UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

PERFIL OXIDATIVO E METABOLISMO RUMINAL DE VACAS LEITEIRAS ALIMENTADAS COM FARELO DE LINHAÇA

Autora: Ana Luiza Bachmann Schogor
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TITULAÇÃO: Doutora em Zootecnia - Área de Concentração Produção Animal

APROVADA em 09 de outubro de 2012.

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Prof. Dr. Geraldo Tadeu dos Santos
(Orientador)

Aos meus pais, Tereza e Francisco,
À Gisele Bonato Muraro, *in memoriam*
DEDICO...

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BIOGRAFIA

Ana Luiza Bachmann Schogor é filha de Tereza Bachmann Schogor e Francisco Schogor Junior, nascida em Guarapuava (PR) no dia 22/01/1982.

Em fevereiro de 2000, iniciou no curso de Zootecnia na Universidade Federal do Paraná, o qual conclui em dezembro de 2004, colando grau no dia 13 de maio de 2005.

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Em julho de 2006, ingressou no Programa de Pós-Graduação em Agronomia na Universidade de São Paulo, "Escola Superior de Agricultura Luiz de Queiroz" onde em 30 de setembro de 2008 recebeu o título de Mestre em Agronomia, na área de concentração Ciência Animal e Pastagens.

Em maio de 2009, ingressou no Programa de Pós-Graduação em Zootecnia, na Universidade Estadual de Maringá, em nível de Doutorado, na área de concentração Produção Animal. Entre os meses de novembro de 2010 e novembro de 2011, realizou estágio-sanduíche no *Dairy and Swine Research and Development Centre, Agriculture and Agri-Food Canada*, em Sherbrooke, Quebec.

No dia 9 de outubro de 2012, submeteu-se à defesa da Tese de Doutorado, sendo aprovada para o recebimento do título de Doutora em Zootecnia, na área de concentração de Produção Animal.

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RESUMO

O farelo de linhaça (FL) é uma rica fonte de antioxidantes, e ruminantes são capazes de converter lignanas em lignanas mamíferas no rúmen. Entretanto, poucas informações estão disponíveis sobre os efeitos da suplementação com FL sobre indicadores do perfil oxidativo de vacas leiteiras, sobre a produção de enterolactona (EL) e suas correlações entre os distintos fluidos corporais quando vacas são então alimentadas com FL, e quais bactérias poderiam ser responsáveis pela conversão de SDG em EL no rúmen. Oito vacas da raça Holandesa, canuladas no rúmen, foram usadas em um experimento em duplo quadrado Latino, com quatro períodos experimentais de 21 dias cada, com as seguintes dietas: controle, sem FL; e dietas contendo 5, 10 e 15% de FL, com base na matéria seca. A primeira abordagem foi a avaliação do perfil oxidativo. A produção de TBARS foi menor no leite de vacas alimentadas com 5 e 10% de FL. Não foi observado efeito de tratamento sobre a produção de TBARS no plasma e no líquido ruminal, nem sobre a atividade sequestrante do DPPH no leite, no plasma e no líquido ruminal, ambas somente decrescendo após a alimentação. A atividade da glutationa peroxidase aumentou no plasma de acordo com a suplementação de FL. Os tratamentos não apresentaram efeito sobre a atividade da superoxido dismutase no leite, plasma e líquido ruminal. A atividade da catalase não foi modificada no leite e no líquido ruminal, e não apresentou resultados consistentes no plasma, uma vez que vacas alimentadas com 5 e 10% de FL aumentaram sua atividade; porém, a suplementação com 15% de FL apresentou resultado semelhante à dieta controle. Os dados sobre o perfil de fermentação ruminal mostraram que a

concentração de EL na urina, no líquido ruminal, no leite e no plasma aumentou linearmente de acordo com a inclusão de FL na dieta. Os coeficientes de correlação de Spearman foram significantes para todas as comparações, exceto para apenas uma tendência observada entre a concentração de EL na urina e no fluído ruminal anterior à alimentação. A mais alta correlação foi observada entre a concentração de EL no fluído ruminal 2h após a alimentação e sua concentração no leite. A inclusão de níveis crescentes de FL na dieta, a qual resultou em maior ingestão de lignanas, não teve efeito sobre a atividade da ß-glicuronidase no fluído ruminal e nas fezes. Comparativamente ao observado para animais não-ruminantes, os resultados do presente experimento sugerem que a atividade da ß-glicuronidase no fluído ruminal tem menor importância no processo de absorção de EL e sua transferência ao leite e outros fluídos corporais. Finalmente, a terceira abordagem foi a identificação de potenciais bactérias que poderiam exercer um papel na conversão de lignanas em enterolactona. A concentração do total de genes bacterianos 16S rDNA obtidos com Q-PCR não diferiram entre os tratamentos. Os dendogramas para PCR-T-RFLP não revelaram agrupamentos óbvios da microbiota com base em dieta. Porém, os dendogramas de PCR-DGGE mostram um agrupamento por dieta, para quatro animais. As bandas presentes no DGGE de acordo com a suplementação com 15% de FL (portanto ausentes no tratamento controle) foram sequenciadas, e as bactérias identificadas (não-cultivadas) pertenceram às famílias *Succinivibrionaceae*, *Alphaproteobacteria*, aos gêneros *Prevotella*, *Succinivibrio*, *Lachnospiraceae*, *Bacteroidales*, *Anaerovorax* e às cepas de *Fibrobacter succinogenes*, e H23 da *F. succinogenes*, as quais podem exercer um papel na conversão das lignanas em enterolactona. Os resultados sugerem que a suplementação com FL pode melhorar o perfil oxidativo de vacas no terço médio para final de lactação, pelo aumento da atividade da glutationa peroxidase no plasma dos animais. Adicionalmente, as características de fermentação ruminal mostraram que o pH não limitou o processo de conversão de SDG em EL. Futuras pesquisas se fazem necessárias para proporcionar melhor entendimento sobre a absorção de EL pelos ruminantes, aumentando então sua transferência para o leite. Além disso, esse estudo forneceu informações sobre quais bactérias podem ser potencialmente investigadas por seu papel na produção de EL.

Palavras-chave: antioxidantes, enzimas antioxidantes, enterolactona

ABSTRACT

Flax meal (FM) is a rich source of antioxidant, and ruminants are able to convert lignans into mammalian lignans in the rumen. However, little information is available on the effects of flax meal supplementation on indicators of oxidative stress in dairy cows, the EL production and the correlation of its concentration among milk and other body fluids when cows were fed flax meal, and which ruminal bacterial would be responsible for SDG conversion into EL in the rumen. Eight rumen cannulated cows were used in a double 4 x 4 Latin square design, with four 21-d experimental periods, and fed: a control with no flax meal (FM), and diets with 5%, 10% and 15% FM (on dry matter basis). In a first approach, the oxidative profile was evaluated. The production of TBARS was lower in the milk of cows fed 5FM and 10FM. There was no treatment effect on TBARS production in plasma and ruminal fluid although there was a time effect as shown by the decrease post-feeding in both plasma and ruminal fluid regardless of treatment. DPPH scavenging activity in milk, plasma and ruminal fluid was similar among treatments and decreased overtime for all treatments. GPx activity increased in plasma with FM supplementation. Treatment had no effect on SOD activity in milk, plasma and ruminal fluid. Catalase activity was not modified in milk and ruminal fluid, and did not show consistent results in plasma, because when cows were supplemented with 5 or 10% of FM, CAT activity was increased; however, the inclusion of 15% of FM was similar to the control diet. Regarding the data of ruminal fermentation, Concentrations of EL in urine, ruminal fluid, milk and plasma increased linearly with FM supplementation. Spearman's correlation coefficients were

significant for all comparisons except that only a trend was observed between concentration of EL in urine and that in ruminal fluid before feeding. The highest correlation was observed between EL concentration in ruminal fluid 2 h after feeding and that in milk. Feeding increased proportions of FM in the diet, which may have resulted in greater intake of lignans, had no effect on ß-glucuronidase activity of ruminal fluid and faeces. Unlike to what is observed in non-ruminant animals, results of the present experiment may suggest that the activity of ß-glucuronidase in the rumen is of little importance for the absorption of EL and its transfer in milk and other physiological fluids. Further studies are required to better understand and improve EL production and absorption, which could contribute to enhance animal health and the transfer of antioxidant components in milk. Finally, the third approach was to identify bacterial taxa that potentially play a role in the conversion of plant lignans into enterolactone. The concentration of total bacterial 16S rDNA genes obtained using Q-PCR did not differ among treatments. PCR-T-RFLP based dendrograms revealed no obvious global clustering of the microbiota based on diet. PCR-DGGE did however show clustering by diet within four cows. Bands present following feeding of 15% FM and absent when no FM was fed were sequenced. Sequences revealed that uncultured bacteria belonging to the families *Succinivibrionaceae, Alphaproteobacteria* and genera *Prevotella Succinivibrio, Lachnospiraceae, Bacteroidales, Anaerovorax* and strain of *Fibrobacter succinogenes*, and strain H23 of *F. succinogenes* may play a role in the conversion of plant lignan into enterolactone in the rumen. Altogether, the results suggest that FM supplementation could improve the oxidative status of Holstein cows in mid to late lactation as suggested by increased GPx activity in plasma. In addition, the ruminal fermentation characteristics and EL production, showed that ruminal pH around 6 is not limiting in the process of EL conversion. The higher correlation observed between ruminal fluid and milk than between plasma and milk, suggest that the rumen may have major contribution to EL concentration in milk than plasma. Future research is needed to provide better understanding of the absorption of EL by ruminants, then increasing its transference into the milk. Furthermore, this study provided information about which bacteria can potentially be investigated for its role in EL production.

Keywords: antioxidants, antioxidant enzymes, enterolactone

INTRODUÇÃO

1.1 Metabolismo oxidativo, produção de radicais livres e definição de estresse oxidativo

O metabolismo oxidativo é o que mantém a vida aeróbia. Segundo Andrade Junior et al. (2005), o oxigênio que entra no organismo deve ser metabolizado até sua completa redução à água, incorporando quatro elétrons ao final da cadeia respiratória. Concomitantemente, os nutrientes ingeridos pela dieta são utilizados para a produção de energia por esta mesma cadeia respiratória (ou fosforilação oxidativa) e o metabolismo intermediário a esse processo resulta em incorporações diretas de átomos de oxigênio em determinadas biomoléculas (Sies et al., 2005). Entretanto, se houver redução do oxigênio com número menor de elétrons, haverá produção de radicais livres de oxigênio, sendo que suas principais formas reativas são o oxigênio singlete ($^1O_2$), o ânion radical hidroxila ($-OH^-$), o ânion radical superóxido (O_2^-), o peróxido de hidrogênio (H_2O_2), radicais peroxila (RO_2^-) e alcoxila (RO^-) e o ozônio (O_3) (Andrade Junior et al., 2005).

O ânion radical superóxido (O_2^-) é gerado continuamente por processos celulares, pela redução de um elétron do oxigênio, na cadeia de transporte de elétrons nas mitocôndrias, e/ou por atividade das enzimas xantina oxidase e NADPH oxidase nos microssomos, e/ou pela redução monoeletrônica de O_2 (Vasconcelos et al., 2007). De acordo com Frankel (2005), o ânion radical superóxido é um importante mecanismo antibacteriano utilizado por neutrófilos, monócitos e macrófagos, os quais eliminam bactérias por meio da produção de O_2^-.

O peróxido de hidrogênio é um composto intermediário, formado pela reação de dismutação de O_2^- catalizada pela enzima superoxido dismutase (SOD), pela redução de dois elétrons na molécula de O_2 e/ou pela ação de diversas enzimas oxidases "in vivo", localizadas nos peroxissomos (Vasconcelos et al., 2007). A reação de dismutação ocorre em bactérias, células fagocitárias, mitocôndrias, microssomos e cloroplastos. Pode atravessar membranas, como por exemplo, as dos eritrócitos, e na presença de Fe^{+2} pode formar radicais hidroxila, por meio da reação de Fentom (Andrade Junior et al., 2005). A equação que representa a reação de formação do peróxido de hidrogênio é dada a seguir (Sorg, 2004):

$$2\ O_2^- + 2\ H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

O radical hidroxila ($-OH^-$), o mais reativo e lesivo dos radicais, uma vez formado no organismo não dispõe de mecanismos de defesa (Vasconcelos et al., 2007). Na presença de metais de transição, o peróxido de hidrogênio produz radicais hidroxila, de acordo com a seguinte reação descrita por Frankel (2005):

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH + OH^-$$

Uma vez produzidos "in vivo", podem reagir próximos aos locais de formação (Frankel, 2005), causando modificação no DNA, danos em proteínas, inativação enzimática e peroxidação lipídica (Sordillo & Aitken, 2009).

O oxigênio singlete, do inglês *singlet* ($^1O_2$) é o estado eletronicamente excitado do oxigênio, produzido por reações fotoquímicas ou outras radiações. Pode reagir com diversas biomoléculas, incluindo lipídeos de membrana, iniciando processos de peroxidação. De acordo com Frankel (2005), o oxigênio singlete reage com ácido linoleico cerca de 1.500 vezes mais rápido que o oxigênio em sua forma normal (triplete), formando hidroperóxidos que causam dano às membranas lipídicas da retina. E ainda, o oxigênio singlete causa danos na molécula de α-tocoferol, DNA, colesterol, β-caroteno e proteínas.

Por fim, o ozônio é produzido no ar atmosférico poluído, ou por fonte de luz intensa, sendo extremamente danoso aos pulmões, oxidando proteínas, DNA e lipídeos (Vasconcelos et al., 2007).

Dependendo da rota metabólica e dos radicais livres formados, o distúrbio metabólico causado por eles pode ser classificado em estresse oxidativo metabólico, ambiental, foto-oxidativo, dependente de drogas, e nitrosativo (Sies et al., 2005).

Entretanto, a produção de radicais livres de oxigênio, dentre outros radicais livres, é parte integrante do metabolismo dos animais e pode ser observada em diversas condições fisiológicas (Valko et al., 2006).

Quando em baixas concentrações, essas substâncias são essenciais em diversos processos fisiológicos (Sies et al., 2005), pois intermediam funções como fosforilação de proteínas, ativação dos fatores de transcrição, diferenciação celular, apoptose, maturação de oócitos, imunidade celular e defesa contra microrganismos (Miller et al., 1993; Celi, 2010), pela sua participação na transferência de elétrons em diversas reações bioquímicas.

Percebe-se, então, que naturalmente há um fluxo constante de elétrons em líquidos biológicos, organelas e células, sendo que esses elétrons se interconvertem entre o estado reduzido e oxidado, resultando em um balanço redox (Vasconcelos et al., 2007). A esse estado de equilíbrio das funções internas do organismo, o qual é atingido de maneira dinâmica, e não-estática, é nominado de homeostase, ou homocinese (Robertshaw, 1996). Todavia, pelas circunstâncias patológicas ou não, ou a deficiência em substâncias naturais que protegem o organismo ou exposição excessiva a estimuladores de produção de radicais livres de oxigênio (Miller et al., 1993), moléculas de radicais livres podem ser produzidas em quantidades elevadas no organismo, gerando uma desordem metabólica denominada estresse oxidativo (Castillo et al., 2006). Em outras palavras, seria o desequilíbrio entre a formação e a remoção de radicais livres no organismo, decorrente da diminuição dos antioxidantes endógenos ou do aumento da geração de espécies oxidantes, o qual gera um estado pró-oxidante que favorece a ocorrência de lesões oxidativas em macromoléculas e estruturas celulares (Rover Júnior et al., 2001).

De acordo com Miller et al. (1993), quando os radicais livres de oxigênio não são removidos do organismo de forma segura e eficiente, o estresse oxidativo pode prejudicar a saúde de vacas leiteiras, direta e indiretamente. Efeitos diretos seriam reações de peroxidação em lipídeos e macromoléculas. Efeitos indiretos seriam aqueles induzidos por radicais livres de oxigênio em membranas celulares e alguns componentes que poderiam modificar rotas metabólicas, resultando em uma alteração na fisiologia do animal e o aparecimento de possíveis patologias. O estresse oxidativo pode ser particularmente perigoso porque há ausência de sinais clínicos, sendo o diagnóstico dado por métodos analíticos (Celi et al., 2010).

A detecção dos danos causados pelos radicais livres e a proteção contra os mesmos têm se tornado uma prática rotineira (Castillo et al., 2003). Estudos do perfil oxidativo dos animais pode refletir seus estados de saúde, reprodutivo e alimentar (Castillo et al., 2006), fornecendo informações que podem evitar ou atenuar o efeito desses distúrbios metabólicos e proporcionar um ambiente adequado para o bom desempenho produtivo do animal.

1.2 Antioxidantes, enzimas antioxidantes e sistema antioxidante não-enzimático

A palavra antioxidante se torna cada dia mais popular na sociedade moderna, pela grande publicidade que é feita sobre seus efeitos benéficos à saúde. Como denotação tradicional, pode ser definida como uma substância natural ou sintética, adicionada a produtos para prevenir ou retardar sua deterioração por ação do oxigênio (Huang et al., 2005). Ou ainda, um composto ou substância química capaz de inibir a oxidação (estabilizar ou desativar radicais livres; Rahman, 2007), e que quando presente em baixas concentrações comparadas ao do substrato oxidável diminui ou inibe sua oxidação (Abdalla, 1993). São diversas as classes de antioxidantes, cada qual com suas propriedades e funções distintas. Antioxidantes capazes de sequestrar radicais livres são uma das classes mais importantes desses compostos, e a avaliação dessa capacidade em sequestrá-los (capacidade antioxidante) tem sido o objetivo de extensos estudos e argumentações (Niki, 2010).

Os animais possuem um complexo sistema antioxidante (enzimático e não-enzimático) o qual trabalha de forma sinérgica, e a combinação entre eles protege as células e órgãos contra os danos causados pelos radicais livres. Os antioxidantes podem ser endógenos, a exemplo de enzimas que apresentam atividade antioxidante em diversos organismos, ou exógena, quando substâncias antioxidantes fazem parte da dieta. Alguns dos antioxidantes advindos das dietas podem não neutralizar diretamente os radicais livres, mas podem melhorar a atividade endógena contra os mesmos, podendo então também ser classificados como antioxidantes.

1.2.1 Enzimas antioxidantes

A enzima superóxido dismutase (SOD) pode ser encontrada em meio intra e extracelular (Fridovich, 1997), e catalisa a dismutação do ânion superóxido em oxigênio

e peróxido de hidrogênio, sendo esse ânion superóxido seu único substrato (Donnelly et al., 1989). A SOD forma uma família de metaloenzimas, podendo se ligar com cobre e zinco no citosol de células eucarióticas, com magnésio nas mitocôndrias e com ferro e níquel em bactérias. A análise da SOD pode ser feita de maneira indireta, por adição da amostra ao sistema xantina – xantina oxidase como fonte de O_2^- e um composto que seja reduzido pelo O_2^- (Vasconcelos et al., 2007).

A glutationa peroxidase (GPx) é uma enzima intracelular, que remove o peróxido de hidrogênio do seu meio por seletividade, utilizando-se de glutationa (GSH) (Frankel, 2005). A GSH é um tripeptídeo formado por ácido glutâmico, cisteína e glicina. A cisteína contém selênio, covalentemente ligado ao restante da enzima (Rover Júnior et al., 2001) e a glicina seria o aminoácido - o doador de hidrogênio (Frankel, 2005). Duas moléculas de glutationa, por meio do seu grupamento –SH da cisteína, formam uma ponte dissulfeto, que oxida a glutationa e libera uma molécula de água. A glutationa oxidada é novamente reduzida pela glutationa redutase, e a proporção entre glutationa reduzida e glutationa oxidada deve se manter maior que dez em células normais (Vasconcelos et al., 2007). A equação que descreve essa reação é mostrada a seguir (Rover Júnior et al., 2001):

$$2\ H_2O_2 + 2\ GSH \rightarrow GSSG + H_2O$$

O fato de o Se fazer parte da estrutura da enzima, indica que o elemento é um cofator, e deve ser indicado como necessário em dietas para humanos e animais (Frankel, 2005).

A quantificação de GSH pode indicar uma possível correlação entre a diminuição das atividades de enzimas antioxidantes, como a GPx, e o aumento nos níveis de bases de DNA lesados pelo dano oxidativo (Rover Júnior et al., 2001).

A catalase é uma enzima encontrada nos peroxissomos, mitocôndrias, e no citosol dos eritrócitos, responsável pela inativação intracelular do peróxido de hidrogênio (Ferro et al., 2010). A atividade da catalase é monitorada pela redução na concentração de H_2O_2 ou na geração de oxigênio (Vasconcelos et al., 2007).

1.2.2 Sistema antioxidante não-enzimático

O sistema antioxidante não-enzimático é basicamente caracterizado por serem antioxidantes provenientes da dieta. São moléculas capazes de sequestrar radicais livres de oxigênio diretamente, incluindo os tocoferóis, ascorbato, carotenoides, tióis

(moléculas que possuem o grupamento – SH), compostos fenólicos, dentre outros micronutrientes (Sies et al., 2005).

O α-tocoferol (forma ativa da vitamina E) atua como um sequestrador de radicais livres (Jordão Junior et al., 1998), podendo reagir com ânion superóxido, oxigênio singlete e compostos carboxílicos (Prassad, 2000). A pró-vitamina A (β-caroteno) exibe boa capacidade de capturar radicais livres (Rover Júnior et al., 2001). A vitamina E, uma molécula lipossolúvel, se concentra no interior das membranas e age de forma sinérgica com ascorbato, pois pode sequestrar radicais pela doação de um íon hidrogênio, com a formação radical tocoferoxil, o qual é regenerado à sua forma reduzida pela vitamina C (Sordillo & Aitken, 2009).

Os compostos fenólicos são um grupo muito diversificado de fitoquímicos, originados do metabolismo secundário das plantas, derivados dos aminoácidos fenilanalina e tirosina. Possuem anel aromático com um ou mais grupamentos hidroxila. Dentre os compostos fenólicos destacam-se os flavonides, os ácidos fenólicos, os fenóis simples, cumarinas, taninos, ligninas e tocoferóis (Soares, 2002; Angelo & Jorge, 2007). De acordo com Vasta & Luciano (2011), a proporção de diferentes classes de compostos fenólicos pode variar amplamente entre diferentes fontes, sendo que ensaios "in vitro" podem não refletir sua efetividade "in vivo", sendo que esta última também necessita ser investigada.

1.3 Avaliações do perfil oxidativo e caracterização do estresse oxidativo em ruminantes

O estresse oxidativo é um tema que vem sendo amplamente discutido, haja vista o grande número de publicações recentes acerca do assunto. Desde 2003, Castillo et al. (2003) afirmam que tanto práticas que são desenvolvidas para detecção de radicais livres, bem como métodos de proteção contra os mesmos são amplamente publicadas. Entretanto, nenhum teste é padronizado, o que dificulta a comparação entre resultados (Castillo et al., 2006; Celi, 2010), mesmo para estudos similares (Celi, 2010).

Parâmetros metabólicos comuns podem ser utilizados para avaliar a homeostase do animal, bem como sua alteração, quadro que poderia sugerir o estresse oxidativo. Alguns exemplos são teores de glicose, ácidos graxos não-esterificados, triglicerídeos, proteínas totais, albumina, ureia, creatinina e colesterol total. Esses parâmetros foram avaliados por Castillo et al. (2005) e Turk et al. (2008).

Castillo et al. (2006) demonstraram que houve intensa produção de radicais livres no início do período de lactação em vacas leiteiras, por meio de testes para avaliar os níveis de malonaldeído (MDA; produto da degradação de lipídeos pela peroxidação). Os altos níveis de MDA, detectados na primeira semana de lactação, caracterizaram estresse oxidativo; entretanto, decaíram cerca de 50% a partir da segunda semana. O mesmo teor elevado na produção de MDA foi detectado por Turk et al. (2008), embora em todo o primeiro trimestre de lactação, quando comparado ao restante do ciclo produtivo, incluindo o período seco subsequente.

Celi et al. (2010) testaram dois níveis energéticos na dieta de cabras leiteiras no período pré-parto e não encontraram alteração do perfil oxidativo no plasma dos animais em resposta a esses níveis de energia. Por outro lado, sugeriram que as cabras passaram por estresse oxidativo no período de transição, de acordo com os resultados obtidos para atividades das enzimas GPx e SOD. A atividade da GPx no sangue retornou a menores níveis a partir da segunda semana pós-parto, bem como a atividade da SOD, reduzida a partir da quarta semana pós-parto. Ademais, os resultados de Adela et al. (2006) corroboram os de Celi et al. (2010), porém, em vacas leiteiras, em que a atividade mais intensa de GPx no sangue dos animais ocorreu até a segunda semana de lactação, decrescendo após esse período. O mesmo padrão de resposta para a enzima GPx, ou seja, o aumento na atividade enzimática imediatamente pós-parto, seguida de decréscimo, foi observado por Aitken et al. (2009). Porém, essa atividade foi detectada na enzima especificamente presente no citosol de células do tecido mamário de vacas em início de lactação. Conjuntamente, essas situações sugerem que o organismo predispõe de respostas fisiológicas que podem protegê-lo contra processos pró-inflamatórios no período de transição.

Wullepit et al. (2012) avaliaram os efeitos da suplementação com uma alga marinha específica, rica em ácidos graxos poli-insaturados, sobre o perfil oxidativo de vacas leiteiras. O objetivo era aumentar o teor energético da dieta. Observaram, porém, que a atividade da GPx no plasma dos animais aumentou e a capacidade antioxidante total do plasma, bem como os níveis de α-tocoferol diminuíram no pós-parto, caracterizando o quadro de estresse oxidativo. Segundo os autores, esse resultado decorreu do enriquecimento da dieta com ácidos graxos poli-insaturados, sendo recomendada, então, a adição de antioxidantes na dieta.

O estresse oxidativo pode ocorrer no momento após a alimentação, pois o período pós-prandial é caracterizado por um aumento na susceptibilidade do organismo

a danos oxidativos, após o consumo de dietas ricas em lipídios e/ou carboidratos (Sies et al., 2005). Entretanto, os resultados e recomendações quanto à adição ou uso de antioxidantes na dieta dos animais são feitas por diferentes autores, os quais acabam divergindo entre si e muitas vezes acabam sendo contraditórios (Castillo et al., 2003).

1.4 Doenças metabólicas relacionadas ao estresse oxidativo

O estresse oxidativo pode contribuir ou induzir o animal ao aparecimento de problemas de saúde. De acordo com Miller et al. (1993), o estresse oxidativo está relacionado às doenças como mastite, retenção de placenta e edema de úbere.

Vacas com mastite apresentam níveis aumentados de radicais livres no sangue e diminuição da atividade da GPx em eritrócitos, os quais estão negativamente correlacionados com a prevalência da doença (Ellah, 2010). Em adição, os neutrófilos polimorfonucleares, que atuam na glândula mamária em defesa do organismo contra a mastite, produzem radicais livres de oxigênio, mais especificamente o ânion superóxido, o qual pode desencadear produção de outros radicais livres (Lauzon et al., 2006).

A insuficiência hepática foi associada ao decréscimo da atividade de mecanismos antioxidantes intracelulares, como a GPx, resultando no aumento da produção de radicais livres de oxigênio, especialmente H_2O_2 (Ellah, 2010).

Segundo Castillo et al. (2005), o período de transição apresenta consideráveis desafios metabólicos à homeostase do animal, pela produção de substâncias estressoras, as quais podem contribuir para o aparecimento de diversas desordens. Miller et al. (1993) e Bernabucci et al. (2002) descreveram a variação nos antioxidantes fisiológicos presentes no plasma e nos eritrócitos de vacas em transição. De acordo com Petit (2009), o período de transição tem importantes efeitos na saúde, produção e rentabilidade das vacas leiteiras. O principal problema enfrentado nesse período é a ingestão insuficiente de energia pelo animal, a qual não atinge níveis exigidos para manutenção e produção, resultando em um balanço energético negativo. Esse quadro leva à mobilização de gorduras de reserva corporal e pronunciada alteração no perfil oxidativo do animal (Barnabucci et al., 2005). Vacas durante o período de transição expostas ao calor moderado apresentaram valores elevados de SOD, GPx, tióis intracelulares e maiores níveis de TBARS no plasma, quando comparadas às vacas expostas às temperaturas mais amenas (Bernabucci et al., 2002).

1.5 Antioxidantes usados em dietas de animais leiteiros

O objetivo comum quanto ao uso de antioxidantes nas dietas dos animais leiteiros visa melhorar e/ou aumentar a "carga" ou a defesa do organismo, pelo maior aporte de substâncias antioxidantes, aliado a uma dieta balanceada.

Nesse contexto, um estudo recente foi publicado por Gobert et al. (2009), no qual vacas foram alimentadas com dietas ricas em ômega 3 suplementadas com antioxidantes. A suplementação dietética realizada com vitamina E juntamente com extratos de plantas ricas em polifenóis foi capaz de reduzir a liperoxidação, particularmente no plasma, em vacas em lactação após o pico de produção. A vitamina E foi utilizada na dose de 375 UI por quilo de MS (7.500 UI/vaca/dia) e o extrato de plantas (patenteado pelo *INRA*, França) à base de alecrim, uva, *grapefruit* e calêndula na dose de 10 g/kg de MS. Foram reportadas reduções na produção de malonaldeído e aumento da concentração de vitamina E no plasma, indicando o papel preventivo da utilização desses antioxidantes contra a peroxidação lipídica.

Vázquez-Añón et al. (2008) suplementaram vacas leiteiras com óleo de soja como fonte de gordura, fresco ou oxidado na proporção de 2% da dieta, e em adição o uso de um antioxidante comercial (Agrado Plus®, Novus International, St. Louis, MO). Este composto foi capaz de aumentar a atividade plasmática da SOD e da GPx, entretanto quando combinado com o fornecimento de óleo de soja fresco.

Substâncias como a trealose e a celobiose foram testadas com intuito de proteger vacas leiteiras contra o estresse oxidativo por Aoki et al. (2010). Os dissacarídeos foram fornecidos na dose de 1% da dieta em MS. Tais produtos aumentaram o poder antioxidativo e a atividade da enzima SOD do líquido ruminal a partir de 2h após a alimentação, sendo que e a trealose elevou esse mesmo poder antioxidativo no plasma dos animais. Ambos os dissacarídeos diminuíram a peroxidação lipídica no plasma e no líquido ruminal, estimados em função da produção de MDA. Nesse estudo, particularmente, a trealose foi considerada proveitosa como suplemento para reduzir o estresse oxidativo.

Megahed et al. (2008) publicaram um estudo sobre efeito de um antioxidante comercial, composto por vitamina E e selenito de sódio sobre o perfil oxidativo e a taxa de prenhez em búfalas. Os pesquisadores avaliaram os animais durante os períodos de

verão e inverno, quanto aos níveis plasmáticos de progesterona, estradiol, cortisol e SOD, bem como avaliaram a taxa de prenhez dos animais ao final de cada período. Durante o inverno, a taxa de prenhez dos animais foi superior quando comparada ao verão. Entretanto, durante o verão, os animais que foram tratados com o antioxidante (duas vezes por semana durante seis semanas; dose de 150 mg de vitamina E e 1,67 mg de selenito de sódio) apresentaram maior atividade para a enzima SOD, e níveis mais elevados de estradiol. A taxa de prenhez foi 12,5% superior para animais que receberam antioxidante. Portanto, houve melhora no perfil oxidativo dos animais e elevação dos níveis produtivos em búfalas.

Fontes orgânicas e inorgânicas de Zn, Cu e Se foram testadas por Cortinhas et al. (2010) quanto à sua prevenção contra mastite subclínica, no número de casos de mastite clínica, na contagem de células somáticas (CCS) e na atividade plasmática das enzimas SOD, GPx e ceruloplasmina. A ingestão foi controlada pela administração intraesofágica das quantidades pré-estabelecidas dos minerais. A atividade das enzimas não foi modificada de acordo com a fonte de minerais utilizada, embora fontes orgânicas tenham reduzido o número de mastites subclínicas e a CCS.

Até a atualidade, poucos estudos foram publicados sobre os efeitos antioxidantes advindos da linhaça para vacas leiteiras. Côrtes et al. (2012) suplementaram vacas leiteiras com casca de linhaça e/ou óleo de linhaça e investigaram seus efeitos sobre a atividade das enzimas SOD, CAT e GPx no plasma e na glândula mamária dos animais. A suplementação dietética com casca de linhaça aumentou a atividade no plasma e no tecido mamário da enzima CAT. Os autores também investigaram a abundância do RNA mensageiro relativo à produção das enzimas oxidativas no plasma e na glândula mamária, e verificaram que a casca da linhaça contribui para o aumento de alguns dos genes envolvidos na produção de enzimas no tecido mamário, protegendo esse local contra o estresse oxidativo. Os autores concluíram que a inclusão de casca de linhaça poderia proteger o animal do estresse oxidativo. Entretanto, foi observado que a infusão de óleo de linhaça no abomaso dos animais resultou em decréscimo na atividade da GPx no plasma. Os resultados de da Silva et al. (2007), Liu et al. (2010) e Côrtes et al. (2010) corroboram os de Côrtes et al. (2012), pois mostraram que embora a suplementação lipídica com dietas ricas em ácidos graxos poli-insaturados seja uma estratégia para melhorar a qualidade nutricional do leite e de produtos lácteos; essa técnica pode aumentar o risco de peroxidação do plasma, com consequências deletérias sobre a saúde do animal (Gobert et al., 2009).

De acordo com as informações apresentadas, percebe-se que são poucos os dados publicados sobre a utilização da linhaça e de seus subprodutos, e seus efeitos como antioxidantes. Portanto, ainda não são permitidas inferências sobre sua utilização, e quais os benefícios sobre o perfil oxidativo dos animais.

1.6 Linhaça: composição, propriedades antioxidantes e metabolismo microbiano

A linhaça, de nome botânico *Linum usitatissimum* L., é a semente oleaginosa do linho, pertencente à família das Lináceas. É uma planta herbácea anual, e sua semeadura ocorre nos meses de outono e inverno e sua colheita nos meses de novembro a janeiro, no Estado do Rio Grande do Sul. De clima temperado, necessita de temperaturas perto de 0ºC para floração (Vieira et al., 2012). Os maiores produtores mundiais, em quantidade produzida são o Canadá (com 930 mil toneladas na safra 2009/2012, de acordo com o *Flax Council of Canada*), Estados Unidos, a Índia e China; na América do Sul, os maiores produtores são Argentina, Uruguai e Brasil (Trucom, 2006).

A semente de linhaça contém cerca de 25% de proteína bruta, 34% de fibra solúvel em detergente neutro, 21% de fibra solúvel em detergente ácido e, 31% de extrato etéreo (Petit, 2002). Entretanto, de acordo com Morris (2007), a linhaça é pobre em carboidratos (açúcares e amidos), sendo sua quantia cerca de 1%, assim pouco contribuindo sobre a ingestão total de carboidratos. É rica em ácidos graxos essenciais, e do total de ácidos graxos 50 a 55% são do ácido graxo insaturado α-linolênico (18:3n-3), e ainda contém ácido linoleico, ácidos graxos monoinsaturados e saturados (Galvão et al., 2008). A linhaça é usada basicamente como fonte de óleo e farelo, o qual é utilizado para alimentação animal. A composição bromatológica do farelo de linhaça, adicionalmente comparada a outros concentrados proteicos utilizados na alimentação animal, está descrita na Tabela 1.

Outra característica que justifica o grande interesse pela linhaça é a presença de lignanas vegetais, as quais são compostos fenólicos classificados como fitoestrógenos, os quais podem diminuir a incidência dos sintomas da menopausa, cânceres hormônio-dependentes, doenças cardiovasculares, osteoporose e diabetes em humanos (Prasad, 1997; Prasad, 2000; Raffaelli et al., 2002; Hu et al., 2007; Jin & Hattori, 2011).

Os compostos fenólicos são antioxidantes classificados como primários, pois interrompem a cadeia de reações de oxidação, por meio ou da doação de elétrons ou de átomos de hidrogênio aos radicais livres. Assim, os converte em produtos estáveis ou

podem formar complexos lipídio-antioxidantes, os quais combatem outros radicais livres (Angelo & Jorge, 2007).

Tabela 1. Composição bromatológica do farelo de linhaça, de algodão, de canola e de soja (valores em % da matéria seca; aminoácidos em % da proteína bruta)

	Farelo linhaça[1]	Farelo de algodão[2]	Farelo de Canola[2]	Farelo de soja[2]
MS	91,6	90,2	90,1	88,6
PB	34,3	35,0	40,0	48,8
EE	1,32	1,38	1,32	1,71
FDN	25,0	28,5	30,7	14,6
FDA	16,4	28,87	21,77	9,86
Aminoácidos				
Lisina	3,85	1,45	2,36	2,82
Metionina	1,86	0,62	0,83	0,63
Cistina	1,56	0,51	1,02	0,66
Treonina	3,65	1,27	1,67	1,8
Triptofano	1,66	0,55	0,48	0,67
Fenilalanina	4,93	2,00	1,56	2,34
Leucina	6,00	2,21	2,69	3,62
Isoleucina	4,18	1,11	1,41	2,07
Valina	4,99	1,64	1,85	2,16
Histidina	2,15	1,00	1,04	1,16
Arginina	9,10	3,94	2,36	3,32
Tirosina	2,71	1,21	1,29	1,5
Alanina	4,50	1,64	1,71	2,06
Aspartato	9,14	3,17	2,78	5,5
Glutamato	18,3	7,1	7,0	8,7
Glicina	5,84	1,64	1,98	1,97
Serina	3,88	1,66	1,75	2,47

[1]Eastwood (2008); [2]Valadares Filho et al. (2006).

Particularmente, as isoflavonas (pertencentes à classe dos flavonoides), as lignanas vegetais e mamíferas (sendo as mamíferas o composto fenólico resultante da metabolização bacteriana das lignanas vegetais; Cordeiro et al., 2009) e as cumestanas (derivados das cumarinas) são categorizados como fitoestrógenos. Nestes, o grupamento fenólico pode agir de forma semelhante ao hormônio estrogênio (Wang, 2002).

As lignanas vegetais matairesinol (MAT) e secoisolariciresinol (SECO) são as principais precursoras para formação duas lignanas mamíferas, enterolactona (EL) e enterodiol (ED). São denominados lignanas mamíferas ou enterolignanas, por esses compostos serem produzidos em animais (Raffaelli et al., 2002). Tanto a EL (“trans-2,3-bis(3-hidroxibenzil)-γ-butirolactona”) quanto o ED (“2,3-bis(3-hidroxibenzil)butano-1,4-diol”), são considerados os dois mais importantes

fitoestrógenos dentre as lignanas mamíferas, pois são as lignanas mais recorrentes no soro, urina, bile e líquido seminal de humanos e animais (Wang, 2002). Entretanto, as formas glicosídicas de MAT e SECO também são possíveis precursores de ED e EL, sendo: matairesinosídeo, secoisolariciresinol diglucosídeo (SDG), secoisolariciresinol 4-*O*-β-$_D$-glucopiranosídeo e mais seis outros compostos (Wang et al., 2002).

A mais rica fonte de lignanas vegetais é a linhaça (*Linum usitatissimum*; Prasad, 1997), pois possui as maiores concentrações de SECO, quando comparadas a qualquer outro alimento (de 28 a 369 mg/100 g), além de pinoresinol e MAT (Raffaelli et al., 2002). De acordo com Morris (2007), a linhaça é também fonte mais rica de SDG, um glicosídeo da SECO. A SDG representa mais de 95% de todas as suas lignanas e é, principalmente, encontrada na porção fibrosa da linhaça (Adlercreutz & Mazur, 1997). Consequentemente, a proporção de SDG é maior na casca comparativamente às sementes inteiras (Côrtes *et al*., 2008), e resultados de pesquisas sugerem uma correlação negativa entre a presença de óleo e seu conteúdo de SDG (Novello & Pollonio, 2009). Alguns teores de SECO e MAT podem ser observados na Tabela 2, e teores de EL e ED na Tabela 3.

Tabela 2. Quantidade de lignanas secoisolariciresinol (SECO) e matairesinol (MAT) em alguns alimentos

Alimento	SECO	MAT
	(μg lignanas / 100 g alimento em matéria seca)	
Centeio	47	65
Cevada	58	0
Linhaça	369.900	1087
Chá verde chinês	2890	195

Fonte: adaptado de Raffaelli et al. (2002).

Tabela 3. Produção de lignanas mamíferas a partir de alguns alimentos*

Alimento	Enterolactona	Enterodiol
	(μg lignanas / 100 g alimento em matéria natural)	
Farelo de linhaça	8.517	59.024
Farinha de linhaça	11.818	40.861
Soja	693	170
Girassol	201	195
Trigo	411	79
Sorgo	199	56
Milho	199	31
Centeio	69	91
Cevada	41	74

Baseado em técnica "in vitro", utilizando-se como inóculo fezes humanas, para simulação de fermentação colônica. Fonte: Adaptado de Thompson et al. (1991).

A atividade antioxidante dos compostos advindos da linhaça (SDG, SECO, ED e EL) foram comparados à vitamina E e mensurados por Prasad (2000). Foi testada a capacidade desses compostos em reduzir a quimioluminescência de leucócitos polimorfonucleares, os quais produzem ânions superóxido, peróxido de hidrogênio, radicais hidroxila e oxigênio singlete. Esse método monitorou a produção dessas substâncias reativas ao oxigênio e a capacidade sequestrante dos compostos da linhaça. Apesar de o estudo não descrever especificamente quais compostos sequestram quais substâncias reativas ao oxigênio, de maneira geral a SECO, ED, EL e SDG tiveram capacidades antioxidantes 4,86, 5,02, 4,35 e 1,27 vezes maiores comparativamente à vitamina E. Ademais, a EL se mostrou mais eficiente em prevenir a foto-oxidação e oxidação por calor comparada à SDG, quando avaliadas em produtos lácteos (Matumoto-Pintro et al., 2010).

Em monogástricos, a SDG é convertida em SECO sob ação dos micro-organismos intestinais. Eles convertem SECO principalmente em ED e EL, as lignanas mamíferas (Clavel *et al*., 2006; Morris, 2007).

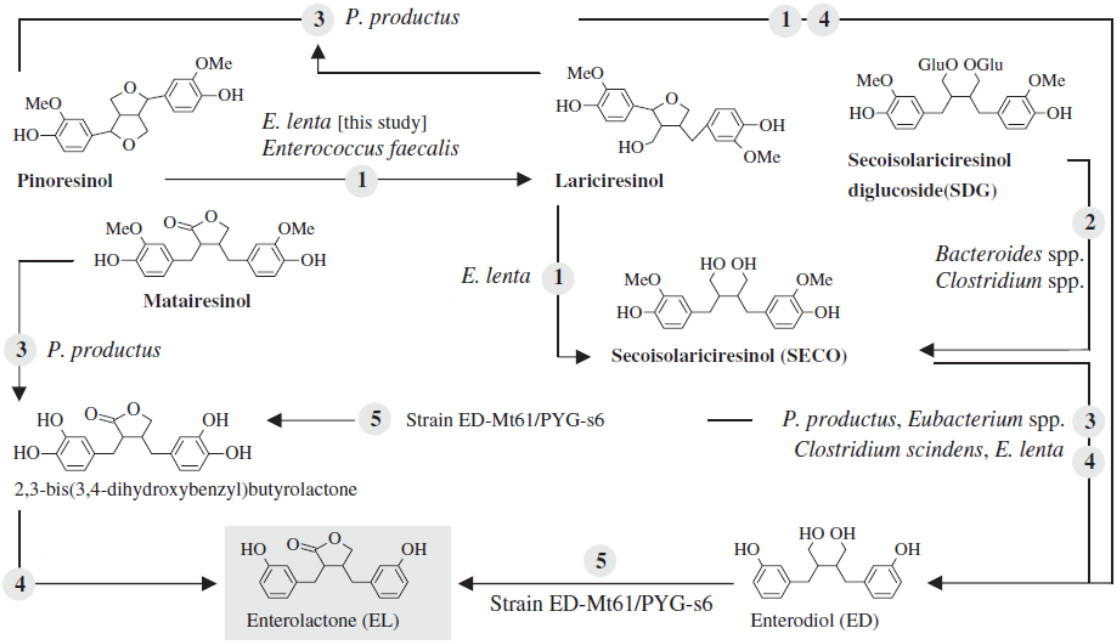

Figura 1. Rotas para produção de EL de várias fontes vegetais. Números indicam as reações catalisadas pelas bactérias intestinais dos humanos: (1) redução, (2) deglicolisação, (3) demetilação, (4) dehidroxilação, (5) desidrogenação
Fonte: adaptado de Clavel et al. (2006).

A conversão da lignana SDG em lignanas mamíferas em humanos pode ser descrita por quatro tipos de reações catalíticas: O-deglicolisação, O-demetilação, desidrogenação e desidroxilação (Wang *et al.*, 2000; Clavel *et al*., 2006). Algumas das

bactérias intestinais de humanos envolvidas nas reações catalíticas descritas são cepas de *Klebsiella* (Wang *et al*., 2010), *Bacteroides distasonis, B. fragilis, B. ovatus*, *Clostridium cocleatum*, *Clostridium* sp. SDG-Mt85-3Db, *Butyribacterium methylotrophicum, Eubacterium callendari, E. limosum, Peptostreptococcus productus, Clostridium scindens, Ruminococcus productus*, *Eggarthella lenta* and ED-Mt61/PYG-s6 (Wang, 2002; Clavel *et al*., 2005; Clavel *et al*., 2006). A figura elaborada por Clavel et al. (2006) compilou diversos estudos demonstrando rotas, reações e micro-organismos envolvidos na conversão de lignanas vegetais em lignanas mamíferas.

Estudos conduzidos por Côrtes *et al.* (2008), Petit & Gagnon (2009) e Zhou *et al*. (2009) demonstraram que os microrganismos ruminais pode metabolizar as lignanas da linhaça em EL, sendo semelhante à situação do trato gastrintestinal humano. Em estudo posterior, utilizando vacas fistuladas no rúmen, foi comprovado que o rúmen é o principal local em que se dá o metabolismo das lignanas da linhaça em ruminantes (Gagnon et al., 2009) e a EL é a maior lignana mamífera que pode ser encontrada no leite (Petit & Gagnon, 2009). Esses estudos corroboram os de Lundh et al. (1990) os quais reportaram que a absorção dos fitoestrógenos ocorriam no rúmen.

Zhou et al. (2009) avaliaram o metabolismo das lignanas em cabras, administrando-as via fístula ruminal na quantia de 1 mg de SDG por quilo de peso vivo. Os autores encontraram no líquido ruminal maiores quantias de ED comparativamente à EL, entretanto, ambos considerados baixos. Após 14 dias de administração de SDG, os níveis de EL no plasma foram duas vezes maiores que os níveis de ED. Ademais, os autores utilizaram a técnica de reação em cadeia da polimerase (do inglês *Polimerase chain reaction*, PCR) e de eletroforese em gel de gradiente desnaturante (do inglês *Denaturing Gradient Gel Electrophoresis*, DGGE) para identificar possíveis microrganismos ruminais envolvidos na conversão de SDG em EL e ED. A principal bactéria identificada, por meio de técnicas moleculares empregadas no estudo, foi a *Ruminococcus gnavus*. Esta mesma bactéria foi caracterizada pela produção da enzima β-glicuronidase em humanos (Beaud & Anba-Mondoloni, 2005), enzima esta capaz de clivar as lignanas mamíferas, permitindo sua posterior absorção intestinal (Gagnon et al., 2009).

A EL produzida no rúmen é excretada na urina, sangue e no leite, indicando que os fitoestrógenos podem ser transferidos aos fluidos corporais (Gagnon et al., 2009). De fato, a concentração de EL no leite de vacas aumenta quando produtos de linhaça são incorporados à dieta dos animais, e a concentração de EL no leite aumenta linearmente

de acordo com a inclusão de farelo de linhaça na dieta de vacas leiteiras (Petit et al., 2009; Petit & Gagnon, 2009; 2011). Entretanto, o estudo conduzido por Zhou et al. (2009b) comprovou que a SDG, por sua infusão via cânula ruminal, pode ser absorvida pela parede ruminal, uma vez que esta substância foi encontrada no plasma dos animais.

Uma vez que a EL tem mostrado atividade antioxidante (Prassad, 2000), níveis elevados de EL no leite poderiam prevenir sua oxidação (Matumoto-Pintro et al., 2011) e aumentar o tempo de prateleira do leite e produtos lácteos. Entretanto, maiores esforços devem ser realizados no sentido de aumentar a concentração de EL no leite, pois o estudo de Matumoto-Pintro et al. (2010) demonstraram que em vacas alimentadas com 15% de farelo de linhaça (com base na matéria seca), a transferência de EL para o leite foi baixa, não permitindo detectar seu efeito de proteção da oxidação do produto.

Dentre todas as informações expostas, verifica-se que pesquisas com uso de antioxidantes na alimentação animal devem continuar, pois se mostram de grande importância tanto para melhora do sistema produtivo, promovendo melhor desempenho, melhores condições de saúde e de bem-estar aos animais, quanto visam melhorar o produto dos mesmos como, por exemplo, o leite. Entretanto, percebe-se que ainda serão necessários grandes esforços, multidisciplinares, para que os resultados esperados sejam obtidos.

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OBJETIVOS GERAIS

Avaliar o efeito de níveis crescentes de farelo de linhaça na dieta de vacas leiteiras sobre a produção e a composição do leite e a ingestão de matéria seca; o perfil oxidativo dos animais; produção e concentração de enterolactona em diferentes fluídos corporais bem como a relação entre elas; o perfil fermentativo das dietas; e, avaliar quais as possíveis bactérias ruminais responsáveis pela conversão da lignanas vegetais presentes no farelo de linhaça, em lignanas mamíferas, em especial a enterolactona.

CAPÍTULO I

(British Journal of Nutrition)

Mammary gene expression and activity of antioxidant enzymes and oxidative indicators in blood, milk, mammary gland and ruminal fluid of dairy cows fed flax meal

Short title: Flax meal and antioxidant enzymes

Abstract

The objectives of the study were to evaluate the effects of dietary supplementation of flax meal (FM) on the activity of antioxidant enzymes (superoxide dismutase, SOD), glutathione peroxidase (GPX) and catalase (CAT)) in blood, mammary gland tissue and ruminal fluid, on the production of thiobarbituric acid reactive substances (TBARS) and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity in milk, plasma and ruminal fluid of dairy cows. The mRNA abundance of antioxidant enzymes and oxidative stress related genes was also assessed in the mammary gland tissue. Eight Holstein cows were used in a double 4 × 4 Latin square design. There were four treatments: control with no FM (CON), or 5% FM (5FM), 10% FM (10FM), and 15% FM (15FM) in the diet. Treatment had no effect on SOD activity. GPX activity increased in plasma upon FM supplementation, presenting linear tendency (P=0.13) for a FM effect 3 h after feeding, as a result of an interaction between treatment and time. There was an increased CAT activity in mammary gland tissue and erythrocytes and a decreased TBARS production in milk only for cows fed 5FM and 10FM. Cows fed 15FM had higher CAT and nuclear factor (erythroid-derived 2)-like 2 **(**NFE2L2) mRNA abundance in the mammary gland compared with those fed CON. These findings suggest that FM supplementation could improve the oxidative status of Holstein cows in mid to late lactation as suggested by increased GPx activity in plasma and increased CAT and NFE2L2 mRNA abundance in the mammary gland.

Keywords: Catalase, DPPH, Glutathione peroxidase, Superoxide dismutase, TBARS

High-yielding dairy cows are prone to oxidative stress due to the intensive metabolic demands for maintenance and production. This condition can be exacerbated under certain environmental, physiological, and dietary factors[2]. Although lipid supplementation of ruminant diets with *n*-3 polyunsaturated fatty acids (PUFA) is a strategy to improve the nutritional quality of dairy products, this approach could increase the risk of plasma peroxidation with deleterious consequences on animal health[3]. Peroxidation results from the oxidative metabolism, which is essential for survival of cells. However, a side effect of this phenomenon is the production of free radicals and other reactive oxygen species that can cause oxidative damages[2]. Normally, the body is protected by a wide range of antioxidant systems working in concert with intracellular enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT), which remove superoxides and peroxides before they react with metal catalysts to form more reactive compounds[4].

Lactation performance and antioxidant status of cows fed oxidized fat are enhanced when antioxidants are included in the diet[5], which may be due to scavenging of peroxides and reduced peroxidation of fatty acids[6]. Supplementing antioxidants such as vitamin E and sodium selenite from early summer (*e.g.*, period characteristic of heat stress) may improve fertility through a decrease in cortisol secretion and oxidative stress, which enhances pregnancy rates in buffalo cows[7]. Moreover, strong positive correlations between several antioxidant enzymes (*e.g.*, GPX) and proinflammatory-vascular adhesion molecules suggest a protective response of antioxidants to an enhanced proinflammatory state in transition dairy cows[8]. In addition, the study of Gobert *et al.*[3] has shown that the association of plant polyphenols and vitamin E reduces plasma lipoperoxidation in dairy cows supplemented with a rich PUFA diet. Antioxidants then could contribute to enhance defense mechanisms against oxidative stress with various immunity, reproductive, and health benefits.

Plant lignans are natural strong antioxidants and flax (*Linum usitatissimum*) is known as the richest dietary source of lignans[9]. Prasad[10] reported that millimolar concentrations of the plant lignan secoisolariciresinol diglucoside (SDG) and its mammalian metabolites enterodiol and enterolactone inhibit reactive oxygen species following an *in vitro* incubation of venous blood. This is of particular interest as many chronic diseases are characterized by an oxidative stress component[11]. More recently, Côrtes *et al.*[12] have shown that flax hulls increase SOD activity and SOD1 mRNA abundance in the mammary gland of dairy cows. These findings corroborate those of

Rajesha *et al.*[13] who reported that flax antioxidants enhance rats' endogenous defense by upregulating the expression of genes encoding for antioxidant enzymes such as SOD, CAT and GPX. As flax meal (FM) contains less than 5% of residual oil[14], it is richer in plant lignans than flax seed on a dry matter (DM) basis, which makes it a better source of antioxidants. To our knowledge, there is no information available on the effect of FM on the activity and expression of antioxidant enzymes in dairy cows. We hypothesized that dietary FM decreases oxidative stress indicators in physiological fluids (*e.g.*, blood, ruminal fluid and milk) and enhances those of oxidative status. Therefore, the present study investigated the effects of increased concentrations of FM on the activity of SOD, CAT and GPX enzymes, the DPPH radical scavenging activity and lipid peroxidation (TBARS production) in blood, milk, mammary gland and ruminal fluid of dairy cows, and the mRNA abundance of oxidative stress related genes in mammary gland tissue.

Material and Methods

Animals and diets

Eight multiparous Holstein cows fitted with ruminal cannula (10 cm, Bar Diamond Inc., Parma, ID, USA) were assigned to four treatments in a double 4 × 4 Latin square design with four diets and four 21 d periods balanced for residual effects. Cows averaged 686 (SE 35) kg of body weight and 112 (SE 21) d in milk at the start of the experiment. The cows were housed in individual stalls and had free access to water. Diets were offered in equal amounts twice daily at 08.30 and 15.30 hours for *ad libitum* intake (10% refusals on as-fed basis) and cows were milked twice daily at 08.00 and 19.00 hours. The diets were isonitrogenous and isoenergetic and they were formulated to meet requirements for cows having 657 kg of body weight and producing 37.7 kg of milk with 3.8% fat per d[15]. National guidelines for the care and use of animals were followed as recommended by the Canadian Council on Animal Care[16]. The four treatments (Table 1) were control with no FM (CON), or pre-planned inclusion of 5% (5FM), 10% (10FM), and 15% (15FM) FM in the DM. The FM used in this study was prepared using the expeller meal method (*i.e.*, mechanical extraction of meal), which leaves around 5% of residual oil[14].

Experimental procedures

Feed intake and milk yield were measured daily throughout the experiment and data were averaged over the 7 days of the third week of each period and subjected to analysis of variance. Samples of the diet were taken once weekly and pooled within period. All

samples were frozen at -20°C for subsequent drying at 55°C and further analysis. On day 21, milk samples were taken from the two consecutive milkings and pooled to the corresponding milk yield. One sample was stored at 4°C with a preservative (bronopol-B2; DNF Company, Dublin, CA, USA) until analyzed for fat, lactose, protein, and urea N. Another sample was taken and 0.02% (w/w) of sodium azide was added and kept frozen at -80°C for further analysis of TBARS and DPPH.

On day 20 of each period, blood samples (60 mL) were collected before the morning meal and 3 h post-feeding from the caudal vein into vacutainer tubes (Becton Dickinson and Cie, Rutherford, NJ, USA) containing K_3-EDTA (0.47 mol/L)[3]. Plasma was isolated from blood by centrifugation at 3,000 ***g*** for 12 min at 4°C and stored at −80°C to determine enzyme activities, TBARS and DPPH. The remaining erythrocytes were stored at -80°C for subsequent analysis of enzyme activity. Biopsies of the mammary gland were taken on day 21 of each period using the method of Farr *et al*.[17] and alternating between the left and right hindquarters. Although inflammation was restricted to a very small area and disappeared within two days, a site at least 10 cm apart from the first one was chosen when a quarter was used for the second time. Tissue obtained from the biopsies was rinsed in sterile saline solution to remove all traces of blood, cut into two parts: one was immediately frozen in liquid N and stored at -80°C for gene expression analyses. The other half was ground immediately with a rotor-stator homogenizer and stored at -80°C for further analysis of antioxidant enzyme activity.

On day 20, 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) to obtain representative samples. The ruminal contents were then strained through four layers of cheesecloth, and the filtered ruminal fluid of each sampling time was stored at -80°C for further chemical analysis. Ruminal liquid was thawed later and one portion was used to determine TBARS production. Another portion was centrifuged at 800 ***g*** for 10 min at 4°C to remove protozoa; the supernatant was taken and centrifuged at 13,700 ***g*** for 25 min at 4°C to remove debris of bacteria (Sorvall RC-6 Plus Superspeed Centrifuge, Thermo Scientific, ON, Canada) and the supernatant was used directly to analyze activity of antioxidant enzymes. For DPPH analysis, 75 μL of the supernatant was mixed with methanol (2.55 mL) during 30 sec and centrifuged at 9,809 ***g*** for 15 min and the upper layer was used to perform DPPH analysis.

Chemical analysis

Concentrations of DM, ether extract, acid-detergent fibre (ADF), neutral-detergent fibre (NDF) and total N in diets were analyzed according to the procedures described by Côrtes *et al*.[12]. Fat, lactose, protein, and urea N concentrations in milk samples were analyzed by infrared spectrophotometry (System 4000 Milkoscan; Foss Electric of Hillerod, Denmark) following procedure 972.16 of AOAC (1990). Somatic cells were counted using an optical somatic cell counter (Fossomatic 90; Foss Electric of Hellerod).

The activity of GPX (EC 1.11.1.9), SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) in plasma, erythrocytes, ruminal fluid and mammary gland was determined enzymatically. Activities of SOD, CAT and GPX were analyzed using commercial assay kits (Cayman Chemical, Ann Harbor, MI, USA) according to the manufacturer's instructions. Details of these analyses were described previously[12]. The maximum intra- and inter-assay coefficients of variation for SOD and GPX analysis were 10% and 10%, respectively, while the maximum intra- and inter-assay coefficients of CAT were 12.5% and 10.1%. Total protein concentration in plasma, erythrocytes, ruminal fluid and mammary gland was determined with a bicinchoninic acid protein assay (Sigma-Aldrich, St. Louis, MO, USA).

Determination of DPPH in plasma, ruminal fluid and milk was done according to the procedures of Brand-Williams *et al*.[18] and Martinez *et al*.[19] using a stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), with some modifications. Concentration of the DPPH (Sigma-Aldrich D9132) solution was 200 μM in methanol, prepared 1 h before use, and samples were read in polypropylene 96 well-plates. Concentration of the DPPH solution was the same for plasma, milk and ruminal fluid. Plasma samples were prepared according to Martinez *et al*. [19]. Milk extracts were prepared by mixing 0.75 ml of milk and 3 ml of methanol. Samples were then vortex mixed for 15 sec at high speed and kept at -20°C for 48 h. The milk extract was then centrifuged at 2000 ***g*** for 15 min at 4°C and the supernatant was used to perform the DPPH analysis. For each sample, five different volumes of milk extract (50, 100, 125, 250, and 500 μL) were added to 500 μL of the DPPH solution and dilutions were done in duplicate. An aliquot of 250 μL of each dilution was placed in a 96 well-plate as well as 250 μL of the DPPH solution (control) and 250 μL of methanol (blank). The reduction of DPPH was determined at 515 nm after 30 min for plasma and milk, and after 25 min for ruminal fluid. The assay was performed in triplicate. The antioxidant capacity was calculated

according to the method of Li *et al.*[20]. A linear relationship was obtained between antioxidant capacity and the volume dilution of samples as described by Smet *et al.*[21] and the EC_{50} was calculated. Lipid peroxidation was assessed in plasma, ruminal fluid and milk in original samples using a commercially available TBARS assay kit (OXI-TEK TBARS Assay Kit, Zepto Metrix Co., New York, NY, USA) according to the manufacturer's instructions.

RNA extraction and complementary DNA synthesis

Total RNA was extracted from mammary gland tissue and complementary DNA synthesis was performed as previously described[22]. Integrity and purity of extracted RNA was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

Real-time PCR amplifications of studied genes

The relative mRNA abundance of studied genes was determined using real-time PCR amplification. PCR amplification, detection and data analyses were performed using an ABI 7500 Fast Real-time PCR System (PE Applied BioSystems, Foster City, CA, USA). Primer pairs were designed using the Primer Express software 3.0 (PE Applied BioSystems). A detailed description of primer sequences, GenBank accession numbers and amplified products size of the CAT, SOD1, SOD2, SOD3, GPX1 and GPX3 genes was published previously[12]. Forward 5'-GTACCCCTGGAAATGTCAAACAG-3' and reverse 5'-TGTGATGACGACAAAGGTTGGA-3' primers (NM_001011678, 88 bp amplicon) were used for the *NFE2L2* gene and the following primer pair: forward 5'-CTCAAAGCAGCAGGAGCAGA-3' and reverse 5'-CGGTACGACCCCTTCATCC-3' (NM_001076409, 102 bp amplicon) was used for the nuclear factor of kappa light polypeptide gene enhancer in B-cells (*NFKB*) gene. The PCR amplifications were carried out in a 10 µl reaction volume containing primers (final concentrations ranging from 150 to 900 nM), 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). Cycling conditions were 2 min at 50ºC, followed by 10 min at 95ºC and 40 cycles of 3 sec at 95ºC and 30 sec at 60ºC. Specificity of amplified fragments was determined for all genes using the melting curve analysis. Amplification of the reference genes (RG) glyceraldehyde-3-phosphate dehydrogenase (GAPDH), peptidylpropyl isomerase A (PPIA), actin beta (ACTB)[12] and polyubiquitine (forward

5'-TGGAGCCCAGTGACACCAT-3' and reverse 5'-GGCCATCTTCCAGCTGCTT-3' primers, NM_174133, 111 bp amplicon) was also performed and the NormFinder algorithm[23] was then used to identify the least affected by treatments. Polyubiquitine was identified as the best RG for normalization of the current study data sets. PCR amplifications were performed in triplicate and standard curves were established in duplicate for each gene. Standard curves were composed of serial dilutions of complementary DNA pools[22] and were used to obtain the relative mRNA abundance of studied genes using the standard curve method as described by Applied BioSystems[24].

Statistical analysis

All data were analyzed as a 4 × 4 double Latin square design using the MIXED procedure of SAS, release 9.2 (SAS 2002; SAS Institute, Cary, NC, USA) according to the model:

$$Y_{ijkl} = \mu + T_i + P_j + Q_{lk} + A/Q_k + e_{ijkl}$$

Where Y_{ijkl} = the response variable, μ = overall mean, T_i = global effect of treatment (i = CON, 5FM, 10FM and 15FM), P_j = the fixed effect of period (j = 1 to 4), Q_k = fixed effect of square (k = 1, 2), A/Q_{lk} = random effect of cow within square, and e_{ijkl} = residual error. Treatments were compared by contrasts in order to test the polynomial effects (linear, quadratic and cubic) of FM. Data on TBARS, DPPH and enzyme activities in plasma, ruminal fluid, erythrocytes and milk were analyzed as repeated measurements and the compound symmetry was used as the covariance structure. When a significant interaction ($P \leq 0.10$) occurred between treatment and time, the effect of treatment was examined within each time group, and then treatment effects were compared at the relevant time. Similar approach was performed by Barret et al.[25]. The relative mRNA abundance values, collected from mammary gland tissue were analyzed using a one-way ANOVA followed by multiple comparisons of all treatments (5FM, 10FM and 15FM) to the control (CON). Differences were declared when $P \leq 0.10$, and a trend when $0.10 < P \leq 0.15$.

Results

There was a linear effect of treatment on DM intake as a result of higher intake with an increased level of FM in the diet (Table 2). Concentration of FM in the diet had no effect on milk production and composition and yield of milk components (Table 2). The only exception was the proportion of lactose in milk that showed linear (P=0.10),

quadratic (*P*=0.03) and cubic (*P*=0.09) effects with an increasing level of FM in the diet.

Production of TBARS, expressed in terms of malondialdehyde (MDA) equivalents (nmol/ml), was lower in the milk of cows fed 5FM and 10FM (20.2 and 22.1, respectively) than in the milk of those fed CON and 15FM (24.5 and 25.3, respectively) as shown by quadratic (*P*=0.009) and cubic (*P*=0.006) effects of treatment (Figure 1).

Flax meal supplementation had no effect on TBARS production in plasma (*P*=0.43), with average values of 3.60, 4.05, 3.90, and 4.05 nmol/ml (SE 0.28) for CON, 5FM, 10FM, and 15FM, respectively. However, concentration of TBARS in plasma was reduced (*P*=0.04) 3 hours after feeding regardless of FM supplementation, with mean values of 4.1 and 3.7 nmol/ml at 0 and 3 hours, respectively.

There was an interaction (*P*=0.01) between time and treatment for TBARS measurement in ruminal fluid; when cows were supplemented with FM, there was a linear (*P*=0.01) reduction in TBARS at 2 hours after feeding and levels rose thereafter to become similar among treatments. Cows fed CON showed no reduction in TBARS production after feeding.

Radical scavenging activities determined in milk, plasma and ruminal fluid by the DPPH technique was not affected by FM supplementation. Values of EC_{50} were similar among diets and averaged 26.70, 46.50 and 8.67 µl/ml, respectively, in milk, plasma, and ruminal fluid. Regardless of treatment, there was a time effect on DPPH in ruminal fluid (*P*<0.0001). The mean values of EC_{50} in ruminal fluid at 0, 2, 4, and 6 h after feeding were 11.1, 10.8, 10.6, and 10.8 µl/ml, respectively. Supplementation with FM had no effect on SOD, CAT and GPx activity in the mammary gland and erythrocytes.

Supplementation with FM had no effect on the activity of SOD in plasma, erythrocytes, ruminal fluid and mammary gland, with values that averaged, respectively, 0.13, 16.33, 11.58 and 33.71 U/mg of protein. Values of GPX activity were similar among diets and averaged 33.68 and 1.18 nmol/min/mg of protein in mammary gland tissue and erythrocytes, respectively. However, there was an interaction (*P*=0.03) between treatment and time (Figure 2) for plasma GPX activity. There were quadratic (*P*=0.002) and cubic (*P*=0.07) effects of FM supplementation before feeding, and a linear tendency (*P*=0.13) for a FM effect 3 h after feeding. Cows supplemented with 5% and 10% FM had an increase in GPX activity over time (0 *versus* 3 hours) while the

absence and the inclusion of 15% FM in the diet decreased GPX activity from 0 to 3 hours. Supplementation with 15FM was able to maintain higher values of GPX activity in plasma when compared with the CON diet and other treatments.

The activity of CAT was not altered by treatments in mammary gland tissue and erythrocytes, and values averaged 47.95 and 207.92 U/mg, respectively. There was an interaction (P=0.04) between treatment and time for CAT activity in plasma (Figure 2). Before feeding, there were quadratic (P=0.01) and cubic (P=0.01) effects of FM supplementation and there was no effect of FM supplementation (P>0.15) 3 hours post-feeding. When 5% and 10% FM were supplemented, activities of GPX and CAT were increased post-feeding while a decrease was observed with the CON and 15FM diets.

There was a trend for an overall treatment effect (P=0.106) for NFE2L2 mRNA abundance in the mammary gland when using the one-way ANOVA, with the 15FM treatment showing higher NFE2L2 mRNA abundance values when compared with the CON treatment (Figure 3, P=0.048) when using the multiple comparisons of treatments to CON. In contrast, the GPX1, GPX3, SOD1, SOD2, SOD3 and NFKB mRNA abundance was not affected by treatment. Cows that were fed 15FM had higher levels of CAT mRNA abundance than those fed the CON diet (Figure 3, P =0.098).

Discussion

The oxidative status of dairy cows was monitored in the present study by the production of TBARS, which represents peroxidation of lipids. The MDA production quantified through the TBARS assay is one of the biomarkers of the damage caused in the body by reactive oxygen and nitrogen substances[26]. Flax meal supplemented cows presented an initial reduction in TBARS of ruminal fluid, but the levels returned or exceeded initial concentrations over time. According to Sadeghian & Kojouri[27], a greater period of time required to reach basal levels of MDA depend upon antioxidants supplementation and could indicate enhanced antioxidant activity. Cows fed no FM showed no reduction in TBARS production in ruminal fluid after feeding. Therefore, this could suggest that within the first few hours (*i.e,* < 4) of feed consumption, antioxidants present in FM may have contributed to protect dietary lipids against oxidation in the rumen as suggested by the linear decrease in TBARS with the inclusion of FM in the diet.

Production of TBARS was lower in the milk of cows fed 5FM and 10FM than in the milk of those fed the CON and 15FM diets and there was no difference among treatments for TBARS production in plasma. However, all diets resulted in lower

plasma TBARS production after feeding, which can be associated with the consumption of certain feed ingredients. Indeed, many feedstuffs contain glutathione, tocopherols, ascorbate, uric acid, and β-carotene, which may improve plasma non-enzymatic antioxidant indicators[26].

Radical scavenging activity was assessed by the DPPH technique, which measures modifications in antioxidant activity[18]. Supplementation with FM had no effect on the DPPH determined in milk, plasma and ruminal fluid although there was a time effect in ruminal fluid. According to the method used in the present study and described previously by Smet *et al.*[21], the lower the EC_{50} value, the higher the content of antioxidants. To our knowledge, only two studies have previously observed an increase in antioxidant activity of ruminal fluid measured by the DPPH method 2 h after feeding. The first one is that of Gizi *et al.*[28] who compared faunated *versus* defaunated cattle (protozoa free) and the second one is that of Aoki *et al.* [29] who supplemented cows with trehalose and cellobiose, two disaccharides with and without antioxidant activity, respectively. According to Gizi *et al.* [28], the level of antioxidant in ruminal fluid increases due to the supply of lipids or fat-soluble compounds which contain carotenoids and vitamins, and/or to antioxidants obtained from the metabolism of ruminal microorganisms. In the present study, a similar increase in antioxidant activity was observed in ruminal fluid after feeding although this occurred regardless of treatment, thus corroborating the results of GIzi *et al.*[28] that distinct nutrients and microbial production of antioxidants may be responsible for increased antioxidant activity in ruminal fluid.

Superoxide dismutase is considered the first intracellular defense against reactive oxygen molecules[30]. This enzyme causes a dismutation of the superoxide anion radical to hydrogen peroxide[26], which is further degraded by the catalase and peroxidase actions. In the present study, the addition of FM had no effect on the activity of SOD. These results then may suggest that the same amount of hydrogen peroxides was produced in body fluids and tissues of animals fed or not FM. According to Hosada *et al.*[31], a lack of alteration in antioxidant enzymes could be due to a sufficient pool of non-enzymatic antioxidant substances likely present in the defense system of ruminants. Activity of SOD in plasma of dairy cows has been shown to increase with antioxidant supplementation, but the effect was dependent on the type of fat fed, with the highest activity being observed when oxidized fat was added to the diet[5]. This is corroborated with the fact that supplementation of non-oxidized lipids in the form of flax oil infusion

in the abomasum has had no effect on plasma, erythrocytes and mammary gland SOD activity of dairy cows[12].

Values of GPX activity were similar among diets although there was an interaction between treatment and time for plasma GPX activity. The highest values of GPX activity in plasma maintained with 15FM may suggest that a proportion of 15% FM in the diet may provide enough antioxidants to improve the oxidative status of cows. An increase in plasma GPX activity of dairy cows has also been observed by Vazquez-Anon *et al*[5] who fed diets containing antioxidants and oxidized or non-oxidized soybean oil. Wullepit *et al.* [32] fed marine algae (source of *n*-3 fatty acids) to cows and they attributed the increase in plasma GPX activity to the PUFA supply from algae and its induction of lipoperoxidation. On the other hand, Côrtes *et al*. [12] observed a decrease in plasma GPX activity when they fed *n*-3 PUFA to lactating dairy cows. Taken altogether, these results suggest that the ingestion of antioxidant compounds may act directly on the GPX enzyme system, where they can protect against plasma lipoperoxidation even when PUFA are supplied in the diet.

The same treatment effects were observed on both plasma GPX and CAT activities, which are enzymes degrading hydrogen peroxides. According to Celi[33], when SOD activity increases production of hydrogen peroxides, a protection from reactive oxygen substances would only be provided by a simultaneous increase in CAT and GPX activity and availability of glutathione. However, in the present study, the simultaneous increase observed for GPX and CAT activities in plasma likely was not due to an increase in SOD activity as SOD activity was not affected by treatment.

Supplementation with FM had no effect on SOD, CAT and GPX activity in the mammary gland and erythrocytes. Although the red cells are one rich source of antioxidant enzymes, they are also a significant source of superoxide generation in biological systems[26]. As the production of superoxide ions and oxygen peroxides occurs under oxidative stress[28], this may suggest that the lack of a treatment effect on enzyme activities was a result of the cows that were in good health, mid-lactation, and positive energy balance. Changes in the activity of erythrocyte antioxidant enzymes in dairy cattle have been reported only when animals were in the transition period, which may result from cows that are under oxidative stress[1].

The highest NFE2L2 mRNA abundance was observed in the mammary gland of cows fed 15FM while the mRNA abundance of GPX1, GPX3, SOD1, SOD2, SOD3 and NFKB was similar among diets. The NFE2L2 gene, also known as NF-E2-related factor

2 (NRF2), encodes for a transcription factor that plays essential roles in cellular defense against oxidative stress[34]. This transcription factor activates the expression of a series of genes having an antioxidant response element (ARE) in their promoters, including antioxidant proteins and phase 2 detoxifying enzymes[35, 36]. Of interest, flax lignans are composed of polyphenolic compounds present in plants as glycoside conjugates[37] and many natural polyphenol containing compounds have the ability to modulate NRF2-mediated cellular events[38, 39]. In this study, cows that were fed 15FM had higher levels of CAT mRNA abundance than those fed the CON diet. Since CAT is a known downstream target of NFE2L2, it can be hypothesized that polyphenolic compounds present in FM activate NFE2L2, which would then bind to the CAT ARE, thus increasing CAT mRNA abundance. The reason for the lack of a treatment effect on GPX1, GPX3, SOD1, SOD2 and SOD3 genes remains to be determined since SOD1, CAT and GPX are all considered as established Nrf2-regulated genes[40]. Moreover, the SOD2 gene was recently identified as a potential novel transcription regulatory target of Nrf2 in mouse's lungs although it seems that Nrf2 may negatively regulate its expression[41]. It was earlier demonstrated that the basal activity level of CAT was lower in $Nrf2^{-/-}$ mice cardiac fibroblasts as compared with those of $Nrf2^{+/+}$ cells, whereas there were no differences in the basal SOD and GPX activities[36]. When $Nrf2^{+/+}$ cells are incubated with D3T, a chemoprotective agent, significant increases in CAT, SOD and GPX activities are observed and the increase is completely abolished in $Nrf2^{-/-}$ cells for CAT and SOD but not for GPX. This observation suggests that D3T upregulation of GPX activity in cardiac fibroblasts is independent of Nrf2 activation, thus demonstrating that CAT, SOD and GPX respond differently to Nrf2 activation. Taken altogether, these results suggest that Nrf2 activation of antioxidant enzymes may vary according to the cell type and to the different antioxidant compounds used. In a previous study, we have reported that cows fed 9.88% flax hulls, a rich source of the plant lignan SDG, have higher levels of CAT, GPX1 and SOD1 mRNA in the mammary gland and lower mRNA abundance of GPX3, SOD2 and SOD3 than cows fed no flax hulls[12]. These discrepancies may be explained by the higher oil content found in flax hulls (30% of ether extract in the DM) compared with FM (5% of residual oil content in the DM)[14]. Since we have shown that flax oil modulates antioxidant enzymes mRNA abundance in the cows' mammary gland, differences in the lipid content between flax hulls and flax meal may indeed account for some of the observed discrepancies between our data and those of Côrtes *et al.*[12].

In conclusion, the present study shows that flax meal supplementation can improve the oxidative status of Holstein cows in mid to late lactation as suggested by increased GPX activity in plasma and NFE2L2/Nrf2 and CAT mRNA abundance in the mammary gland. Further studies are required to clarify the role of flax meal on the oxidative status of cows during the transition and early-lactation periods as cows are more prone to oxidative stress during these periods. Any beneficial effects of flax meal on the oxidative status of cows could lead to a prophylactic strategy against diseases affecting the health status of dairy cattle.

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Table 1. Ingredient and nutrient composition of total mixed diets of Holstein cows fed no flax meal (CON), or 5 (5FM), 10 (10FM) and 15% (15FM) flax meal

	Treatments			
	CON	5FM	10FM	15FM
Ingredients (g/100 g DM)				
Maize silage	292.2	290.8	289.8	290.6
Grass silage	315.3	315.7	317.5	313.9
Ground maize grain	211.3	202.3	191.6	189.0
Soybean meal	107.6	74.5	41.4	20.4
Flax meal	0	47.9	95.3	140.6
Top supplement[*]	17.4	17.4	17.9	8.5
Beet pulp	34.3	29.6	25.0	16.1
Calcium carbonate	5.5	5.5	5.4	5.8
Minerals and vitamins[†]	16.4	16.3	16.3	15.3
Chemical composition[‡§]				
DM (g/100 g diet)	37.7	37.9	37.6	38.1
Crude protein (g/100 g DM)	17.0	17.4	17.6	17.9
Fat (g/100 g DM)	2.4	2.4	2.4	2.4
Acid-detergent fibre (g/100 g DM)	18.3	18.5	19.2	19.3
Neutral-detergent fibre (g/100 g DM)	28.4	28.6	29.5	29.6
Net energy for lactation (MJ/kg DM) [§]	6.65	6.61	6.61	6.61

[*]Contained 20% of canola meal, 30% of corn gluten meal, 20% of soybean meal, and 30% of brewer's corn.

[†‡]The premix contained (per kg of premix): 92 g Ca, 47.9 g P, 47.8 g Mg, 15.2 g S, 137.2 g Na, 13.7 g K, 19.5 mg Se, 23 mg I, 2013 mg Fe, 1068 mg Cu, 1796 mg Mn, 2657 mg Zn, 57 mg Co, 265 mg F, 442,000 IU(463.1 µmol/l) of vitamin A, 56,670 IU (3536208 nmol/l) of vitamin D_3, and 2630 IU (40986 µmol/l) of vitamin E.

[‡]Mean of four samples that were prepared by compositing 3 samples collected once a week and pooled within period.

[§]Calculated using published values of feed ingredients (National Research Council, 2001).

Table 2. Dry matter intake (DMI), milk yield and milk composition of Holstein cows fed no flax meal (CON), or 5 (5FM), 10 (10FM) and 15% (15FM) flax meal

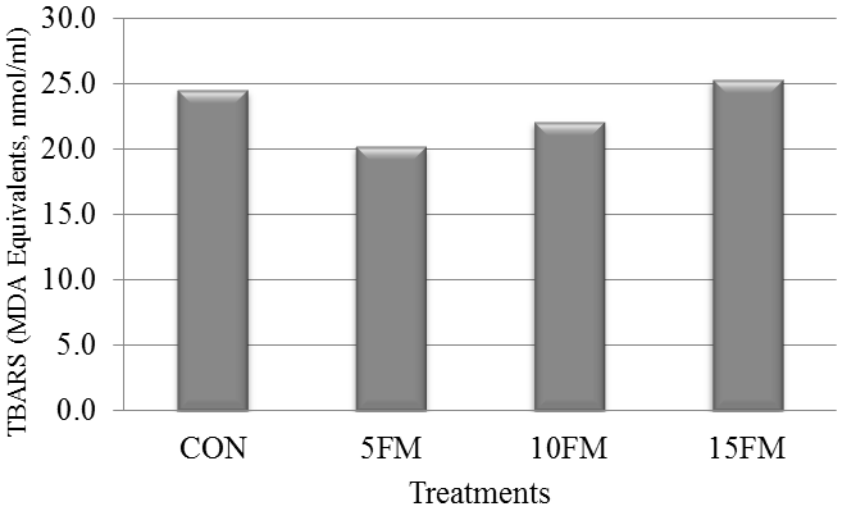
	Treatments					
	CON	5FM	10FM	15FM	SEM	*P*
DMI (kg/d)	21.03	20.63	21.15	22.02	0.315	0.03*
DMI (% of body weight)	3.03	3.03	3.17	3.27	0.071	0.07*
Milk yield (kg/d)	35.5	36.5	36.0	36.0	0.772	0.85
Milk composition (%)						
Fat	3.69	3.57	3.40	3.63	0.091	0.16
Lactose	4.49	4.60	4.57	4.56	0.025	0.03†
Protein	3.58	3.52	3.55	3.53	0.031	0.50
Urea N	10.82	10.67	10.39	11.27	0.639	0.80
Total solids	12.74	12.67	12.49	12.69	0.097	0.30
Yield of milk components (kg/d)						
Fat	1.32	1.27	1.24	1.29	0.033	0.38
Lactose	1.63	1.66	1.69	1.65	0.037	0.70
Protein	1.28	1.25	1.29	1.25	0.032	0.70
Total solids	4.58	4.53	4.58	4.54	0.092	0.96
Somatic cell score‡	1.19	1.61	2.03	2.02	0.378	0.37

*linear (*P*=0.01) effect of treatment.

†linear (*P*=0.10), quadratic (*P*=0.03) and cubic (*P*=0.09) effects of treatment.

‡ Log_{10} (somatic cell score).

(a)



(b)

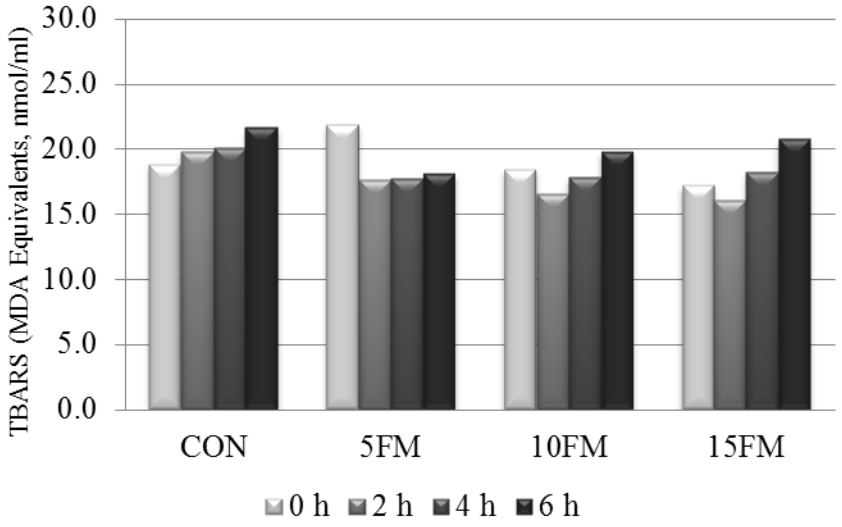
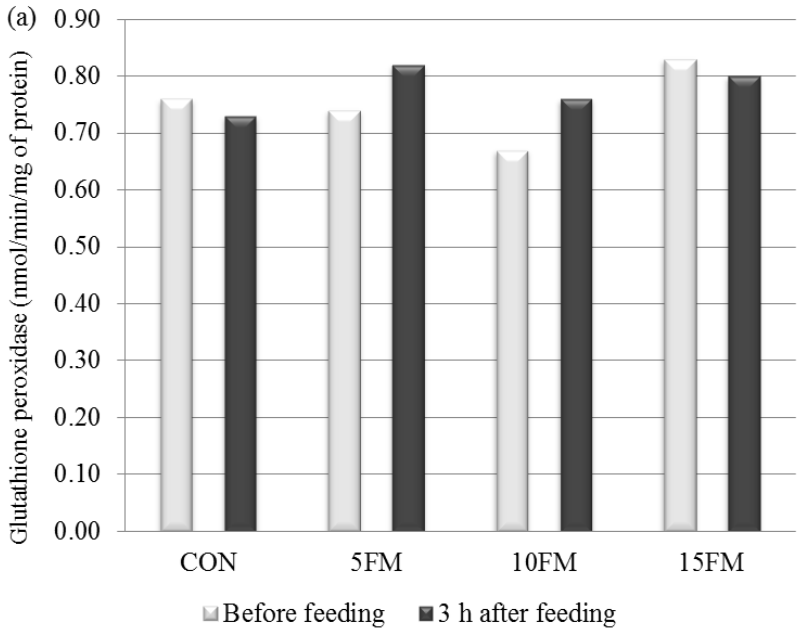


Fig. 1. TBARS production (MDA equivalents) in milk (a) and ruminal liquid (b) of Holstein cows fed no flax meal (CON), or 5 (5FM), 10 (10FM) and 15% (15FM) flax meal in the diet. Standard errors were 1.28 for milk. Standard errors were 1.39, 0.85, 0.99 and 1.10 for 0, 2, 4 and 6 h after feeding, respectively, for ruminal liquid. Production of TBARS was lower in the milk of cows fed 5FM and 10FM than in the milk of those fed the CON and 15FM diets as a result of quadratic (P=0.009) and cubic (P=0.006) effects of treatment. There was an interaction (P= 0.01) between time and treatment for TBARS in ruminal fluid.

(a)



(b)

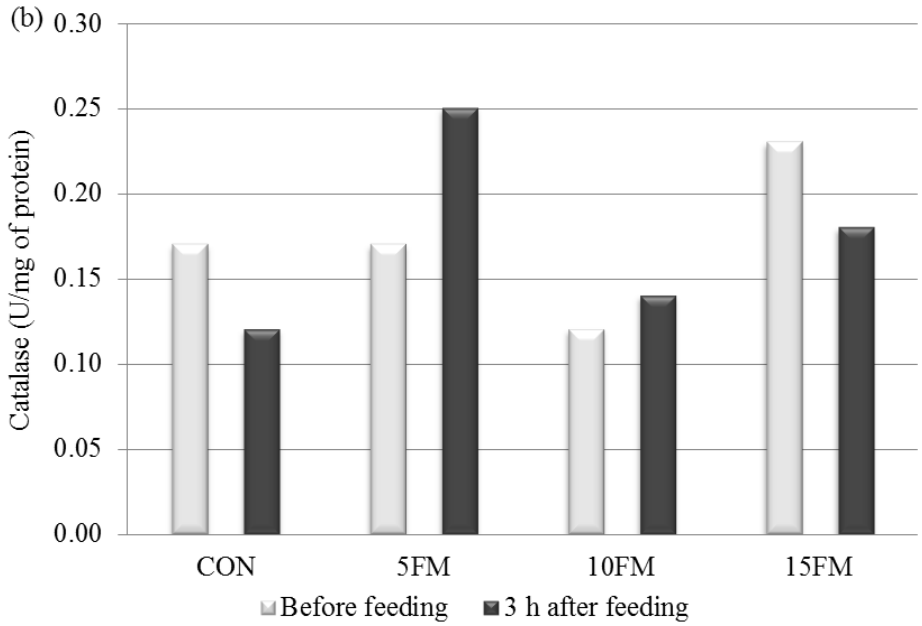


Fig. 2. Activity of glutathione peroxidase (GPx) (a) and catalase (CAT) (b) in plasma of Holstein cows fed no flax meal (CON), or 5 (5FM), 10 (10FM) and 15% (15FM) flax meal in the diet. Standard errors were 0.02 for GPx and 0.02 and 0.04 for CAT at 0 and 3 h after feeding, respectively. There was an interaction between treatment and time for plasma GPx and CAT activities (P=0.03 and 0.04, respectively).

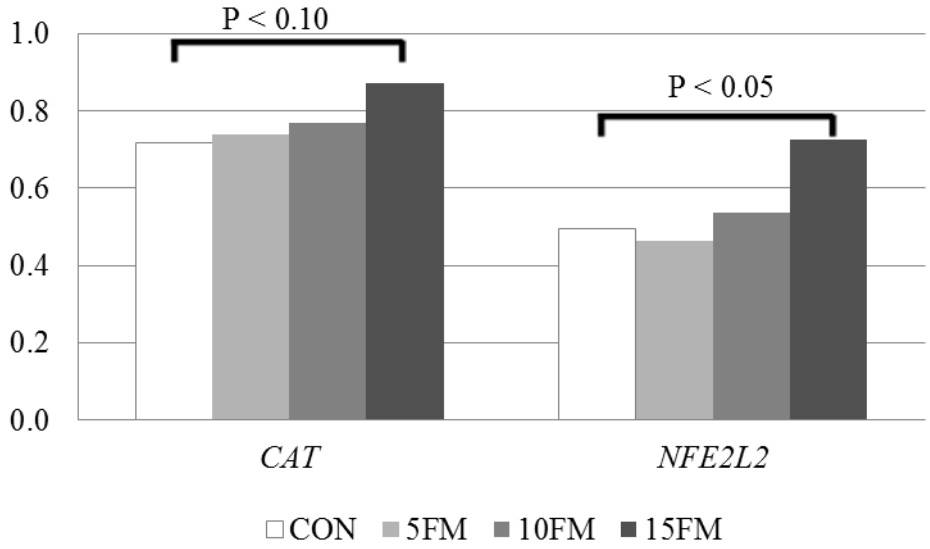


Fig. 3. Relative mRNA abundance of the catalase (CAT) and nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) genes in mammary gland biopsies of Holstein cows fed no flax meal (CON), or 5 (5FM), 10 (10FM) and 15% (15FM) flax meal in the diet. Results are presented as least-squares means of 7 animals from analysis performed in triplicates. The maximum standard errors of the mean were 0.063 and 0.101 for CAT and NFE2L2, respectively. There was a significant difference between CON and 15FM for CAT and for NFE2L2 (P=0.098 and 0.048, respectively).

CAPÍTULO 2
(British Journal of Nutrition)

β-glucuronidase activity and enterolactone concentration in ruminal fluid, plasma, urine, and milk of Holstein cows fed increased levels of flax (*Linum usitatissimum*) meal

Short title: Flax meal and ruminal metabolism

Abstract

Ruminants are able to convert plant lignans into mammalian lignans in the rumen. The main mammalian lignan present in milk of dairy cows fed flax products is enterolactone (EL). However, the relationship between concentration of EL in milk and other body fluids when cows are fed increased proportions of flax meal (FM) in the diet is unknown. The objectives of the present study were to investigate the effects of increased levels of FM on EL concentration in milk, plasma, urine and ruminal fluid. The activity of ß-glucuronidase, which plays an important role for absorption of mammalian lignans in non-ruminant animals, was also determined in ruminal fluid and faeces. Eight multiparous rumen-cannulated cows were assigned to a double 4 × 4 Latin Square design with four 21-d periods and four diets: a control diet with no FM (CON) or a diet with 5, 10 and 15% of FM. Concentrations of EL in urine, ruminal fluid, milk and plasma increased linearly with FM supplementation. Spearman's correlation coefficients were significant for all comparisons except that only a trend was observed between concentration of EL in urine and that in ruminal fluid before feeding. The highest correlation was observed between EL concentration in ruminal fluid 2 h after feeding and that in milk. Feeding increased proportions of FM in the diet, which may have resulted in greater intake of lignans, had no effect on ß-glucuronidase activity of ruminal fluid and faeces. Unlike to what is observed in non-ruminant animals, results of the present experiment may suggest that the activity of ß-glucuronidase in the rumen is of little importance for the absorption of EL and its transfer in milk and other physiological fluids. Further studies are required to better understand and

improve EL production and absorption, which could contribute to enhance animal health and the transfer of antioxidant components in milk.

Flax products are interesting feed ingredients for inclusion in the diet of lactating dairy cows as a source of both energy and protein[1]. Flax meal contains about 366 g/kg of crude protein, 47 g/kg of ether extract, 289 g/kg of neutral detergent fiber and 1.64 g/kg of secoisolariciresinol diglucoside (SDG)[2]. According to Morris[3], flax is a poor source of carbohydrates (sugars and starches), with concentrations around 1%. Flax then is not considered a glycemic ingredient[4], although the mucilage presents in flax contains glucose, xylose, rhamnose and galactose[5]. Indeed, Knegsel et al.[6] consider flax meal as a lipogenic ingredient, once possess lipogenic nutrients as long chain fatty acids with degrees of saturation. Flax contains also high levels of arginine, aspartate and glutamate[4], and these high proportions of amino acids are similar in flax meal[7].

Flax is a well-known source of antioxidant compounds, mainly due to the presence of lignans[8]. Lignans, such as isoflavones and coumestans, are phytoestrogens, which are substances with a phenolic group that make them similar in structure to the estrogenic steroids hormones[9], such as 17β-estradiol[10]. Phytoestrogens have been linked to lower incidence of monopausal symptoms, hormone-dependent cancers, cardiovascular diseases, diabetes and osteoporosis in humans[11-15].

The SDG is one of the most abundant plant lignan[16]. Flax is the richest source of SDG[11], with more than 95% of the total lignans in flax as SDG[17-18]. The proportion of SDG is higher in hulls than seeds[19] as lignans are concentrated in the outer fiber-containing layers of flax. In humans, SDG is converted in secoisolariciresinol (SECO) under the action of the microbial enzyme glycosidases in the intestine[20]. The SECO is converted mainly into enterodiol (ED) and enterolactone (EL)[3,16]. The ED and EL reach the intestine and are conjugated as sulfate and glucuronide that are further excreted in body fluids[21]. Deconjugation enhances ED and EL reabsorption, through the cleavage by microbial ß-glucuronidase enzymes[13,22]. The use of oral antimicrobials decreases serum EL concentration in humans, demonstrating the fundamental role of intestinal bacteria for the conversion of plant into mammalian lignans[15].

Phytoestrogens of the isoflavones family, formonentin and daidazen, are absorbed in the rumen as shown by Lundh et al.[23]. Therefore, the ruminal microbiota plays an important role in the metabolism of phytoestrogens as shown by the conversion of flax lignans into EL that occurs mainly in the rumen [2, 19, 24-25]. The main mammalian lignan present in the milk of cows fed flax products is EL[2] and EL concentration in milk increases when flax products are fed to dairy cows. Petit and Gagnon[2,26-27] demonstrated that EL concentration in milk increases in parallel with whole flax seed or flax meal inclusion in the diet of dairy cows and that the increase in milk EL concentration was higher with the latter. Linear increases also were observed in EL concentration of plasma, milk and urine of cows when flax hulls were placed in the rumen (via cannula), demonstrating that rumen is the main site for metabolism of flax lignans[24]. However, the relationships between concentrations of EL in milk and other body fluids when cows are fed increased proportions of flax meal in the diet are unknown. Moreover, in ruminant animals, importance of the activity of ß-glucuronidase, which enhances absorption of mammalian lignans in non-ruminant mammals, is unknown. Therefore, the objectives of the present study were to investigate the relationships between concentrations of EL in milk and other body fluids when cows are fed increased proportions of flax meal in the diet and to determine the role of ß-glucuronidase for EL absorption.

Materials and methods

Animals and experimental diets

The experiment procedures were approved by the Canadian Council on Animal Care[28]. The study was conducted using eight multiparous Holstein rumen-cannulated (10cm, Bar Diamond Inc., Parma, ID, USA) cows that averaged 686 (SE 35) kg and 112 (SE 21) days in milk. The animals were assigned in a double 4 × 4 Latin Square design with four 21-d periods and four treatments. The cows had free access to water and were housed in individual stalls. The diets were offered in equal amounts twice daily at 08.30 and 15.30 hours for *ad libitum* intake (10% refusals on as fed basis) and were milked twice daily at 08.00 and 19.00 hours. The diets were formulated according to NRC[29] for cows that weighed 657 kg and produced 37.7 kg/d of milk with 3.8% of fat. The experimental diets

were isonitrogenous and isoenergetic (Table 1) and consisted of a control diet (CON) with no flax meal or inclusion (dry matter basis) of 5% FM (5FM), 10% (10FM) and 15% FM (15FM) in the diet.

Sampling, pH monitoring and volatile fatty acids analysis

Milk samples were taken twice daily on day 21, pooled to the corresponding milk yield, and frozen at -20°C for determination of EL concentration. Blood samples were also collected into K_3EDTA-vacutainer tubes (Becton Dickinson and Cie, Rutherford, NJ, USA) from the caudal vein 6 h after the morning meal. Plasma was isolated from blood by centrifugation at 3,000 ***g*** for 12 min at 4^{o}C and the samples were kept frozen at -20^{o}C until EL analysis. Samples of urine were collected on day 21, 2 h after the morning meal by hand stimulation of the perineal region and kept frozen at -20°C for EL analysis. Ruminal contents were collected 0, 2, 4, and 6 h after the morning meal from different locations within the rumen (the cranial dorsal, cranial anterior ventral, medium ventral, caudal dorsal and caudal ventral locations) to obtain a representative sample. Ruminal pH was monitored immediately after sample collection with a portable pH meter (Oakton; Eutech Instruments Pte Ltd, Singapore). The ruminal contents were then strained through four layers of cheesecloth. A 350-ml sample of strained ruminal fluid was mixed with ruminal retentate (26 g) and frozen at -20^{o}C until assay for ß-glucuronidase activity. Another portion of filtered ruminal fluid for each sampling time was acidified to pH 2 with 50% H_2SO_4 and frozen at -20^{o}C for later determination of volatile fatty acids (VFA) and ammonia N concentrations. Ruminal ammonia concentration was determined according to Weatherburn[30] modified for use with a plate reader. The quantification of VFA in the rumen was performed using a gas chromatograph (model 5890; Hewlett-Packard Labs, Palo Alto, CA) with a capillary column (30-m × 0.32-mm i.d., 1-µm phase thickness, Zebron ZB-FAAP; Phenomenex Inc., Torrance, CA, USA) fitted with a flame ionization detector. The oven temperature was 170°C held for 4 min, which was then increased by 5°C/min to 185°C, and then by 3°C/min to 220°C, and held at this temperature for 1 min. The injector temperature was 225°C, the detector temperature was 250°C, and the carrier gas was helium. Another portion of ruminal fluid was kept at -20°C and freeze-dried for further analysis of EL. In addition, ruminal samples for the three post-feeding times (2, 4

and 6 h) were pooled within cow and period as previously carried out by Gagnon et al.[24] to obtain only one composite sample for EL analysis. Moreover, fecal grab samples (250 g) were collected directly from the rectum 2, 4, 6, and 8 h post-feeding on day 21. Faecal pH was monitored immediately after sample collection. Faecal samples were pooled on a fresh weight basis and kept frozen at -20°C for further ß-glucuronidase analysis.

Lignan extraction, enterolactone and β-glucuronidase analysis

Lignans in ruminal fluid, plasma, urine and milk samples were hydrolysed, extracted and analyzed as described by Gagnon et al.[24]. Determination of EL was performed using an EIA kit (Cayman Chemical, Ann Arbor, MI, USA). Faecal and ruminal samples were treated and analyzed as described by Gagnon et al.[24] for determination of ß-glucuronidase activity. The protein content of faeces and ruminal fluid was determined by a bicinchoninic acid protein kit (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis

Data on EL concentrations were transformed (log) as previously performed by Nesbitt et al.[25]. All data were analyzed as a double 4 × 4 double Latin square using the MIXED procedure of SAS (SAS 2000; SAS Institute, Cary, NC, USA) according to the model:

$$Y_{ijklm} = \mu + T_i + P_j + Q_k + R_l + TR_{il} + A/Q_{lk} + e_{ijklm}$$

Where Yijklm = dependent variable; μ = overall mean, Ti = fixed effect of treatment (i = CON, 5%FM, 10%FM and 15% FM), Pj = fixed effect of period (j = 1 to 4), Qk = fixed effect of square (k = 1, 2), Rl = fixed effect of time (l = 0, 2), TRil = interaction between treatment and time, A/Qlk = cow within square, and eijklm = random residual error. The model was augmented with time and time by treatment interaction for repeated measurements and values are reported with their adjusted mean values with standard errors. Significance was declared at $P<0.05$ and trend at $P<0.10$. The Spearman's correlation test was used to determine strength of the relationships among samples analyzed with the CORR procedure of SAS (2000; SAS Institute, Cary, NC, USA).

Results

Enterolactone production and ß-glucuronidase activity

Concentrations of EL in urine and ruminal fluid (Fig 1a) and milk and plasma (Fig 1b) increased linearly (P=0.01, <0.0001, <0.0001 and 0.0001, respectively) with higher concentration of FM in the diet. The Spearman's correlation coefficients (ρ) were statistically significant for all tested combinations (Table 2) except for that between EL concentration in urine and ruminal fluid at time 0 (immediately before feeding) where only a trend (P=0.1002) was observed. The highest correlation coefficient was between concentration of EL in the rumen 2 h after feeding and that of EL in milk (0.76), followed by correlation between EL in ruminal fluid at time 0 (immediately before feeding) and 2 h after feeding (0.75). The lowest coefficient of correlation was observed between EL concentrations in urine and ruminal fluid at time 0.

There was no interaction between treatment and sampling time and no effect of time on the activity of ß-glucuronidase (nmol phenolphthalein/min/mg) in ruminal fluid and faeces. ß-glucuronidase activity in faeces and ruminal fluid averaged, respectively, 8.20 and 2.48 nmol phenolphthalein/min/mg and it was similar among diets.

Ruminal fermentation characteristics and pH of ruminal fluid and faeces

There were an interaction (P=0.02, 0.01, and 0.008, respectively) between sampling time and treatment for propionate, isovalerate, and caproic acid (data not shown). The interaction between sampling time and treatment for the proportion of propionate in the rumen was a result of lower proportion before feeding for cows fed the FM diets compared to those fed the CON diet. Although there were interactions between sampling time and treatment for the proportions of isovalerate and caproic acid, values varied only from 1.26 and 1.68 and from 0.40 to 0.54, respectively, which likely was of little biological significance. The concentrations of total and individual VFA were similar among treatments (Table 3). Therefore, only mean values for all treatments, according to the sampling time, are presented in Table 3. There was no significant treatment effect or interaction between treatment and sampling time for ruminal fluid and faecal pH. However,

regardless of treatment, there was a decrease and an increase (P<0.0001), respectively, in ruminal fluid pH and faecal pH overtime.

Ammonia concentration in ruminal fluid

There was no interaction between hour and treatment (P=0.19) for ammonia N concentration in ruminal fluid. Cows fed with FM tended (P=0.05) to have higher ammonia N concentration in ruminal fluid when compared with those fed no FM. A time effect was observed for all treatments (P<0.0001), with concentrations of ammonia increasing 2 h after feeding and decreasing afterwards (Figure 2).

Discussion

Previous studies have shown that increasing dietary levels of FM[2], whole flaxseed[27] and flax hulls[28] increases linearly EL concentration in milk although higher concentrations are observed when flax hulls or meal compared to flax seed are supplemented[2,28]. However, to our knowledge, this is the first *in vivo* study investigating the relationship between concentration of EL in milk and other body fluids when cows are fed increased proportions of FM in the diet.

The highest concentration of EL among the physiological fluids studied was found in urine as previously reported by Gagnon et al.[24]. However, prior to being excreted in the urine, mammalian lignans such as EL are absorbed by the intestine and then reach body fluids[21,32] and different organs. Indeed, Saarinen et al.[20] have shown the presence of lignans in liver, kidney, breast tumors, spleen, skin, lung, brain, uterus and adipose tissues of rats. Lignans are polyphenolic compounds with antioxidant properties[12], and there is an inverse relationship between blood concentrations of EL and the incidence of cardiovascular diseases[33]. Taken altogether, these results then suggest that a greater concentration of EL in tissues may contribute to enhance antioxidant function and health of mammal animals.

Under the action of microflora, the plant lignan SDG is converted mainly in the two mammalian lignans enterodiol and EL[3,16]. In humans, EL is absorbed by the intestine as sulphates and glucuronides conjugates[21] and then excreted in physiological fluids, or they

return to the intestinal lumen via enterohepatic circulation[34]. They also can be excreted in urine and faeces[35]. In the present experiment, concentration of EL in milk presented the highest correlation with EL concentration in ruminal fluid at 2 hours after feeding, suggesting that the conversion of plant into the mammalian lignan EL in the rumen is responsible for the increased EL concentration in milk. Therefore, these data may indicate that EL is absorbed directly through the rumen wall and that the concentration of EL in milk depends directly on the concentration of EL in ruminal fluid. Lundh et al.[23] and Adams[36] have previously found that isoflavones, which also belong to the class of phytoestrogens as lignans and coumestans, are absorbed through the ruminal wall. Similarly, a pure infusion of the plant lignin SDG in the rumen of goats has increased EL concentration in the rumen and blood[37], and also SDG was found in plasma after infusion, further indicating that lignans are metabolized in the rumen and can be absorbed through the rumen wall.

In non-ruminant animals, the activity of ß-glucuronidase plays an important role for optimal absorption of mammalian lignans[13]. In humans, microbial ß-glucuronidase activity is positively correlated with the level of plant lignans intake and urinary excretion of mammalian lignans[22]. However, in the present experiment, feeding increased proportions of FM in the diet, which may have resulted in greater intake of lignans, had no effect on ß-glucuronidase activity of ruminal fluid and faeces. Unlike to what is observed in non-ruminant animals, results of the present experiment may suggest that the activity of ß-glucuronidase in the rumen is of little importance for the absorption of EL in the rumen. This is corroborated by the fact that lower ruminal ß-glucuronidase activity has no effect on the conversion of flax lignans in EL in the rumen of dairy cows[38]. However, the importance of deconjugation in the rumen for further absorption of mammalian lignans is not well understood in ruminant animals.

The weak correlation (P=0.10) between EL concentration in urine and ruminal fluid before feeding can result of the lower concentration of EL in ruminal fluid before feeding than after feeding (data not presented).Concentration of EL in urine was quantified 2 hours after feeding and time is required for absorption and transfer in the organs and fluids.

The data set presented on the fermentative profile of cows fed increasing levels of dietary flax meal suggest that the pattern of ruminal fermentation was little altered by the inclusion of FM and that EL production was not limited by variations in ruminal pH.

Ruminal pH around 6 could slightly decrease fiber digestion and change the ruminal microbiota, mainly the fibrolytic bacteria[39]. In the present experiment, ruminal pH ranged between 5.9 (four hours after feeding) and 6.3 (immediately before feeding) (Table 3). This low variation may have resulted from the use of calcium carbonate as a buffering substance, which ensures low oscillations in ruminal pH. Therefore, it is unlikely that the pH was a limiting factor for the conversion of plant into mammalian lignans. Then, the reactions required for SDG conversion into EL, such as deglycolysation, demethylation, dehydroxylation and dehydrogenation[16,40] are not affected by the change in ruminal pH overtime.

Increased levels of FM in the diet had little effect on VFA production in the rumen, suggesting that the substitution of soybean meal for FM had little effect on ruminal fermentation. The trend (P=0.06) for higher ruminal isobutyrate concentration with increased dietary levels of FM was likely a result of higher concentrations of amino acids such as arginine, aspartate and glutamate in FM than soybean meal[4]. Indeed, isoacids production in the rumen of dairy cows increases with higher ruminal digestibility of flax protein[41]. Moreover, protein ruminal degradability of FM is greater than that of soybean meal[42], which may further enhance availability of amino acids for isoacids production in the rumen. This higher ruminal degradability of protein from FM than soybean meal also could explain the trend (P=0.05) for increased concentration of ammonia N in the rumen. According to Zhou et al.[37], a crude extract of SDG promotes the utilization of non-protein N in the rumen as shown by decreased ammonia N concentration and increased microbial protein synthesis in the rumen.

This study provided information on EL concentration in the rumen when feeding increased levels of FM in the diet of dairy cows. There were significant correlations between EL concentrations of milk, urine, plasma and ruminal fluid taken 2 h after feeding. The highest correlation was between concentration of EL in ruminal fluid and that in milk. Unlike to what is observed in non-ruminant animals, results of the present experiment may suggest that the activity of ß-glucuronidase in the rumen is of little importance for the

absorption of EL in the rumen and its transfer in milk and other physiological fluids. Further studies are required to better understand and improve EL production and absorption, which could contribute to enhance animal health and the transfer of antioxidant components in milk.

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Table 1. Ingredient and chemical composition of total mixed diets of Holstein cows fed no flax meal (CON) or 5 (5FM), 10 (10FM) and 15% (15FM) flax meal in the DM

Ingredients (g/kg DM)	Diets			
	CON	5FM	10FM	15FM
Corn silage	292.2	290.8	289.8	290.6
Grass silage	315.3	315.7	317.5	313.9
Ground corn	211.3	202.3	191.6	189.0
Soybean meal	107.6	74.5	41.4	20.4
Top supplement[†]	17.4	17.4	17.9	8.5
Beet pulp	34.3	29.6	25.0	16.1
Calcium carbonate	5.5	5.5	5.4	5.8
Mineral and vitamins[‡]	16.4	16.3	16.3	15.3
Flax meal	0	47.9	95.3	140.6
Chemical analysis[§]				
DM (%)	37.7	37.9	37.6	38.1
CP (% DM)	17.0	17.4	17.6	17.9
ADF (%DM)	19.6	19.9	20.3	20.3
NDF (% DM)	31.2	31.5	31.9	31.9
Net energy for lactation (MJ/kg DM)[¶]	6.65	6.61	6.61	6.61

[†]Contained 20% of canola meal, 30% of corn gluten meal, 20% of soybean meal, and 30% of brewer's corn

[‡]The premix contained (per kg): 92 g Ca, 47.9 g P, 47.8 g Mg, 15.2 g S, 137.2 g Na, 13.7 g K, 19.5 mg Se, 23 mg I, 2013 mg Fe, 1068 mg Cu, 1796 mg Mn, 2657 mg Zn, 57 mg Co, 265 mg F, 442,000 IU vitamin A (463 µmol/l); 56,670 IU vitamin D, (3536 µmol/l); 2630 IU vitamin E (40 986 µmol/l).

[§]Mean of three samples that were prepared by compositing weekly samples collected once per week and pooled within period

[¶]Calculated using published values of feed ingredients[(29)]

Table 2. Spearman's correlation coefficients between EL concentrations of milk, plasma, urine and ruminal fluid (Rum) of Holstein cows fed no flax meal or 5, 10 and 15% flax meal in the diet.

Variables	EL Milk	EL plasma	EL urine	EL Rum 0 h	EL Rum 2 h
EL Milk	-				
EL plasma	0.61¶	-			
EL urine	0.48$^{|}$	0.64¶	-		
EL Rum 0 h	0.56¶	0.4§	0.30‡	-	
EL Rum 2 h	0.76¶	0.55¶	0.39§	0.75¶	-

Significance level: $^{‡}P<0.10$; $^{§}P<0.05$; $^{|}P<0.01$; $^{¶}P<0.001$.

Table 3. Mean values of volatile fatty acids concentration in ruminal fluid, according to the sampling time, and pH of ruminal fluid and faeces, from Holstein cows fed no flax meal or 5, 10 and 15% of flax meal in the diet (mean of four diets, in dry matter basis)

	Sampling time (hours after feeding)					
	0	2	4	6	SEM	*P*
Total VFA (mmol/l)	104.44	104.36	103.31	106.48	2.433	0.92
Molar proportions (mmol/mol)						
Acetate	596.2	605.5	599.4	599.6	3.94	0.79
Propionate‡	242.3	238.1	245.7	244.2	4.77	0.93
Butyrate	113.0	108.9	109.3	108.3	2.01	0.75
Isovalerate‡	1.54	15.7	13.1	15.5	0.47	0.14
Valerate	14.5	14.5	14.4	16.3	0.87	0.31
Isobutyrate	8.3	9.2	8.6	9.5	0.18	0.06
Lactate	5.6	3.0	5.4	1.5	1.68	0.17
Caproic Acid‡	4.7	5.1	4.2	5.2	0.24	0.44
Molar ratio						
Acetate: Propionate	2.4	2.5	2.4	2.4	0.82	0.10
P/ (A+B)	0.3	0.3	0.3	0.3	0.80	0.31
pH						
Ruminal Fluid	6.32	6.00	5.92	6.00	0.035	0.49
Faeces	6.51	6.57	6.66	6.67	0.028	0.77

‡Interaction between treatment and time

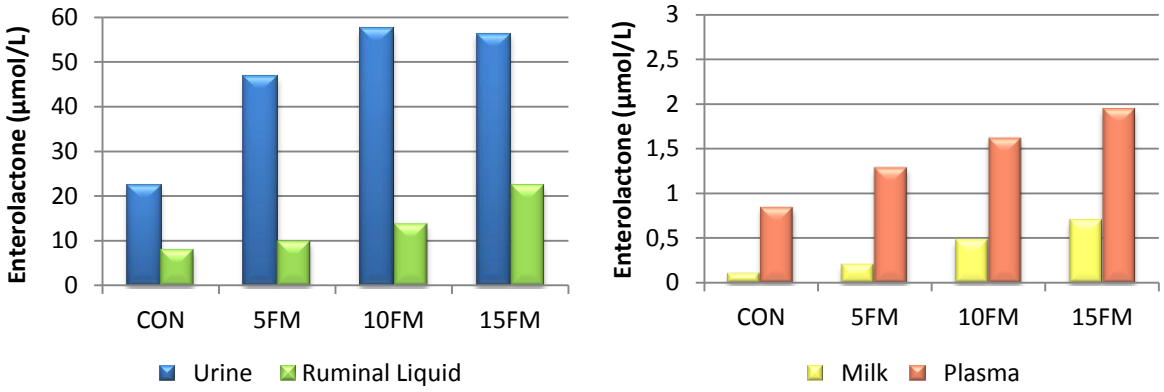

Figure 1. Concentration of enterolactone (µmol/L) in urine and ruminal fluid (a) and milk and plasma (b) in Holstein cows fed no flax meal (CON) or 5 (5FM), 10 (10FM) and 15% (15FM) flax meal in the diet. There was a linear effect of treatment, with *P* values of 0.0153, <0.0001, 0.0001 and <0.0001 for urine, ruminal fluid, milk and plasma, respectively. Standard errors were 0.001, 1.15, 0.0012 and 0.0011 for urine, ruminal fluid, milk and plasma, respectively.

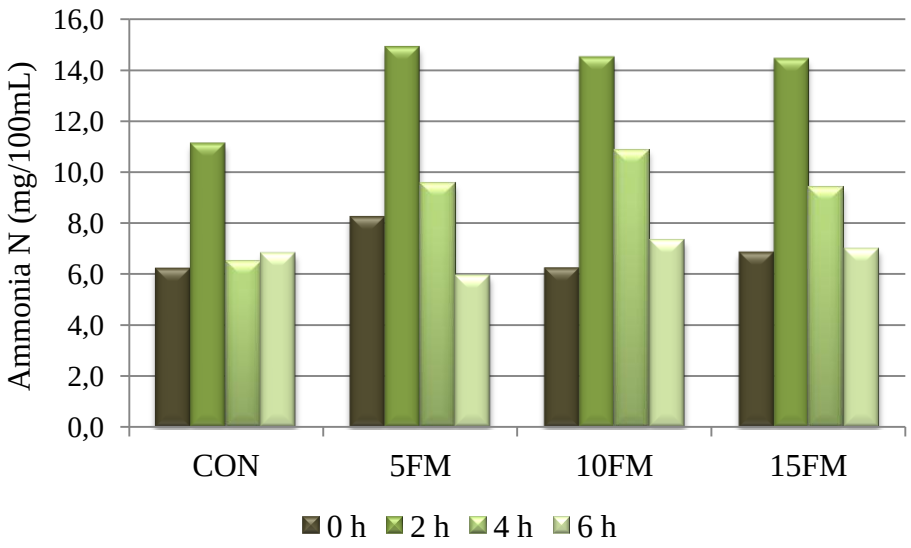

Figure 2. Ammonia N concentration (mg/100 mL) in ruminal fluid of Holstein cows fed no flax meal (CON) or 5 (5FM), 10 (10FM) and 15% (15FM) flax meal in the diet. The effect of treatment tended (P=0.05) to be linear and the time effect was significant (P<0.0001). The standard error was 1.00 for all sampling times.

CAPÍTULO 3

Potential role of rumen bacteria belonging to numerous taxa in the conversion of plant lignans to the mammalian lignan enterolactone

(Journal of Applied Microbiology)

Running Title: Lignan conversion by dairy cows

Aims: To identify bacterial taxa that potentially play a role in the conversion of plant lignans into enterolactone (EL) in ruminants using biomolecular analysis.

Methods and Results: Eight rumen cannulated cows were used in a double 4 × 4 Latin square design and fed a control diet with no flax meal (FM) or a diet with 5%, 10% and 15% FM. Concentration of EL in ruminal fluid increased linearly with FM inclusion. The concentration of total bacterial 16S rDNA genes obtained using Q-PCR was similar among treatments. PCR-T-RFLP based dendrograms revealed no obvious global clustering of the microbiota based on diet. PCR-DGGE did show clustering by diet within four cows. Bands present following feeding of 15% FM and absent when no FM was fed were sequenced.

Conclusions: Sequences revealed that uncultured bacteria belonging to the families *Succinivibrionaceae*, *Alphaproteobacteria* and genera *Prevotella Succinivibrio*, *Lachnospiraceae, Bacteroidales, Anaerovorax* and strain of *Fibrobacter succinogenes*, and strain H23 of *F. succinogenes* may play a role in the conversion of plant lignans into EL in the rumen.

Significance and Impact of the Study: This study provides much needed insight into which rumen microbiota require further investigation for their role in EL production and contributes to fundamental understanding of flax lignans conversion in ruminants.

Keywords

Enterodiol, enterolignans, flax, PCR-DGGE, rumen, secoisolariciresinol, secoisolariciresinol-diglucoside.

Introduction

Several human studies have revealed that ingestion of plant lignans, which are polyphenolic compounds classified as phytoestrogens, can decrease the incidence of menopausal

symptoms, hormone-dependent cancers, cardiovascular diseases, osteoporosis and diabetes (Prasad 1997; Prasad 2000; Raffaelli *et al*. 2002; Hu *et al.* 2007; Jin and Hattori 2011). Flax (*Linum usitatissimum*) is the richest source of lignans (Prasad 1997), with secoisolariciresinol diglucosidase (SDG) representing more than 95% of all flax lignans. Lignans are mainly found in the fiber portion of flax (Adlercreutz and Mazur 1997), thus resulting in higher concentration of lignans in hulls than seeds (Côrtes *et al*. 2008).

In non-ruminant animals, SDG is converted into secoisolariciresinol (SECO) under the action of intestinal glycosidases and the colonic microbiota convert SECO to the mammalian lignans enterodiol and enterolactone (EL; Clavel *et al*. 2006; Morris 2007). Prasad (2000) showed that the beneficial effects of flax lignans on human health may be due to their antioxidant activity as SECO, enterodiol, EL, and SDG are 4.86, 5.02, 4.35, and 1.27 times more potent than vitamin E. The conversion of plant SDG into mammalian lignans in humans can be described by four catalytic reactions: O-deglycosylation, O-demethylation, dehydrogenation and dehydroxylation (Wang *et al.* 2000; Clavel *et al*. 2006). Some of the human intestinal bacteria involved in the catalytic reactions are strains of *Klebsiella* (Wang *et al*. 2010), *Bacteroides distasonis, B. fragilis, B. ovatus*, *Clostridium cocleatum, Clostridium* sp. SDG-Mt85-3Db, *Butyribacterium methylotrophicum, Eubacterium callendari, E. limosum, Peptostreptococcus productus, Clostridium scindens, Ruminococcus productus*, *Eggerthella lenta* and ED-Mt61/PYG-s6 (Wang 2002; Clavel *et al*. 2005; Clavel *et al*. 2006).

Previous studies carried out by Côrtes *et al.* (2008), Petit and Gagnon (2009a; 2009b) and Zhou *et al*. (2009) have demonstrated that the rumen microbiota metabolize flax lignans mainly into EL, akin to the situation in the human gastrointestinal tract. Later studies have shown that the metabolism of flax lignans occurs mainly in the rumen and not in the intestine (Gagnon *et al*. 2009) and that EL is the major mammalian lignan in milk (Petit and Gagnon, 2009b). The EL is present in urine, blood, and milk, indicating that phytoestrogens can be transferred to physiological fluids (Gagnon *et al.*, 2009). Indeed, EL concentration in milk increases by feeding flax products, with higher concentrations observed with 15% inclusion of FM in the diet (Petit *et al*. 2009; Petit and Gagnon 2009b; 2011). Since EL has shown antioxidant activity (Prasad 2000), high levels of EL in milk

may prevent oxidation (Matumoto-Pintro *et al*. 2011) and increase shelf life of milk and dairy products.

The rumen microbiota responsible for the conversion of flax lignans into EL are nonetheless unknown. Therefore, the aims of this study were to evaluate the effects of inclusion of FM in the diet of dairy cows on the rumen microbiota using terminal restriction fragment length polymorphisms (T-RFLP), denaturing gradient gel eletrophoresis (PCR-DGGE) and quantitative polymerase chain reaction (Q-PCR). Ultimately this study aimed to identify the rumen bacteria potentially responsible for the conversion of flax lignans into the mammalian lignin EL.

Material and methods

Animals and treatments

Eight lactating multiparous Holstein cows fitted with ruminal cannulas averaging 686 kg of body weight and 112 days in milk were assigned to a double 4 × 4 Latin Square design with four treatments and four 21-d periods. The cows were kept in individual stalls and had free access to water. Cows were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993). Diets were offered in equal amounts twice daily at 0830 and 1530 h for *ad libitum* intake (10% refusals as served). Cows were milked twice daily (0800 and 1900 h) and fed a control diet (CON) with no flax meal (FM) or a diet with 5% (5FM), 10% (10FM) and 15% FM (15FM) on a dry matter basis. The four total mixed diets (Table 1) were equal in protein and energy of lactation and were formulated to meet nutrient requirements for cows that average 657 kg of body weight and produce 37.7 kg/d of milk with 3.8% of fat (NRC, 2001).

Sampling and preparation

On day 21 of each period, ruminal contents were collected 0, 2, 4 and 6 h after the morning meal from different locations within the rumen (the cranial dorsal, cranial anterior ventral, medium ventral, caudal dorsal and caudal ventral locations) to obtain a representative sample. The ruminal contents were strained through four layers of cheesecloth. One portion was kept at -20°C and freeze-dried for EL analysis. Another portion of 1 l of strained

ruminal fluid was taken 2 h post-feeding for microbial isolation as described by Lee *et al.* (2002). The resultant microbial pellets were freeze-dried, ground with a mortar and frozen at -80°C for molecular analysis.

Enterolactone analysis

Analysis of EL was performed using an EIA kit (Cayman Chemical, Ann Arbor, MI, USA). The detailed procedures of extraction and analysis were described by Gagnon *et al.* (2009). Ruminal samples for the three post-feeding times (2, 4 and 6 h) were pooled within cow and period as previously carried out by Gagnon *et al.* (2009) to obtain only one post-feeding composite sample for EL analysis.

DNA extraction

DNA was extracted from approximately 10 mg of freeze-dried ruminal samples using the FastDNA Spin Kit for Soil (QBiogene, Cambridge, UK) following the manufacturer's guidelines, although 3 x 30S beating with 1 min intervals on ice was employed. The quality and quantity of DNA were determined using a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA) (260 and 280 nm).

PCR-T-RFLP analysis of the total bacterial population

PCR T-RFLP was conducted in triplicate following the procedures described by Huws *et al.* (2011) with subsequent pooling and use of the restriction enzymes Hae III or Msp I (Promega, Madison, WI, USA). Restriction digestions were run on a ABI3130xl DNA sequencer (Applied Biosystems, Foster City, California, USA) and T-RFs checked and exported using Genemapper software (Applied Biosystems, CA, USA). Peaks < 0.5% of the cumulative peak height were removed (Blackwood *et al*. 2003). Data were imported into Bio-Rad fingerprinting (Bio-Rad Laboratories, Hertfordshire, UK) and clustering analysis was undertaken using separation criteria based on a 0.5 bp size difference in peaks and the Pearson's coefficient.

PCR-DGGE analysis of the total bacterial population

V6-V8 16S rDNA PCR was performed as described by Huws *et al.* (2007; 2011) and Kim *et al.* (2008). Amplicons were loaded onto 6% polyacrylamide gels with a 35-60%

denaturating parallel gradient and the electrophoresis performed in a D-Code system (Bio-Rad Laboratories; Kim *et al.* 2008), running for 10 min at 200V at 60°C and then for 16 h at 85V and 60°C. Gels were then stained with silver nitrate (Sanguinetti *et al.* 1994), scanned using a GS-710 calibrated imaging densitometer (Bio-Rad, Hemel Hempstead, UK) and the saved image imported into the software package Fingerprinting (Bio-Rad Laboratories) for analysis (Kim *et al.* 2008: Huws *et al.* 2011). UPGMA dendrograms were constructed using the Dice coefficients and a position tolerance of 0.5% and an optimization parameter of 1%. The band number was calculated using generated binary data. Bands of interest were cut, re-amplified and cloned for subsequent sequencing using pGEM-T easy vector system (Promega, Southampton, UK). Clones obtained were sequenced using an ABI3130xl DNA sequencer (Applied Biosystems). These sequences have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers HQ849553-567. Sequences were compared with deposited sequences within Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the ribosomal database project (RDP – II Release 10; Cole *et al*., 2005; http://rdp.cme.msu.edu), which gives taxonomic information.

Quantitative PCR

To investigate total bacteria DNA concentration, 16S rDNA was quantified within ruminal digesta in triplicate according to Kim *et al.* (2008) and Huws *et al.* (2011).

Statistical analysis

Band and peak numbers from TRFLP and Q-PCR and EL concentration were analyzed using the MIXED procedure of SAS (2000, SAS Institute, Cary, NC, USA). Treatment 5FM was excluded for molecular bacterial analysis (PCR-DGGE, PCR-T-RFLP and Q-PCR) as the main objective was to compare the microbiota present under the control treatment *versus* high inclusions of FM in the diet (10% and 15%). Therefore, for the statistical analysis of DGGE band numbers, the number of T-RFLP peaks, and Q-PCR, the statistical model was a double incomplete 4 × 4 Latin square design while a 4 × 4 double Latin square was considered for EL with the general model:

$$Y_{ijklm} = \mu + T_i + P_j + Q_k + A/Q_k + e_{ijk}$$

Where Y_{ijklm} = the response variable, μ = overall mean, T_i = global effect of treatment (i = CON, 5FM, 10FM and 15FM), P_j = the fixed effect of period (j = 1 to 4), Q_k = fixed effect of square (k = 1, 2), A/Q_{lk} = random effect of cow within square, and e_{ijklm} = residual error. Enterolactone data were treated according to Côrtes *et al.* (2008). Normality and homogeneity were analyzed with the procedure UNIVARIATE of SAS (2000, SAS Institute, Cary, NC, USA). Statistical differences were declared at $P < 0.10$.

Results

Diet composition

Flax meal was fed at 47.9, 95.3 and 140.6 g/kg of dry matter, respectively, for the treatments with 5, 10, and 15% of FM, amounts closed to percentages planned for the experiment (Table 1).

Ruminal concentration of EL

There was no interaction ($P = 0.19$) between diet and sampling times for EL concentration in ruminal fluid. Flax meal supplementation increased linearly ($P < 0.0001$) concentration of EL in ruminal fluid (Fig. 1) before feeding and in the pool of post-feeding times.

Bacterial 16S rDNA quantity and diversity

Total bacterial 16S rDNA concentration was similar ($P = 0.1505$) among treatments. The values averaged 6.46, 7.65, and 7.27 ng g^{-1} 16S rDNA (S.E. = 0.41) for treatments CON, 10FM, and 15FM, respectively.

T-RFLP-derived unweighted pair group method with arithmetic mean (UPGMA) dendrograms did not show any global clustering dependent on diet neither for Hae III nor MSP I (Fig. 2A and 2B). When animals were analyzed separately, again dendrograms did not show any global clustering dependent on diet neither for Hae III nor MSP I (data not shown). A higher number of peaks for restriction enzyme MSP I was observed for cows supplemented with FM, but no treatment effect was observed with respect to the number of peaks obtained from Hae III (Table 2). The DGGE-based unweighted pair group method with arithmetic mean dendrograms demonstrated two distinct situations. First, when all

cows were considered, there was no clear clustering by treatment due to the individual variation among animals (Fig. 3A). However, when some cows that were obviously masking the treatment effect were removed, bacterial communities were seen to cluster separately, dependent on diet (approx. 68% similarity seen between bacterial diversity present on the CON and 15FM diets; Fig. 3B), suggesting a treatment effect on bacterial population. There was no treatment effect (P = 0.21) on band numbers from V6-V8 16S rDNA PCR-DGGE of bacteria obtained from ruminal samples of cows receiving either the CON, 10FM or 15FM diets (Table 2).

For sequencing purposes, DGGE gels were run contrasting the CON treatment with 15FM for the 4 cows that demonstrated a clear effect of diet on ruminal microbiota. The objective was to elucidate the effect of FM inclusion on ruminal microorganisms and identify bands of interest present following 15% FM inclusion and absent when feeding the CON diet (Fig. 4). Therefore, DNA bands that were absent under the CON diet but present on the 15% FM diet were chosen on an individual animal basis. The DNA extracted from bands of interest were cloned and sequenced. Sequences revealed that bacteria associated with EL production belonged to uncultured bacteria phylogenetically classified as *Prevotella*, *Succinivibrionaceae*, *Alphaproteobacteria* and uncultured rumen bacterium *Succinivibrio*, *Lachnospiraceae*, *Bacteroidales*, *Anaerovorax* and *Prevotella*, and strain of *Fibrobacter succinogenes* (Table 3).

Discussion

The presence of EL in ruminal fluid on all diets may indicate that the conversion of SDG into enterolignans occurred in the rumen through the metabolism of its microbiota as first reported by the *in vitro* experiment of Côrtes *et al.* (2008). Dietary feed ingredients such as soy and corn also contain lignans (Penalvo *et al.* 2005; Steinshamm *et al.* 2008), which may lead to the production of EL as reported by Thompson et al. (1991) and explain the small amounts of EL for cows fed the CON diet. However, in the present experiment, FM was clearly the main source of lignans (i.e., SDG) as flax is known to be one of the richest source of plant lignans (Thompson *et al.* 1991). Substitution of FM with soybean meal in diets was unlikely responsible for increased EL concentration of ruminal fluid with higher dietary proportion of FM. Indeed, previous results have shown that feeding 3.8% compared

to 0.6% soybean meal in the diet resulted in EL concentration five times lower in the milk of cows fed the former proportion (Gagnon *et al.* 2009). In addition, Wang *et al.* (2010) have shown that a source of nitrogen is required for the conversion by ruminal microbiota of flax lignans into enterodiol, which is the precursor of EL. In the present study, proportion of nitrogen in the diet was similar among treatments supplied either by FM or soya meal. Based on these results, we postulated that the production of EL resulting from increased proportion of FM in the diet may lead to numerical and/or diversity shifts in the ruminal microbiota, which could be detected using biomolecular techniques.

Global changes were observed in the ruminal microbiota upon FM feeding when the PCR-DGGE technique was applied. Observing each cow individually, four animals clustered closely based on diets (Fig. 3b), and distinct bands appeared when FM was supplemented (Fig. 4). Thus, in order to investigate and identify which bacteria may be involved in SDG conversion and consequently EL production, the bands present when the FM diet was fed compared to the CON diet were cut and sequenced on an individual animal basis.

The T-RFLP technique did not show any global change in the bacterial communities following FM supplementation, even when animals were compared separately. This discrepancy in T-RFLP data compared to DGGE has previously been noted. This may be due to the fact that a different amplicon was used for both techniques. Nonetheless, Smalla *et al.* (2007) compared both techniques for samples obtained from soil using the same DGGE V6-V8 target amplicon and a longer 16S rDNA target amplicon for T-RFLP and they reported similarity between the two data sets. There are also many reports that show that DGGE results in comparable results or in some instances gives more information on the bacterial diversity present (Casamayor *et al.* 2002; Enwall and Hallin 2009). The T-RFLP peak identification using a sequencer is sensitive and amplicons of low intensity may not be visualised using DGGE-based silver staining. Indeed, our data showed that both MSP1 and Hae III gave more peak information with Hae III being superior as we have found previously (Huws *et al.* 2011). Nonetheless, in order to assess global changes in the dominant bacterial communities for sequencing purposes, DGGE was required.

In this study, we demonstrated that rumen bacteria belonging to the families *Succinivibrionaceae*, *Alphaproteobacteria* and genera *Prevotella Succinivibrio*,

Lachnospiraceae, *Bacteroidales*, *Anaerovorax*, and strain of *Fibrobacter succinogenes* potentially have the ability to convert plant lignans to the human health beneficial EL, which is subsequently secreted into milk as reported by Petit *et al.* (2009) and Petit and Gagnon (2011).

Interestingly, the genus *Bacteroides*, which belongs to the order *Bacteroidales*, was identified by Clavel *et al.* (2006) as one of the human intestinal bacterium responsible for the catalytic conversion of SDG to SECO via deglycosylation (β-glycosidases). The family *Prevotellaceae* and the genus *Prevotella* also belong to the order *Bacteroidales*. As the genus *Prevotella* within the family *Prevotellaceae* represented more than 45% of the identified bands in the current study, this may suggest that they play a predominant role in SDG conversion into EL. *F. succinogenes* is a fibrolytic bacteria, and its enzymes are the most studied among rumen bacteria; it possesses endoglucanases, cellobiosidase, cellodextrinase, xylanases, and β-glucosidase activities (Krause *et al.* 2003). β-glucosidase activity has been demonstrated by *F. succinogenes* S85 (Krause *et al.* 2003) and is reported as the enzyme required for the conversion of SDG to SECO (Wang *et al.* 2002). Since *F. succinogenes* and the unclassified bacteria *Lachnospiraceae* belong to the predominant ruminal bacteria (Kim *et al.* 2011), it is probable that these two species also play an important role in SDG conversion. In another recent study, Zhou *et al.* (2009) found that *Ruminococcus gnavus* was potentially responsible for the conversion of plant lignans to EL in goats. However, ruminococci were not implicated in the present study as having the ability to convert lignans into enterolignans.

In summary, inclusion of FM increased the concentration of the health beneficial EL in the rumen whilst altering the rumen microbiota as demonstrated using DGGE. Sequencing of key bands present upon inclusion of 15% FM and absent when no FM was fed showed that diverse rumen bacterial taxa may play a role in the production of EL. Further studies are required to verify if these bacteria, families, genera or strains are involved in the production of EL, nonetheless this provides information regarding which bacteria to further investigate and contributes to our fundamental understanding of EL production in ruminants.

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Table 1 Ingredient and chemical composition of experimental diets

	Control*	5FM*	10FM*	15FM*
Ingredients, % of dry matter (DM)				
Corn silage	29.22	29.08	28.98	29.06
Grass silage	31.53	31.57	31.75	31.39
Ground corn	21.13	20.23	19.16	18.90
Soya meal	10.76	7.45	4.14	2.04
Top Suplement†	1.74	1.74	1.79	0.85
Beet pulp	3.43	2.96	2.50	1.61
Calcium carbonate	0.55	0.55	0.54	0.58
Mineral and vitamins‡	1.64	1.63	1.63	1.53
Flax meal	0	4.79	9.53	14.06
Chemical analysis¶				
DM, %	37.7 ± 1.60	37.9 ± 1.60	37.6 ± 1.60	38.1 ± 1.60
Crude protein, % of DM	17.0 ± 0.15	17.4 ± 0.15	17.6 ± 0.15	17.9 ± 0.15
Acid detergent fiber, % of DM	18.3 ± 0.34	18.5 ± 0.34	19.2 ± 0.34	19.3 ± 0.34
Neutral detergent fiber, % of DM	28.4 ± 0.31	28.6 ± 0.31	29.5 ± 0.31	29.6 ± 0.31
Ether extract, % of DM	2.4 ± 0.07	2.4 ± 0.07	2.4 ± 0.07	2.4 ± 0.07

*Control diet with no flax meal (FM) or a diet with 5%, 10% and 15% FM (DM basis).
†Contained 20% of canola meal, 30% of corn gluten meal, 20% of soybean meal, and 30% of brewer's corn.
‡Contained 9.2 % Ca; 4.79 % P; 4.78 % Mg; 1.52 % S; 13.72 % Na; 1.37 % K; 19.5 mg/kg Se; 23 mg/kg I; 2013 mg/kg Fe; 1068 mg/kg Cu; 1796 mg/kg Mn; 2657 mg/kg Zn; 57 mg/kg Co; 265 mg/kg Fl; 442000 UI/kg vitamin A; 56670 UI/kg vitamin D; and 2630 UI/kg vitamin E.
¶Values with standard errors of the mean.

Table 2 Mean peak/band number following HaeIII- and MSP1-based 16S rDNA T-RFLP and V6 – V8 PCR-DGGE of rumen bacteria within rumen samples obtained from Holstein cows fed a control diet with no flax meal (CON), 10% (10FM) and 15% (15FM) of flax meal (FM) in the dry matter

	Treatments			S.E.M.	*P*-value
	CON	10FM	15FM		
Hae III	101.75	94.12	97.12	4.26	0.5222
MSP I	75.62	99.62	82.25	3.79	0.0027
PCR-DGGE	46.87	42.25	40.75	2.4	0.2149

Table 3 Taxonomic identification of DGGE bands associated with enterolactone production in ruminal fluid

Band position (clone no.)	Nearest match (accession number; Maximum% sequence similarity)	Ribosomal Database Project Classification
Cow 1 (band position A clone 1)	Uncultured bacterium isolate 16S ribosomal RNA gene (EU624093.1; 99%)	unclassified_*Succinivibrionaceae*
Cow 3 (band position A clone 1)	Uncultured rumen bacterium clone CF23 16S ribosomal RNA gene (EU871348.1; 94%)	unclassified_"*Lachnospiraceae*"
Cow 3 (band position A clone 2)	Uncultured bacterium clone p-1030-a5 16S ribosomal RNA gene (AF371866; 96%)	unclassified_*Alphaproteobacteria*
Cow 4 (band position A clone 1)	Uncultured rumen bacterium clone YRC13 16S ribosomal RNA gene (EU259389.1; 98%)	unclassified_"*Bacteroidales*"
Cow 4 (band position B clone 1)	Uncultured rumen bacterium clone BF399 16S ribosomal RNA gene (EU850583.1; 96%)	genus *Anaerovorax*
Cow 4 (band position B clone 2)	Uncultured rumen bacterium 5C3d-4 gene for 16S rRNA (AB034106.1; 99%)	genus *Prevotella*
Cow 4 (band position B clone 3)	Uncultured rumen bacterium clone P5_D21 16S ribosomal RNA gene (EU381799.1; 98%)	genus *Succinivibrio*
Cow 4 (band position B clone 4)	*Fibrobacter succinogenes* strain H23 16S ribosomal RNA gene (JF970205.1; 99%)	genus *Fibrobacter*
Cow 4 (band position C clone 1)	Uncultured rumen bacterium clone TWBRB64 16S ribosomal RNA gene, partial sequence (FJ028779.1; 94%)	unclassified_*"Prevotellaceae"*
Cow 4 (band position C clone 2)	Uncultured rumen bacterium clone BE5 16S ribosomal RNA gene (AY244922.1; 96%)	genus *Prevotella*
Cow 4 (band position C clone 3)	Uncultured bacterium clone NED5F11 16S ribosomal RNA gene (EF445279.1; 99%)	genus *Prevotella*
Cow 4 (band position C clone 4)	Uncultured rumen bacterium clone CTRS1H03 16S ribosomal RNA gene (GQ327793.1; 99%)	genus *Prevotella*
Cow 5 (band position A clone 1)	Uncultured rumen bacterium clone CTRS1H03 16S ribosomal RNA gene (GQ327793.1; 97%)	genus *Prevotella*

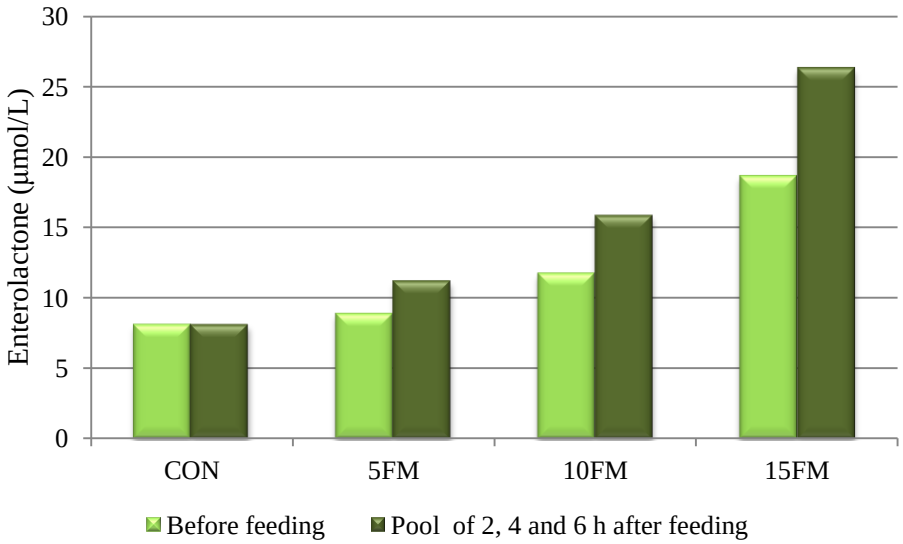

Figure 1 Concentration of enterolactone (µmol/L) in ruminal fluid of Holstein There was a linear effect of treatment ($P < 0.0001$) before feeding and in the post-feeding pool of ruminal fluid. The standard error was 0.001 for all sampling times.

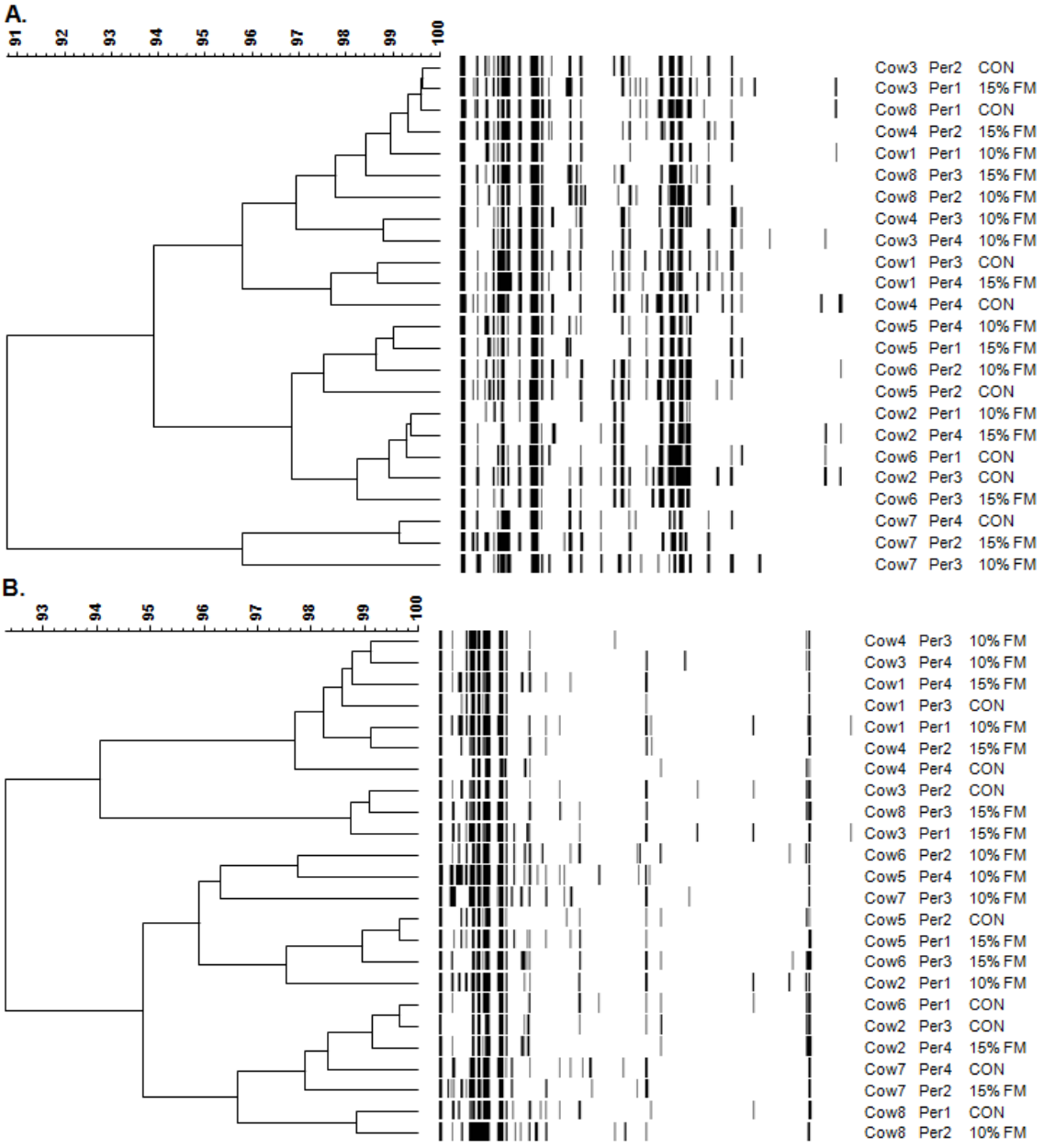

Figure 2 Unweighted pair group method with arithmetic mean (UPGMA) dendrograms showing the effect of flax meal (FM) inclusion on the rumen microbiota following T-RFLP based on Hae III (A) and MSP I (B) restriction enzymes. Cows were fed a control diet (CON) or a diet with 10% flax meal (FM) and 15% FM. Scale relates to percent similarity and data are presented per period (Per).

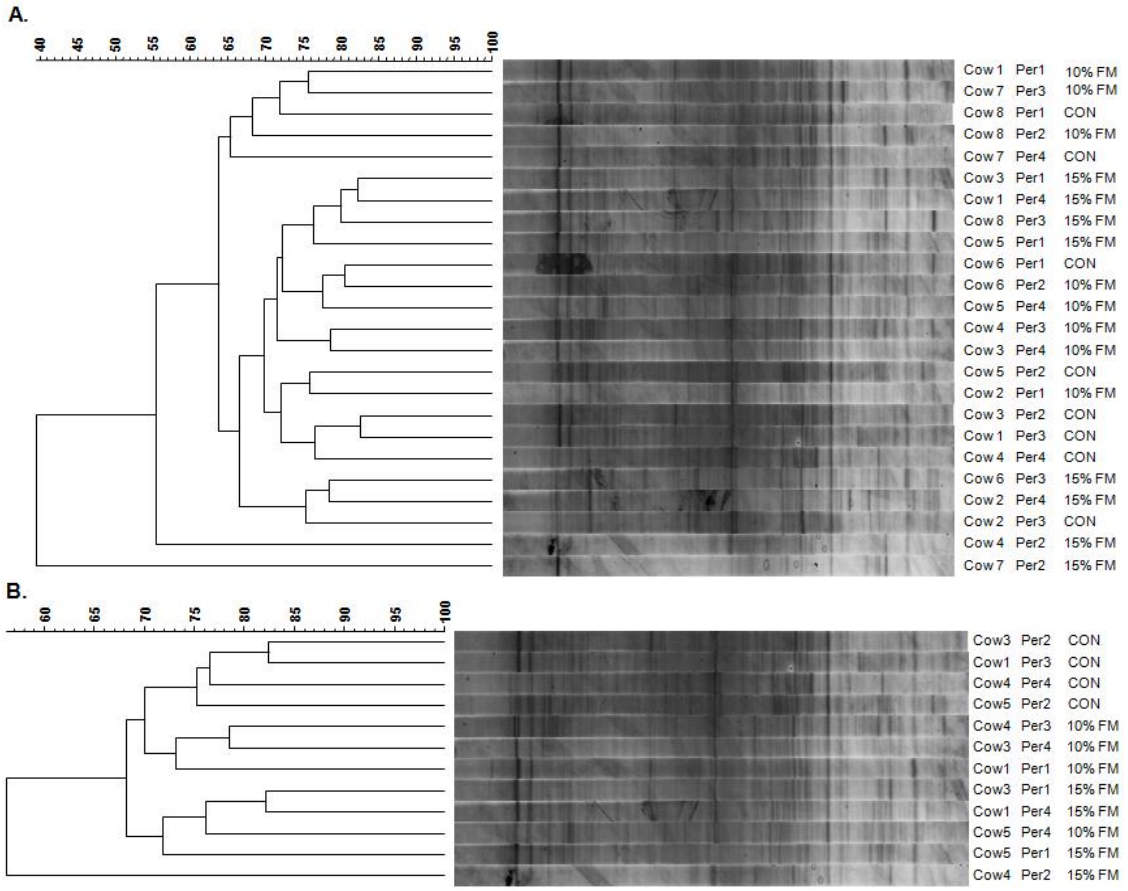

Figure 3 Unweighted pair group method with arithmetic mean (UPGMA) dendrograms showing the effect of flax meal (FM) inclusion on the rumen bacteria following 16S rDNA PCR-DGGE for all cows (A) and for four cows (B). Cows were fed a control diet (CON) or a diet with 10% flax meal (FM) and 15% FM. Scale relates to percent similarity and data are presented per period (Per).

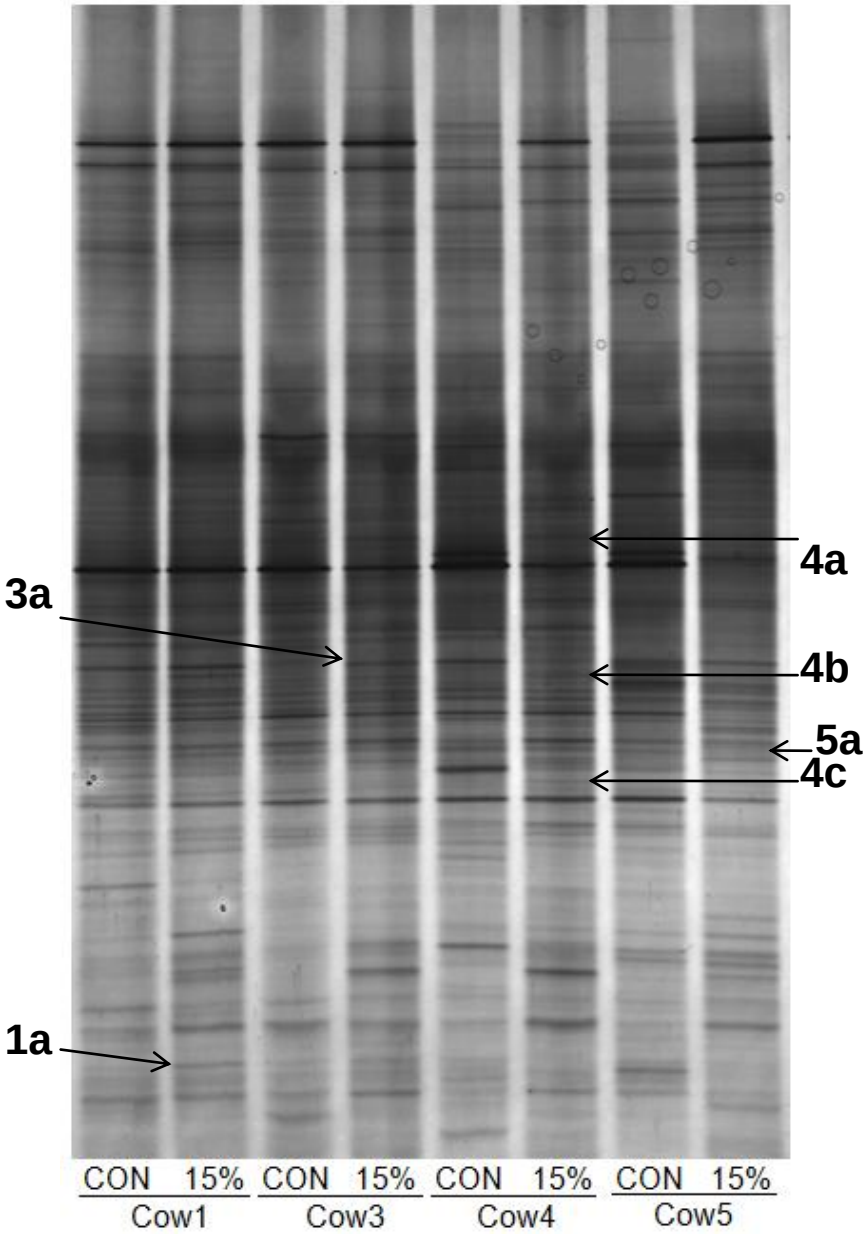

Figure 4 DGGE gel showing rumen bacterial diversity within cows fed no flax meal or 15% flax meal in the diet (dry matter basis). Arrows show bands that were cut and sequenced.

CONSIDERAÇÕES FINAIS

A complexidade com que acontecem as interações entre as substâncias antioxidantes, seja da linhaça, de seus coprodutos ou de qualquer outra fonte, torna essa linha de pesquisa desafiadora, e os esforços para a obtenção de resultados expressivos e eficientes devem ser continuados, com o intuito de trazer benefícios aos animais e aos humanos.

A inclusão do farelo de linhaça foi capaz de melhorar o perfil oxidativo de vacas leiteiras da raça Holandesa, pelo aumento da atividade enzimática da glutationa peroxidase no plasma dos animais. Entretanto, futuros estudos deverão ser realizados para se verificar o efeito do farelo de linhaça fornecido aos animais durante o período de transição, no qual as vacas estão mais susceptíveis ao estresse oxidativo.

A absorção por parte do ruminante, das enterolignanas produzidas no rúmen, está diretamente ligada tanto ao perfil oxidativo dos animais, quanto à sua transferência para o leite. Uma vez que há comprovada possibilidade de produção de elevadas quantias de enterolactona no rúmen, e que a concentração de enterolactona tanto no rúmen quanto no plasma dos animais influenciam diretamente a quantidade de enterolactona que potencialmente será transferida para o leite, estudos devem ser realizados com o intuito de melhor se compreender como se dá essa absorção da enterolactona produzida, e como essa transferência poderia ser mais efetiva. Assim, tanto o animal seria beneficiado por ter maiores concentrações de enterolactona circulante, podendo agir como substância antioxidante, quanto o leite poderia adquirir características nutricionais adicionais, seja pelo aumento de antioxidantes em sua composição, seja pelo efeito protetor dos antioxidantes sobre a gordura do leite.

Adicionalmente, a identificação de bactérias potencialmente conversoras de linanas vegetais em enterolignanas poderia promover avanços nas pesquisas acerca do assunto. Maior produção de enterolactona no rúmen poderia por consequência aumentar sua absorção, trazendo os benefícios esperados dos antioxidantes ou até mesmo, proporcionar que técnicas “in vitro” sejam desenvolvidas, com o intuito de se produzir antioxidantes naturais, prontamente disponíveis para o consumo humano, que poderiam ser incorporados em diferentes alimentos trazendo então à população os benefícios que podem ser esperados dos antioxidantes presentes na linhaça.