 1980; Saarinen et al., 2002). No caso de vacas leiteiras, os microrganismos do rúmen desempenham um importante papel no metabolismo das lignanas da linhaça (Gagnon et al., 2009). Côrtes et al. (2012b) observaram maior concentração de enterolactona na urina, plasma e leite em vacas suplementadas com casca de linhaça. Rajesha et al. (2006), usando ratos como modelo de estudo, relataram que as lignanas oriundas da linhaça regulam positivamente a expressão dos genes das enzimas SOD, CAT e GPX. Estes resultados são corroborados pelo estudo realizado por Côrtes et al. (2012a), no qual foi observado aumento na abundância de mRNA dos genes CAT, SOD, GPX na glândula mamária de vacas suplementadas com casca de linhaça.

Após a extração do óleo a partir do grão de linhaça, o farelo resultante é usado como suplemento proteico na alimentação animal. Além de sua finalidade como alimento proteico, o farelo de linhaça é também rico em lignanas vegetais e quando este alimento foi inserido na dieta de vacas em lactação houve aumento linear na concentração de enterolactona no leite (Petit et al., 2009). De acordo com Prasad (2000), concentrações milimolares de lignanas mamíferas já são suficientes para inibir oxidação induzida por ROS avaliada por incubação *in vitro* de sangue venoso. Desta forma, a fornecimento de farelo de linhaça pode ser eficiente na proteção do organismo animal e o leite em condições de predisposição à oxidação.

Assim, o estudo do fornecimento de fontes naturais de antioxidantes, tais como a polpa cítrica e o farelo de linhaça, para vacas em lactação suplementadas com fontes de ácidos graxos poli-insaturados, poderá ser de grande utilidade na busca pela produção do leite como alimento funcional e na busca pela melhora do *status* antioxidante de vacas leiteiras.

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(Normas: Animal Feed Science and Technology)

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II - OBJETIVOS GERAIS

Este trabalho foi realizado com os objetivos de avaliar os efeitos da suplementação com antioxidantes provenientes da polpa cítrica sobre o desempenho, qualidade do leite e *status* oxidativo do leite de vacas recebendo dietas ricas em ácidos graxos ômega-6 oriundos do óleo de soja e avaliar os efeitos da suplementação com antioxidantes do farelo de linhaça sobre o desempenho, qualidade do leite, lipoperoxidação no plasma e no leite, atividade de enzimas antioxidantes (catalase, superóxido dismutase, glutationa peroxidase) no sangue e tecido mamário, abundância de mRNA de genes que codificam para enzimas antioxidantes e lipogênicas no tecido mamário de vacas recebendo dietas ricas em ácidos graxos ômega-3 provenientes do óleo de linhaça.

III - Effect of abomasal or ruminal supplementation of citrus pulp and soybean oil on production performance, fatty acid profile and antioxidant properties of milk and ruminal fermentation characteristics of dairy cows

(Normas: Animal Feed Science and Technology)

Abstract

Soybean oil (SBO) is rich in polyunsaturated fatty acids (FA) and rumen bypass of SBO can contribute to increase polyunsaturated FA proportion in milk. Citrus pulp (CPP) is a source of antioxidants but there is little information on its effects on milk properties and milk production and composition. The aim of this research was to investigate the effects of supplementing two products, SBO (0.2 kg/d) or SBO+CPP (0.2+1.0 kg/d), at two different sites, rumen or abomasum, on the transfer of antioxidant properties to milk, milk FA profile, milk production, and ruminal fermentation. Four ruminally fistulated lactating Holstein cows were assigned to a 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments: 1) SBO administered in the rumen; 2) SBO infused in the abomasum; 3) SBO+CPP administered in the rumen; and 4) SBO+CPP infused in the abomasum. Intake of dry matter (DM) of the basal diet was decreased due to administration of SBO+CPP in the rumen. Basal DM intake and total DM input tended to be decreased with abomasal infusion of SBO and SBO+CPP. Product and site of supplementation had no effect on milk production and composition. Concentrations of total polyphenols and flavonoids, reducing power and production of conjugated diene (CD) hydroperoxides in milk were not affected by products but infusion in the abomasum compared to administration in the rumen increased production of CD. Milk fat FA profile was not affected by products. However, cows infused in the abomasum compared to those administered in the rumen showed lower proportions of short-chain and monounsaturated FA and higher proportions of polyunsaturated, omega 3 and omega 6 FA, which resulted in enhanced health-promoting index (HPI) of milk. Higher plasma concentrations of high- and low-density lipoproteins and total cholesterol were observed when cows were infused in the abomasum. Ruminal pH and ammonia N concentration were similar among treatments, but total volatile FA production was reduced when SBO was administered in the rumen.

There was no advantage to supplement SBO and CPP in the rumen or the abomasum on milk antioxidant properties although rumen bypass of SBO increased the proportion of polyunsaturated FA in milk fat and enhanced milk HPI.

Keywords: flavonoids, polyphenols, conjugated diene, health-promoting index, abomasal infusion, fat supplementation

Abbreviations: FA, fatty acids; SBO, soybean oil; CPP, citrus pulp; DMI, dry matter intake; DM, dry matter; CD, conjugated diene; EE, ether extract; GAE, gallic acid equivalent; HPI, health-promoting index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; QE, quercetin equivalent; SCC, somatic, cell count; TTAD, total tract apparent digestibility; VFA, volatile FA.

1.Introduction

Milk fat is an important dietary source of nutrients and energy. However, milk consumption has been linked to higher risk of cardiovascular diseases in humans because of its high proportion of saturated fatty acids (FA) (~72%) and low proportions of monounsaturated and polyunsaturated FA (~24% and 4%, respectively; (Bu et al., 2007). Therefore, recent research has focused on the importance of supplying lactating dairy cows with polyunsaturated FA to increase their proportion in milk fat since they are considered health promoting (Ulbricht and Southgate, 1991). However, supplementation with dietary lipids such as soybean oil (SBO) may result in both positive and adverse changes in the nutritional and dietetic properties of milk as shown by increased unsaturated FA proportion and increased oxidation susceptibility (Chen et al., 2004; Bobe et al., 2007). The oxidative deterioration of milk fat containing a high unsaturated FA proportion can increase the development of rancid odors and flavors in milk (Timmons et al., 2001), which results in products of lower nutritional quality and safety due to the formation of secondary, potentially toxic compounds (Moure et al., 2001; Chen et al., 2004).

However, the presence of antioxidants such as α-tocopherol may prevent oxidation of milk enriched in polyunsaturated FA and the development of oxidized flavor as shown by Barrefors et al. (1995). Recent studies have demonstrated that antioxidants are effectively transferred from the diet to milk when cows are fed flaxseed

byproducts (Petit et al., 2009; Côrtes et al., 2012). Moreover, although Gagnon et al. (2009) reported that rumen microorganisms play an important role in the transfer of antioxidants from flaxseed hulls to milk there is little information on the importance of the rumen for the absorption of antioxidants. Gladine et al. (2007) has reported that, contrary to monogastrics, ruminants can benefit from antioxidants and that the ruminal microbiota may be involved in the metabolism of antioxidants although further experiments should be performed to better understand the role of microorganisms and the metabolic pathways of antioxidants in ruminants.

Agricultural and industrial byproducts are attractive sources of natural antioxidants (Moure et al., 2001). Citrus pulp (CPP) is a byproduct from the food processing industry that has been widely used as a high energy feed in diets of lactating dairy cows (Bampidis and Robinson, 2006). Citrus pulp is also a source of the flavonoids hesperidin and naringin, which are antioxidants (Williams et al., 2004; Bampidis and Robinson, 2006). However, there is little information on the effects of CPP on the properties of milk and the role of the rumen on the absorption of antioxidant from CCP. Thus, the overall aim of carrying out this research was to investigate the effect of supplementing soybean oil (SBO) or soybean oil + citrus pulp (SBO+CPP) in the rumen or the abomasum on the transfer of antioxidant in milk and on its properties. Performance, milk production and composition, plasma parameters and ruminal fermentation were also evaluated.

2.Material and methods

2.1 Cows, diets, and experimental procedures

Four multiparous lactating Holstein cows fitted with ruminal cannulas (10 cm, Bar Diamond Inc., Parma, ID, USA) were assigned to a 4 × 4 Latin square design with a 2 × 2 (two products and two sites of supplementation) factorial arrangement of treatments and four 21 d periods balanced for residual effects. At the beginning of the experiment, the cows averaged 92 ± 13 days in milk, 29.0 ± 2.2 kg of milk/d, and 559 ± 67 kg of body weight. Cows were housed in individual stalls with free access to water. All cows were fed with the same total mixed ration (Table 1) twice a day (07:00 and 14:00 h) for *ad libitum* intake (10% of refusals on as fed basis). The diet was formulated to meet requirements for cows producing 30 kg/d of milk with 38 g/kg of fat (NRC, 2001).

Table 1

Ingredients, chemical composition, and fatty acid profile of the total mixed diet, citrus pulp, and soybean oil.

Item	Total mixed diet	Citrus pulp	Soybean oil
Ingredient (g/kg DM)			
Corn silage	600.2		
Ground corn	183.1		
Soybean meal	197.0		
Mineral supplement[1]	15.0		
Dicalcium Phosphate	0.1		
Limestone	0.9		
Ca bicarbonate	3.7		
Chemical analysis[2]			
DM (g/kg)	525.1	887.5	
CP (g/kg DM)	163.7	58.4	
Ether extract (g/kg DM)	25.2	17.7	
aNDF (g/kg DM)	389.5	189.3	
ADF (g/kg DM)	220.2	160.2	
NE_L (MJ/kg DM)[3]	7.11	8.28	
Total polyphenols (mg/100g)	-	783.5	
Flavonoids (mg/100g)	-	161.5	
Fatty acid[2] (g/kg of total fatty acids)			
14:0	2.4	8.4	13.5
16:0	165.5	295.5	117.9
18:0	37.0	50.3	20.0
*cis*9-18:1	301.1	202.2	257.6
*cis*9,*cis*12-18:2	432.0	341.9	545.0
*cis*6,*cis*9,*cis*12-18:3	62.0	79.0	46.1
20:0	ND[4]	5.3	ND
*cis*13-22:1	ND	5.7	ND
24:0	ND	11.8	ND

[1]Contained (per kg, as-is basis): Ca 240 g, P 60 g, Mg 15.0 g, S 18.0 g, Na 78.0, Fe 2,200 mg, Zn 3.800 mg, Cu 680 mg, Mn 1.105 mg, I 40 mg, Co 10 mg, Se 25 mg, vitamin A 100,000 IU, vitamin D3 66,700 IU, and vitamin E 1,000 IU.

[2]One sample obtained from four pool samples prepared by compositing eight daily samples from d 14 to 21.

[3]Calculated using described published values of feed ingredients (NRC, 2001).

[4]ND = not detected.

Milking times were 06:30 and 16:00 h, and milk yield was recorded at each milking. The Animal Care and Use Committee of the Universidade Estadual de Maringá approved all animal procedures.

The experimental treatments consisted of: 1) 0.2 kg/d of SBO administered in the rumen and 15.0 kg/d of tap water infused in the abomasum; 2) 0.2 g/d of SBO and 15.0 kg/d of tap water infused in the abomasum; 3) 0.2 kg/d of SBO and 1.0 kg/d of CPP administered in the rumen and 15.0 kg/d of tap water infused in the abomasum; 4) 0.2 kg/d of SBO, 1.0 kg/d of CPP and 14.0 kg/d of tap water infused in the abomasum.

To perform abomasal infusions, an infusion line was inserted through the ruminal cannula and the sulcus omasi as described by Gressley et al. (2006). Plastisol discs (12 cm in diameter and 9 mm in height) were used to anchor the infusion line, and placement of infusion lines was monitored daily to ensure postruminal delivery. Two different infusion lines were used to pump oil and water, each at a constant flow rate, in a larger line using a Y-shape connection line leading to the abomasum as previously reported by Kazama et al. (2010). Abomasal infusions consisted of 15 kg/d of solution (water or water + CPP) and they were daily prepared using an electric mixer (Fisatom, São Paulo, SP, Brazil). Dried citrus pulp is a commercially available product from orange (*Citrus sinensis*) juice industrie and was ground through a 1 mm screen for the infusion.

Variable-speed peristaltic pumps (Provitec, São Paulo, SP, Brazil) were used to deliver the infusion mixtures at a rate of 1.25 kg/h. During the first 7 days of each period, only 50% of the experimental dose of solution and oil was supplied over a 6-h period (from 07:00 to 13:00 h). From day 8 to 21, the abomasal infusion was conducted with 100% of the experimental dose of solution and oil over a 12 h (from 07:00 to 19:00 h) period. Oil was pumped into the abomasum using a peristaltic pump. The ruminal administration was performed by adding one-third each of oil dose and citrus pulp 3 times daily (07:00, 13:00, and 19:00 h).

Samples of RTM and citrus pulp were taken daily from day 14 to 21 and pooled within period. Samples of orts were taken daily from day 14 to 21 of each period and feed intake was recorded. Samples of diet, citrus pulp and orts were frozen at -20^{o}C for subsequent drying at 55^{o}C. The samples were ground through a 1 mm screen in a Wiley mill and afterward were composited by cow within period for further analysis.

From day 11 to 19, a capsule of chromic oxide (Cr_2O_3) was inserted in the rumen once daily at 09:00 h, supplying a total of 10 g of Cr_2O_3/d. Fecal grab samples were taken twice daily at 08:30 and 16:30 h from day 15 to 19 and a portion (about 100 g) was dried for 48 h at 55°C and composited by cow within period for later component analysis. Chromic oxide was dosed to the animals according to Williams et al. (1962) to predict fecal output and total tract apparent digestibility (TTAD).

Milk samples were collected from two consecutive milkings on day 18 and kept separately for all analyses. Samples without preservative and with Na azide (0.2 g/kg) were kept frozen at −20°C for determination of FA profile, and antioxidants (total polyphenols and flavonoids) and conjugated diene (CD) hydroperoxides, respectively. Another milk sample was stored at +4°C with a preservative (bronopol-B2) until analysis for normal composition.

Blood was collected from all cows on day 18 one hour after the morning milking to determine concentrations of very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol, triacylglycerol and glucose. Blood was withdrawn from the tail vein into vacutainer tubes (Becton Dickinson and Cie, Rutherford, NJ, USA) containing heparin. Tubes were immediately centrifuged at 3,000 × *g* for 20 min. Plasma was separated and frozen at -20^{o}C for subsequent analysis.

On day 21, ruminal contents were collected from different locations within the rumen (the anterior dorsal, anterior ventral, medium ventral, posterior dorsal, and posterior ventral locations) 0, 2, 4, and 6 h after the morning meal. Rumen pH was measured immediately after sample collection with a portable pH meter (Tecnal, Piracicaba, SP, Brazil). The ruminal contents were then strained through four layers of cheesecloth and two aliquots of strained ruminal fluid were acidified to pH 2 with 50% H_2SO_4 and frozen at -20°C for later determination of volatile fatty acids (VFA) and ammonia concentrations.

2.2 Chemical analysis

Dry matter in RTM, orts and feces samples was evaluated according to method no. 934.01 of AOAC (1998). Organic matter was determined by combustion in a muffle furnace according to method no. 942.05 of AOAC (1998). Total N was determined with a Tecnal TE-036/1 (Tecnal, Piracicaba, SP, Brazil) following method no. 988.05 of the

AOAC (1998) and crude protein (CP) was estimated as N × 6.25. Ether extraction (EE) in diets was conducted with Tecnal TE-044/1 according to procedure no. 920.39 of AOAC (1998). The neutral detergent fiber (aNDF) was evaluated as described by Mertens (2002) using a heat-stable α-amylase and sodium sulphite. The ADF content was determined according to method 973.18 of AOAC (1998). The aNDF procedure was adapted for use of an Ankom^{200} Fiber Analyzer (Ankom Technology Corp., Fairport, NY, USA).

Protein, lactose, fat and urea N concentrations in milk were analyzed by infrared spectrophotometry (Bentley model 2000; Bentley Instrument Inc., Chaska, MN, USA). Milk somatic cells counts (SCC) were obtained using an electronic counter (Somacount 500, Chaska, MN, USA). Fat in milk was separated by centrifugation as described by Murphy et al. (1995) and FA were methylated according to method 5509 of ISO (1978) using KOH/methanol (Synth, São Paulo, Brazil) and n-heptane (Vetec, Rio de Janeiro, RJ, Brazil). Fatty acid methyl esters were quantified by gas chromatography (Trace GC Ultra, Thermo Scientific, West Palm Beach, Florida, USA) equipped with an autosampler, a flame-ionization and a Rt-2560 fused-silica capillary column (100 m and 0.25 mm i.d., 0.20 µm film thickness). The column parameters were as follows: initial column temperature of 65°C was maintained for 8 min; the temperature was then programmed at 50°C/min to 170°C; this temperature was maintained for 40 min and then increased 50°C/ min to 240°C and remained for 28.5 min. Injector and detector temperatures were 220 and 245°C, respectively. The gas flow was 1.5 ml/min for hydrogen (carrier gas), 30 ml/min for N_2 (auxiliary gas), 35 ml/min for H_2 and 350 ml/min for compressed air. Fatty acid peaks were identified using pure methyl ester standards (Sigma, São Paulo, SP, Brazil).

Content of total polyphenols of citrus pulp and milk was determined using the Folin–Ciocalteu technique (Singleton and Rossi, 1965) with elimination of interfering substances using polyvinylpolypyrrolidone as reported by Han et al. (2011). Polyphenols were extracted from citrus pulp by mixing 1 g of sample (ground through 1 mm screen) with methanol/water (90:10, v/v) and the volume was made up to 100 mL. From milk, polyphenols were extracted by mixing 1 mL of each sample with methanol/water (90:10, v/v), the volume was made up to 10 mL). The extracts were then filtered on a 0.22 µm PTFE membrane filter (Spritzen, Shanghai, China) into a tube protected from light. The assay was performed using a UV-Vis spectrophotometer

(Spectrum SP2000, Shanghai Spectrum, China). The phenolic compound content was reported as gallic acid equivalents (GAE; µg/mL of milk and mg /100g of citrus pulp).

Flavonoid content of citrus pulp and milk samples was measured at 425 nm by spectrophotometry after reaction with aluminum chloride as described by Woisky and Salatino (1998) and modified by Sánchez et al. (2010) . Flavonoids were extracted from citrus pulp and milk samples using the procedure described for polyphenols. Quercetin was used as standard for the calibration curve and results were reported as quercetin equivalents (QE; µg/mL of milk and mg/100 g of citrus pulp).

Total reducing power was determined as described by Zhu et al. (2002) with some modifications. Milk proteins were precipitated by adding 1 mL of a trichloroacetic acid solution (20%; v/v) to 1 mL of milk. The mixture was vortex-mixed for 10 min and centrifuged at 1,058 × *g* for 10 min at 20°C. Absorbance was measured at 700 nm on a UV-Vis spectrophotometer (Spectrum SP2000, Shanghai Spectrum, China) and reducing power was reported as GAE (µg/mL). Production of conjugated diene (CD) hydroperoxides in milk was used to measure lipid oxidation according to the method of Kiokias et al. (2006) with some modifications. Briefly, samples (50 µL) were added to a mixture of 2.5 mL isooctane/2-propanol (2:1 v/v) and vortexed for 10 sec. Samples then were filtered on a 0.22 µm PTFE membrane filter (Spritzen, Shanghai, China). The absorbance was measured at 232 nm using a UV-Vis (Spectrum SP2000, Shanghai Spectrum, China). The production of CD was calculated as follows: CD (mmol/kg of fat) = (A/27)/[(a*b)/100000*(c + b/1000)]; where: A = absorbance at 232 nm; a = milk fat proportion (g/100 g); b = sample volume (µL); and c = mixture volume (mL).

The health-promoting index (HPI) was calculated as the inverse of the atherogenic index (Ulbricht and Southgate, 1991) according to the equation described by Chen et al. (2004), where the concentration of total unsaturated FA is divided by the sum of 12:0, 16:0, and 4 × 14:0.

Plasma triacylglycerol concentration was determined by an enzymatic colorimetric method using glycerol-3-phosphate oxidase at 500 nm (kit 15710; Diasys Diognostic Systems, Holzheim, Germany). The HDL and LDL concentrations were analyzed by colorimetric assays at 600 nm (kits 13521 and 14121, respectively; Diasys Diognostic Systems). The VLDL concentration was estimated by dividing TG concentration by five as described by Friedewald et al. (1972). Concentrations of total cholesterol (kit 11300; Diasys Diognostic Systems) and glucose (kit 12500; Diasys Diognostic Systems) were

analyzed at 500 nm using enzymatic photometric assays and urea concentration (kit 1301; Diasys Diognostic Systems) was determined at 340 nm.

Ruminal concentration of ammonia N was analyzed as described by Preston (1995). Samples of ruminal fluid were centrifuged at 15,000 × *g* at 4°C for 50 min before determination of volatile FA (VFA) concentration. A 0.1 µl aliquot was injected into a HP 5890 Series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) with a silic column (2 m × 0.2 mm i.d., 0.20 µm film thickness) packed with 10% SP 1200/1% H_3PO_4 on 80/100 Chromasorb W AW silica column (2 m × 2 mm i.d.) packing and fitted with an autosampler (Hewlett Packard 6890 Series Injector, Milford, MA, USA) and flame-ionization detection. The column, inlet, and detector temperatures were maintained at 115, 160, and 190ºC, respectively. The carrier gas (N_2) flow rate was 25 mL/min and total time of the analysis was 15 minutes.

2.3 Statistical Analysis

All results were analyzed using the MIXED procedure of SAS (SAS 2000; SAS Institute) within a 2 × 2 factorial arrangement of treatments. Data were analyzed using a 4 × 4 Latin square design with the following general model:

$$Y_{ijkl} = \mu + C_i + P_j + T_k + e_{ijk}$$

Where: Y_{ijkl}, the dependent variable; µ, overall mean; C_i, random effect of cow (i = 1 to 4); P_j, fixed effect of period (j = 1 to 4); T_k, fixed effect of treatment and e_{ijk}, random residual error. Factorial contrasts were used to test the main effects of supplement (soybean oil *versus* soybean oil + citrus pulp), site of supplementation (rumen *versus* abomasum), and their interaction. Data on milk production and milk composition were initially analyzed as repeated measurements and further analyzed without the time effect when time was not significant. Data on ruminal fermentation (pH, ammonia N and VFA) were analyzed as repeated measurements and covariance structures were modeled. The most appropriate covariance structure was fitted based on the smallest values of fit statistics for Akaike's information criterion, and Akaike's information criterion corrected. Results are reported as least squares means and SEM. When the interaction between product and site was significant, the SLICE option of the MIXED procedure was used to examine the differential response of infusion of oil in the

abomasum to FM supplementation. Significant differences were set at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

3.Results

There was no interaction between product and site for intake of DM and TTAD of nutrients (Table 2). Dry matter intake of the basal diet, expressed in kg/day and percentage of body weight, was significantly reduced when cows where supplemented with SBO+CPP compared to SBO. Infusion in the abomasum tended (P=0.07) to decrease the basal DM intake and total DM input expressed in kg/day, and they were significantly decreased when expressed as a percentage of body weight. There was no significant effect of product and site on TTAD of DM and aNDF (Table 2). However, TTAD of CP tended (P=0.09) to be lower when products were administered in the rumen compared to when they were infused in the abomasum. There was a significant effect of product for TTAD of EE with higher TTAD for cows supplemented with SBO+CPP compared to those supplemented with SBO only.

Table 2

Dry matter intake (DMI) and total tract apparent digestibility (TTAD) of Holstein cows supplemented with soybean oil (SBO) or soybean oil + citrus pulp (SBO+CPP) in the rumen (RUM) or in the abomasum (ABO).

	Treatments							
	SBO		SBO+CPP			*P*		
Item	RUM	ABO	RUM	ABO	SEM	Product	Site	P × S
Basal DMI (kg/d)	17.64	16.93	16.64	16.37	0.84	0.01	0.07	0.36
Total DM input (kg/d)	17.84	17.13	17.78	17.51	0.84	0.49	0.07	0.36
Basal DMI (g/kg of BW)	32.9	31.2	31.4	30.1	2.20	0.01	0.01	0.74
Total DM input (g/kg of BW)	33.2	31.6	33.5	32.2	2.20	0.23	0.01	0.79
TTAD (g/kg MS)								
Dry matter	656.2	682.7	640.6	655.4	14.6	0.16	0.18	0.68
Crude protein	668.8	699.4	653.2	670.9	15.5	0.12	0.09	0.61
Ether Extract	753.6	758.6	796.3	785.3	17.0	0.03	0.82	0.55
Neutral detergent fiber	496.0	518.2	440.0	464.7	23.1	0.11	0.36	0.96

Abomasal infusion was carried out only over a 12-h period. Therefore, samples of milk were analyzed separately for the morning and evening samples to determine if the response to treatment was similar for both milkings. As there was no interaction between sampling time (a.m. and p.m.) and treatment for milk composition, only average values are reported (Table 3).

Table 3

Milk production (kg/d) and milk composition of Holstein cows supplemented with soybean oil (SBO) or soybean oil + citrus pulp (SBO+CPP) in the rumen (RUM) or in the abomasum (ABO).

	Treatments							
	SBO		SBO+CPP			*P*		
Item	RUM	ABO	RUM	ABO	SEM	Product	Site	P × S
Milk production	24.5	24.4	24.0	23.5	2.78	0.33	0.62	0.80
Milk composition (g/kg)								
Protein	31.1	30.4	30.8	30.7	1.22	0.98	0.28	0.42
Fat	37.8	33.8	35.8	35.0	2.31	0.80	0.19	0.35
Lactose	45.8	46.1	46.4	46.0	1.94	0.30	0.57	0.15
Total solids	124.9	119.3	122.4	120.8	4.42	0.80	0.11	0.32
Urea N (mg/dL)	14.3	15.1	14.1	14.6	0.73	0.63	0.39	0.85
Milk yield (kg/d)								
Protein	0.76	0.74	0.74	0.72	0.07	0.30	0.18	1.00
Fat	0.93	0.83	0.87	0.84	0.11	0.46	0.17	0.30
Lactose	1.14	1.14	1.14	1.10	0.16	0.54	0.64	0.49
Total solids	3.07	2.93	2.98	2.87	0.37	0.34	0.14	0.82
SCS[1]	2.03	1.84	1.82	2.02	0.33	0.94	0.99	0.33
Polyphenols[2]	47.3	52.1	49.4	49.2	2.63	0.89	0.39	0.35
Flavonoids[3]	119.4	112.0	118.6	129.6	13.9	0.56	0.90	0.52
Reducing power[4]	37.6	29.2	28.1	44.5	6.01	0.63	0.51	0.17
CD[5] (mmol/kg fat)	29.8	31.3	28.3	29.6	2.99	0.11	0.04	0.89

[1]Somatic cell score = log somatic cell count; [2]Total Polyphenols = gallic acid equivalent (µg GAE/mL); [3]Flavonoids = quercetin equivalent (µg EQ/mL); [4]Reducing power = gallic acid equivalent (µg GAE/mL); [5]Conjugated Diene.

There was no interaction between product and site for milk production and composition, yield and concentrations of milk components, concentration of total

polyphenols and flavonoids, reducing power and production of CD hydroperoxides. Product and site of supplementation had no effect on milk production, proportion and yield of milk components, concentration of total polyphenols and flavonoids, and reducing power. However, there was a significant effect of site for CD production in milk, with infusion in the abomasum resulting in higher production compared to administration in the rumen.

There was no significant interaction between product and site for milk FA profile (Table 4). Milk fat from cows infused in the abomasum tended to have higher proportions of 4:0, 8:0, 10:0 and 12:0 than milk fat from those supplemented in the rumen (Table 4). Proportions of 11:0; 13:0; 14:0; *cis*9-14:1; 15:0; 15:1; 17:0; 18:0; *trans*9-18:1; *trans*9,*trans*12-18:2; *cis*9,*trans*11-18:2, 20:0; 20:1; *cis*11,*cis*14,*cis*17-20:3 and *cis*5,*cis*8,*cis*11,*cis*14-20:4 in milk fat were similar among treatments (Table 4). The proportions of 16:0, *cis*9-16:1, 17:1 and *cis*9-18:1 were decreased and those of *cis*9,*cis*12-18:2 and *cis*9,*cis*12,*cis*15-18:3 were increased when cows were infused in the abomasum compared to the rumen. There was a trend (P=0.06) for an interaction between product and site for *cis*6,*cis*9,*cis*12-18:3 as a result of lower proportion for cows infused with SBO+CPP in the abomasum compared to those infused SBO only while administration of SBO and SBO+CPP in the rumen resulted in similar proportions but higher ones than when they were infused in the abomasum. Cows infused in the abomasum had higher proportions of short-chain, polyunsaturated, omega 3, and omega 6 FA than those administered with products in the rumen and the inverse was observed for proportions of monounsaturated FA. There was also a tendency (P=0.06) for lower proportions of saturated FA in milk fat when infusion was performed in the abomasum compared to administration in the rumen. Cows infused with products in the abomasum showed higher milk health-promoting index (HPI) compared to those which received products in the rumen.

There was no interaction between product and site for blood parameters and treatments had no effect on concentrations of glucose, triacylglycerol and VLDL in plasma (Table 5). Plasma concentrations of HDL, LDL and total cholesterol were not affected by product but cows infused in the abomasum compared to those administered with products in the rumen had higher concentrations of HDL, LDL and total cholesterol.

Table 4

Milk fatty acid concentration (g/kg of total fatty acids) of Holstein cows supplemented with soybean oil (SBO) or soybean oil + citrus pulp (SBO+CPP) in the rumen (ABO) or in the abomasum (ABO).

	Treatments							
	SBO		SBO+CPP			*P*		
Item	RUM	ABO	RUM	ABO	SEM	Product	Site	P × S
4:0	3.4	4.6	3.8	4.9	0.62	0.48	0.07	0.90
6:0	7.3	17.9	1.5	21.9	2.23	0.14	0.007	0.52
8:0	5.7	6.9	6.2	8.4	1.01	0.27	0.08	0.48
10:0	6.1	6.3	5.2	9.5	1.43	0.33	0.09	0.10
11:0	2.8	1.5	2.5	4.5	1.41	0.27	0.75	0.20
12:0	20.6	21.3	20.9	26.7	2.75	0.14	0.09	0.14
13:0	2.4	1.9	2.4	2.4	0.42	0.45	0.59	0.53
14:0	1.6	1.3	1.8	1.5	0.21	0.57	0.36	0.73
cis-9 14:1	111.9	110.1	116.1	116.6	6.73	0.12	0.89	0.34
15:0	8.6	6.7	8.9	6.9	1.24	0.85	0.14	0.95
15:1	12.6	12.2	13.0	11.9	8.2	0.98	0.44	0.81
16:0	337.3	303.9	335.9	308.6	14.7	0.91	0.01	0.35
cis-9 16:1	13.6	10.1	13.3	9.4	3.30	0.40	0.0007	0.99
17:0	6.3	6.7	6.6	6.9	0.32	0.61	0.15	0.68
17:1	1.3	0.7	1.2	0.4	0.24	0.30	0.004	0.78
18:0	130.0	123.2	127.5	111.2	8.61	0.27	0.15	0.67
*cis*9-18:1	277.8	260.8	277.6	241.4	15.43	0.22	0.02	0.50
*trans*9-18:1	20.8	22.5	23.3	17.0	1.89	0.43	0.31	0.10
*cis*9,*cis*12-18:2	18.9	65.8	20.7	74.0	9.51	0.54	0.0009	0.65
*trans*9,*trans*12-18:2	1.8	1.5	1.9	2.1	0.24	0.23	0.99	0.36
*cis*9,*trans*11-18:2	4.0	4.5	4.3	3.7	0.31	0.36	0.96	0.15
*cis*9,*cis*12,*cis*15-18:3	1.7	5.7	2.0	6.5	0.84	0.51	0.002	0.67
*cis*6,*cis*9,*cis*12-18:3	0.8[a]	0.5[b]	0.7[a]	0.2[c]	0.14	0.01	0.0002	0.06
20:0	1.4	1.9	1.6	1.7	0.23	0.86	0.10	0.71
20:1	0.0	0.3	0.0	0.2	0.21	0.75	0.24	0.75
*cis*11,*cis*14,*cis*17-20:3	0.3	0.2	0.2	0.2	0.22	0.83	0.99	0.71
*cis*5,*cis*8,*cis*11,*cis*14-20:4	0.9	0.8	0.9	1.1	0.31	0.18	0.54	0.14
SFA[1]	554.8	524.6	552.7	535.7	14.76	0.49	0.06	0.27
MUFA[2]	415.6	393.0	416.4	372.9	9.31	0.34	0.005	0.41
PUFA[3]	29.7	82.4	30.9	91.4	10.53	0.54	0.0009	0.67
SCFA[4]	16.9	30.5	25.6	36.6	3.52	0.07	0.01	0.79
MCFA[5]	538.0	494.7	533.7	518.3	26.74	0.46	0.11	0.27
LCFA[6]	445.1	474.8	440.7	445.1	28.71	0.37	0.26	0.60
Omega 3[7]	1.7	5.7	2.0	6.5	0.83	0.51	0.002	0.67
Omega 6[8]	22.7	69.0	24.5	77.7	9.92	0.54	0.001	0.64
HPI[9]	1.20	1.39	1.22	1.32	1.03	0.59	0.03	0.37

[1]MUFA = monounsaturated fatty acids; [2]PUFA = polyunsaturated fatty acids; [3]SFA = saturated fatty acids; [4]SCFA = short-chain fatty acids; [5]MCFA = medium-chain fatty acids; [6]LCFA = long-chain fatty acids; [7]*cis*9,12,15-18:3 + *cis*5,8,11,14,17-20:5 + 22:5. [8]*cis*9,12-18:2 + *cis*6,9,12-18:3 + *cis*11,14-20:2 + *cis*8,11,14-20:3 + *cis*5,8,11,14-20:4.
[9]Health-promolting index: (sum of % of unsaturated fatty acids)/[12:0 + (4 × 14:0) + 16:0]; (Chen et al., 2004).

Table 5

Blood parameters (mg/100mL) of Holstein cows supplemented with soybean oil (SBO) or soybean oil + citrus pulp (SBO+CPP) in the rumen (RUM) or in the abomasum (ABO).

	Treatments							
	SBO		SBO+CPP			*P*		
Item	RUM	ABO	RUM	ABO	SEM	Product	Site	P × S
Glucose	62.0	66.0	66.0	63.5	2.31	0.76	0.76	0.21
Triacylglycerol	10.8	14.5	12.3	12.3	1.92	0.84	0.35	0.35
HDL	79.8	85.3	79.3	86.3	3.23	0.83	0.002	0.53
LDL	61.8	83.8	68.0	84.0	7.90	0.43	0.003	0.47
VLDL	2.2	2.9	2.5	2.5	0.38	0.85	0.35	0.35
Total cholesterol	143.8	171.8	149.8	172.8	10.25	0.38	0.0005	0.53

The pH and ammonia N concentration in the rumen were similar among treatments (Table 6). There was an interaction between product and site of supplementation for VFA in ruminal fluid; the SLICE procedure showed that SBO+CPP increased (P=0.035) VFA concentration when administered in the rumen, while it had no effect (P=0.74) when infused in the abomasum.

A significant interaction between product and site was observed for acetate, propionate and butyrate molar proportions, and acetate to propionate ratio, as well. The SLICE procedure showed that ruminal administration of SBO+CPP reduced acetate (P=0.007) molar proportion and acetate to propionate ratio (P=0.004), and increased propionate (P=0.005) and butyrate (P=0.04) molar proportions. A significant interaction between treatment and sampling time for ruminal proportions of acetate (P=0.002), butyrate (P<0.0001) and valerate (P<0.0001). Considering the mean across all sampling times, there was no difference for isobutyrate and isovalerate molar proportions whereas valerate molar proportion tended to be lower when products were administered in the abomasum.

Table 6

Rumen fermentation characteristics of Holstein cows supplemented with soybean oil (SBO) or soybean oil + citrus pulp (SBO+CPP) in the rumen (RUM) or in the abomasum (ABO).

	Treatments							
	SBO		SBO+CPP			*P*		
Item	RUM	ABO	RUM	ABO	SEM	Product	Site	P × S
pH	6.29	6.38	6.33	6.34	0.05	0.98	0.67	0.69
Ammonia N (mg/100ml)	20.98	19.31	19.92	19.60	3.84	0.98	0.71	0.77
VFA (mmol/L)	135.99	164.67	156.58	163.15	3.13	0.02	0.001	0.01
Molar proportions (mmol/mol)								
Acetate*	66.87	65.55	65.67	65.78	0.22	0.07	0.03	0.02
Propionate	18.67	19.89	19.81	19.43	0.25	0.23	0.14	0.02
Butyrate*	9.58	10.19	10.02	10.43	0.12	0.05	0.006	0.05
Isobutyrate	1.18	0.78	0.87	0.92	0.07	0.19	0.12	0.13
Valerate*	1.46	1.61	1.56	1.61	0.04	0.33	0.06	0.32
Isovalerate	2.23	1.97	2.08	1.96	0.10	0.41	0.11	0.51
Acetate:propionate	3.58	3.30	3.32	3.39	0.06	0.17	0.09	0.02

*Interaction between treatment and sampling time ($P \leq 0.05$).

4. Discussion

The lower basal DM intake with SBO+CPP supplementation compared with SBO was likely a result of the additional energy provided by the citrus pulp, which may be confirmed by the lack of a product effect on total DM input. However, infusion in the abomasum compared to administration in the rumen decreased DM intake. Over the years, it has been recognized that abomasal infusion of oils has had negative effects on DM intake (Benson et al., 2001; Martin et al., 2008; Côrtes et al., 2011). According to these reports, such negative effects are related to the increased concentration of polyunsaturated FA in the small intestine, which was provided by the abomasal infusion of soybean oil in the present experiment. The postruminal delivery of polyunsaturated FA has been previously reported to increase plasma concentration of gut hormones, such as cholecystokinin and pancreatic polypeptide (Choi and Palmquist, 1996) and glucagon-like peptide-1 (Relling and Reynolds, 2007), which are related to postprandial

satiety signals and gut motility reduction (Litherland et al., 2005) and decreased DM intake as observed in the present experiment.

The trend for lower TTAD of CP when the products were administered in the rumen suggests that soybean oil was responsible for the depression in digestion of protein. Similar decreases have been reported when sheep were supplemented with flax (Ikwuegbu and Sutton, 1982) and corn (Jenkins and Fotouhi, 1990) oils. However, the response to free oil supplementation in the rumen seems to be variable and unclear so far, since no effects have been observed when cows were supplemented with soybean (Bateman and Jenkins, 1998) and flax (Kazama et al., 2010) oils. Other factors such as the basal diet and a reduction in protozoa number have been known also to influence TTAD of CP (Ikwuegbu and Sutton, 1982).

Supplementation with SBO+CPP compared to SBO led to higher TTAD of EE. Although citrus pulp has a low ether extract content (Table 1), the actual administration of oil was increased by 20 g/d. Lipids from citrus pulp contain mainly unsaturated FA (Table 1) which are known to be more digestible than saturated FA (Borsting et al., 1992). Therefore, the additional amount of digestible EE from citrus pulp may have contributed to the increase TTAD of EE.

The lack of a treatment effect on milk production and composition disagrees with the effects of abomasal infusion of high polyunsaturated FA oil on milk components usually reported. For example, Drackley et al. (2007) infused increasing amounts (0, 250, 500, 750, and 1,000 g/d) of high oleic sunflower oil in the abomasum and reported increased proportions of fat and total solids in milk. Bremmer et al. (1998) also reported an increase in milk fat proportions due to the infusion of 445 g/d of soybean oil in the abomasum of dairy cows. On the other hand, 400 g/d of flax oil administered three times daily in the rumen or infused over a 23 h period in the abomasum has had no effect on milk yield and composition (Kazama et al., 2010) as observed in the present experiment.

Although citrus pulp is rich in antioxidants (Manthey and Grohmann, 1996), its supplementation in the rumen and abomasum led to similar antioxidant properties of milk. Gladine et al. (2007) suggested that the hydrolysis of the glycosidic fraction of naringin, which like hesperidin is found in citrus, is required before absorption although that, in contrast with monogastrics, it is likely that the hydrolysis of the glycosidic fraction occurred in the rumen rather than in the large intestine. However, the presence

of naringin in the plasma of sheep was obtained following administration in the rumen of an acute dose of citrus extract in the experiment of Gladine et al. (2007), which suggests that a greater amount of citrus pulp may be required in the diet to positively affect antioxidant properties of milk.

It is also possible that the absorption of flavonoids occurs similarly in the rumen and the intestine as formonentin and daidzein from the isoflavone family are absorbed in the rumen (Lundh, 1990) and the small intestine (Walsh et al., 2009). Infusing citrus pulp in the rumen or directly in the small intestine would then lead to similar antioxidant properties of milk as observed in the present experiment.

Production of CD represents the ability of antioxidants to delay oxidation of polyunsaturated FA (Gladine et al., 2007). Our results showed that milk from cows infused with products in the abomasum was more susceptible to lipoperoxidation than milk from those administered with products in the rumen, which agrees with the higher polyunsaturated FA proportion in milk fat when cows were on the former treatment. As previously shown by Shiota et al. (1999) and Chen et al. (2004) milk fat richer in polyunsaturated FA is more prone to oxidation.

Changes in FA profile of milk fat were typical of those reported in studies where cows were infused in the abomasum *versus* the rumen with supplemental fats rich in polyunsaturated FA (Drackley et al., 2007; Kazama et al., 2010; Côrtes et al., 2011). The increased proportion of *cis*9-18:1 when cows were administered with products in the rumen was likely related to the higher ruminal supplementation of linoleic acid (18:2), which increases biohydrogenation of 18:2 into 18:0 (Bauman and Griinari, 2003). Thereafter, 18:0 can be desaturated to *cis*9-18:1 by the action of the mammary stearoyl-CoA (Δ9) desaturase (Kinsella, 1972). About 40% of the stearic acid taken up by the mammary gland is desaturated, thus contributing to more than 50% of the oleic acid that is secreted into milk fat (Enjalbert et al., 1998; Bretillon et al., 1999). Furthermore, biohydrogenation may be incomplete with the production of *trans* isomers of 18:1 that are transferred to milk (Bauman and Griinari, 2003). Although they were not detected in the present experiment, some unidentified *trans* isomers of 18:1 may have been among the total 18:1. About 5 to 15% of total 18:1 is of *trans* configuration in milk (Storry and Rook, 1965; Selner and Schultz, 1980). An increased supply of 18:1 to mammary cells decreases *de novo* synthesis of short- and medium-chain FA (Chilliard et al., 2003), which may explain the lower proportion of short-chain FA and

the trend for decreased 10:0 and 12:0. A mass-action effect on esterification also has been reported by (Drackley et al., 2007) as a possible explanation for reductions of short and medium-chain FA when mammary cells are highly supplied with 18:1. According to these authors, esterification of oleic acid at all 3 *sn*-positions of glycerol to synthetize milk triglycerides would compete with short-chain FA that are found almost exclusively at the sn-3 position, thus decreasing the short-chain FA proportion.

Cows infused with soybean oil in the abomasum had higher milk fat proportions of *cis*9,*cis*12-18:2, *cis*6,*cis*9-18:3 and omega 3 and omega 6 FA than those administered with oil in the rumen as expected from the rumen bypass of oil. Indeed, soybean oil is a great source of *cis*9,*cis*12-18:2 (54.27 g/100g; Table 1) with a significant concentration of *cis*6,*cis*9-18:3 (4.57 g/100g), which may contribute to improve milk FA profile. Moreover, as HPI is the inverse of the atherogenic index (Chen et al., 2004), results of the present experiment may suggest that enrichment of milk due to the rumen bypass of SBO and SBO+CPP could result in milk and dairy products with benefits to human health, such as the prevention of cardiovascular diseases (Noakes et al., 1996; Poppitt et al., 2002)

That plasma total cholesterol increased with abomasal infusion of soybean oil agrees with the results of Drackley et al. (1992) who infused long-chain FA in the abomasum and with those of Weiss and Wyatt (2003) who fed a protected source of polyunsaturated FA as roasted whole soybeans to cows. Higher plasma concentration of total cholesterol with abomasal infusion of soybean oil is typical of cows fed supplemental fat (Grummer and Carroll, 1991) due to the contribution of LDL and HDL concentrations (Storry et al., 1980), which were also increased by abomasal infusion. According to Rindsig and Schultz (1974), increased plasma cholesterol concentration is required to support the transport of larger amounts of circulating 18:2 FA and total lipids, which may be the case in the present experiment as abomasal infusion of soybean oil likely increased the amount of 18:2 reaching the small intestine due to rumen bypass.

Over the years, it has been reported that supplemental soybean oil affects rumen VFA production in dairy cows (Jenkins et al.,1996; Yang et al.,2009). Gagnon et al. (2009) also reported lower VFA concentration when high polyunsaturated FA oil was administered in the rumen *versus* the abomasum. These changes in rumen VFA production may be a result of an increased availability of free polyunsaturated FA in the rumen which can modulate the microbial population and thus affecting ruminal

fermentation (Yang et al., 2009). However, in the present study, ruminal administration of SBO+CPP led to higher VFA compared to ruminal SBO only. This suggests that citrus pulp supported total VFA synthesis and compensated the negative effect of unprotected soybean oil placed in the rumen. It has been well established that citrus pulp is degraded very rapidly and extensively in the rumen owing to its high concentration of pectin, about 223 g/kg (Bampidis and Robinson, 2006). Ruminal fermentation of pectin produces mainly galacturonic acid and then VFA unlike starch which leads to a high yielding of lactate. Thus, the additional amount of fermentable carbohydrates provided by citrus pulp could explain the higher total VFA concentration in rumen fluid.

The observed changes in molar proportions of VFA in rumen fluid may indicate that unsaturated FA from soybean oil could have interfered with ruminal fermentation. Data on literature yields variable results on the effect of dietary oil supplementation on ruminal VFA molar proportions have been variable. For example, Sullivan et al. (2005) reported that the acetate molar proportion and the acetate to propionate ratio increases linearly with increased dietary free FA from whole cottonseed. Similarly, Avila et al. (2000) reported that supplemental fat from yellow grease or tallow tended to increase ($P<0.10$) molar proportions of acetate, decreased that of butyrate ($P=0.02$), and increased ($P<0.01$) the acetate to propionate ratio. In contrast, Yang et al. (2009) reported increased propionate and decreased butyrate molar proportions, whereas the acetate proportion was not affected by supplementing oil from soybean or flax. However, when there is a change in normal ruminal fermentation patterns due to the toxic effect of unsaturated FA in the rumen (Yang et al., 2009), there is also a decrease in fiber digestibility and milk fat production (Jenkins, 1993). The lack of difference in TTAD and milk yield among treatments in the present study may suggest that the changes in ruminal fermentation characteristics were of little biological importance.

5. Conclusions

Supplementation of soybean oil and citrus pulp (0.2+1.0 kg/d) in the rumen or the abomasum had no influence on milk antioxidant properties. However, the abomasal infusion of soybean oil increased the proportion of polyunsaturated FA in milk fat and resulted in enhanced HPI.

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IV - Effect of antioxidants from flax meal on the production performance and oxidative status of dairy cows infused with flax oil in the abomasum

(Normas: Animal Feed Science and Technology)

Abstract

Flax oil (FO) is rich in omega-3 fatty acids (FA) and its rumen bypass contributes to increase polyunsaturated FA proportion in milk. Flax meal (FM) is a source of antioxidants that could reduce oxidative damage in cows given omega-3 FA. The aim of this research was to investigate the effects of dietary antioxidants from FM on performance and antioxidant status in dairy cows infused with FO in the abomasum. Eight ruminally fistulated lactating Holstein cows were assigned to a double 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments: 1) no FM in the diet and no abomasal infusion of FO; 2) diet containing 124 g FM/kg (dry matter (DM) basis) and no abomasal infusion of FO; 3) no FM in the diet + abomasal infusion of 250 g /d FO; 4) diet containing 124 g /kg FM (DM basis) + abomasal infusion of 250 g/d FO. Intake of DM and total DM input were increased for cows fed FM and reduced for cows infused with FO. Milk production and milk composition did not differ among treatments except for lactose concentration that was increased by FO. Milk fat from cows fed FM had higher monounsaturated and lower omega-6 FA proportions. Abomasal infusion of FO increased proportions of polyunsaturated, omega-6 and omega-3 FA. When cows where fed only FM, the omega-6 to omega-3 FA ratio in milk fat was higher compared to those infused with FO only or infused with FO and fed FM. Milk fat from cows infused with FO showed the highest health promoting index. Feeding FM did not change plasma and milk TBARS concentration, whereas FO increased these values. The lag phase duration of formation and oxidation rate of conjugated dienes in plasma were not affected by treatment, but FO reduced the lag phase duration. Neither FM nor FO affected total antioxidant capacity in plasma. Overall, the results suggest that FM supplementation to dairy cows receiving a source of polyunsaturated FA that bypasses the rumen does not provide any benefits for protecting cows and milk against lipoperoxidation.

Keywords: flaxseed, conjugated diene, health-promoting index, abomasal infusion, fat supplementation, omega-3

Abbreviations: DM, dry matter; DMI, dry matter intake; CD, conjugated diene; HPI, health-promoting index; FA, fatty acids; FM, flax meal; FO, flax oil; MDA, malondialdehyde; SCC, somatic cell counts; TBARS, thiobarbituric acid reactive substances; TEAC, trolox equivalents antioxidant capacity; VFA, volatile fatty acids.

1. Introduction

Consumption of omega-3 fatty acids (FA) has been associated with a decrease in risk factors of coronary diseases (Simopoulos, 2002a) and cancer (Simopoulos, 2002b). Scientific investigations have shown increased polyunsaturated FA, including omega-3 FA, in milk fat of dairy cows infused with flax oil into the abomasum (Kazama et al., 2010; Côrtes et al., 2011). Although a higher proportion of polyunsaturated FA in milk fat is desirable for consumers due to potential health benefits, this also increases oxidation of milk fat (Shiota et al., 1999; Chen et al., 2004). Indeed, milk enriched in omega-3 FA may be more susceptible to oxidation with the development of rancid odors and flavors (Timmons et al., 2001; Puppel et al., 2012), thus decreasing the nutritional quality and shelf life of milk and dairy products.

Additionally, feeding omega-3 diets to dairy cows may render tissues susceptible to free radical-mediated lipid peroxidation which is further aggravated in high producing cows that are naturally prone to oxidative stress (Bernabucci et al., 2005; Castillo et al., 2005). However, studies have demonstrated that inclusion of antioxidants in the diet diminishes the negative effects of oxidized fat by scavenging peroxides and reducing peroxidation of FA (Frankel, 2005) and enhances lactation performance and antioxidant status of cows (Vazquez-Anon et al., 2008). For example, plant polyphenols associated with vitamin E decrease lipoperoxidation damage generated by the oxidative stress generated by diets containing flax oil (FO) (Gobert et al., 2009). Similarly, supplementation with the antioxidants ethoxyquin and tertiary butylhydroquinone improves the oxidative status of dairy cows fed rumen-inert FA (Wang et al., 2010).

Flax products are rich in plant lignans, which have strong antioxidant properties (Landete, 2012). Plant lignans are metabolized in the two main mammalian lignans, enterodiol and enterolactone, under the action of the rumen microbiota (Gagnon et al.,

2009a). Feeding flax hulls to cows increases concentration of enterolactone in urine, plasma and milk (Côrtes et al., 2012) and there is a linear increase in milk enterolactone concentration with the proportion of flax meal (FM) in the diet (Petit et al., 2009). Previous results have shown that antioxidant activity of plant and mammalian lignans is greater than that of vitamin E (Prasad, 2000). Moreover, Rajesha et al. (2006) demonstrated in a rat model that flax lignans upregulate the expression of hepatic genes encoding for enzymes such as superoxide dismutase, catalase, and glutathione peroxidase which are involved in defence mechanisms against oxidative stress. Therefore, we hypothesized that dietary antioxidants from FM reduce plasma and milk oxidation damage in dairy cows supplemented with omega-3 FA. Thus, the aim of the experiment was to investigate the effects of dietary antioxidants from FM on performance and antioxidant status in dairy cows infused with flax oil in the abomasum.

2. Material and methods

2.1 Cows, diets, and experimental procedures

Eight multiparous lactating Holstein cows fitted with ruminal cannulas (10 cm, Bar Diamond Inc., Parma, ID, USA) were assigned to a replicated 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments and four 21 d periods balanced for residual effect. The experimental treatments were as follows: 1) no FM in the diet and no abomasal infusion of FO (CO-0); 2) diet containing 124 g/kg FM (dry matter (DM) basis) and no abomasal infusion of FO (FM-0); 3) no FM in the diet + abomasal infusion of 250 g/d FO (CO-250); 4) diet containing 124 g/kg FM(DM basis) + abomasal infusion of 250 g/d FO (FM-250). Flax oil contained, on total fatty acids basis, 50 g/kg of 16:0, 42 g/kg of 18:0, 174 g/kg of 18:1cis-9, 149 g/kg of 18:2cis-9,cis-12, 534 g/kg of 18:3cis-9,cis-12,cis-15 and 51 g/kg of others. Flax meal contained, on DM basis, 374 g/kg of crude protein, 263 g/kg of neutral-detergent fiber, 182 g/kg of acid-detergent fiber, 13.2 g/kg of fat lipids and 1.4 g/kg of secoisolariciresinol diglucoside (SDG).

Cows were weighed on the first and last day of each experiment period. Cows averaged 108 ± 39 days in milk, 32.6 ± 5.6 kg of milk/d, and 759 ± 44 kg of body weight at the beginning of the experiment. Cows were housed in individual stalls with free access to water and were fed twice a day (08:00 and 19:00 h) for *ad libitum* intake (100 g/kg of refusals as fed). The diets (Table 1) were formulated to meet requirements

for cows producing 30 kg/d of milk with 35 g/kg of fat (NRC, 2001). The oil was stored at +4ºC and was mixed before infusion. Milking times were 06:30 and 19:30 h, and milk yield was recorded at each milking. Cows were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993) and all experimental procedures were approved by the local Animal Care Committee.

Table 1

Composition of total mixed diets of Holstein cows fed no flax meal (CO) or 124 g/kg flax meal (FM) in the dry matter (DM).

Item	CO	FM
Ingredient (g/kg DM) [a]		
Grass, silage	301	300
Corn, silage	303	301
Corn, grain (cracked)	185	183
Beet pulp	66	28
Soybean, meal (480 g/kg CP, solvent)	88	-
Flax, meal	-	124
Top supplement[b]	40	46
Mineral[c]	13	14
Ca carbonate	4	4
Chemical Analysis		
DM (g/kg)	530	529
CP (g/kg DM)	189	196
Fat (g/kg DM)	33.8	34.3
aNDF (g/kg DM)	340	344
ADF (g/kg DM)	241	240
NE_L (MJ/kg DM)[e]	6.9	6.9

[a]Actual values obtained by precise weighing of dietary ingredients.

[b]Contained (per kg as is basis): Contained 200 g of canola meal, 250 g of corn gluten meal, 340 g of soybean meal, and 210 g of brewer's corn

[c]Contained (per kg, as-is basis): Ca 92.3 g, P 48.0 g, Mg 47.8 g, S 15.2 g, Na 53.9, K 13.7, Fe 2,014 mg, Zn 2,657 mg, Cu 1,068 mg, Mn 1,796 mg, I 23 mg, Co 57 mg, Se 19.6 mg, vitamin A 441,606 IU, vitamin D3 66,700 IU, and vitamin E 2,630 IU.

[d]One sample obtained from four pool samples prepared by compositing seven daily samples from days 15 to 21. DM = dry matter, CP = crude protein, aNDF = neutral detergent fiber inclusive of residual ash, ADF = acid detergent fiber inclusive of residual ash, NE_L= net energy for lactation.

[e] Net energy for lactation as calculated using published values of feed ingredients (NRC, 2001).

To perform abomasal infusions, an infusion line was inserted through the ruminal cannula and the sulcus omasi as described by Gressley et al. (2006). Plastisol discs (12 cm in diameter and 9 mm in height) were used to anchor the infusion line, and placement of infusion lines was monitored daily to ensure postruminal delivery. Variable-speed peristaltic pumps (Masterflex L/S; Cole-Parmer Canada Inc., Montreal, QC, Canada) were used to deliver FO in the abomasum at a rate of 10.86 g/h. Cows were infused from days 8 to 21 with 100% of the experimental dose of oil over a 23-h period (from 13:00 to 12:00 h).

Feed intake was recorded daily and samples of total mixed diets and FM were taken daily from days 15 to 21 and pooled by cow within period. All samples were frozen at -20°C for subsequent drying at 55° C and ground through a 1 mm screen in a Wiley mill for further analyses.

On day 19, ruminal contents were collected 0, 2, 4, and 6 h after the morning meal from different locations within the rumen (the anterior dorsal, anterior ventral, medium ventral, posterior dorsal, and posterior ventral locations). Ruminal pH was monitored immediately after sample collection with a portable pH meter (OAKTON; Eutech Instruments, Singapor). The ruminal contents were then strained through four layers of cheesecloth. Two aliquots of strained ruminal fluid from each sampling time were acidified to pH 2 with H_2SO_4 (0.5, v/v) and frozen at -20°C for later determination of volatile fatty acids (VFA) and ammonia concentrations. Aliquots of strained ruminal fluid were stored at -80^{o}C with Na azide (0.2 g/kg) for further analysis of thiobarbituric acid reactive substances (TBARS).

Milk samples were collected from two consecutive milkings on day 20 and pooled within cow and period relative to yield at each milking. Milk samples were stored at +4°C with a preservative (2-bromo-2-nitropropan-1,3 diol) and then sent to a commercial laboratory (Valacta Laboratories Inc., Ste-Anne-de-Bellevue, QC, Canada) for analyses of fat, protein, lactose, urea N and somatic cell counts (SCC). Milk FA profile was determined on samples pooled on milk yield basis and frozen without preservative at -20ºC. Another milk sample was kept at -80ºC with Na azide (0.2 g/kg) for TBARS analysis.

On day 20 of each period, blood samples were collected from the tail vein before (0 h) and 3 h after the morning meal using vacutainer tubes (Becton Dickinson and Cie, Rutherford, NJ, USA) containing K_3-EDTA (0.47 M) for determination of TBARS,

plasma lipids and FA profile (only at 3 h), lithium heparin (18.75 IU/ml) for determination of total antioxidant status (TAS) and Na citrate (105 mM) for measurement of the kinetic of *in vitro* copper induced conjugated diene (CD) production (only at 0 h). Plasma and erythrocytes samples were isolated from blood by centrifugation at 1800 × *g* for 12 min at +4ºC and stored at -80ºC until TBARS, TAS and CD analysis.

2.2 Chemical analysis

The DM of the diets was determined in a forced-air oven according to procedure 934.01 of AOAC (1990). Total N content was determined by combustion according to procedure 990.03 of AOAC (1990) and crude protein (CP) was estimated as N × 6.25. Concentrations of neutral detergent fiber (aNDF) inclusive of residual ash were measured according to procedure 5.1 of NFTA (1993) using a heat-stable α-amylase, but without sodium sulphite in the neutral detergent solution. Concentrations of acid detergent fiber (ADF) inclusive of residual ash were determined according to procedure 4.1 of NFTA (1993). Concentrations of ether extract in diets were conducted according to procedure Ba 3-38 of AOCS (1997). The analysis of SDG in flax meal was performed according to the procedures described by Muir and Westcott (2000).

Concentration of VFA in ruminal fluid was measured using a gas-liquid chromatograph (Hewlett Packard Model 6890, Agilent Technologies, Santa Clara, CA, USA) fitted with auto sampler, flame ionization detector, and split inlet (split ratio 1:50). Chromatography was performed using a Zebron ZB-FFAP (Phenomenex, Torrance, CA, USA) fused silica capillary column (30 m, 0.32 mm i.d., 1.0 µm film thickness). The initial column temperature was 150°C, and then it was increased to 210°C at 15°C/min, then held for 2 min. Injector and detector temperatures were 190 and 250^{o}C, respectively. The carrier gas was Helium at 1.5 ml/min. Hydrogen flow to the detector was 30 ml/min, airflow was 350 mL/min, and the flow of N_2 make-up gas was 25 ml/min. VFAs were identified by comparison of the retention times of the sample with that of the standards (Sigma-Aldrich , Oakville, ON, Canada).

Protein, fat, lactose, and urea N concentrations in milk samples were analyzed by infrared spectrophotometry (System 6000 MilkoScan, Foss Electric, Hillerød, Denmark) following method 972.16 of AOAC (1990). Milk SCC was obtained using an electronic counter (Foss FC, Foss Electric, Hillerød, Denmark).

Fat in milk was separated and methylated according to method 996.06 of AOAC (1990). Fatty acids in FO were methylated by *in situ* transesterification according to method 996.06 of AOAC (1990). Fatty acid methyl esters were quantified using a gas chromatograph (Agilent 6890 PLUS, Agilent Ltd., Mississauga, ON, Canada) equipped with an autosampler, a flame-ionization and a SP^{TM}-2560 fused-silica capillary column (100 m and 0.25 mm i.d., 0.20 µm film thickness). The temperature program was: level one, 140^{o}C held for 5 min; level two, 140^{o}C to 240^{o}C at 4.0^{o}C/min increments, then held for 15 min. Injector temperature was set at 250^{o}C, and the detector was set at 260^{o}C. Column head pressure was set at 40.24 psi. Gas flow rates were: helium (carrier) 1.1 ml/min, helium (make up) 25 ml/min, compressed air 350 ml/min, and hydrogen 35 ml/min. The carrier gas was helium at the flow rate of 1.1mL/min, linear velocity of 20 cm/s and split ratio of 100:1. Fatty acid peaks were identified using pure fatty acids methyl ester standards (Supelco 37 component FAME mix and Nu-Check prep GLC Reference Standard #569 and 463).

The health-promoting index (HPI) was calculated as the inverse of the atherogenic index (Ulbricht and Southgate, 1991) according to the equation described by (Chen et al., 2004) where concentration of total unsaturated FA is divided by the sum of 12:0, 16:0, and 4 × 14:0. The measurement of TBARS in milk, plasma and rumen fluid was performed at 532 nm using a kit (OXItek, ZeptoMetrix Corporation, Buffalo, NY, USA). Results were expressed in terms of malondialdehyde (MDA) equivalents (nmol/mL).

To determine FA profiles in plasma, samples (200 µL) were accurately weighed in culture tubes. Toluene (1 mL) containing 0.1 mg/mL of heneicosanoic acid (21:0), as internal standard, and 0.05 mg/mL of butylated hydroxytoluene was added to each tube followed by the addition of 2 mL of sodium methoxyde (0.5 M in methanol). Tubes were carefully vortexed at 700 rpm for 10 sec and incubated at 70°C for 60 min. After cooling down for 5 min, 3 mL of HCl 5% (v/v) were added and tubes were vortexed (700 rpm; 10 sec) and incubated at 50°C for 30 min. After cooling down for 5 min, 1 mL of hexane and 4 mL of a saturated NaHCO3 solution were added. Tubes were then centrifuged at 832 × *g* for 5 min. The upper organic phase of each tube was transferred to a new tube through a disposable Pasteur pipet packed with silica gel. A second extraction with 2 mL of hexane was performed and both organic phases were combined.

Solvent was evaporated and FA methyl esters were dissolved in 200 µL of hexane and transferred to a 500-µL GC vial insert and the vial capped prior to analysis.

Fatty acid methyl esters were quantified using a gas chromatograph (Agilent 7890A; Agilent Technologies, Santa Clara, CA, USA) equipped with a 100-m CP-Sil-88 capillary column (0.25 µm i.d., 0.20µm film thickness; Agilent Technologies Canada Inc., Mississauga, Canada), and a flame ionisation detector as described by Gervais et al (2009).

Determination of peroxidation susceptibility of plasma polyunsaturated FA was based on the production of CD according to the method of Schnitzer et al. (1995) with some modifications. Briefly, plasma samples were diluted 50 fold and degassed with 0.01M PBS (pH 7.4). Thereafter, the oxidation reaction was induced at 37°C by adding 10 mM of a freshly prepared aqueous copper chloride solution (Gobert et al., 2009). The CD absorbance was continuously recorded at 245 nm for 140 min at 37ºC using a Biochrom Libra (Biochrom, Holliston, MA, USA) double-beam spectrophotometer (Scislowski et al., 2005; Gobert et al., 2009). The kinetic of CD generation was divided into two phases (Esterbauer et al., 1989), from which two parameters were calculated: The first parameter was the length of lag phase (min), which is the resistance time of polyunsaturated FA against peroxidation and the second one was the maximum rate of peroxidation (A_{245}/min), which corresponds to the slope of the curve and denotes the speed of the propagation phase.

Plasma TAC was determined at 405 nm using a commercial kit (CS0790, Sigma-Aldrich , Oakville, ON, Canada) based on the formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxides, which oxidizes ABTS (2,2´-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) to produce a radical cation, $ABTS^{\bullet+}$. Trolox was used as standard for the calibration curve and results were reported as Trolox equivalents antioxidant capacity (mmol/l TEAC).

2.3 Statistical Analysis

All results were analyzed using the MIXED procedure of SAS (SAS 2000; SAS Institute) within a 2 × 2 factorial arrangement of treatments. Data were analyzed using a double 4 × 4 Williams Latin square design with the following general model:

$$Y_{ijkl} = \mu + S_i + C_{j(i)} + P_k + T_l + e_{ijkl}$$

Where: Y_{ijkl}, the dependent variable; μ, overall mean; S_i, fixed effect of square (i = 1 to 2); $C_{j(i)}$, random effect of cow within square (i = 1 to 4); P_k, fixed effect of period (k = 1 to 4); T_l, fixed effect of treatment, and e_{ijkl}, random residual error. Treatments were compared to provide factorial contrasts: 1) with *versus* without FM, 2) with *versus* without abomasal infusion of FO, and 3) the interaction between FM and abomasal infusion of FO. Data on ruminal fermentation characteristics (*i.e.*, pH, ammonia N and VFA), TBARS in plasma and ruminal fluid and TEAC were analyzed as repeated measurements and covariance structures were modeled separately for each variable. Values of fit statistics for Akaike´s information criterion (AIC) and Akaike´s information criterion corrected (AICC) were used to determine the most appropriate covariance structures. Results were reported as least squares means and standard error of the mean (SEM). When the interaction between FM and FO was significant, the SLICE option of the MIXED procedure was used to examine the differential response of infusion of oil in the abomasum to FM supplementation. Significant differences were set at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

3. Results

The actual amounts of oil infused in the abomasum averaged 248 g/d compared to the preplanned amount of 250 g/d. There was no interaction between FM and FO for total input and intake of DM (Table 2). Intake of DM, expressed in kg/day and as a percentage of body weight, was significantly increased for cows fed FM. Conversely, DM intake was reduced when cows were infused with FO in the abomasum. Total input of DM was increased by FM and decreased by FO infusion.

There was no interaction (P>0.10) between sampling time and treatment for ruminal fermentation characteristics (data not shown). Therefore, only mean values are presented (Table 2). Ruminal pH, ammonia N and total VFA were not affected by FM. However, molar proportion of acetate tended (P=0.09) to be reduced, whereas that of propionate was increased and of isovalerate was decreased with FM supplementation. The acetate to propionate ratio was lower in cows fed FM than in those fed the control diets. Abomasal infusion of FO had no effect on ruminal concentration of ammonia N but tended (P=0. 07) to increase ruminal pH and decrease total VFA concentration. Abomasal infusion of FO tended to increase molar proportion of acetate (P=0.10) and isobutyrate (P=0.09) and the acetate to propionate ratio (P=0.08).

Table 2

Total input of DM (intake of DM + flax oil infused into the abomasum), intake of DM and ruminal fermentation characteristics of Holstein cows fed total mixed diets containing no flax meal (CO) or 124 g/kg flax meal (FM) in the DM and infused or not with flax oil (250 g/d) in the abomasum.

	Treatments							
	CO		FM			*P*		
Item	0	250	0	250	SEM	Meal	Oil	M × O
Total input of DM (kg/d)	31.5	30.4	32.6	32.1	0.35	0.001	0.03	0.38
Intake of DM (kg/d)	31.5	30.2	32.6	31.8	0.35	0.001	0.01	0.38
Intake of DM (% BW)	3.9	3.8	4.1	4.0	0.05	0.01	0.02	0.93
pH	6.31	6.40	6.32	6.41	0.041	0.86	0.07	0.95
Ammonia N (mg/100ml)	12.8	12.5	13.5	13.2	0.82	0.42	0.68	0.98
VFA (mmol/L)	131.8	125.5	132.3	128.0	2.21	0.50	0.03	0.66
Molar proportions (mmol/mol)								
Acetate	63.7	64.4	63.2	63.7	0.35	0.09	0.10	0.82
Propionate	19.8	19.1	20.2	20.0	0.28	0.04	0.13	0.35
Butyrate	12.0	11.9	12.3	12.0	0.19	0.31	0.42	0.57
Isobutyrate	1.1	1.2	1.1	1.2	0.03	0.56	0.09	0.38
Valerate	1.6	1.5	1.6	1.5	0.04	0.82	0.24	0.38
Isovalerate	1.8	1.8	1.6	1.6	0.07	0.03	0.65	0.88
Acetate:propionate	3.3	3.4	3.2	3.2	0.05	0.04	0.08	0.44

DM = dry matter.

Table 3

Milk production and composition of Holstein cows fed total mixed diets containing no flax meal (CO) or 124 g/kg flax meal (FM) in the dry matter and infused or not with of flax oil (250 g/d) in the abomasum.

	Treatments							
	CO		FM			*P*		
Item	0	250	0	250	SEM	Meal	Oil	M × O
Milk production (kg/d)	31.2	31.4	32.9	32.0	1.72	0.19	0.73	0.55
Milk composition (g/kg)								
Protein	36.3	36.6	36.7	36.8	0.80	0.24	0.84	0.73
Fat	42.6	38.7	40.9	37.0	3.00	0.51	0.14	0.99
Lactose	44.1	45.2	44.1	45.4	0.50	0.66	0.001	0.71
Urea N (mg/dL)	14.3	14.1	14.1	14.1	0.73	0.70	0.88	0.97
Milk yield (kg/d)								
Protein	1.12	1.12	1.20	1.17	0.055	0.11	0.64	0.70
Fat	1.33	1.17	1.32	1.17	0.102	0.97	0.15	0.96
Lactose	1.38	1.43	1.45	1.46	0.089	0.23	0.56	0.61
SCS[a]	2.11	1.96	2.22	2.01	0.174	0.52	0.18	0.82

[a] Somatic cell score = log somatic cell count.

There was no interaction between FM and FO for production of milk and concentration and yield of milk components (protein, fat and lactose). Milk production was similar among treatments (Table 3). Concentration of milk components was not affected by treatments, except for lactose which was increased by abomasal infusion of FO. Urea N concentration and log somatic cell scores were not affected by treatments.

There was no significant interaction between FM and FO for milk FA profile (Table 4). Proportions of 6:0, 8:0, 10:0, 12:0, 17:1, 18:0, *cis*11-18:1, *trans*9-18:1, 20:0, *cis*11,*cis*14,*cis*17-20:3, 24:0 and *cis*15-24:1 in milk fat were similar among treatments. Dietary FM increased milk fat proportion of *cis*9-18:1, reduced that of *cis*9,*cis*12-18:2 and tended to decrease proportion of 16:0.

Proportions of monounsaturated and omega-6 FA were higher in milk fat of cows fed FM. Milk fat from cows infused with FO in the abomasum had lower proportions of 11:0, 14:0, *cis*9-14:1, 15:0, 16:0, *cis*9-16:1, 17:0, *trans*11-18:1, *cis*6,*cis*9,*cis*12-18:3, 19:0, *cis*8,*cis*11,*cis*14-20:3 and higher proportions of *cis*9,*cis*12-18:2, *cis*9,*cis*12,*cis*15-18:3, *cis*5,*cis*8,*cis*11,*cis*14,*cis*17-20:5, 22:0, *cis*13-22:1 and *cis*13,*cis*16-22:2. Infusion of FO increased proportions of polyunsaturated, long-chain, omega-3, and omega-6 FA and lowered those of saturated, monounsaturated and medium-chain FA in milk fat. There was a significant interaction between FM and FO for the omega-6 to omega-3 FA ratio in milk fat; FM supplementation reduced ($P<0.0001$) omega-6 to omega-3 FA ratio in the absence of FO abomasal infusion whereas it had no effect ($P=0.99$) when FO was infused in the abomasum. Milk fat from cows infused with FO in the abomasum had the highest milk HPI.

Neither FM dietary FM nor abomasal infusion of affected concentrations of TBARS in ruminal fluid 0 and 2 h after feeding but it was reduced by FM supplementation 4 and 6 h after feeding (Table 5). Feeding FM had no effect on plasma and milk TBARS concentration, whereas abomasal infusion of FO increased plasma TBARS concentration before feeding and 3 h after feeding. Milk TBARS concentration was increased by abomasal infusion of FO.

Table 4

Milk fatty acid concentration (g/kg of total fatty acids) in milk fat of Holstein cows fed total mixed diets containing no flax meal (CO) or 124 g/kg flax meal (FM) in the dry matter and infused or not with of flax oil (250 g/d) in the abomasum.

Item	Treatments				SEM	*P*		
	CO		FM					
	0	250	0	250		Meal	Oil	M × O
6:0	3.0	2.8	3.2	3.0	0.20	0.18	0.15	0.72
8:0	7.3	7.4	7.4	7.4	0.30	0.74	0.84	0.83
11:0	3.3	2.8	3.3	3.0	0.10	0.35	0.0003	0.36
11:0	3.3	2.8	3.3	3.0	0.10	0.35	0.0003	0.36
12:0	41.8	41.1	42.4	42.6	1.6	0.25	0.80	0.62
14:0	134.8	118.2	133.8	120.9	0.28	0.64	<0.0001	0.31
*cis*9-14:1	10.8	6.6	9.0	7.7	0.89	0.67	0.0009	0.05
15:0	13.9	10.5	13.9	11.0	0.60	0.53	<0.0001	0.46
16:0	365.9	305.3	357.2	296.6	7.11	0.07	<0.0001	0.99
*cis*9-16:1	12.3	9.5	12.0	9.2	1.43	0.78	0.02	0.99
17:0	5.0	4.7	5.4	4.0	0.42	0.66	0.02	0.14
17:1	0.49	0.16	0.00	0.00	0.131	0.08	0.37	0.37
18:0	105.1	110.6	111.8	111.5	3.9	0.14	0.31	0.25
*cis*9-18:1	198.4	193.5	204.9	201.2	4.74	0.01	0.12	0.82
*cis*11-18:1	4.9	4.4	4.5	4.3	0.34	0.33	0.20	0.73
*trans*9-18:1	12.4	10.3	11.6	10.8	1.12	0.88	0.17	0.53
*trans*11-18:1	9.5	9.1	9.9	8.9	0.59	0.75	0.04	0.34
*cis*9,*cis*12-18:2	22.9	42.1	20.4	40.0	0.13	0.0002	<0.0001	0.77
*trans*9,*trans*12-18:2	2.5	2.0	2.7	2.6	0.28	0.12	0.141	0.39
*cis*9,*cis*12,*cis*15-18:3	6.5	78.3	7.8	74.0	0.24	0.49	<0.0001	0.19
*cis*6,*cis*9,*cis*12-18:3	4.7	3.7	4.6	3.7	0.19	0.84	<0.0001	0.81
19:0	1.2	0.8	1.1	0.8	0.2	0.48	0.04	0.76
*cis*11,*cis*14,*cis*17-20:3	0.1	0.0	0.01	0.07	0.05	0.23	0.89	0.23
*cis*8,*cis*11,*cis*14-20:3	1.3	0.5	1.2	0.7	0.09	0.54	<0.0001	0.24
*cis*5,*cis*8,*cis*11, *cis*14, *cis*17-20:5	0.2	0.8	0.0	0.9	0.11	0.65	<0.0001	0.50
22 :0	0.2	2.1	0.08	1.9	0.22	0.27	<0.0001	0.65
*cis*13-22 :1	0.3	0.5	0.4	0.5	0.12	0.14	0.01	0.40
*cis*13,*cis*16-22:2	0.3	0.9	0.0	1.0	0.13	0.35	<0.0001	0.19
Others[a]	2.0	1.8	2.0	1.8	0.21	0.87	0.38	0.88
SFA[b]	712.4	637.5	710.9	634.7	6.71	0.61	<0.0001	0.88
MUFA[c]	248.9	234.0	252.0	242.5	4.5	0.52	0.0003	0.33
PUFA[d]	38.7	128.5	37.1	122.8	3.34	0.15	<0.0001	0.41
SCFA[e]	10.3	10.2	10.6	10.4	0.43	0.39	0.54	0.77
MCFA[f]	611.7	523.5	600.7	521.2	9.13	0.29	<0.0001	0.46
LCFA[g]	378.0	466.3	388.7	468.4	9.74	0.32	<0.0001	0.48
Omega-3[h]	6.8	79.2	7.8	75.0	2.41	0.45	<0.0001	0.23
Omega-6[i]	29.2	47.2	26.3	45.3	1.21	<.0001	<0.0001	0.31
Omega-6/Omega-3	4.3	0.6	3.4	0.6	1.22	0.0005	<0.0001	0.0005
HPI[j]	0.30	0.45	0.31	0.45	0.013	0.93	<0.0001	0.73

[a]Others = 20:0 + 24:0 + *cis*15-24:1; [b]MUFA = monounsaturated fatty acids; [c]PUFA = polyunsaturated fatty acids; [d]SFA = saturated fatty acids; [e]SCFA = short-chain fatty acids; [f]MCFA = medium-chain fatty acids; [g]LCFA = long-chain fatty acids.

[h]*cis*9,12,15-18:3 + *cis*5,8,11,14,17-20:5 + 22:5.

[i]*cis*9,12-18:2 + *cis*6,9,12-18:3 + *cis*11,14-20:2 + *cis*8,11,14-20:3 + *cis*5,8,11,14-20:4.

[j]Health-promolting index: (sum of unsaturated fatty acids)/[12:0 + (4 × 14:0) + 16:0].

Table 5

Concentration of TBARS (nmol MDA equivalent/ml)[1] in biological fluids of Holstein cows fed total mixed diets containing no flax meal (CO) or 124 g/kg flax meal (FM) in the dry matter and infused or not with of flax oil (250 g/d) to the abomasum.

	Treatments							
	CO		FM			*P*		
Item	0	250	0	250	SEM	Meal	Oil	M × O
Ruminal fluid								
0 h	22.2	23.7	20.1	21.7	1.21	0.13	0.28	0.87
2 h	18.7	21.0	19.0	19.4	1.31	0.61	0.29	0.49
4 h	23.0	20.5	20.5	17.8	1.26	0.008	0.17	0.92
6 h	20.5	22.0	19.6	17.7	1.24	0.01	0.81	0.13
Plasma								
0 h	5.71	6.42	5.69	6.52	0.322	0.89	0.03	0.85
3 h	4.62	5.05	5.69	6.52	0.212	0.85	0.01	0.94
Milk	32.3	35.0	32.2	35.6	1.51	0.87	0.05	0.82

[1]MDA = malondialdehyde.

Dietary FM supplementation did not affect plasma concentration of saturated and polyunsaturated FA, but increased monounsaturated FA and reduced omega-6 FA concentrations (Table 6). Abomasal infusion of FO reduced saturated, monounsaturated and omega-6 FA whereas it increased polyunsaturated FA and polyunsaturated to saturated FA ratio. There was a significant interaction between FM and FO for omega-3 FA; FM supplementation increased (P=0.006) omega-3 FA in the absence of FO abomasal infusion whereas it had no effect (P=0.54) when FO was infused in the abomasum. The peroxidizability index, the duration of the lag phase of CD formation and oxidation rate of CD in plasma were not affected by FM supplementation (Table 6) but infusion of FO in the abomasum reduced the lag phase duration. Neither dietary FM nor abomasal infusion of FO affected plasma antioxidant capacity.

Table 6

Fatty acid composition, lipid susceptibility to peroxidation and antioxidant capacity in plasma of Holstein cows fed total mixed diets containing no flax meal (CO) or 124 g/kg flax meal (FM) in the dry matter and infused or not with of flax oil (250 g/d) into the abomasum.

	Treatments							
	CO		FM			*P*		
Item	0	250	0	250	SEM	Meal	Oil	M × O
Fatty acids (g/100g of total methyl esters)								
SFA	38.4	33.8	38.9	34.6	0.91	0.29	<.0001	0.82
MUFA	8.3	6.4	9.2	6.7	0.29	0.04	<.0001	0.34
PUFA	53.3	59.8	51.9	58.7	1.08	0.12	<.0001	0.89
n-6	46.0	40.3	43.4	38.9	1.06	0.01	<.0001	0.42
n-3	7.3	19.5	8.5	19.8	0.32	0.02	<.0001	0.10
PUFA/SFA	1.4	1.9	1.3	1.7	0.06	0.14	<.0001	0.82
Peroxidizability index	71.3	86.2	71.4	85.1	1.4	0.61	<.0001	0.49
Conjugated dienes								
Lag phase (min)	15.9	13.6	15.2	13.9	0.49	0.69	0.001	0.34
Oxidation rate (A_{245}/min)	7.4	7.6	6.8	7.4	0.30	0.23	0.18	0.55
Antioxidant capacity (mmol/L TEAC)[1]								
0 h	0.91	0.93	0.93	1.04	0.057	0.31	0.29	0.43
3 h	1.09	1.06	1.04	1.00	0.070	0.36	0.58	0.99

[1]Trolox equivalent antioxidant capacity.

4. Discussion

That FM supplementation increased DM intake and total input of DM agrees with the results of Gagnon et al. (2009b) for cows fed 200 g/kg FM (DM basis). It suggests that flax meal has a positive effect on intake likely as a result of good nutrient composition and good acceptability. Conversely, abomasal infusion of FO lowered DM intake and total input of DM, which was likely a result of the increased concentration of polyunsaturated FA in the small intestine provided by the rumen bypass of oil. Indeed, postruminal delivery of polyunsaturated FA increases plasma concentration of gut hormones such as cholecystokinin, pancreatic polypeptide (Choi and Palmquist, 1996) and glucagon-like peptide-1 (Relling and Reynolds, 2007) that are related to postprandial satiety signals and gut motility reduction (Litherland et al., 2005).

Feeding FM tended to decrease molar proportions of acetate and increased propionate in the rumen, which accounted for the reduction in the acetate to propionate ratio. Results of the present study were consistent with those previously found in dairy

cows fed flax products. Silva-Kazama et al. (2011) reported similar effects for cows fed flax hulls and Ueda et al. (2003) reported higher propionate proportion when cows were supplemented with 30 g/kg FO. These results are consistent with those reported by Gonthier et al. (2004), where lower acetate to propionate ratio was observed in cows fed flax seed-based diets. Changes in ruminal fermentation characteristics may be partly due to the residual content of polyunsaturated FA in flax meal after flax processing for oil extraction, and mainly to that of linolenic acid. Indeed, this fatty acid has been shown to reduce population of *Fibrobacter succinogenes and Ruminococcus flavefaciens, Ruminococcus albus and Butyrivibrio fibrisolvens* in the rumen (Yang et al., 2009) which are responsible for cellulose and hemicellulose fermentation (Russell et al., 2009), which may directly affect acetate synthesis (Russell and Rychlik, 2001).

Cows infused with FO in the abomasum had lower total VFA concentration, which likely accounted for the higher ruminal pH. Abomasal infusion of polyunsaturated FA does not change ruminal fermentation due to the rumen bypass of the fat source (Gagliostro and Chilliard, 1991; Christensen et al., 1994; Bremmer et al., 1998). However, as abomasal infusion of FO lowered DM intake, changes in ruminal fermentation characteristics were likely associated with the negative effect of FO on DM intake. Although DM intake decreased with FO infusion, milk production of cows was not affected by treatments, which supports the concept that milk production drives DMI rather than the other way around (NRC, 2001; Collier et al., 2006).

Although milk lactose concentration is barely influenced by diet, the increase in lactose concentration obtained for cows infused with FO in the abomasum agrees with some experiments where dairy cows were fed flax products. For example, Petit and Côrtes (2010) reported higher lactose concentration in milk from cows supplemented with whole or ground flax seed at 72 g/kg DM compared to those fed no flax seed. Another study showed that milk lactose concentration was increased when cows were fed 104-108 g/kg DM whole flax seed compared to those fed 38-40 g/kg DM calcium salts of palm oil (Petit, 2002). Linolenic acid, which represents more than 534 g/kg of total FA in flax seed, could have interfered with the metabolism of glucose, thus increasing gluconeogenesis and lactose concentration in milk. Indeed, the highest rate of gluconeogenesis among long chain FA has been reported for linolenic acid (Mashek and Grummer, 2003).

Even though FM contains little fat (13.2 g/kg DM), feeding 124 g/kg DM was enough to increase milk fat proportion of *cis*9-18:1 and decrease that of *cis*9,*cis*12-18:2 with a trend (P=0.07) for lower proportion of 16:0. As a result, proportions of monounsaturated and omega-6 FA were higher and lower, respectively, in milk fat of cows fed FM. As flax products are rich in linolenic acid compared to other vegetable feed ingredients (He and Armentano, 2011; Kazama et al., 2010; Mustafa et al., 2002) linolenic acid supplied by FM may have increased the proportion of *cis*9-18:1 in milk fat. Indeed, ruminal biohydrogenation of linolenic acid produces 18:0 (Lock and Bauman, 2004), which can be transformed into *cis*9-18:1 in the mammary gland by stearoyl-CoA (Δ9) desaturase enzyme (Kinsella, 1972). A possible negative correlation between proportions of *cis*9-18:1 and 16:0 could explain the observed tendency to lower 16:0 proportion in milk fat as it have been shown in goat milk (Chilliard et al., 2003). The lower proportion of *cis*9,*cis*12-18:2 in milk fat of cows fed FM was likely due to the replacement of a feed ingredient richer in linoleic acid (*i.e.*, soybean meal) for FM.

Changes in milk FA profile are consistent with those found previously in dairy cows infused with FO in the abomasum (Kazama et al., 2010; Côrtes et al., 2011) or fed whole flax seed (Petit and Côrtes, 2010). As concentration of FA in milk fat depends on the source and amount of fat reaching duodenum (Chilliard et al., 2000), rumen bypass of FO will directly affect milk FA profile. The increase in *cis*9,*cis*12-18:2 and *cis*9,*cis*12,*cis*15-18:3 proportions in milk fat coupled with a sharp reduction in 16:0 may account for the lower proportion of medium-chain FA in cows infused with FO. Increases in long-chain FA at the expense of medium-chain FA such as 16:0 have been previously reported for dairy cows supplemented with FO (Petit and Côrtes, 2010; Côrtes et al., 2011) and may be explained by a mass-action effect on esterification processes (Drackley et al., 2007). As long-chain FA (*e.g.*, 18:2) may occupy the same *sn*-positions as 16:0 in triacylglycerol molecules (Christie and Clapperton, 1982), a potential competition for the *sn*-positions promoted by the increased long-chain FA supply to mammary cells could reduce 16:0 proportion in milk fat.

That FM supplementation reduced omega-6 to omega-3 FA ratio just in the absence of FO abomasal infusion may be explained at least partially by a possible higher supply of linolenic acid in the small intestine provided by dietary supplementation of FM compared to those not fed FM, which agrees with the results of Côrtes et al. (2011) which fed flax hulls for dairy cows infused with FO in the

abomasun. Moreover, due soybean meal replacing by FM, the supply of linoleic acid was likely lower for cows fed FM than for those fed CO, which may have reduced the proportion of *cis*9,*cis*12-18:2 in milk fat and may accounted for the lower omega-6 to omega-3 FA ratio.

Polyunsaturated FA (especially n-3 FA) have potential antiatherogenic and anticarnogenic roles while some saturated FA (12:0; 14:0 and 16:0) have negative effects on human health (Kinsella et al., 1990; Molkentin, 1999; Parodi, 1999; Simopoulos, 2002b). In the present study, abomasal infusion of FO sharply increased and decreased, respectively, proportions of polyunsaturated saturated FA which improved the HPI of milk fat from cows infused FO in the abomasum.

Lower TBARS concentration 4 and 6 h after feeding in rumen fluid of cows fed FM suggests that antioxidants from FM could prevent free radical activity in rumen fluid from 4 h postfeeding. Conversely, flax antioxidants were not efficient to inhibit lipoperoxidation in plasma and milk of cows infused with FO as suggested by the lack of treatment effects on TBARS, TAC and CD production. Although FM (Petit et al., 2009) and flax products (Gagnon et al., 2009a; Côrtes et al., 2012) have been shown to increase antioxidant molecules such as enterolactone in milk and plasma of dairy cows , results of the present experiment suggest that higher amounts of FM may be required to inhibit lipid oxidation in blood and milk. This would agree with the results of Matumoto-Pintro et al. (2011) who reported that although enterolactone concentration was increased in milk when cows were fed 150g/kg FM, milk oxidation was not affected.

That higher TBARS concentrations in plasma and milk when cows were infused with FO in the abomasum suggest that polyunsaturated FA from FO increased lipoperoxidation susceptibility. This is supported by the higher plasma concentration of polyunsaturated FA coupled with the higher plasma peroxidizability index showed by cows after abomasal infusion. Additionally, the reduced lag phase for CD production in plasma due to FO abomasal infusion may help to explain this higher lipoperoxidation susceptibility. A reduced lag phase denotes less resistance to initiation of lipoperoxidation, and our results are consistent with those found previously in dairy cows fed extruded flax seeds (Gobert et al., 2009) and in sheep infused with FO in the duodenum (Gladine et al., 2007) where increased susceptibility to plasma lipoperoxidation was observed.

5. Conclusions

Similar to previous studies, abomasal infusion of FO increased milk fat proportion of polyunsaturated FA and susceptibility to lipoperoxidation as shown by Côrtes et al. (2011) and Gladine et al. (2007). Plasma susceptibility to lipoperoxidation also increased with abomasal infusion of FO. Feeding 124 g/kg DM FM to dairy cows has no effect on milk yield and composition and does not improve the oxidative status. Feeding FM to dairy cows receiving a source of polyunsaturated FA that bypasses the rumen has no benefits when the main objective is to protect cows and milk from lipoperoxidation. However, further studies are required with higher amounts of FM in the diet (124 g/kg DM) to determine if beneficial effects on lipoperoxidation in milk and plasma could be observed at such proportions to improve the oxidative status of cows.

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V - Effect of antioxidants from flax meal on gene expression and activity of antioxidant enzymes in mammary tissue and blood of dairy cows infused with flax oil in the abomasum

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Abbreviations: ACACA, acetyl-coenzyme A carboxylase alpha; ACTB, actin beta; CAT, catalase; CD36, cluster of differentiation 36; CO, control diet; DM, dry matter; FASN, fatty acid synthase; FM, flax meal; FO, flax oil; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX, glutathione peroxidase; LPL, lipoprotein lipase; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PPIA, peptidylprolyl isomerase A; PPARG1, peroxisome proliferator-activated recepetor-γ1; PPARG2, peroxisome proliferator-activated recepetor-γ2; PPPARα, peroxisome proliferator-activated receptor alpha; PUFA, polyunsaturated fatty acids; RG, reference genes; ROS, reactive oxygen species; SCD, stearoyl-coA desaturase; SOD, superoxide dismutase; SREBP1, sterol regulatory element binding transcription factor 1; UBQ, ubiquitine.

Abstract

The effects of antioxidants from flax meal (FM) and abomasal infusion of flax oil (FO) on the activity of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase (GPX)) in blood and mammary tissue and the mRNA abundance of antioxidant and lipogenic-related genes in mammary tissue of dairy cows were determined. Eight ruminally fistulated lactating Holstein cows were assigned to a double 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments: 1) no FM in the diet (control); 2) diet containing 12.4% FM; 3) no FM in the diet and 250 g FO/d infused in the abomasum; 4) diet containing 12.4% FM and 250 g FO/d infused in the abomasum. Catalase activity in erythrocytes tended to increase when cows were fed FM. Abomasal infusion of FO had no effect on activity and gene expression of antioxidant enzymes and gene expression of lipogenic-genes in mammary tissue, except for an increase in GPX1 expression in the absence of FM. The results suggest that feeding 12.4% FM and infusing 250 g of FO/d in the abomasum of dairy cows does not

induce significant changes in the activity of antioxidant enzymes in blood and mammary tissue, and expression of antioxidant and lipogenic-genes in mammary tissue. However, more studies are required to determine any beneficial effects of natural antioxidants such as FM on the oxidative status of cows supplemented with polyunsaturated fatty acids, which could lead to feeding strategies to prevent diseases affecting the health status of dairy cattle.

Key words: L**ignan: Flaxseed: mRNA abundance: Omega-3**

Introduction

Supplying rumen bypass vegetable oils to dairy cows changes milk composition and enhances the concentration of polyunsaturated fatty acids (PUFA) in milk fat[1; 2; 3]. However, changes in the amount or composition of dietary fat impacts cellular processes, which affect many physiological systems. Diets with a high PUFA content may be easily oxidized[4] and their consumption may render tissues susceptible to lipid peroxidation as previously reported for the plasma of dairy cows[5] as a result of increased production of free radicals such as reactive oxygen species (ROS)[6].

Studies in which rats were used as a model have shown that PUFA, especially those from the omega-3 FA family, play a role in the down-regulation of lipogenic-related genes and the up-regulation of an extensive network of genes involved in fatty acid oxidation[7; 8]. Although oxidative metabolism is essential for cell survival, a side effect of this dependence is the production of free radicals and other ROS that cause oxidative changes. Such conditions may be further aggravated by a putative down-regulation of genes involved in free radical scavenging provided by PUFA as reported for rats[9]. This is corroborated by results from a recent investigation[10] with dairy cows where catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD) genes were down-regulated by infusion of flax oil (FO) in the abomasum. Conversely, up-regulation of the expression of genes encoding for enzymes involved in free radical scavenging, which is achieved by supplementing antioxidants[11; 12], is thought to be a way to preserve the oxidative status of dairy cows[6]. Flax lignans, such as secoisolariciresinol diglucoside, are strong antioxidants[13], have been shown to up-regulate the expression of genes encoding for SOD, CAT and GPX in the liver of rats[14]. Moreover, Côrtes *et al.*[10] have reported an increase in mRNA abundance of

CAT, GPX1 and SOD1 genes in mammary tissue of dairy cows when diets were supplemented with flax lignans.

Flax meal (FM) is a protein supplement with higher concentration of lignans than flax seed owing to oil extraction. Under the action of the rumen microbiota, flax lignans are metabolized in two main mammalian lignans, enterodiol and enterolactone[15], which have stronger antioxidant properties than their precursors. Moreover, increasing proportions of FM in the diet of dairy cows have been reported[16] to have a linear positive effect on milk enterolactone concentration. We hypothesized that dietary antioxidants from FM could up-regulate the expression of genes encoding for enzymes involved in free radical scavenging in mammary tissue of dairy cows supplemented with FO, a rich source of omega-3 fatty acids. Infusion of FO in the abomasum was used as a tool to increase lipoperoxidation of tissues as previously carried out[17]. Therefore, the present study was performed to evaluate the effects of dietary antioxidants from FM and abomasal infusion of high omega-3 FA oil on: (1) the activity of antioxidant enzymes (SOD, CAT and GPX) in blood and mammary tissue; (2) the mRNA abundance of antioxidant genes in mammary tissue; and (3) the mRNA abundance of lipogenic-related genes in mammary tissue of dairy cows.

Materials and methods

Animal, diets and experimental treatments

Eight multiparous lactating Holstein cows fitted with ruminal cannulas (10 cm, Bar Diamond Inc.) were assigned to a replicated 4 × 4 Latin square with a 2 × 2 factorial arrangement of treatments and four 21 d periods balanced for residual effect. The experimental treatments were: 1) control diet with no FM (CO); 2) diet containing 12.4% FM (FM); 3) CO and infusion of 250 g FO/d in the abomasum; 4) FM and infusion of 250 g FO/d in the abomasum. The CO and FM diets were designed to have similar composition (Table 1). Flax oil (Brenntag Canada, Inc.) contained, expressed as a percentage of total fatty acids, 5.0% of C16:0, 4.2% of C18:0, 17.4% of C18:1*cis*-9, 14.9% of C18:2*cis*-9,*cis*-12, 53.4% of C18:3*cis*-9,*cis*-12,*cis*-15 and 5.1% of others. Flax meal contained, expressed as a percentage of DM, 37.4% of crude protein, 26.3% of neutral-detergent fiber, 18.2% of acid-detergent fiber, 1.3% of fat and 0.14% of secoisolariciresinol diglucoside (SDG).

At the beginning of the experiment, cows averaged 108 (SE 39) d in milk, 32.6 (SE 5.6) kg of milk/d, and 759 (SE 35) kg of body weight. Cows were housed in individual stalls with free access to water. Diets were offered twice a day (08.00 and 19.00 hours) for *ad libitum* intake (10% of refusals on as fed basis) and milking times were 06.30 and 19.30 hours. Feed intake and milk yield were recorded daily throughout the experiment and data from day 15 to day 21 of each period were averaged over the 7 days and subjected to analysis of variance.

Table 1. Ingredient and nutrient composition of experimental diets for lactating cows

Item	CO	FM
Ingredient (g/kg DM) [*]		
Grass, silage	301	300
Corn, silage	303	301
Corn, grain (cracked)	185	183
Beet pulp	66	28
Soybean, meal (480 g/kg CP, solvent)	88	-
Flax meal	-	124
Top supplement[†]	40	46
Mineral[‡]	13	14
Ca carbonate	4	4
Chemical composition[§]		
DM (g/kg)	530	529
Crude protein (g/kg DM)	175	181
Fat (g/kg DM)	31.3	31.7
Neutral-detergent fibre (g/kg DM)	315	319
Acid-detergent fibre (g/kg DM)	224	222
NE_L (MJ/kg DM)[¶]	6.9	6.9

CO, cows fed no flax meal; FM, cows fed flax meal.

[*] Actual values obtained by precise weighing of dietary ingredients.

[†] Contained (per kg as is basis): Contained 200 g of canola meal, 250 g of corn gluten meal, 340 g of soybean meal, and 210 g of brewer's corn

[‡] Contained (per kg, as-is basis): Ca 92.3 g, P 48.0 g, Mg 47.8 g, S 15.2 g, Na 53.9, K 13.7, Fe 2,014 mg, Zn 2,657 mg, Cu 1,068 mg, Mn 1,796 mg, I 23 mg, Co 57 mg, Se 19.6 mg, vitamin A 441,606 IU, vitamin D3 66,700 IU, and vitamin E 2,630 IU.

[§] One sample obtained from four pool samples prepared by compositing seven daily samples from days 15 to 21. DM = dry matter, CP = crude protein, aNDF = neutral detergent fiber inclusive of residual ash, ADF = acid detergent fiber inclusive of residual ash, NE_L= net energy for lactation.

[¶] Net energy for lactation as calculated using published values of feed ingredients (NRC, 2001).

The diets were formulated to meet requirements for cows producing 30 kg of milk/d with 35 g of fat/kg[18]. The oil was stored at 4ºC and mixed before being infused. Cows were cared for in accordance with the guidelines of the Canadian Council on Animal Care[19] and all experimental procedures were approved by the local Animal Care Committee.

To perform abomasal infusions, an infusion line was inserted through the ruminal cannula and the sulcus omasi as described by Gressley et al. (2006). Plastisol discs (12 cm in diameter and 9 mm in height) were used to anchor the infusion line, and placement of infusion lines was monitored daily to ensure postruminal delivery. Variable-speed peristaltic pumps (Masterflex L/S; Cole-Parmer Canada Inc.) were used to deliver flax oil in the abomasum at a rate of 10.86 g/h. Cows were infused from day 8 to day 21 with 100% of the experimental dose of oil over a 23 h period (from 13.00 to 12.00 hours).

Sampling

Samples of total mixed diets and FM were taken daily from day 15 to day 21 and pooled by cow within period. All samples were frozen at -20 °C for subsequent drying at 55°C and analyzed following the procedures used by Côrtes *et al.*[21]. The analysis of SDG in flax meal was performed according to the procedures described by Muir and Westcott[22].

Blood samples from each cow were collected from the tail vein on day 20 before (0 h) and 3 h after the morning meal using 10 ml vacutainer tubes (Becton Dickinson and Cie) containing K_3-EDTA (0.47 M). Plasma and erythrocytes were isolated from blood by centrifugation at 1800 ***g*** for 12 min at 4ºC and stored at -80ºC until analysis for activity of antioxidant enzymes.

On day 21, samples of mammary tissue were obtained by biopsies from the upper portion of the hindquarters of the udder. To avoid inflammation problems, the left and right hindquarters were alternated from one period to another and a site at least 10 cm apart from the first one was chosen when the quarter was used for the second time. After biopsies, mammary tissues were immediately rinsed with sterile saline solution (phosphate buffered saline (pH 7.4) + heparin) to remove any red blood cells and clots, and then cut into 2 portions. One portion was immediately frozen in liquid N and stored at −80°C for analysis of relative mRNA abundance. The other portion was immediately

divided in three parts (200 mg) and suspended separately in 2 ml of collecting buffer for CAT (50 mM potassium phosphate (pH 7.0) + 1 mM EDTA), GPX (50 mM Tris-HCl (pH 7.5) + 5 mM EDTA + 1mM DTT) and SOD (1 mM EGTA + 210 mM mannitol + 70 mM sucrose; pH 7.2). Samples were then homogenized on ice for 15 sec using a Polytron homogenizer (Kinematica Inc.) and centrifuged at 10,000 ***g*** for 15 min (4ºC) for CAT and GPX analyses and at 1,500 ***g*** for 5 min (4ºC) for SOD analysis. The supernatants were collected and stored at -80ºC for all assays.

Enzyme Activity

The activities of CAT, GPX and SOD in plasma, erythrocytes and supernatants obtained from mammary tissue were determined as reported previously by Côrtes *et al.*[10] using commercial assay kits (kits 707002, 703102 and 706002, respectively; Cayman Chemical). Total protein concentration was determined in samples of plasma, erythrocytes and supernatants using a bicinchoninic acid protein assay (kits BCA1 and B 9643, Sigma-Aldrich) as reported previously by Côrtes *et al.*[10]. Data were normalized per milligram of total protein content for each sample.

RNA extraction and complementary DNA synthesis

Extraction of total RNA from mammary tissue and cDNA synthesis was performed as previously described by Labrecque *et al.*[23]. Integrity and putrity of extracted RNA was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.).

Real-time PCR amplifications

The target genes were: SOD1, SOD2, SOD3, GPX1, GPX3, CAT, nuclear factor Kappa-B1 (NF-κB1) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2), acetyl-coenzyme A carboxylase alpha (ACACA), lipoprotein lipase (LPL), stearoyl-coA desaturase, sterol regulatory element binding transcription factor 1 (SREBP1), fatty acid synthase (FASN), cluster of differentiation 36 (CD36), peroxisome proliferator-activated receptor-γ1 (PPARG1), peroxisome proliferator-activated recepetor-γ2 (PPARG2), and peroxisome proliferator-activated receptor-α (PPARα).

Table 2. Primer sequences used for real-time PCR amplifications of reference gene and genes encoding for antioxidant enzymes in mammary gland biopsies

Genes*	Primer sequences † (5'→3')	GenBank Accession no.	Product size (nt)	Primer (nM)	Amplification efficiency (%)
GAPDH	(F) TGACCCCTTCATTGACCTTCA	NM_001034034	66	150	97.83
	(R) AACTTGCCGTGGGTGGAAT			300	
PPIA	(F) GAGCACTGGAGAGAAAGGATTTG	NM_178320	71	300	95.12
	(R) GGCACATAAATCCCGGAATTATT			150	
UBQ	(F) TGGAGCCCAGTGACACCAT	NM_174133	111	300	96.48
	(R) GGCCATCTTCCAGCTGCTT			300	
ACTB	(F) GCGTGGCTACAGCTTCACC	NM_173979.3	54	900	93.20
	(R) TTGATGTCACGGACGATTTC			900	
NF-κB1	(F) CTCAAAGCAGCAGGAGCAGA	NM_001076409	102	300	97.68
	(R)CGGTACGACCCCTTCATCC			900	
Nrf2	(F) GTACCCCTGGAAATGTCAAACAG	NM_001011678	88	900	98.94
	(R) TGTGATGACGACAAAGGTTGGA			900	
CAT	(F) GCTCCAAATTACTACCCCAATAGC	NM_001035386	104	900	94.34
	(R) GCACTGTTGAAGCGCTGTACA			300	
SOD1	(F) TGTTGCCATCGTGGATATTGTAG	NM_174615	102	900	98.93
	(R) CCCAAGTCATCTGGTTTTTCATG			900	
SOD2	(F) CGCTGGAGAAGGGTGATGTT	NM_201527	99	900	95.20
	(R) GATTTGTCCAGAAGATGCTGTGAT			900	
SOD3	(F) GCAGCAGATGGGCTCCAA	NM_001082610	80	900	92.17
	(R) GCATCATCTCCTGCCAGATCTC			900	
GPX1	(F) GCAAGGTGCTGCTCATTGAG	NM_174076	82	900	109.67
	(R) CGCTGCAGGTCATTCATCTG			900	
GPX3	(F) GTCAACGTGGCCAGCTACTGA	NM_174077	93	900	90.73
	(R) CAGAATGACCAGACCAAATGGTT			900	

*ACTB, actin beta; CAT, catalase; FA, fatty acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX, glutathione peroxidase; NF-κB1, nuclear factor Kappa-B1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PPIA, peptidylprolyl isomerase A SOD, superoxide dismutase; UBQ, ubiquitine.

Table 3. Primer sequences used for real-time PCR amplifications of lipogenic-related genes in mammary gland biopsies

Genes*	Primer sequences† (5'→3')	GenBank Accession no.	Product size (nt)	Primer (nM)	Amplification efficiency (%)
ACACA	GAGTTCCTCCTTCCCATCTACCA	NM_174224	123	900	96.87
	GGTGCGTGAAGTCTTCCAATC			900	
CD36	ACCTCCTGGGCCTGGTAGA	NM_174010	89	300	94.54
	TGATCTGCATGCACAATATGAAATC			900	
LPL	TAAGGCCTACCGGTGCAATTC	NM_001075120	100	900	98.67
	CTTGTTGATCTCGTAGCCCATGT			900	
PPARG1	AAGTCCCCTTGCTTAGTTGTTCAG	Y12419	102	900	102.14
	AGGAATGACACTTGTTTACGGAAAC			900	
PPARG2	TGAACGGAACTGGCCTTTTG	AF288373	94	900	99.57
	TCCCAGAGTTTCACCCATCAC			900	
PPARα	GACAAAGCCTCTGGCTACCACTA	NM_001034036	80	900	92.47
	TTCAGCCGAATCGTTCTCCTA			900	
SCD	CCTGTGGAGTCACCGAACCT	NM_173959	146	900	96.53
	GGTCGGCATCCGTTTCTG			900	
SREBP1	TTTCTTCGTGGATGGCAACTG	NM_001113302	130	900	93.61
	TGCTCGCTCCAAGAGATGTTC			900	
FASN	AGCCCCTCAAGCGAACAGT	NM_001012669	100	900	97.23
	CGTACCTGAATGACCACTTTGC			900	

*ACACA, acetyl-coenzyme A carboxylase alpha; FA, fatty acid; CD36, cluster of differentiation 36; FASN, fatty acid synthase; LPL, lipoprotein lipase; PPARG1, peroxisome proliferator-activated recepetor-γ1; PPARG2, peroxisome proliferator-activated recepetor-γ2; PPPARα, peroxisome proliferator-activated receptor alpha; SREBP1, sterol regulatory element binding transcription factor 1; SCD, stearoyl-coA desaturase.

The relative mRNA abundance of target genes was determined using real-time PCR amplifications. PCR amplification, detection and data analyses were performed with an ABI 7500 Fast Real-time PCR System (PE Applied BioSystems). All primers (Tables 2 and 3) used in the present study were derived from *Bos Taurus* genome (GenBank) and were designed using the Primer Express software 3.0 (PE Applied BioSystems). The PCR was performed in triplicate using a 10 µl reaction mixture per well, containing 5 µl of 2x Power SYBRGreen Master Mix (PE Applied BioSystems), 3 µl of 15x diluted cDNA, and 0.05 µl of AmpErase (PE Applied BioSystems). Amplification involved one cycle at 50ºC for 2 min, followed by 10 min at 95°C for initial denaturation, and then 40 cycles at 95ºC for 3 sec, followed by annealing at 60ºC for 30 sec. Specificity of amplification was performed by analysis of melting curves after each amplification. Four reference genes (RG), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA), actin beta (ACTB) and ubiquitine were assessed as an endogenous control. Ubiquitine was chosen as internal control based on NormFinder algorithm[24] results, which showed that ubiquitine was the RG least affected by treatments. Data were analyzed using the comparative Ct method, by which the amount of target genes normalized to ubiquitine and relative to a calibrator (known individual value) is calculated as $2^{-\Delta\Delta Ct}$ as described in the User Bulletin #2 (Applied Biosystems 1997; ABI PRISM 7700 Sequence Detection System).

Determination of amplification efficiency

Amplification efficiency (E) for each gene was assessed using a standard curve. A pool of mammary tissue cDNA from all biopsies collected (n = 32) was used to prepare the different dilutions (1, 5, 10, 20, 50, 100, 150 and 200 ng) needed for construction of the standard curve. PCR reactions were performed in duplicate and repeated twice on different days as described above and amplification E for each target gene was determined according to Cikos *et al.*[25].

Statistical Analysis

All results were analyzed using the MIXED procedure of SAS (SAS 2000; SAS Institute) within a 2 × 2 factorial arrangement of treatments. Data were analyzed using a double 4 × 4 Williams Latin square design with the following general model:

$$Y_{ijkl} = \mu + S_i + C_{j(i)} + P_k + T_l + e_{ijkl}$$

Where: Y_{ijkl}, the dependent variable; μ, overall mean; S_i, fixed effect of square (i = 1 to 2); $C_{j(i)}$, random effect of cow within square (i = 1 to 4); P_k, fixed effect of period (k = 1 to 4); T_l, fixed effect of treatment, and e_{ijkl}, random residual error. Treatments were compared to provide factorial contrasts: 1) with *versus* without FM, 2) with *versus* without abomasal infusion of FO, and 3) the interaction between FM and abomasal infusion of FO. Data on enzyme activity were analyzed as repeated measurements and covariance structures were modeled separately for each variable. Values of fit statistics for Akaike´s information criterion (AIC) and Akaike´s information criterion corrected (AICC) were used to determine the most appropriate covariance structures. Data on mRNA abundance were analyzed using the normalized quantities and are presented as relative quantification (RQ) of mRNA abundance using the comparative Ct method comparing all treatment combinations to the CO diet used here as reference. When the interaction between FM and FO was significant, the SLICE option of the MIXED procedure was used to examine the differential response of infusion of oil in the abomasum to FM supplementation. Significant differences were set at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

Results

DM intake and milk production

There was no interaction between FM and FO for DM intake and milk production. Intake of DM was higher for cows fed FM than for those fed CO and values averaged, respectively, 32.2 kg/d and 30.9 kg/d, and 4.1% and 3.9% of body weight. Conversely, DM intake was lower for cows infused with FO in the abomasum than for those not infused, and values averaged 32.1 kg/d and 31.0 kg/d, and 4.0% and 3.9% of body weight, respectively. Milk production averaged 31.9 kg/d and was similar among treatments.

Enzyme activity in plasma, erythrocytes and mammary tissue

There was no interaction between hour and treatment for activity of antioxidant enzymes in plasma and erythrocytes (data not shown). Therefore, only mean values for the 2 h blood sampling period are presented (Table 4).

Table 4. Activity of antioxidant enzymes in plasma, erythrocytes and mammary tissue of Holstein cows fed diets containing no flax meal (CO) or 12.4% flax meal (FM) in the dry matter and infused or not with 250 g of flax oil/d in the abomasum

	Treatments							
	CO		FM			*P*		
Item	0	250	0	250	SEM	Meal	Oil	M × O
Plasma								
CAT (nmol/min per mg of protein)	0.26	0.21	0.18	0.19	0.002	0.008	0.23	0.06
GPx (nmol/min per mg of protein)	0.97	1.08	1.07	0.95	0.094	0.82	0.97	0.23
SOD (U/mg protein)	0.022	0.023	0.026	0.021	0.0022	0.69	0.40	0.17
Erythrocytes								
CAT (nmol/min per mg of protein)	184.5	176.4	191.5	189.8	5.77	0.09	0.41	0.58
GPX (nmol/min per mg of protein)	173.4	182.2	183.2	176.8	2.54	0.15	0.22	0.008
SOD (U/mg protein)	32.2	30.9	29.6	32.2	1.71	0.72	0.69	0.85
Mammary tissue								
CAT (nmol/min per mg of protein)	55.79	47.42	55.42	46.20	5.866	0.92	0.14	0.96
GPx (nmol/min per mg of protein)	48.37	49.16	54.79	45.15	5.827	0.82	0.41	0.33
SOD (U/mg protein)	39.39	40.39	38.61	34.61	2.897	0.27	0.61	0.40

*CAT, catalase; GPX, glutathione peroxidase; SOD, superoxide dismutase.

There was a tendency for an interaction (*P*=0.06) between FM supplementation and infusion of FO in the abomasum for plasma activity of CAT; FM reduced (*P*=0.02) plasma CAT activity in the absence of FO whereas it had no effect (*P*=0.51) when FO was infused in the abomasum. Plasma activity of GPX and SOD was similar among treatments. There was a tendency (*P*=0.09) for increased activity of CAT in erythrocytes when cows were fed FM compared to those fed CO. There was an interaction between FM supplementation and infusion of FO in the abomasum for erythrocytes activity of GPX; FM supplementation increased (*P*=0.01) plasma GPX activity in the absence of infusion of FO whereas it had no effect (*P*=0.15) when FO was infused in the abomasum. SOD activity in erythrocytes was not affected by treatments. Activity of antioxidant enzymes in mammary tissue was similar among treatments.

Table 5. Relative quantification (RQ)* of mRNA from antioxidant-related genes in mammary tissue of Holstein cows fed diets containing no flax meal (CO) or 12.4% flax meal (FM) in the dry matter and infused or not with 250 g of flax oil/d in the abomasum

	Treatments							
	CO		FM			*P*		
Gene	0	250	0	250	SEM	Meal	Oil	M × O
NFKB1	0.29	0.37	0.33	0.34	0.045	0.94	0.31	0.48
NFR2	0.22	0.31	0.27	0.26	0.051	0.99	0.47	0.37
CAT	0.37	0.42	0.37	0.35	0.034	0.27	0.60	0.29
GPX1	0.25	0.35	0.32	0.26	0.035	0.75	0.62	0.04
GPX3	0.07	0.17	0.09	0.08	0.043	0.40	0.32	0.18
SOD1	0.72	0.70	0.63	0.62	0.069	0.23	0.87	0.96
SOD2	0.18	0.19	0.18	0.22	0.058	0.71	0.70	0.84
SOD3	0.39	0.50	0.41	0.41	0.045	0.45	0.22	0.22

CAT, catalase; FA, fatty acid; GPX, glutathione peroxidase; NF-κB1, nuclear factor Kappa-B1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; SOD, superoxide dismutase.

*RQ of mRNA abundance using the comparative cycle threshold method and the control (CO) treatment as reference.

Relative quantification of mRNA from antioxidant and lipogenic-related genes in mammary tissue

Abundance of mRNA of the nuclear factors Nrf2 and NF-κB1 and of the antioxidant enzymes was not affected by treatments except for that of GPX1 which showed an interaction (*P*=0.04) between FM supplementation and infusion of FO in the abomasum (Table 5). FM meal supplementation tended to reduced (*P*=0.09) mRNA abundance of GPX1 but only in the presence of oil. The abundance of mRNA lipogenic-related genes (ACACA, CD36, FASN, LPL, SCD) and that of PPARG1, PPARG2, PPARα and SREBP1 was similar among treatments (Table 6).

Table 6. Relative quantification (RQ) of mRNA from lipogenic-related genes in mammary tissue of Holstein cows fed diets containing no flax meal (CO) or 12.4% flax meal (FM) in the dry matter and infused or not with 250 g of flax oil/d in the abomasum.

	Treatments							
	CO		FM			*P*		
Gene	0	250	0	250	SEM	Meal	Oil	M × O
ACACA	0.37	0.38	0.35	0.35	0.061	0.71	0.97	0.91
CD36	0.64	0.68	0.60	0.58	0.089	0.39	0.9	0.75
FASN	0.38	0.44	0.40	0.38	0.072	0.77	0.81	0.56
LPL	0.48	0.43	0.38	0.37	0.081	0.36	0.76	0.81
PPARG1	0.48	0.45	0.43	0.40	0.058	0.46	0.63	0.99
PPARG2	0.24	0.49	0.28	0.249	0.088	0.27	0.22	0.13
PPARa	0.32	0.41	0.33	0.35	0.030	0.36	0.12	0.26
SCD	0.39	0.45	0.41	0.36	0.068	0.58	0.96	0.43
SREBP1	0.54	0.45	0.44	0.38	0.062	0.19	0.20	0.83

*ACACA, acetyl-coenzyme A carboxylase alpha; FA, fatty acid; CD36, cluster of differentiation 36; FASN, fatty acid synthase; LPL, lipoprotein lipase; PPARG1, peroxisome proliferator-activated recepetor-γ1; PPARG2, peroxisome proliferator-activated recepetor-γ2; PPPARα, peroxisome proliferator-activated receptor alpha; SREBP1, sterol regulatory element binding transcription factor 1; SCD, stearoyl-coA desaturase.

Discussion

The present study was especially designed to evaluate the effect of antioxidants from FM on activity and gene expression of antioxidant enzymes and lipogenic-related genes expression in mammary tissue of dairy cows infused with FO in the abomasum.

Oxidative stress conditions were achieved by infusing FO in the abomasum to provide a higher postruminal delivery of PUFA, which has been shown to increase lipoperoxidation susceptibility of tissues in ruminant[17]. Although our main objective was not to evaluate performance, it is worth noting that FM supplementation led to higher DM intake. It has been suggested that FM has a positive effect on intake likely as a result of good nutrient composition and good acceptability, which is supported by the greater DM intake reported previously[26]. As observed in the present experiment, studies have shown that abomasal infusion of high unsaturated fatty acid sources, such flax[3] and soybean[1] oils, reduces DM intake of dairy cows. Abomasal infusion of FO provides a greater postruminal delivery of PUFA, which accounts for increased plasma concentration of gut hormones such as cholecystokinin, pancreatic polypeptide[27] and glucagon-like peptide-1[28] that are related to postprandial satiety signals and gut motility reduction[1].

Over the years, it has been well recognized that celullar antioxidant enzymes (e.g. CAT, GPX and SOD) are crucial components of the antioxidant defense system in animals[29]. Catalase is involved in the detoxification of hydrogen peroxide, a ROS, which is a toxic product of normal and pathogenic ROS production[30; 31]. GPX catalyzes the reduction of lipid hydroperoxides and free hydrogen peroxides and protects the cell against oxidative damage[32; 33]. Superoxide dismutases are metaloenzymes that catalyze the dismutation of superoxide anions into molecular oxygen and hydrogen peroxides[34; 35].

Literature shows variable results on the effects of dietary antioxydants on the activity of antioxidant enzymes in dairy cows supplemented with PUFA. For example, in a study with dairy cows fed diets containing soybean oil, plasma GPX and SOD activity was increased with antioxidant (ethoxyquin and tertiary-butyl-hydroquinone) supplementation[36]. In contrast, no effect of flax hull antioxidant supplementation has been reported on activity of CAT, GPX and SOD in plasma and erythrocytes whereas SOD activity in mammary tissue tended to increase when FO was infused in the abomasum of dairy cows[10]. In the same study, the authors reported lower plasma GPX activity and a trend for higher CAT activity in mammary tissue with FO infusion in the abomasum. Results of the present experiment are different from those reported in previous studies carried out with dairy cows. In the present study, no benefits owing to dietary FM were observed for antioxidant enzymes in mammary tissue and plasma.

Activity of CAT in plasma decreased with FM supplementation but the decrease was higher in the absence of abomasal infusion of oil as indicated by the interaction that tended to be significant. In this particular situtation, feeding FM showed a depletion effect on the protective role against oxidation played by CAT. This disagrees with the results of Côrtes *et al.*[37] who reported no effect of flax lignans and abomasal infusion of FO on activity of CAT in plasma . At the present time, we do not have an appropriate explanation for this unexpected result. However, in the present experiment, activity of CAT in erythrocytes increased when cows were fed FM. Moreover, FM supplementation increased GPX activity in erythrocytes of cows but only in the absence of abomasal infusion of FO. Since antioxidant enzymes are among the most potent antioxidants[6], our results on CAT and GPX activity in erythrocytes could represent a physiological response of the antioxidant defense system to face an increased lipoperoxidation susceptibility, ensuring that potential oxidants are metabolized and removed more promptly from the organism. However, the specific mechanism by which antioxidants increase the activity of antioxidant enzymes remains unclear. The red blood cells are one rich source of antioxidant enzymes and they are also a significant source of superoxide generation in biological systems[38]. As the production of superoxide ions and oxygen peroxides occurs under oxidative stress[39], this may suggest that cows fed CO were under greater stress conditions when infused with FO in the abomasum as shown by increased GPX activity in erythrocytes compared to those not infused. Indeed, increases in erythrocyte GPX activity have been observed in heat-stressed cows as an indirect compensatory response of cells to increased oxidant challenge during heat stress[40]. However, the lack of difference in activity of GPX in erythrocytes when FM was supplied with FO is not easy to explain; nevertheless, it is possible to suppose that erythrocyte GPX is not involved in the homeostatic response as suggested by Bernabucci *et al.*[40].

Flax antioxidants have been reported to have regulatory effects on the expression of genes encoding for antioxidants enzymes[14]. Flax hulls are rich in lignans and previous research has shown consistent increases in the abundance of mRNA of CAT, GPX1 and SOD1 in mammary tissue of dairy cows[10]. In contrast, the same study showed that flax hulls reduced mRNA abundance of GPX3, SOD2 and SOD3 compared to cows fed no flax hulls. In the present study, increased GPX1 mRNA abundance with FM supplementation was observed only in the absence of FO, which could suggest that FM

antioxidant supplementation combined with infusion of FO in the abomasum maintains GPX1 expression. However, in general, we observed no significant variations in mRNA abundance of antioxidant enzymes, which suggest that FM did not induce gene expression in mammary tissue. This is supported by the lack of treatment effect on mRNA abundance of Nrf2 and NF-κB1, which are involved in modulation of genes encoding for many antioxidant enzymes[12; 41] and modulated by phenolic compounds[42; 12] such as lignans. Furthermore, as lignans are fibre-bound polyphenols[43], they are present in higher amounts in flax hulls (0.99% DM of SDG)[10] than flax meal (0.14% DM of SDG), which could explain discrepancies between our results and those reported previously[10].

In rats, omega-3 fatty acids play a putative inhibition on the expression of antioxidant-related genes[44; 9]. A recent research[10] with dairy cows infused with 500 g FO/d in the abomasum has corroborated this theory. However, the present study failed to provide evidence supporting the inhibition effect of abomasal infusion of FO as the only significant effect of treatment was on the mRNA abundance of GPX1. Lower amount of oil infused in our study (250 g/d) compared to that of the previous study (500 g/d) could explain discrepancies between experiments.

Dietary supplementation of FM and abomasal infusion of FO had no significant effect on mRNA abundance of ACACA, FASN, CD36, LPL and SCD, thus suggesting that lignans and PUFA have no effect on the modulation of lipogenic-related genes in mammary tissue. This hypothesis is supported by the lack of difference in mRNA abundance of PPARG1, PPARG2 and SREBP1 genes, which encode proteins that play roles in the regulation of lipogenic-related genes. Omega-3 fatty acid supplementation has been previously shown to down-regulate lipogenic-related genes in rats[7]. However, studies using rats as a model often imply the consumption of high doses of oil, which could exceed the proportion of oil offered in diets of dairy cows.

In conclusion, the present study suggests that FM added at 12.4% of the diet does not induce significant changes in the mRNA abundance of antioxidant enzymes of dairy cows that are infused in the abomasum with 250 g FO/d, except for a significant increase in GPX activity in erythrocytes and higher GPX1 gene expression in mammary tissue, although the latter was observed only in the absence of abomasal infusion of FO. Abomasal infusion of 250 g FO/d did not induce changes in the activity and gene expression of antioxidant enzymes and gene expression of lipogenic-genes in mammary

tissue, except for a significant increase in the expression of GPX1 in the absence of dietary FM. However, studies such as the present one are useful to determine any beneficial effects of natural antioxidants such as flax meal on the oxidative status of cows supplemented with sources of PUFA, which could lead to feeding strategies to prevent diseases affecting the health status of dairy cattle.

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VI - CONSIDERAÇÕES FINAIS

O fornecimento de fontes *bypass* de ácidos graxos poli-insaturados para vacas leiteiras é eficaz em aumentar a concentração dos mesmos na gordura do leite, mesmo em situações em que a quantidade absoluta de óleo liberado no intestino para absorção não é considerada alta. Este é o caso do presente estudo, no qual foram utilizadas quantidades diárias relativamente baixas de óleo (0,2 kg de óleo de soja e 0,25 kg de óleo de linhaça) e foi observado melhora no índice promotor de saúde da gordura de leite.

As alterações na composição de ácidos graxos da gordura do leite promovidas pela suplementação de fonte *bypass* de ácidos graxos poli-insaturados permitem agregação de valor ao leite como um produto para alimentação humana. Entretanto, esta prática promove, concomitantemente, aumento na susceptibilidade do leite e do plasma à lipoperoxidação.

Por outro lado, os níveis de suplementação de polpa cítrica (1,0 kg MS/d) bem como de farelo de linhaça (120 g/kg MS) estudados no presente trabalho não são suficientes para promover efeito protetivo contra a lipoperoxidação tanto no leite como no plasma de vacas leiteiras.

As alterações na atividade enzimática e expressão gênica no tecido da glândula mamária diante da suplementação de 120 g de farelo de linhaça/kg MS são pouco representativas, mas sugerem que as substâncias antioxidantes presentes no farelo de linhaça poderiam ter efeito modulador da atividade e da expressão gênica de enzimas antioxidantes quando fornecido em maior quantidade.