UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

ANTIOXIDANTES E ÁCIDOS GRAXOS POLI-INSATURADOS NO LEITE DE VACAS EM LACTAÇÃO EM RESPOSTA À INGESTÃO DE LINHAÇA

Autora: Daniele Cristina da Silva Kazama
Orientador: Prof. Dr. Geraldo Tadeu dos Santos
Co-orientadora: Dr[a]. Hélène V. Petit

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

MARINGÁ
Estado do Paraná
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A

Deus que me guia, me conduz, me orienta.

Aos

meus pais João e Maria Vera, meu..... tudo!

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No dia 20 de fevereiro de 2009, submeteu-se à banca para a defesa da Tese.

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LISTA DE ABREVIAÇÕES

ADF	acid detergent fiber
AG	ácido graxo
AGPI	ácido graxo poli-insaturado
AGS	ácido graxo saturado
BW	body weight
CLA	conjugated linoleic acid ou ácido linoleico conjugado
CP	crude protein
DHA	docosahexaenoic acid ou ácido docosahexaenoico
DIM	days in milk
DM	dry matter
DMD	dry matter digestibility
DMI	dry matter intake
ED	enterodiol
EE	ether extract
EIA	enzyme immunoassay
EL	enterolactone ou enterolactona
EPA	eicosapentaenoic acid ou ácido eicosapentaenoico
FA	fatty acid
FCM	Fat-corrected milk
FRAP	ferric reducing antioxidant power

HPLC	high performance liquid chromatography
LCFA	long-chain fatty acid
MCFA	medium-chain fatty acid
MS	matéria seca
MUFA	monounsaturated fatty acid
n-3	ácidos graxos da família ômega 3
n-6	ácidos graxos da família ômega 6
NDF	neutral detergent fiber
NE_L	net energy of lactation
NSC	non-structural carbohydrate
PUFA	polyunsaturated fatty acid
SCFA	short-chain fatty acid
SDG	secoisolariciresinol diglucoside
SECO	secoisolariciresinol
SFA	saturated fatty acid
TMR	total mixed ration

RESUMO

Estudos, *in vitro* e *in vivo*, foram conduzidos para avaliar o potencial da linhaça em transferir lignanas mamíferas para o leite. No Primeiro Experimento, semente e casca de linhaça foram incubadas para determinar, *in vitro*, a conversão de lignanas vegetais em lignanas mamíferas (enterolactona e enterodiol) pela microflora ruminal e fecal. A concentração de lignanas vegetais na semente e na casca de linhaça foi em média 9,2 e 32,0 nmol mg^{-1}, respectivamente. A maior produção de enterodiol, à 72h e 96h de incubação, foi obtida com casca de linhaça incubada com o inóculo fecal. Não houve diferença na produção de enterodiol entre a casca e a semente de linhaça nas primeiras 24h de incubação. Em geral, a produção de enterolactona no decorrer das 96h foi significativamante maior para a casca e a semente de linhaça incubadas com o inóculo ruminal do que com o inóculo fecal. A produção de enterolactona à 72h e 96h de incubação foi maior para a casca do que para a semente de linhaça. No Segundo Experimento, conduzido *in vivo*, quatro vacas da raça Holandesa em lactação foram distribuídas em um delineamento em quadrado latino 4 x 4 nos seguintes tratamentos: controle sem casca de linhaça **(CO)**, controle com casca de linhaça **(CL)**, monensina (16 ppm) sem casca de linhaça **(MO)**, e monensina (16 ppm) com casca de linhaça **(CM)**. A digestibilidade aparente da PB foi maior para as dietas que continham casca de linhaça e para as dietas suplementadas com monensina sódica. Um aumento significativo da digestibilidade do extrato etéreo foi observado para os tratamentos com casca de linhaça comparados com os tratamentos sem casca de linhaça. O fornecimento de casca de linhaça aumentou as

concentrações de ácidos graxos *trans* totais, ácidos graxos mono-insaturados, poli-insaturados, de cadeia longa, n-3 total e a razão poli-insaturados/saturados na gordura do leite. A razão n-6/n-3 na gordura do leite foi menor para as vacas que receberam casca de linhaça em comparação àquelas que não a receberam. A suplementação com monensina sódica aumentou as concentrações dos ácidos graxos *cis*9-16:1 e *cis*9,12-18:2 na gordura do leite. A ingestão de MS foi maior para os tratamentos sem casca de linhaça do que para os tratamentos com casca de linhaça. Além disso, a produção de leite foi diminuída para as vacas que receberam casca de linhaça. A concentração de enterolactona tanto no líquido ruminal como no leite foi maior para as vacas que receberam casca de linhaça quando comparada com as vacas que não receberam casca de linhaça. O poder antioxidante ferro-redutor medido no leite foi similar entre os tratamentos. Um Terceiro Experimento *in vivo* foi conduzido com quatro animais da raça Holandês distribuídos em um delineamento em quadrado latino 4 x 4. Os tratamentos foram: uma dieta controle sem adição de produtos de linhaça **(CO)**, 4,18 g/kg de MS de grãos de linhaça inteiros **(LI)**, 1,87 g/kg de MS de sais de cálcio de óleo de linhaça **(SC)** e uma mistura de 2,3 g/kg de MS de grãos de linhaça inteiros e 0,83 g/kg de MS de sais de cálcio de óleo de linhaça **(LS)**. A ingestão e digestibilidade da MS, produção e composição do leite, com excessão da gordura, a qual foi menor para SC quando comparado ao CO, mas sem diferença entre SC, LI e LS. Os ácidos graxos (AG) intermediários da biohidrogenação no rúmen foram maiores quando sais de cálcio foram fornecidos e o ácido rumênico (*cis*9,*trans*11-18:2) aumentou no tratamento SC comparado ao LI. O AG alfa–linolênico no leite foi maior para SC e LS do que CO, mas não diferiu de LI. O fornecimento de linhaça inteira não alterou a concentração de enterolactona no leite em comparação aos sais de cálcio. Tanto a microflora ruminal como a fecal foram capazes de transformar lignanas vegetais em lignanas mamíferas. Casca de linhaça foi uma boa estratégia para melhorar a composição em ácidos graxos e aumentar a concentração de enterolactona no leite. Sais de cálcio de óleo de linhaça causaram depresão na gordura do leite e grãos de linhaça inteiros não alteraram a concentração de enterolactona no leite.

Palavras-chave: casca de linhaça, enterolactona, gado leiteiro, lignanas, monensina, sais de cálcio de óleo de linhaça

ABSTRACT

In vitro and *in vivo* studies were conduced to evaluate the potential of flaxseed to increase concentration of mammalian lignans in milk. In a first trial, flax seeds and hulls were incubated to determine the *in vitro* conversion of plant lignans from two flax products (hull and seed) into the mammalian lignans enterolactone and enterodiol by bovine ruminal and fecal microbiota. Plant lignans in flax seeds and hulls averaged 9.2 and 32.0 nmol mg^{-1}, respectively. The highest net production of enterodiol at 72 and 96 h of incubation was obtained with flax hulls incubated with fecal microbiota. There was no difference in net production of enterodiol between flax products within the first 24 h of incubation. In general, net production of enterolactone over the 96 h time course was significantly higher for flax products incubated with ruminal than with fecal microbiota. Net production of enterolactone at 72 and 96 h of incubation was greater for flax hulls than flax seeds. A second experiment, was conducted *in vivo* with four lactating Holstein cows assigned to a 4 x 4 Latin square design that were fed one of the four treatments: control with no flaxseed hulls **(CO)**, control with flaxseed hulls **(FH)**, monensin (16 ppm) with no flaxseed hulls **(MO)**, and monensin (16 ppm) with flaxseed hulls **(HM)**. The apparent digestibility of CP was higher for diets containing flaxseed hulls and for diets supplemented with monensin. Significant higher digestibility of ether extract was observed for treatments with flaxseed hulls compared with treatments without flaxseed hulls. Feeding flaxseed hulls increased concentrations of total *trans* fatty acids **(FA)**, monounsaturated FA, polyunsaturated FA, long-chain FA and n-3 FA, and the polyunsaturated to saturated FA ratio in milk fat. The n-

6 to n-3 ratio in milk fat was lower for cows fed flaxseed hulls compared with those fed no flaxseed hulls. Monensin supplementation increased the concentrations of *cis*9-16:1 and *cis*9,12-18:2 in milk fat. Intake of dry matter **(DM)** was higher for treatments without flaxseed hulls than for treatments with flaxseed hulls. Moreover, milk production was decreased for cows fed flaxseed hulls. Concentration of enterolactone in both ruminal fluid and milk was higher for cows fed flaxseed hulls compared with those fed no flaxseed hulls. Concentration of ferric reducing antioxidant power was similar among treatments. A third experiment was conducted *in vivo* with four Holstein cows used in a 4 x 4 Latin square design. The four treatments were: a control diet containing no flaxseed products **(CO)**, 4.18 g/kg DM of whole flaxseed **(WF)**, 1.87 g/kg DM of calcium salts of flaxseed oil **(CF)** or a mixture of 2.30 g/kg DM of whole flaxseed and 0.83 g/kg DM of calcium salts of flaxseed oil **(MF)**. Dry matter intake, digestibility, and milk production and composition were similar among treatments except for milk fat percentage that was lower for CF compared to CO with no difference between CF and WF and MF. Concentrations of intermediates of biohydrogenation of FA in the rumen were higher when calcium salts of flaxseed oil were fed, and rumenic acid (*cis*9,*trans*11-18:2) concentration was higher for CF compared to WF treatment. Milk fat concentration of alpha–linolenic acid was higher for CF and MF than CO, but was similar to WF. Feeding whole flaxseed did not alter enterolactone in milk compare to calcium salts of flaxseed oil. Both ruminal and fecal microbiota are able to transform plant lignans into mammalian lignans. Feeding flaxseed hulls was a good strategy to improve milk FA composition and increase enterolactone concentration in milk. Calcium salts of flaxseed oil depressed milk fat concentration and whole flaxseed had no effect on milk enterolactone concentration.

Key-words: calcium salts of flaxseed oil, dairy cattle, enterolactone, flaxseed hulls, lignans, monensin

INTRODUÇÃO

O leite como alimento funcional

O leite é um produto muito comum à mesa dos brasileiros. Porém, o consumo *per capita* de leite fluído, queijo, manteiga e leite em pó desnatado no Brasil em 2007 (77,0; 3,1; 0,4; e 0,7 kg/pessoa/ano, respectivamente), segundo o Departamento de Agricultura dos Estados Unidos (USDA), citado pela EMBRAPA (2008), ficou abaixo de países grandes consumidores. Como exemplo, temos a Austrália (105,3; 10,5; 2,7 e 1,9 kg/pessoa/ano, respectivamente), Estados Unidos (95,6; 14,9; 2,1 e 1,3 kg/pessoa/ano, respectivamente) e Canadá (93,9; 9,6; 2,3 e 2,1 kg/pessoa/ano, respectivamente).

Segundo Turco (2006) este baixo consumo se deve ao comportamento cultural dos brasileiros e não ao poder aquisitivo da população. Turco (2006) relata que bebidas como refrigerantes e cervejas são consumidas em quantidades maiores do que o leite pelos brasileiros, bebidas essas menos nutritivas, porém com o estímulo de consumo maior do que o leite. Para se estimular o aumento do consumo de leite no Brasil, a produção de um leite com maior qualidade e com componentes benéficos à saúde humana agregados, pode ser uma estratégia importante.

O alimento consumido, além de fornecer nutrientes essenciais para a sobrevivência, pode também contribuir para melhorar a saúde e prevenir futuras doenças através do fornecimento de compostos com funções medicinais preventivas. Neste caso, o alimento seria caracterizado como funcional.

Segundo Roberfroid (2002), um alimento pode ser considerado funcional se este afetar de forma benéfica uma ou mais funções no organismo, além de efeitos nutricionais que sejam relevantes, quer ao estado de bem-estar e à saúde ou à redução de risco de uma doença. Ainda, segundo o mesmo autor, um alimento pode tornar-se funcional aumentando a concentração, adicionando ou melhorando a biodisponibilidade de um componente em particular.

Desta forma, pesquisas podem ser conduzidas com o objetivo de transformar o leite em um alimento funcional e assim, ser um atrativo a mais para seu consumo e melhorar a saúde de quem o consome.

Um nutriente no leite, a gordura, é muito estudado. Antigamente era vista como a vilã deste alimento, porém, hoje se sabe que ela pode contribuir muito para a saúde quando apresentar uma composição ideal em ácidos graxos (AG).

Os AG poli-insaturados (AGPI), ao contrário dos AG saturados (AGS), quando ingeridos por humanos reduzem o risco de doenças cardiovasculares (Bucher et al., 2002; Lorgeril e Salen, 2002). Além disso, Grummer e Carrol (1991) e Petit (2002) também relataram que o aumento de AG n-3 no leite, um importante AGPI, poderia reduzir a incidência de aterosclerose.

Porém, manipular a gordura do leite para a incorporação de AGPI's não é algo tão simples pensando no aspecto da digestão do ruminante. Este, diferente do monogástrico, apresenta um trato digestivo muito complexo capaz de alterar de forma considerável a dieta fornecida.

O fenômeno da biohidrogenação é um entrave para a incorporação de AGPI's no enriquecimento do leite. A transferência, por exemplo, dos AGPI's eicosapentaenoico (EPA) e docosahexaenoico (DHA) da dieta para a gordura do leite é menor do que 4% (Lock e Bauman, 2004). No entanto, nos últimos anos, muito já foi elucidado para amenizar os efeitos da biohidrogenação e alcançar a incorporação destes elementos no leite (Chouinard et al., 1997; Delbechi et al., 2001; Petit, 2002; Neves et al., 2007; da Silva et al., 2007; Juchem et al., 2008). Também muito já foi estudado para evitar parcialmente a biohidrogenação e obter a incorporação do ácido linoleico conjugado (CLA-isômero *cis*9, *trans*11-18:2) no leite (Abughazaleh et al., 2003; Piperova et al., 2004).

O CLA é um importante AGPI exclusivo de animais ruminantes, encontrado na carne e, principalmente, no leite destes animais. Este AG tem sido relacionado com efeitos anticarcinogênicos, antiaterogênicos, aumento da resposta imune, e ainda, efeito antidiabético (Whigham et al., 2000; Tanaka, 2005).

Esses estudos muito contribuíram para o crescimento da ciência, pois, segundo Jenkins e McGuire (2006), a maior oportunidade no horizonte da manipulação da composição do leite será direcionada ao uso do leite como um alimento funcional para melhorar a saúde humana e combater doenças clínicas como a obesidade, intolerância à lactose ou osteoporose.

O fornecimento de AGPI através da linhaça, a qual contém em torno de 53% de AG alfa-linolênico (NRC, 2001) tem aumentado a proporção de AG n-3 na gordura do leite (Petit, 2002). Efeitos benéficos também têm sido encontrados infundindo óleo de linhaça no duodeno para melhorar a composição da gordura do leite (Petit et al., 2002). A concentração de AG n-3 foi aumentada de 1,5%, em média, do total de ácidos graxos em tratamentos com Megalac, linhaça tratada com formaldeído e uma mistura de 50% de linhaça tratada com formaldeído e 50% de óleo de peixe para 13,9% quando óleo de linhaça foi infundido no abomaso.

Potencial da linhaça como antioxidante natural

A linhaça é uma oleaginosa produzida em grande escala no Canadá, a qual é bastante utilizada na alimentação de vacas leiteiras para a produção de um leite enriquecido. O Canadá é o maior produtor mundial de linhaça com 633 500 toneladas produzidas anualmente, muito mais do que a produção americana que é de 149 963 toneladas e a brasileira de apenas 12 000 toneladas (FAO, 2007).

No Canadá, a linhaça pode ser comparada ao uso da soja no Brasil, a qual pode ser utilizada também para a incorporação de ácidos graxos poli-insaturados (Eifert et al., 2006) e ainda apresenta quantidades consideráveis de isoflavonas, o que pode tornar o leite dos animais alimentados com este ingrediente em um alimento funcional (Setchell e Cassidy, 1999).

Os fitoestrógenos são compostos naturais derivados de plantas e são classificados em três principais classes: isoflavonas, coumestanas e lignanas (Stopper et al., 2005). São ainda, compostos fenólicos que apresentam atividade antioxidante devido as propriedades de óxido redução (Degáspari e Waszczynskyj, 2004). Eles estão relacionados com efeitos positivos na prevenção de doenças cardiovasculares (Tikkanen e Adlercreutz, 2000) e sintomas da menopausa (Setchell e Cassidy, 1999).

Na nutrição humana, aumento do consumo de linhaça tem sido associado com a diminuição de doenças cardiovasculares, câncer de próstata e mama, osteoporose e sintomas da menopausa (Oomah e Mazza, 1998). Esses efeitos benéficos da linhaça são parcialmente mediados pelo secoisolariciresinol diglicosídeo (SDG), uma lignana vegetal, precursora de lignanas mamíferas que serão ativas no organismo humano (Adlercreutz, 2002; Wang, 2002).

Sob a ação de glicosidases intestinais, SDG é transformado em secoisolariciresinol (SECO) (Saarinen et al., 2002). Em monogástricos, a flora microbiana converte SECO em lignanas mamíferas, enterodiol (ED) e enterolactona (EL) (Setchell et al., 1980). As lignanas mamíferas são, em seguida, absorvidas e irão para a circulação enterohepática (Borriello et al., 1985).

As lignanas são mais dispersas em diferentes plantas do que isoflavonas. Além da linhaça, outras fontes de lignanas são as algas, frutas tipo “berries”, castanhas e vegetais (Mazur e Adlercreutz, 2000). Porém, a concentração de lignana foi determinada em um estudo e observou-se que o farelo de linhaça possui 75 vezes mais lignanas do que a alga e 804 vezes mais do que as frutas (Thompson, 1991). Desta forma, caracteriza-se a linhaça como a mais rica fonte desta classe de fitoestrógeno.

Em adição aos seus efeitos na saúde como antiestrogênico, o SDG e seus metabólitos (lignanas mamíferas) têm uma grande atividade antioxidante (Kitts et al. 1999). Esta propriedade é muito conhecida como uma evidência de um excelente mecanismo anticarcinogênico. Em um experimento conduzido por Petit et al. (2005) foi demonstrado um aumento linear da concentração de EL no leite quando a concentração de linhaça aumenta na dieta de vacas em lactação.

As lignanas, no grão de linhaça, estão concentradas em camadas que contêm mais fibra (Aldercreutz e Mazur, 1997), ou seja, a casca. E, a casca de linhaça, além de possuir grandes concentrações de lignanas apresenta alta quantidade de óleo (28%), podendo contribuir não só para a incorporação de EL no leite, assim como AGPI.

As lignanas presentes no leite, além de contribuir para a saúde de quem o ingere, podem contribuir também para a preservação dos AGPI transferidos ao leite prevenindo a sua oxidação, já que lignanas possuem propriedades antioxidantes. Porém, é necessário determinar se grandes quantidades de antioxidantes são necessárias para prevenir a oxidação do leite enriquecido com AGPI.

A atividade antioxidante da lignana SDG da linhaça e seus metabólitos, EL e ED, foi avaliada em ambos os sistemas de modelos *in vitro* aquoso e lipídico. Todas as três lignanas inibiram significativamente a peroxidação do ácido linoleico (Kitts et al., 1999).

A produção de um leite enriquecido com AGPI e lignanas seria um passo importante na confecção de um alimento funcional. Essa combinação resultaria em produto com valor agregado, utilizando o leite como um produto fornecedor de componentes com conhecidos efeitos benéficos à saúde humana (Jenkins & McGuire, 2006).

A utilização da Monensina sódica como estratégia de manipulação da fermentação ruminal

A monensina sódica, um ionóforo, tem sido usada extensivamente na dieta de vacas leiteiras, e seus efeitos na produção e composição do leite estão bem documentados (Van der Werf et al., 1998; Phipps et al., 2000; Duffield et al., 2003). A monensina é também conhecida por diminuir a biohidrogenação *in vitro* de AGPI's (Van Nevel and Demeyer, 1995) e aumentar *in vitro* a concentração de CLA (Fellner et al., 1997), sugerindo que a suplementação de monensina na dieta poderia aumentar a concentração de CLA e outros AGPI's no leite. Bell at al. (2006) concluíram que a combinação de óleo de cártamo com monensina fornecidos para vacas em lactação foram efetivos em aumentar o CLA no leite.

Portanto, fornecer uma combinação de monensina sódica e casca de linhaça poderia aumentar as concentrações de AGPI no leite, apesar de o fornecimento desses ingredientes combinados na transferência de antioxidantes para o leite não ser conhecida.

Em um experimento anterior (da Silva et al., 2007) foi encontrado que a suplementação com monensina para vacas em lactação aumentou a concentração de *cis*9, *trans*11-18:2 (CLA) e diminuiu a concentração de AG saturados no leite. Além disso, o fornecimento de grãos de linhaça triturados com monensina sódica resultou em maior concentração de *trans*11-18:1 (precursor do CLA) comparado com o fornecimento de grãos de linhaça inteiros com ou sem monensina sódica ou linhaça triturada sem monensina sódica. Portanto, a suplementação de monensina sódica com uma fonte de AG n-3 contribui para modificar a composição do leite para uma melhor saúde humana.

Sais de Cálcio

O uso de sais de cálcio na dieta pode colaborar para um incremento positivo na concentração de AG no leite aumentando AGPI e n-3 (Chouinard et. al, 1998; Juchem et. al, 2008). Porém, sua utilização pode trazer algumas desvantagens ao processo digestivo.

De maneira geral, quando sais de cálcio são adicionados a dietas, estes podem colaborar para uma diminuição na ingestão de MS por trazer efeitos negativos a motilidade ruminal, ao funcionamento do rúmen e a palatabilidade da dieta quando adicionados em teores maior do que 2% na MS da dieta (Loften and Cornelius, 2004). Além disso, pode ocorrer também uma diminuição da produção de leite (Harvatine e Allen, 2006) e da porcentagem de gordura no leite (Chouinard et al., 1998). Como pode ocorrer dissociação dos sais de cálcio já no rúmen, seguido de biohidrogenação (Castañeda-Gutiérrez et al., 2007), a alta concentração de AGPI e AG intermediários da biohidrogenação presentes no rúmen são causadores da depressão da gordura do leite (Bauman and Griinari, 2003). Segundo Chalupa et al. (1986) quando o pH do rúmen é inferior a 6, dissociação de sais de cálcio de ácido graxo ocorre no rúmen liberando AGPI.

Loor et al. (2005) relataram que ácidos graxos *trans* 18:1 (intermediários da biohidrogenação de AGPI no rúmen) atuam diretamente na diminuição do teor de gordura no leite através da inibição da enzima acetil CoA carboxilase.

Porém, a dissociação de sais de cálcio no rúmen não é total, podendo ainda incorporar AGPI no leite, além de alguns intermediários da biohidrogenação. Sais de cálcio de óleo de peixe têm sido efetivos em aumentar a concentração de AG n-3 no leite. Juchem et al. (2008), utilizando sais de cálcio de óleo de peixe juntamente com sais de cálcio de

óleo de palma em comparação ao sebo bovino, observaram um aumento na concentração dos AG EPA, DHA, CLA (isômero *cis*9,*trans*11-18:2), *trans*9 and *trans*11-18:1 na gordura do leite.

Uma alternativa para o uso de sais de cálcio de óleo de peixe seria o uso de sais de cálcio de óleo de linhaça, o qual, como já relatado é uma rica fonte do AG alfa-linolênico.

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OBJETIVOS GERAIS

Os objetivos deste trabalho foram avaliar:

A eficiência da flora ruminal e fecal na transformação de lignana vegetal em lignanas mamíferas, *in vitro;*

O potencial da casca de linhaça em melhorar a qualidade da gordura do leite pela incorporação de ácidos graxos poli-insaturados, além de lignanas mamíferas com poder antioxidante;

A influência da monensina sódica fornecida juntamente com fonte de ácido graxo poli-insaturado e lignanas vegetais (casca de linhaça) na transferência de ácidos graxos poli-insaturados e lignanas vegetais para o leite;

O potencial de sais de cálcio de óleo de linhaça em transferir ácidos graxos poli-insaturados para o leite;

O incremento de lignanas mamíferas incorporadas ao leite que a linhaça inteira proporciona em comparação ao fornecimento de sais de cálcio de óleo de linhaça.

CAPÍTULO I

(Normas: Journal of Applied Microbiology)

Metabolism of flax lignans by cows

In vitro metabolism of flax lignans by ruminal and fecal microbiota of dairy cows

Abstract

Aims: To determine the *in vitro* conversion of plant lignans from two flax products (hull and seed) into the mammalian lignans enterolactone and enterodiol by bovine ruminal and fecal microbiota .

Methods and Results: Flax seeds and hulls were incubated *in vitro* over a 96-h time course with ruminal or fecal inoculum. Plant lignans in flax seeds and hulls averaged 9.2 and 32.0 nmol mg^{-1}, respectively. The highest net production of enterodiol at 72 and 96 h of incubation was obtained with flax hulls incubated with fecal microbiota. There was no difference in net production of enterodiol between flax products within the first 24 h of incubation. In general, net production of enterolactone over the 96-h time course was significantly higher for flax products incubated with ruminal than with fecal microbiota. Net production of enterolactone at 72 and 96 h of incubation was greater for flax hulls than flax seeds.

Conclusions: Results of the present experiment suggest that, of the metabolites studied, the main mammalian lignan metabolite produced from flax hulls and seeds by ruminal microbiota is enterolactone while fecal microbiota leads mainly to the net production of enterodiol.

Significance and Impact: This research will improve the understanding of the metabolic pathway of mammalian lignans in dairy cows, in order to enable targeted manipulation of their quantities in milk.

Introduction

Phytoestrogens are naturally occurring compounds derived from plants and they belong to three main classes of polyphenolic compounds: isoflavones, coumestans, and lignans

(Stopper *et al.* 2005). In the western diet, lignans are more prevalent than isoflavones (Ganry 2005). Contrary to most isoflavones and coumestans, lignans have no *in vitro* genotoxic effects (Stopper *et al.* 2005). Flaxseed is one of the richest sources of the plant lignan precursor secoisolariciresinol diglucoside (SDG; Axelson *et al.* 1982). Lignans in grain are concentrated in the outer fibre-containing layers (Aldercreutz and Mazur 1997), which would lead to higher concentration of SDG in hulls than seeds. Under the action of intestinal glycosidases, SDG is transformed into secoisolariciresinol (SECO; Saarinen *et al,* 2002). In monogastric animals, colonic microbiota convert SECO to mammalian lignans, mainly enterodiol (ED) and enterolactone (EL; Setchell *et al.* 1980). Plant lignan precursor SDG is first converted to SECO, which is converted to ED and thereafter to EL (Figure 1). Mammalian lignans are subsequently absorbed and undergo enterohepatic circulation (Borriello *et al.* 1985). Production of mammalian lignans from plant lignans is well linked to various beneficial effects including antiestrogenic, anticarcinogenic and antioxidant activities (Kitts *et al.* 1999; Stark and Madar 2002; Thompson and Ward 2002). Mammalian lignan metabolites ED and EL have greater antioxidant activity than vitamin E (Prasad 2000) and it has been shown that people with higher blood concentrations of EL have lower incidence of cardiovascular diseases (Vanharanta *et al.* 1999).

Different studies have confirmed the presence of polyphenolic compounds such as equol, daidzein, and genistein (Bannwart *et al.* 1988; King *et al.* 1998) and mammalian lignan EL (Antignac *et al.* 2004) in the milk of dairy cows. Phytoestrogens such as isoflavones are metabolized by rumen microbes (Dickinson *et al.* 1988) and their absorption takes place mainly in the rumen (Lundh *et al.* 1990). However, the activity of ruminant microbiota for the conversion of flax lignans to mammalian lignans is unknown and there is no information on the different mammalian lignans produced from flax lignans by fecal and rumen microbiota of cattle. Greater concentrations of mammalian lignan metabolites in milk could result in health benefits but more information on the metabolism of flax lignans is required to enable targeted modification of the concentration of mammalian lignans in milk. Therefore, the objectives of this work were to determine the *in vitro* conversion of plant lignans from two flax products (hull and seed) into the mammalian lignans enterolactone and enterodiol by bovine ruminal and fecal microbiota.

Materials and methods

Ruminal inoculum

Two ruminally fistulated, dry Holstein cows were fed only alfalfa hay and minerals and vitamins for at least 4 weeks prior to sampling and no antibiotics were given. Animals were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC 1993). Ruminal inoculum was prepared by collecting rumen contents from the anterior dorsal, anterior ventral, medium ventral, posterior dorsal, and posterior ventral locations within the rumen before the morning feeding (0700 h). Rumen contents from both cows were combined and placed in a pre-warmed (39°C) thermos flask filled with CO_2. Rumen contents were immediately transferred to the laboratory and homogenized for 30 s in a blender flushed with oxygen-free CO_2. The homogenate was then strained through four layers of cheesecloth into an insulated thermos. The strained ruminal contents were mixed with a buffered anaerobic mineral solution (BAMS) previously described by Cone *et al.* (1996) to obtain a mixture containing 10 % filtered rumen contents (v/v). All manipulations were done under continuous flushing with oxygen-free CO_2.

Fecal inoculum

Fecal inoculum was prepared by collecting fresh feces directly from the rectum of cows prior to rumen content sampling. Fresh feces from both cows were combined and placed in a pre-warmed thermos flask filled with CO_2. Feces were immediately transferred to the laboratory, homogenized for 30 s with the BAMS to obtain a first mixture containing 50% feces (w/v), and filtered through four layers of cheesecloth. The strained feces were then diluted with the BAMS to obtain a final mixture containing 10% feces (v/v). All manipulations were done under continuous flushing with oxygen-free CO_2.

In vitro incubations

In vitro incubations were conducted in 16 ml screw cap glass culture tubes (16 x 125 mm; VWR Canlab, Montreal, QC, Canada) in which 140 mg of ground flax products (hulls or seeds) were incubated in 12 ml of buffered filtered rumen contents or buffered feces

mixtures to compare the effects of ruminal and fecal microbiota on the production of mammalian lignans. Flax products were ground in a coffee mill (Krups, Scarborough, ON, Canada) before incubation. Duplicate samples were incubated at 39°C under anaerobic conditions (oxygen-free) for 0, 6, 12, 24, 48, 72, and 96 h. The experiment was repeated on 2 consecutive weeks and conducted as a complete randomized block design with inoculum as the main effect. Ruminal and fecal collections were repeated for each of the 2 weeks. In each series duplicate blanks (filtered rumen contents or fecal material without substrate) were incubated for 0, 12, 48, and 96 h. At the end of each incubation time, the pH was measured. Incubations were placed in the freezer until frozen for freeze-drying and kept at -20°C for further analysis of lignans.

Lignan extraction

Extraction of plant SDG was performed by mixing 225 mg of flax products with 4.0 ml of isopropyl alcohol as described in the patent of Pizzey (2006). After a waiting period of 20 min, 8 ml of 2N NaOH were added. Samples were sonicated (VWR Brand Ultrasonic Cleaners, Model 50D; VWR Canlab) at 40 kHz for 60 min at 60^{o}C. After cooling, 25 ml of methanol/water (40:60) and 6 ml of phosphoric acid (20%) were added. The volume was made up to 50 ml with methanol/water (40:60) and the solution was filtered through a 0.45µm-syringe filter. The samples were run directly on the HPLC after extraction. Lignans SECO, ED, and EL were extracted as described previously by Heinonen *et al.* (2001) with some modifications. Freeze-dried samples of fecal and ruminal incubations were resuspended in Milli-Q purified water (20 mg 0.5 ml^{-1} for 0, 6, and 12-h incubation samples and 2 mg 0.5 ml^{-1} for 24, 48, 72, and 96-h incubation samples) and acidified with 10:l of 6 N HCl. The acidified samples were extracted with 2 ml of diethyl ether. The samples were vortex-mixed twice for 2 min. Organic layer was separated by freezing. The remaining liquid phase was submitted to a second extraction under the same conditions. The organic layers were pooled and evaporated by vacuum (Speed-Vac) at room temperature. The dry extract was dissolved in 375 µl of methanol and 125 µl of 0.2 M sodium acetate buffer (pH 4) by vortexing and filtered on a 0.22-µm PTFE membrane filter (Fisher Scientific, Nepean, ON, Canada) into a HPLC vial. A 20-µl aliquot was injected into the HPLC system.

HPLC analysis

Analyses of SDG in flax products were performed on a HPLC (model 1100, Hewlett Packard, Avondale, PA, USA) set to UV detection at 281 nm as described in the patent of Pizzey (2006). The mobile phase consisted of different ratios of 0.2% H_3PO_4 (solvent A) and acetonitrile (solvent B) mixed together (A:B, v/v): 0 min (90:10), 16 min (70:30), 22 min (10:90), 30 min (0:100) and 35 min (90:10). The mobile phase was filtered on a 0.22-µm nylon membrane filter (Gelman-Sciences, Ann Arbor, MI, USA) and then pumped through the column at a flow rate of 1.15 ml min^{-1}. The column was a C18, 5 µm, 250 × 4.6 mm (Luna ®; Phenomenex, Torrance, CA, USA). Injection volume was set to 10 µl. The SDG peak was identified (retention time: 11.85 min) and quantified by comparison with a standard peak of SDG.

Analyses of lignans SECO, ED, and EL were conducted as described by Nurmi and Adlercreutz (1999) with some modifications. Briefly, lignans were quantified by a HPLC (model 210, Varian, Mississauga, ON, Canada) using a solvent delivery system equipped with a 20-µl injection loop autosampler (model 410, Varian) and an electrochemical detector (Coulachem II; ESA, Concord, ON, Canada) set as follows: guard cell (model 5020; E = +950 mV) and analytical cell (model 5010, E1 = 0 mV; E2 = +900mV). Sensitivity of the detector was set to 10 µA. Analyses were carried out on a C_{18} reversed-phase column (4.6 mm x 250 mm Microsorb-mv; #R0086200C5; Varian). The mobile phase consisted of a mixture of two eluents (50/50): A, 50 mM sodium acetate buffer, pH 4.8/methanol, 80/20; and B, 50 mM sodium acetate buffer, pH 4.8/methanol/acetonitrile, 40/40/20. The mobile phase was filtered on a 0.22-µm nylon membrane filter (Gelman-Sciences, Ann Arbor, MI, USA) and then pumped through the column at a flow rate of 1 ml min^{-1}. All the solvents were HPLC-grade from Fisher Scientific. Concentrations of SECO, ED, and EL were quantified using standards.

Statistical analysis

All data were analyzed as a randomized design with repeated measurements using PROC MIXED of SAS (SAS 2000). The model included the fixed effects of inoculum (feces vs. rumen microbiota), flax product (hulls vs. seeds), inoculum by flax product interaction,

hour, inoculum by hour interaction, flax product by hour interaction, inoculum by flax product by hour interaction, and the residual error. Conversion of plant lignans into mammalian lignans was also analyzed. Data were also analyzed at each incubation time and the model included the fixed effects of inoculum (fecal vs. ruminal microbiota), flax product (hulls vs. seeds), inoculum by flax product interaction. Differences among significant interaction means were assessed using Tukey's multiple-range test. Residuals were plotted to detect assumptions of normality and homogeneity of variance. Data on SECO, ED and EL were transformed (log) as previously performed by Hausner *et al.* (2004) and Nesbitt *et al.* (1999) because of lack of variance homogeneity and variation in lignan concentrations but results in Tables were expressed as the mean value of duplicate runs on the original scale of measurements.

Results

Initial concentration of SDG in ground hulls (GH) and ground seeds (GS) was 32.0 and 9.2 nmol mg^{-1}, respectively. Typical HPLC chromatograms of GH incubated over the 96-h period with ruminal (Figure 2) and fecal (Figure 3) microbiota for one week incubation are presented. Some peaks were off-scale as a number of samples required dilution at certain incubation times while others did not as specified in the Materials and methods section. At initiation of the incubation (0 h), the lignan metabolites produced from GH were not detected. During incubation, plant SDG was metabolized rapidly into SECO and SECO led to formation of ED and EL by both the ruminal and fecal microbiota. At the end of the incubation (96 h), EL was the major metabolite detected with ruminal microbiota (Figure 2) while the major metabolite detected with fecal microbiota was ED (Figure 3). Incubation with ruminal and fecal microbiota resulted in the formation of other metabolites as suggested by the unidentified peaks present on both chromatographic profiles. As only SECO, ED, and EL standards were used in the present experiment, identification of these metabolites was not possible. Similar results were obtained for GS (data not shown). Peaks present in the inoculum blank controls remained generally similar over the 96-h incubation period (Figure 4 and 5).

The quantitative net production of SECO, ED, and EL over time by ruminal and fecal microbiota is summarized in Tables 1, 2, and 3, respectively. At initiation of

incubation (0 h), SECO concentration was below detection level when GH and GS were incubated with ruminal and fecal microbiota. The highest net production of SECO within the first 6 h of incubation was obtained for GH incubated with ruminal microbiota. Net production of SECO was higher for GH than GS for both inocula until 12 h of incubation. At 24 h of incubation, net production of SECO had a strong tendency ($P = 0.05$) to be higher for GH than for GS. No further significant differences were observed after 48 h. At 96 h of incubation, SECO was still present in both flax products incubated with ruminal microbiota but all SECO had disappeared when GS was incubated with fecal microbiota .

Net production of ED was initiated at 6 h of incubation (Table 2) and ruminal microbiota tended ($P = 0.07$) to result in greater ED net production than fecal microbiota. Net production of ED was similar among treatments at 12 and 24 h of incubation. Differences in net production of ED became more apparent from 48 h of incubation. At 48 h, fecal microbiota resulted in greater ED net production than ruminal microbiota and GH tended ($P = 0.07$) to increase ED net production compared to GS. In the last two incubation times (72 and 96 h), the highest net production of ED was obtained when GH was incubated with fecal microbiota.

Net production of EL was similar among treatments within the first 6 h of incubation (Table 3). After 6 h, net production of EL was significantly greater when flax products were incubated with ruminal than with fecal microflora. Net production of EL was similar between GH and GS until 48 h of incubation with differences becoming larger by 72 h, GH producing more EL than GS.

The conversion of plant lignans SDG into mammalian lignans (ED + EL) over time was significantly lower for flax products incubated with ruminal microbiota than for those incubated with feces, which resulted in an interaction ($P = 0.003$) between source of inoculum and time. There was no interaction ($P > 0.05$) between flax product and time and between flax product and inoculum for the conversion of plant SDG into ED and EL. Conversion of plant lignans SDG into mammalian lignans was more important within the first 24 h of incubation for GS than GH and there was no difference later on. From 72 h of incubation, fecal microbiota significantly converted more plant SDG into mammalian lignans (ED + EL) than ruminal microbiota.

Discussion

There was net production of SECO from SDG by both ruminal and fecal microbiota as shown by the HPLC chromatographic profiles, which may result from hydrolyzation of SDG in SECO under the action of intestinal glycosidases (Saarinen *et al.* 2002). Previous results have shown that the activity of intestinal glycosidases is from enzymes of mammalian (Day *et al*, 1998) and microbial (Saarinen *et al.* 2002; Clavel *et al*, 2006) origin. However, the ruminal epithelium is non-secretory and epithelial cells were absent in our *in vitro* model, thus suggesting that the origin of intestinal glycosidases in the present experiment was from microbiota. Metabolites of SDG other than ED and EL have been identified with human microbiota by Wang *et al.* (2000), which may explain the unidentified peaks present on the two chromatographic profiles observed for ruminal and fecal microbiota. Moreover, plant lignans such as matairesinol, pinoresinol, and lariciresinol represent around 2.4% of the total lignans found in flax seed (Liu *et al*. 2006). Therefore, precursors of mammalian lignans other than SDG may also explain the unidentified peaks in our chromatographic profiles. As peaks present in the inoculum blank controls were generally similar over the incubation period, this may suggest that the unidentified peaks that appeared or disappeared during incubation of flax products were mainly the result of flax product metabolism by fecal and ruminal microbiota and not from products present in the microbiota itself. Heinonen *et al.* (2001) reported that SDG was mainly metabolized by human microbiota to EL and ED with no other metabolites being produced. Differences between the present study and other previously reported findings may be due to differences in *in vitro* incubation time and individual variation of microbiota of feces used for *in vitro* fermentation. Large within-day and day-to-day variation may also be important as shown by Hausner *et al.* (2004) for EL levels in human serum (56%) and urine (49%).

Higher net production of SECO for GH than GS incubated with ruminal microbiota may result from the higher initial concentration of SDG in GH than GS (32.0 vs. 9.2 nmol 100 mg^{-1}) as hydrolyzation of SDG is known to produce SECO under the action of intestinal glycosidases from microbial origin (Saarinen *et al.* 2002). Rumen microorganisms readily hydrolyse isoflavones present in the glycoside form (Lundh 1995) and SDG is the glycoside form of SECO (Johnsson *et al.* 2000). Therefore, higher

concentration of SDG in GH than GS is likely cause of the increased concentration of SECO with GH.

Greater net production of ED than EL by ruminal microbiota was observed at 6 h of incubation, thus suggesting that the first step in mammalian lignan production by ruminal microbiota from SECO was ED, the precursor leading to EL production. This would agree with the results of Borriello *et al.* (1985) who have reported that SECO incubated with human fecal microbiota led first to the production of ED. Similarly, a delay between production of ED and that of EL has been reported for flax seed incubated with human fecal microbiota in an *in vitro* fermentation system (Aura *et al.* 2006). However, the net production of EL by ruminal microbiota was higher over time than that of ED. Concentration of EL has been shown to be higher than that of ED in the milk of cows fed flaxseed meal (Petit *et al.* 2005). This may suggest that contrary to what has been previously reported for monogastric mammals such as humans (Heinonen *et al.* 2001; Hausner *et. al.* 2004), rats (Niemeyer *et al.* 2000; Saarinen *et al.* 2000), and pigs (Knudsen *et al.* 2003), absorption of EL may occur either in the rumen or the small intestine of ruminants before further conversion of ED to EL by colon microbes. Similarly, it has been shown that other phytoestrogens of the isoflavones family such as formononetin are metabolized to equol by bovine ruminal microbiota and that equol is available for absorption from the gastrointestinal tract (Dickinson *et al.* 1988). According to Lundh *et al.* (1990), absorption of phytoestrogens takes place mainly in the rumen.

Results of the present experiment indicate that the main mammalian lignan produced by ruminal microbiota from flax products rich in plant SDG is EL. On the other hand, results showed that net production of ED was higher than that of EL by fecal microbiota, suggesting that the main mammalian lignan produced by fecal microbiota from a rich source of plant SDG is ED. Borriello *et al.* (1985) have demonstrated that the production of EL from ED by human fecal flora depends on the numbers of viable bacteria present in stool with high dilutions of bacteria resulting in no conversion of ED to EL. However, there is some controversy regarding the capability of fecal flora of mammals to convert ED to EL. No conversion of ED to EL was observed after the incubation with a rat fecal suspension *in vitro* (Wang *et al.* 2000) while other studies (Aura *et al.* 2006; Liu *et al.* 2006) showed a conversion of SDG to mammalian lignans ED and EL in rats. Difference in

bacterial flora of feces may explain discrepancies between studies. Lower activity of the microbial population present in feces than in ruminal contents has been observed by Mauricio *et al.* (2001), which could contribute to lower net production of EL by fecal than ruminal microbiota.

Results of the present experiment generally indicate that the conversion of plant lignans SDG into mammalian lignans (ED + EL) was higher for flax products incubated with fecal than with ruminal microbiota. Using human fecal inoculum for 24 h incubations, Heinonen et al. (2001) reported a conversion of 72% from pure SDG to ED + EL mammalian lignans, which is higher than values observed in the present experiment.

This study has shown that plant lignans present in flax hulls and flax seeds are converted by ruminal and fecal microbiota of dairy cows to mammalian lignans. However, the kinetics of plant lignan metabolism was affected by the type of microbiota (ruminal vs. fecal) and flax product (hulls vs. seeds). The main mammalian lignan produced by ruminal microbiota was enterolactone while fecal microbiota led mainly to the production of enterodiol. More research is required to advance the understanding of the metabolic pathway of mammalian lignans in dairy cows, in order to enable targeted manipulation of their quantities in milk.

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FIGURES CAPTIONS

Figure 1. Chemical structures of the plant lignan secoisolariciresinol diglucoside and its metabolites.

Figure 2. High-performance liquid chromatography obtained from *in vitro* incubations (0 to 96 h) of ground flax hulls incubated with ruminal microbiota. The lignans, secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (EL) were identified using standards and peak retention times are presented above peaks.

Figure 3. High-performance liquid chromatography obtained from *in vitro* incubations (0 to 96 h) of ground flax hulls incubated with fecal microbiota. The lignans, secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (EL) were identified using standards and peak retention times are presented above peaks.

Figure 4. High-performance liquid chromatography obtained from *in vitro* incubations (0 to 96 h) of blank controls incubated with ruminal microbiota without substrate. The lignans, secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (EL) were identified using standards and peak retention times are presented above peaks.

Figure 5. High-performance liquid chromatography obtained from *in vitro* incubations (0 to 96 h) of blank controls incubated with fecal microbiota without substrate. The lignans, secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (EL) were identified using standards and peak retention times are presented above peaks.

Fig. 1

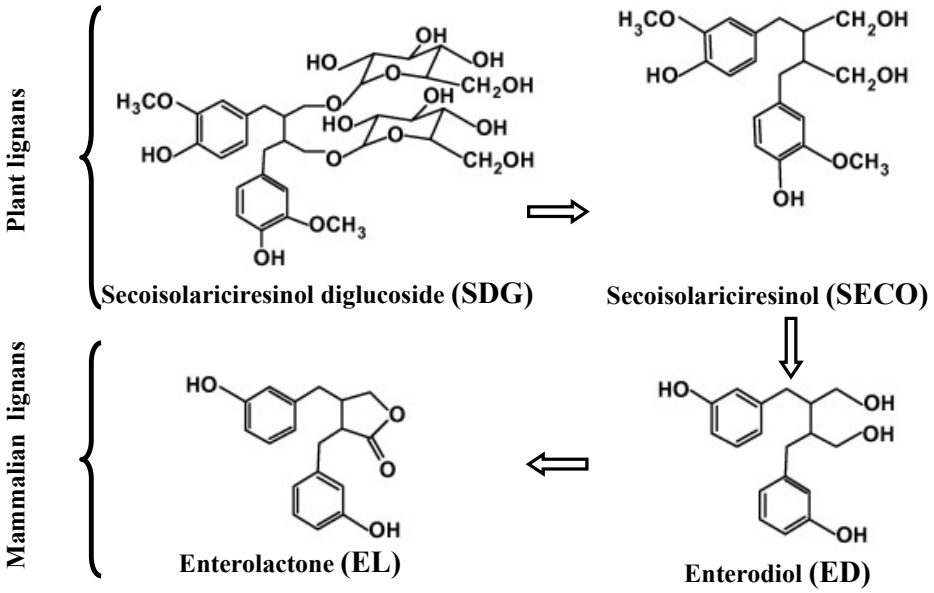
Plant lignans
Mammalian lignans
Secoisolariciresinol diglucoside (SDG)
Secoisolariciresinol (SECO)
Enterolactone (EL)
Enterodiol (ED)

Fig. 2

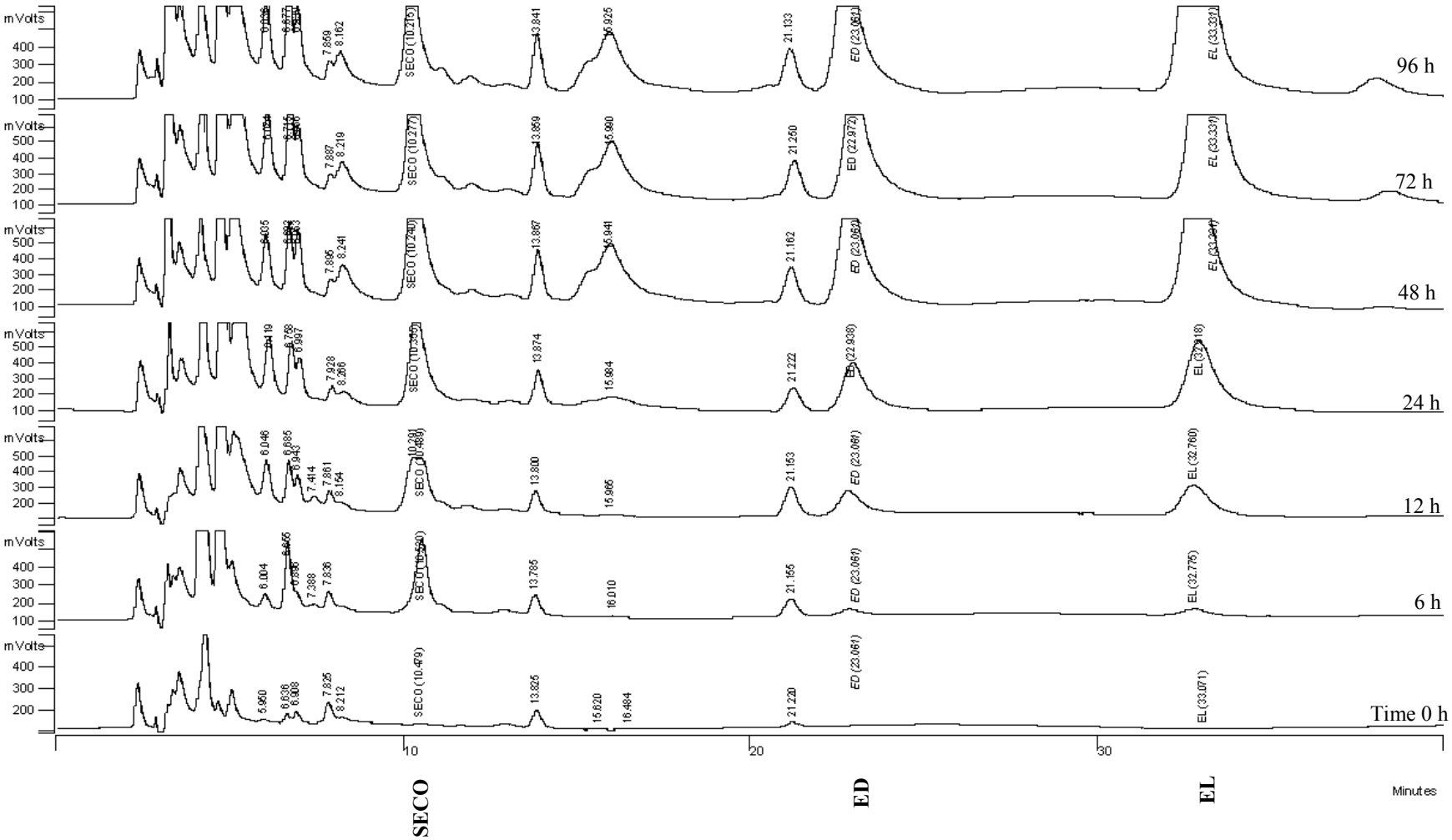
mVolts
96 h
72 h
48 h
24 h
12 h
6 h
Time 0 h
SECO
ED
EL
Minutes
10
20
30

Fig. 3

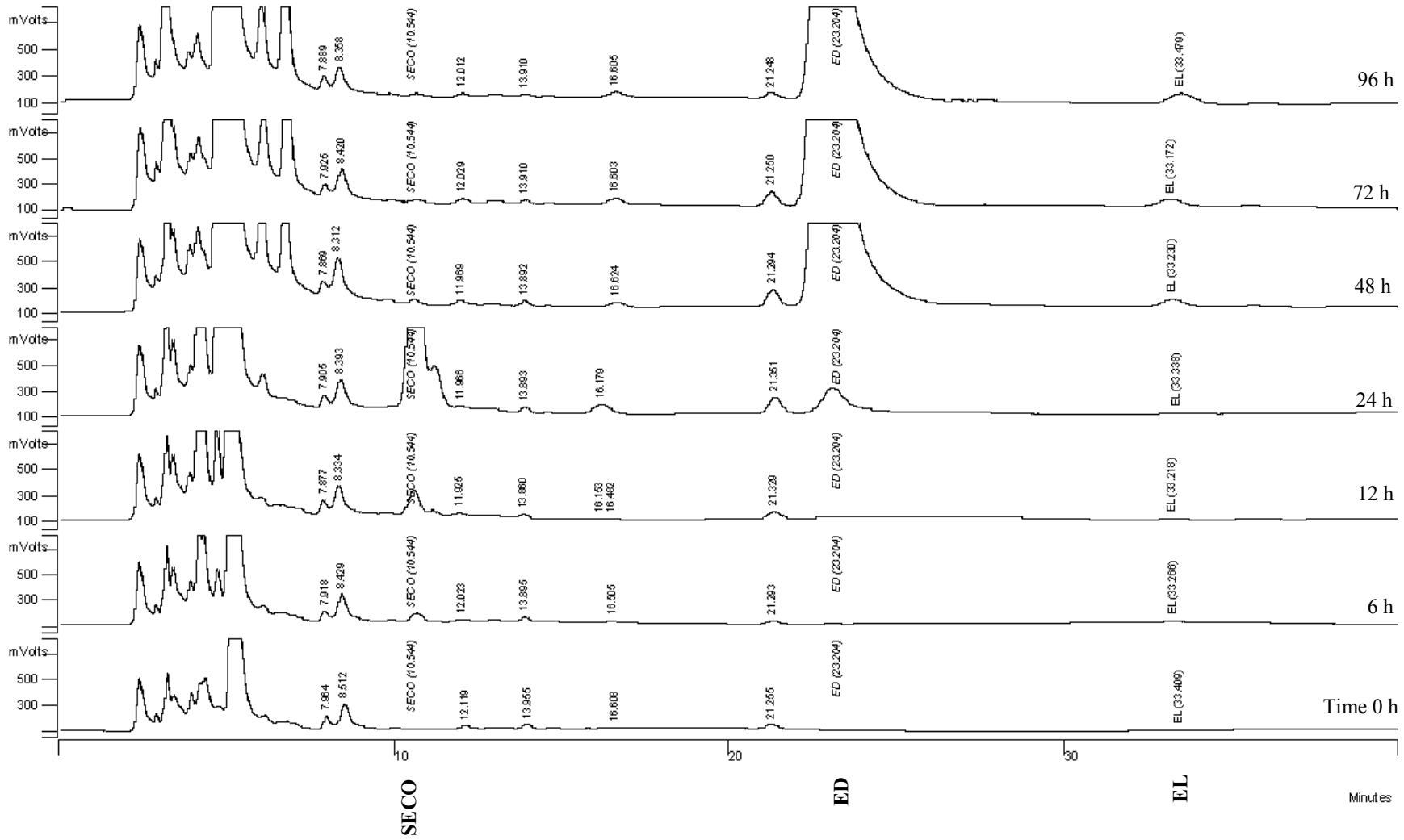
mVolts
500
300
100
7.889
8.358
SECO (10.544)
12.012
13.910
16.605
21.248
ED (23.204)
EL (33.479)
96 h
7.925
8.420
12.029
16.603
21.260
EL (33.172)
72 h
7.889
8.312
11.969
13.892
16.624
21.284
EL (33.230)
48 h
7.905
8.393
11.966
13.893
16.179
21.351
EL (33.338)
24 h
7.877
8.334
11.925
13.860
16.153
16.482
21.329
EL (33.218)
12 h
7.918
8.429
12.023
13.895
16.605
21.293
EL (33.266)
6 h
7.964
8.512
12.119
13.955
16.608
21.255
EL (33.409)
Time 0 h
10
20
30
SECO
ED
EL
Minutes

Fig. 4

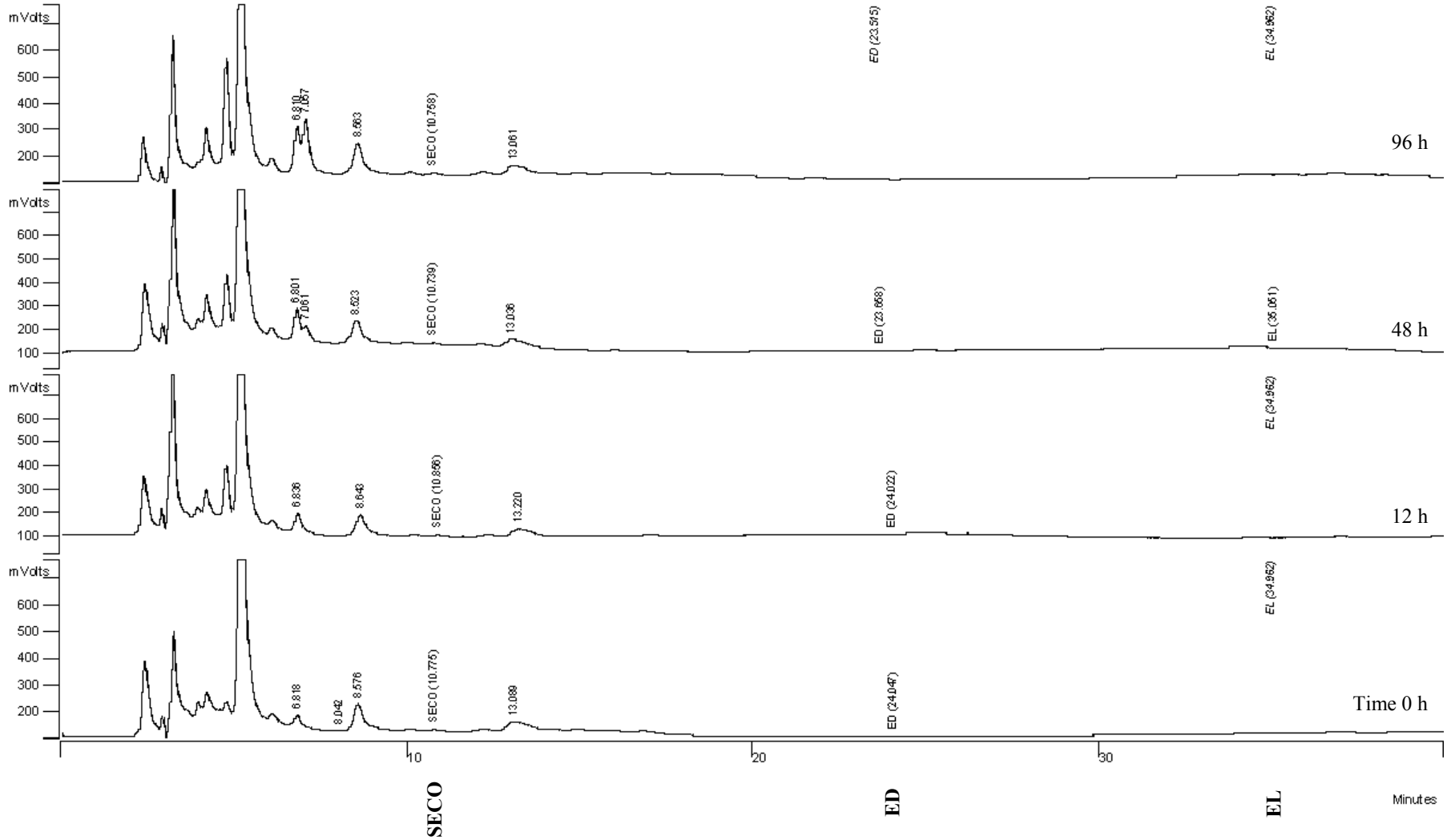
mVolts
96 h
48 h
12 h
Time 0 h
SECO (10.758)
SECO (10.739)
SECO (10.866)
SECO (10.775)
ED (23.515)
ED (23.668)
ED (24.022)
ED (24.047)
EL (34.962)
EL (35.051)
10
20
30
Minutes
SECO
ED
EL

Fig. 5

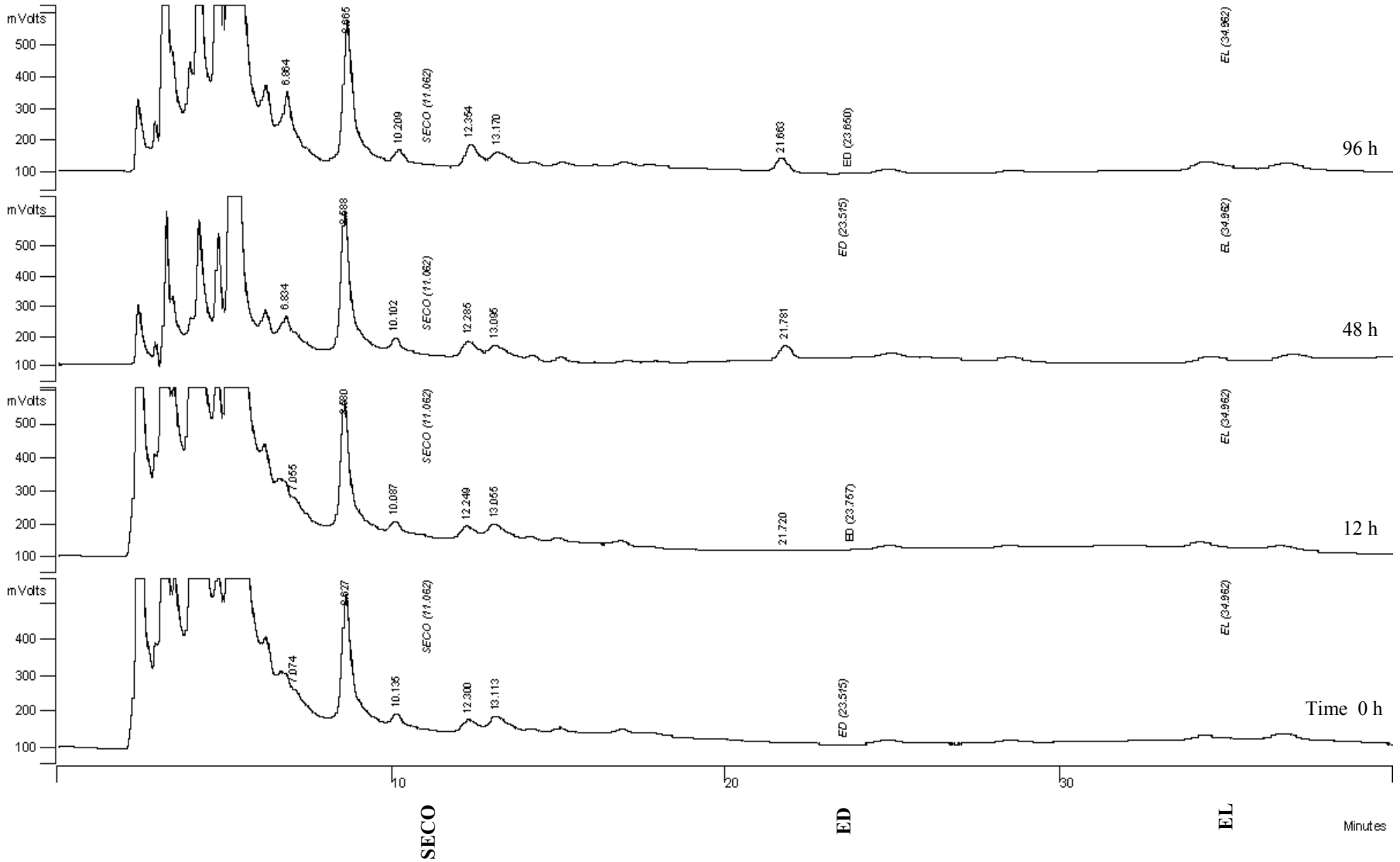
mVolts
500
400
300
200
100
6.864
9.865
10.209
SECO (11.062)
12.354
13.170
21.663
ED (23.650)
EL (34.962)
96 h
6.834
9.688
10.102
SECO (11.062)
12.285
13.095
21.781
ED (23.545)
EL (34.962)
48 h
7.055
9.680
10.087
SECO (11.062)
12.249
13.055
21.720
ED (23.757)
EL (34.962)
12 h
7.074
9.627
10.135
SECO (11.062)
12.300
13.113
ED (23.545)
EL (34.962)
Time 0 h
10
20
30
SECO
ED
EL
Minutes

Table 1 Quantitative high-performance liquid chromatography results of net production of secoisolariciresinol (SECO) from ground flax hulls (GH) and flax seeds (GS) incubated with ruminal or fecal microbiota*

Hours	SECO (nmol 100 mg $^{-1}$ of substrate)				*P* value		
	GH		GS				
	ruminal	fecal	ruminal	fecal	Flax product	Microbiota	Interaction
0	N.D.	N.D.	N.D.	N.D.			
6	80.29[a]	11.07[b]	14.00[b]	10.64[b]	0.02	0.01	0.02
12	116.21	47.93	14.50	19.00	0.01	0.10	0.08
24	81.29	95.00	3.50	19.00	0.05	0.64	0.98
48	81.86	25.86	4.36	4.57	0.21	0.44	0.44
72	58.36	7.21	4.86	6.14	0.28	0.31	0.29
96	109.78	5.79	16.93	N.D.	0.31	0.23	0.

*Data are presented as non-transformed values expressed as the mean value of duplicate runs (n = 2 per assay) on the original scale of measurements.

N.D., not detected.

[a,b]Means within a row with different superscripts are significantly different ($P < 0.05$).

Table 2 Quantitative high-performance liquid chromatography results of net production of enterodiol (ED), a mammalian lignan, from ground flax hulls (GH) and flax seeds (GS) incubated with ruminal or fecal microbiota*

Hours	ED (nmol 100 mg $^{-1}$ of substrate)				*P* value		
	GH		GS				
	ruminal	fecal	ruminal	fecal	Flax product	Microbiota	Interaction
0	N.D.	N.D.	N.D.	N.D.			
6	9.50	4.71	13.29	3.07	0.74	0.07	0.43
12	29.07	26.57	95.21	25.36	0.20	0.16	0.18
24	120.36	135.57	147.93	134.28	0.83	0.99	0.81
48	305.64	1812.56	134.50	472.92	0.07	0.04	0.14
72	215.64^{bc}	2222.27^{a}	138.64^{c}	560.07^{b}	<0.001	<0.001	<0.001
96	234.35^{b}	2193.20^{a}	128.21^{b}	699.07^{b}	<0.001	0.001	0.01

*Data are presented as non-transformed values expressed as the mean value of duplicate runs (n = 2 per assay) on the original scale of measurements.

N.D., not detected.

^{a,b,c}Means within a row with different superscripts are significantly different ($P < 0.05$).

Table 3 Quantitative high-performance liquid chromatography results of net production of enterolactone (EL), a mammalian lignan, from ground flax hulls (GH) and flax seeds (GS) incubated with ruminal or fecal microbiota*

Hours	EL (nmol 100 mg $^{-1}$ of substrate)				*P* value		
	GH		GS				
	ruminal	fecal	ruminal	fecal	Flax product	Microbiota	Interaction
0	N.D.	N.D.	N.D.	N.D.			
6	2.79	1.79	2.71	1.57	0.93	0.48	0.99
12	33.43	1.71	23.00	1.43	0.60	0.05	0.62
24	92.00	6.71	106.00	4.79	0.81	0.01	0.74
48	400.43	61.50	239.57	23.86	0.32	0.03	0.52
72	787.35	127.50	313.43	30.14	0.06	0.01	0.17
96	827.64	221.57	292.14	45.36	0.03	0.01	0.17

*Data are presented as non-transformed values expressed as the mean value of duplicate runs (n = 2 per assay) on the original scale of measurements.

N.D., not detected.

Table 4 Conversion of plant lignan (SDG) to mammalian lignans (ED + EL) from ground flax hulls (GH) and flax seeds (GS) incubated with ruminal microbiota or fecal microbiota

Hours	Conversion (%)				*P* value		
	GH		GS				
	ruminal	fecal	ruminal	fecal	Flax product	Microbiota	Interaction
0	N.D.	N.D.	N.D.	N.D.			
6	0.4	0.2	1.7	0.5	0.05	0.08	0.17
12	2.0	0.9	12.9	2.9	0.06	0.09	0.15
24	6.6	4.4	27.7	15.2	0.02	0.19	0.33
48	22.0	58.5	40.8	54.2	0.56	0.09	0.37
72	31.3	73.4	49.3	64.4	0.68	0.05	0.26
96	33.2	75.4	45.8	81.2	0.36	0.01	0.72

CAPÍTULO II

(Normas : Journal of Dairy Science)

Monensin and Flaxseed Hulls on Apparent Digestibility and Milk Fatty Acid Composition in Late-lactating Dairy Cows

ABSTRACT

Four rumen fistulated multiparous Holstein cows averaging 665 ± 21 kg of body weight and 190 ± 5 d in milk were assigned to a 4 x 4 Latin square design with four 28-d experimental periods to determine the effects of feeding monensin and flaxseed hulls on milk quality. The treatments were: 1) control (no flaxseed hulls and monensin; CO), 2) diet containing (dry matter basis) 20% flaxseed hulls (FH), 3) diet with monensin (16 mg/kg of dry matter; MO), and 4) diet containing 20% (dry matter basis) flaxseed hulls and 16 mg/kg monensin (HM). The apparent digestibility of CP was higher for diets containing flaxseed hulls and for diets supplemented with monensin. Feeding flaxseed hulls decreased the apparent digestibility of ADF. Significant higher digestibility of ether extract was observed for treatments with flaxseed hulls compared with treatments without flaxseed hulls. Feeding flaxseed hulls increased concentrations of total *trans* fatty acids **(FA)**, monounsaturated FA, polyunsaturated FA, long-chain FA and n-3 FA, and the polyunsaturated to saturated FA ratio in milk fat. The n-6 to n-3 FA ratio in milk fat was lower for cows fed flaxseed hulls compared with those fed no flaxseed hulls. Monensin supplementation increased the concentrations of *cis*9-16:1 and *cis*9,12-18:2 in milk fat. Feeding flaxseed hulls is a good strategy to improve milk FA profile for better human health and monensin had no effect on milk FA profile.

Key Words : dairy cows, digestibility, milk quality, n-3, PUFA

INTRODUCTION

Flaxseed is an excellent source of alpha-linolenic acid and feeding flaxseed to cows increases concentration of polyunsaturated fatty acids (PUFA) in milk (Petit, 2002). Many studies have showed a transfer of FA from flaxseed to milk (Goodridge et al., 2001; Petit,

2002; Petit et al., 2002; Gonthier et al., 2005; Da Silva et al., 2007). Moreover, dairy cows infused with flaxseed oil directly in the abomasum have 14% of total fatty acids (FA) in milk fat in the form of alpha-linolenic acid (Petit et al., 2002). Flaxseeds, which are produced in Canadá on a large scale, have more than 28% oil and 55% of total FA are in the form of alpha-linolenic acid. Therefore, flax products rich in oil can provide a good source of dietary PUFA for their transfer in milk.

Monensin is known to decrease in vitro ruminal biohydrogenation of PUFA (Van Nevel and Demeyer, 1995) and to increase in vitro total conjugated linoleic acid (CLA) concentration (Fellner et al., 1997), thus suggesting that dietary supplementation of monensin could increase milk concentrations of CLA and other PUFA. Da Silva et al. (2007) observed that monensin supplementation increased concentration of cis9,trans11-18:2 (rumenic acid, CLA isomer) and decreased that of saturated FA in milk fat of lactating dairy cows. Also, feeding ground flaxseed with monensin resulted in higher concentration of *trans*11-18:1 (CLA precursor) compared to feeding whole flaxseed with or without monensin and ground flaxseed without monensin. Therefore, the main objective of the experiment was to determine the effect of feeding a combination of monensin and flaxseed hulls on digestibility and milk FA composition.

MATERIALS AND METHODS

Cows and Diets

Four rumen fistulated multiparous Holstein cows averaging 665 ± 21 kg of BW and 190 ± 5 DIM were assigned to a 4 x 4 Latin square design to determine the effects of monensin and flaxseed hulls supplementation on digestibility and milk FA composition. The four TMR (Table 1) consisted of four different diets with a 2 x 2 factorial arrangement of treatments: control with no flaxseed hulls **(CO)**, control with flaxseed hulls **(FH)**, monensin (16 ppm) with no flaxseed hulls **(MO)**, and monensin (16 ppm) with flaxseed hulls **(HM)**. Flaxseed hulls were added at 20% of the DM and all diets were equal in protein and energy. Cows were cared for in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1993).

Diets were formulated with similar chemical composition to meet nutrient requirements of 615 kg cow producing 29 kg/d of milk containing 3.9 % of fat (NRC,

2001). Dietary formulations were adjusted to account for changes in ingredient DM content. Cows were housed in tie stalls, fed individually for ad libitum intake (10% refusals) twice a day (0830 and 1530), and milked twice daily at 0800 and 1900 h. Milk production was recorded at every milking. Cows were weighed on the first and last day of each experiment period.

Experimental Procedures

Each experimental period consisted of 21 d of adaptation to the diets and 7 d of total collection of feces and urine for digestibility determination. Feed intake and milk yield were measured daily. Samples of the TMR were taken daily during the digestibility week and pooled within period for each cow. All samples were frozen at -20^{o}C for subsequent drying at 55^{o}C. Milk samples were obtained from each cow for 14 consecutive milkings during the digestibility trial and pooled on a yield basis. One sample was kept frozen at -20°C without preservative for further analyses of milk FA composition. Cows were fitted with harnesses and tubes allowing the collection of feces and urine separately on d 20. From d 21 to 28, feces were collected from a rubber mat placed behind the animals and stored in plastic containers. Daily feces were weighed and mixed thoroughly. A 2%-subsample was taken and stored at -20^{o}C for subsequent freeze drying. Total daily urine was collected in stainless steel containers via Gooch tube (BF Goodrich Co., Kitchener, ON, Canada) attached to the cow with a nylon netting covered with neoprene (Spall Bowan Ltd, Guelph, ON, Canada) affixed to the vulva. A 1%-daily subsample was taken and kept frozen until analysis. Urine was acidified daily with 100 ml of 10*N* H_2SO_4.

Chemical Analyses

Dry matter of the diets and feces were determined in a forced-air oven according to the procedure 934.01 (AOAC, 1990). Total mixed diets and freeze dried feces were ground to pass a 1-mm screen in a Wiley mill before analyses of N, ether extract, ADF, and NDF. Total N content of TMR and feces was determined by thermal conductivity (LECO model FP-428 Nitrogen Determinator, LECO, St. Joseph, MI) and CP was calculated as N x 6.25. The concentration of NDF in TMR and feces was determined as described by Van Soest et

al. (1991) with the use of sodium sulfite and with the inclusion of heat stable α-amylase. The ADF content in TMR and feces was determined according to AOAC (1990; Method 973.18). The NDF and ADF procedures were adapted for use in an ANKOM^{200} Fibre Analyzer (ANKOM Technology Corp., Fairport, NY). Ether extraction in diets and feces was conducted with Tecnal TE–044/1 (Piracicaba, São Paulo, Brazil) according to the method No. 7.060 (AOAC, 1990). The concentration of N in acidified urine samples was determined by micro-Kjeldahl analysis (AOAC, 1990; Method 960.52). Concentrations of protein in milk samples were analyzed by infrared spectrophotometer (System 4000 Milkoscan; Foss Electric of Hillerod, Denmark). Milk FA were extracted and methylated according to the method described by Chouinard et al. (1997) while in situ transesterification was performed on diets according to Park and Goins (1994). Fatty acid methyl esters were measured by GLC on a Hewlett-Packard 6890 chromatograph (Hewlett-Packard Lteé, Montreal, Qc, Canada) with a G1315A auto sampler equipped with a flame ionization detector and a split-splitless injector as described by Delbecchi et al. (2001).

Statistical Analysis

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

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

where: Y_{ijkl} = the dependent variable, μ = overall mean, C_i = random effect of cow (i = 1 to 4), P_j = fixed effect of period (k = 1 to 4), T_k = fixed effect of treatment (k = 1 to 4), and e_{ijk} = random residual error. Treatments were compared to provide factorial contrasts: 1) with vs. without monensin, 2) with vs. without flaxseed hulls, and 3) the interaction between monensin and flaxseed hulls. The residual effect was initially included in the model but was removed when it was not significant. Results are reported as least squares means. Significance was declared at $P \leq 0.05$ and a trend at $P < 0.10$ unless otherwise stated.

RESULTS and DISCUSSION

Feed Composition and consumption

The chemical composition of the TMR (Table 1) was generally similar among diets with the exception of DM and EE, which were greater for FH and HM than for CO and MO ($P < 0.01$). As expected, ether extract concentration was higher ($P < 0.0001$) for diets with than without flaxseed hulls as a result of high fat concentration in flaxseed hulls. Also, the NSC was lower for diets with flaxseed hulls as a result of lower percentage of corn grain in these diets. Concentrations of 12:0, 14:0, 16:0, 20:0, 22:0 and 24:0 were lower in diets containing flaxseed hulls, thus resulting also in lower SFA concentrations. Concentration of *cis*9,12-18:2 was higher for CO and MO than for FH and HM while the inverse was observed for *cis*9,12,15-18:3, showing that diets containing flaxseed hulls provide n-3 FA while those without flaxseed hulls provide more n-6 FA. As a result, the n-6:n-3 ratio was lower for diets containing flaxseed hulls.

Diet intake, digestibility and N Balance

There was no interaction ($P > 0.10$) between flaxseed hulls and monensin supplementation for DMI, expressed in kilograms per day or as a percentage of BW (Table 2). Intake of DM, expressed in kilograms per day or as a percentage of BW, was higher ($P = 0.04$ and $P = 0.05$, respectively) for treatments without flaxseed hulls (CO and MO) than for treatments with flaxseed hulls (FH and HM). Lower NSC content in diets with flaxseed hulls, which contains more structural carbohydrates, may likely limit feed intake by distension within the gastrointestinal tract (Allen, 2000). Intake of dry matter was similar ($P>0.10$) for cows supplemented or not with monensin.

There was no significant interaction ($P > 0.05$) between flaxseed hulls and monensin supplementation for diet digestibility and N balance (Table 2). Supplementation with flaxseed hulls had no effect on the apparent digestibility of DM but monensin tended ($P = 0.08$) to increase it. Apparent digestibility of CP was higher for diets containing flaxseed hulls and for cows fed diets supplemented with monensin compared to those not receiving monensin($P = 0.002$ and $P = 0.05$, respectively). Earlier studies showed inconsistent effects of monensin on N digestibility in dairy cows. For example, Plaizier et al. (2000) reported an increase in N digestibility when early lactating dairy cows were fed

monensin in the post-calving period while Ali-Haimoud et al. (1995) observed no effect of monensin on N digestibility. Discrepancies between studies could partly be due to differences in diet composition. According to Plaizier et al. (2000), monensin administration increases N digestibility in cows fed high concentrate diets while it has no effect in those fed high forage diets. Diets used in the present experiment contained approximately 57% concentrate (Table 1), which would corroborate that hypothesis.

The apparent digestibility of ADF was higher for CO and MO diets than for FH and HM diets ($P < 0.01$). Diets containing whole flaxseed have usually lower ADF digestibility than those without flaxseed (Petit, 2002; Petit et al., 2005). Apparent digestibility of ADF and NDF was not affected by monensin supplementation. This would agree with the results of Plaizier et al. (2000) who reported that monensin increased the apparent digestibilities of NDF and ADF in cows fed high forage diets while there was no effect on fibre digestion in cows fed high concentrate diets. Digestibility of NDF was similar for cows supplemented or not with flaxseed hulls (Table 2). Significant higher digestibility of ether extract ($P < 0.001$) was observed for treatments with flaxseed hulls (FH and HM) compared with treatments without flaxseed hulls (CO and MO). Diets without flaxseed hulls contained more saturated FA, which have lower digestibility than unsaturated FA present in flaxseed (Byers and Schelling, 1993). Monensin had no effect on digestibility of ether extract.

Intake and output of N in urine were similar among treatments (Table 2). Output of N in feces was significantly greater ($P < 0.01$) for cows fed CO and MO compared with those fed FH and HM as a result of lower digestibility of CP for cows fed the former diets. Output of N in milk was significantly lower ($P = 0.04$) for cows fed FH and HM compared with those fed CO and MO. Retention of N, expressed in grams per day, tended ($P = 0.09$) to be higher for cows supplemented with both flaxseed hulls and monensin, which may be a result of the positive effects of feeding monensin and flaxseed hulls on CP digestibility. Monensin premix supplementation has increased N retention in post-calving cows, but not in precalving cows (Plaizier et al., 2000). The increased N retention has usually been explained by improved N digestibility (Tedeschi et al., 2003) as observed in the present experiment.

Milk Fatty Acid Composition

In general, there were no interactions between flaxseed hulls and monensin supplementation on milk concentrations of individual FA with the exception of 4:0 and *trans*9,12-18:2 (Table 3). Feeding HM led to the lowest 4:0 concentration in milk fat, showing an interaction between flaxseed hulls and monensin supplementation ($P = 0.04$). Feeding flaxseed hulls compared with no flaxseed hulls decreased significantly concentrations of all short-chain FA (4:0 to 13:0) with the exception of *cis*11-12:1 and 13:0, which were similar among treatments. There was a significant decrease in concentrations of most medium-chain FA (14:0, *cis*9-14:1, 15:0, 16:0, *cis*9-16:1 and 17:0) for cows fed flaxseed hulls with the exception of *trans*9-16:1, which increased when adding flaxseed hulls to the diet. Cows fed flaxseed hulls compared with those fed no flaxseed hulls had significant higher concentrations of 18:0, *trans*9-18:1, *trans*11-18:1, *cis*6-18:1, *cis*9-18:1, *cis*11-18:1, 19:0, *trans*9,12-18:2, *cis*9,12,15-18:3, and *cis*9,*trans*11-18:2 in milk fat.

Feeding diets with flaxseed hulls compared with feeding diet without flaxseed hulls increased significantly concentrations of total *trans* FA, monounsaturated, polyunsaturated, and long-chain FA in milk fat. Concentrations of unknown FA were similar among treatments. Concentrations of saturated, short-chain, and medium-chain FA were significantly decreased for cows fed flaxseed hulls. The polyunsaturated to saturated FA ratio in milk fat was significantly higher for cows fed flaxseed hulls compared with those fed no flaxseed hulls. Changes in milk FA composition were generally similar to those observed when feeding whole flaxseed (Petit, 2002; Petit et al., 2004). The concentration of alpha-linolenic acid (*cis*9,12,15-18:3) in milk fat was significantly higher for cows fed flaxseed hulls compared with those fed no flaxseed hulls. Concentration of *cis*5,8,11,14-20:4 in milk fat was higher for cows fed no flaxseed hulls. The n-6 to n-3 ratio in milk fat was significantly lower for cows fed flaxseed hulls (FH and HM) compared with those fed no flaxseed hulls (CO and MO). Similarly, a decrease in the n-6 to n-3 FA ratio in milk has been reported by Petit (2002) when cows were fed whole flaxseed compared to calcium salts of palm oil and whole flaxseed decreases the n-6 to n-3 FA in milk compared to sunflower seed (Petit, 2003).

Monensin supplementation had little effect on milk FA composition and the only effects were increased (P = 0.03) concentrations of *cis*9-16:1 and *cis*9, 12-18:2 in milk fat. Concentration of CLA in milk fat was similar for cows supplemented or not with MO, which would agree with the results of Mutsvangwa et al. (2003). There has been some suggestions that MO can modify milk FA composition by inhibiting biohydrogenation of linoleic acid ($C_{18:2}$) and increasing the content of monounsaturated $C_{18:1}$ FA (Fellner et al., 1997; Sauer et al., 1998). Fellner et al. (1997) observed higher concentrations of linoleic acid ($C_{18:2}$), *trans* $C_{18:1}$, and CLA in continuous cultures of ruminal bacteria following infusion of ionophores (monensin, migericin, or tetronasin). Feeding monensin had similar effects on enhancing $C_{18:2}$ and *trans* FA in milk of lactating cows (Sauer et al., 1998). Jenkins et al. (2003) suggested that monensin decreased the concentration of unsaturated FA (e.g. $C_{18:2}$) in the rumen when the diet contained grains with lower rates of ruminal digestion such as corn as compared to barley. In the present study, the amount of starch supplied by corn (grain and silage) was higher in diets CO and MO than diets FH and HM (Table 1). Differences in feed ingredient composition may then explain the lack of effect of monensin on the concentrations of $C_{18:2}$ and $C_{18:3}$ in milk fat.

CONCLUSIONS

Feeding flaxseed hulls enhanced digestibility of CP and EE. Moreover, monensin supplementation increases CP digestibility. Feeding flaxseed hulls increased concentrations of *cis*9,*trans*11-18:2, MUFA, PUFA, n-3 FA, and the n-6 to n-3 FA ratio and decreased concentrations of saturated fatty acids in milk fat. As a result, supplementation with flaxseed hulls improves the milk fatty acid profile for better human health. On the other hand, monensin supplementation had no effect on milk fatty acid profile.

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Table 1. Ingredient and chemical composition of total mixed diets of Holstein cows fed no flaxseed hulls and no monensin (CO), flaxseed hulls and no monensin (FH), no flaxseed hulls with monensin (MO) or a mixture of flaxseed hulls and monensin (HM)

	Treatment					*P*-value		
	CO	FH	MO	HM	SE[1]	Flaxseed hulls	Monensin	Interaction
Ingredient, % of DM								
Grass silage	33.1	30.1	33.2	30.1				
Corn silage	27.0	24.3	27.0	24.3				
Broken corn grain	18.2	8.8	18.2	8.8				
Ground barley	9.4	9.1	9.4	9.1				
Soybean meal	7.0	3.1	7.0	3.1				
Monensin	0	0	0.0016	0.0016				
Flaxseed hull[2]	0	19.8	0	19.8				
Protein supplement[3]	3.2	3.1	3.2	3.1				
Mineral[4]	2.1	1.7	2.1	1.7				
Chemical analysis								
DM, %	43.6	46.7	43.5	46.7	0.4	0.01	1.00	0.97
CP, % of DM	15.1	15.2	14.7	15.3	0.2	0.23	0.66	0.42
Ether extract, % of DM	2.1	6.9	2.0	6.9	0.1	< 0.0001	0.62	0.46
NDF, % of DM	32.5	30.6	34.3	32.0	1.2	0.19	0.29	0.89
ADF, % of DM	26.4	23.6	27.5	25.7	0.8	0.06	0.13	0.55
NSC, % of DM	44.9	41.6	43,2	39.6	1.0	0.007	0.10	0.89
NE_L, Mcal/kg of DM[5]	1.56	1.54	1.56	1.54				

Fatty acids, % of total FA								
12:0	0.92	0.42	0.90	0.42	0.08	0.01	0.76	0.89
14:0	0.83	0.32	0.85	0.31	0.07	0.005	0.65	0.64
16:0	18.48	9.65	18.03	9.75	0.16	< 0.0001	0.18	0.06
18:0	2.03	2.40	2.28	2.32	0.08	0.09	0.57	0.14
*cis*9-18:1	16.98	18.55	16.54	18.81	0.50	0.03	0.97	0.55
*cis*11-18:1	1.07	0.93	1.08	0.96	0.02	0.01	0.54	0.98
*cis*9,12-18:2	44.41	24.69	44.07	24.88	0.23	< 0.0001	0.65	0.40
*cis*9,12,15-18:3	10.28	40.96	11.18	40.42	0.44	< 0.0001	0.83	0.30
20:0	0.60	0.28	0.38	0.28	0.04	0.02	0.18	0.08
22:0	0.61[a]	0.30[b]	0.39[b]	0.33[b]	0.06	0.01	0.12	0.05
24:0	0.39	0.29	0.36	0.30	0.02	0.03	0.62	0.32
Others	3.79	1.49	4.29	1.52	0.18	< 0.0001	0.18	0.23
n-6[6]:n-3[7]	4.42	0.60	4.11	0.62	0.22	0.001	0.89	0.58

[1]Least squares means with pooled standard error (SE).
[2]Contained 26.6% Ether extract, 20.8% CP, 24.0% ADF and 32.4% NDF of DM.
[3]Contained 20% of canola meal, 30% of corn gluten meal, 20% of soybean meal, 30% of brewer's corn.
[4]Contained 9.02% Ca, 4.90% P, 4.89% Mg, 1.76% S, 14% Na, 1.43% K, 2068 mg/kg Fe, 2718 mg/kg Zn, 447 mg/kg Cu, 1814 mg Mn, 69 mg/kg I, 7 mg/kg Co, 20 mg/kg Se, 452,000 IU/kg of vitamin A, 58,000 UI/kg of vitamin D_3, and 2692,000 IU/kg of vitamin E.
[5]Calculated using published values of feed ingredients (NRC, 2001).
[6]*cis*9,12-18:2.
[7]*cis*9,12,15-18:3.

Table 2. Intake of dry matter (DMI), apparent digestibility, and N output of Holstein cows fed total mixed diets containing no flaxseed hulls and no monensin (CO), flaxseed hulls and no monensin (FH), no flaxseed hulls with monensin (MO) or a mixture of flaxseed hulls and monensin (HM)

	Treatment					*P*-value		
	CO	FH	MO	HM	SE[1]	Flaxseed hulls	Monensin	Interaction
DMI, kg/d	20.1	19.0	20.0	18.6	0.4	0.04	0.56	0.72
DMI, % of BW	3.02	2.86	3.01	2.82	0.05	0.05	0.62	0.83
Digestibility, %								
DM	65.0	65.1	66.4	65.8	0.4	0.64	0.08	0.45
CP	65.7	70.0	66.6	71.9	0.4	0.002	0.05	0.35
ADF	55.3	44.0	57.6	47.2	1.5	0.01	0.17	0.78
NDF	46.2	43.9	49.7	45.9	2.1	0.24	0.27	0.75
Ether extract	77.1	91.4	75.7	91.7	1.0	0.001	0.63	0.45
N balance, g/d								
N intake	486	462	474	456	15	0.26	0.58	0.85
N in feces	166	139	158	129	5	0.01	0.16	0.83
N in urine	189	195	180	179	9	0.81	0.26	0.74
N in milk	162	144	156	135	5.3	0.04	0.28	0.75
N retained	-31	-16	-20	13	4.9	0.02	0.02	0.09

[1]Least squares means with pooled standard error (SE).

Table 3. Fatty acid profile in milk fat (percentage of total fatty acids) of Holstein cows fed total mixed diets containing no flaxseed hulls and no monensin (CO), flaxseed hulls and no monensin (FH), no flaxseed hulls with monensin (MO) or a mixture of flaxseed hulls and monensin (HM)

	Treatment					*P*-value		
	CO	FH	MO	HM	SE[1]	Flaxseed hulls	Monensin	Interaction
4:0	5.25[a]	4.58[a]	5.01[a]	3.43[b]	0.13	0.003	0.01	0.04
5:0	0.05	0.02	0.05	0.01	0.01	0.04	0.80	0.71
6:0	2.64	1.76	2.46	1.38	0.12	0.004	0.10	0.48
7:0	0.10	0.02	0.10	0.02	0.02	0.02	0.90	0.86
8:0	1.51	0.98	1.39	0.74	0.09	0.01	0.14	0.62
9:0	0.11	0.03	0.11	0.01	0.02	0.03	0.91	0.76
10:0	3.42	1.76	3.02	1.36	0.26	0.01	0.23	0.99
11:0	0.15	0.03	0.13	0.04	0.02	0.02	0.96	0.65
12:0	4.22	2.17	3.70	1.78	0.33	0.01	0.26	0.86
*cis*11-12:1	0.22	0.21	0.22	0.11	0.04	0.25	0.31	0.30
13:0	0.17	0.16	0.16	0.11	0.02	0.27	0.35	0.46
14:0	12.65	7.86	11.71	7.54	0.55	0.004	0.34	0.61
*cis*9-14:1	1.35	0.87	1.32	0.98	0.34	0.001	0.27	0.13
15:0	1.26	0.86	1.21	0.92	0.34	0.002	0.97	0.22
16:0	31.77	18.47	31.27	20.14	0.74	0.001	0.49	0.24
*trans*9-16:1	0.08	0.32	0.12	0.31	0.05	0.03	0.84	0.70
*cis*9-16:1	1.87	1.39	2.03	1.62	0.05	0.003	0.03	0.53

17:0	0.65	0.58	0.70	0.53	0.02	0.01	0.93	0.15
18:0	9.27	14.90	9.45	13.50	0.64	0.01	0.42	0.32
*trans*9-18:1	0.22	0.68	0.27	0.72	0.03	0.001	0.20	0.87
*trans*11-18:1	1.03	4.41	1.30	5.23	1.05	0.04	0.64	0.81
*cis*6-18:1	0.51	3.93	0.87	4.20	0.16	0.0002	0.14	0.79
*cis*9-18:1	16.35	24.09	17.30	25.37	0.74	0.002	0.23	0.82
*cis*11-18:1	0.52	0.68	0.56	0.82	0.06	0.03	0.22	0.44
19:0	0.20	1.69	0.38	1.68	0.13	0.002	0.52	0.51
*trans*9,12-18:2	0.04[c]	0.25[a]	0.13[b]	0.21[ab]	0.01	0.001	0.21	0.01
*cis*9,12-18:2	2.02	2.10	2.12	2.15	0.02	0.07	0.04	0.29
20:0	0.21	0.33	0.27	0.18	0.06	0.76	0.50	0.18
*cis*6,9,12-18:3	0.07	0.005	0.07	0	0.01	0.02	0.93	0.70
*cis*8-20:1	0.19	0.27	0.22	0.21	0.03	0.28	0.50	0.20
*cis*11-20:1	0.11	0.20	0.13	0.14	0.03	0.19	0.56	0.23
*cis*9,12,15-18:3	0.56	1.86	0.72	1.78	0.06	0.0002	0.51	0.11
*cis*9,*trans*11-18:2	0.49	1.84	0.63	2.40	0.49	0.05	0.53	0.70
*trans*10,*cis*12-18:2	0.00	0.03	0.01	0.01	0.01	0.13	0.43	0.21
*cis*11,14-20:2	0.13	0.03	0.14	0.02	0.03	0.05	0.99	0.74
22:0	0.05	0.16	0.13	0.05	0.05	0.73	0.76	0.15
*cis*8,11,14-20:3	0.15	0.13	0.14	0.05	0.02	0.04	0.09	0.11
*cis*5,8,11,14-20:4	0.17	0.14	0.17	0.11	0.01	0.05	0.29	0.36
*cis*5,8,11,14,17-20:5	0.08	0.08	0.10	0.04	0.04	0.56	0.73	0.49

*cis*5,7,10,13,16-22:5	0.18	0.12	0.18	0.10	0.06	0.37	0.89	0.84
Total trans	1.85	7.52	2.44	8.87	1.57	0.03	0.58	0.83
Unknown	0.77	1.63	1.20	1.46	0.27	0.14	0.66	0.35
MUFA[2]	22.44	37.02	24.36	39.69	1.80	0.004	0.29	0.85
PUFA[2]	3.91	6.74	4.42	6.91	0.47	0.01	0.52	0.74
SFA[2]	73.85	56.59	71.52	53.58	2.25	0.004	0.32	0.89
PUFA/SFA	0.05	0.12	0.06	0.13	0.01	0.01	0.39	0.96
SCFA[2]	17.83	11.69	16.34	8.99	0.92	0.01	0.11	0.56
MCFA[2]	48.92	29.73	47.57	31.49	1.17	0.001	0.87	0.28
LCFA[2]	33.24	58.59	36.13	59.52	1.92	0.001	0.40	0.65
n-3[3]	0.64	1.94	0.82	1.82	0.08	0.001	0.76	0.14
n-6[4]	2.72	2.53	2.82	2.43	0.03	0.61	0.18	0.29
n-6: n-3	4.25	1.30	3.44	1.34	0.23	0.004	0.41	0.30

[1]Least squares means with pooled standard error (SE).
[2]MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; SFA = saturated fatty acids; SCFA = short-chain fatty acids; MCFA = medium-chain fatty acids; LCFA = long-chain fatty acids.
[3]*cis*9,12,15-18:3 + *cis*5,8,11,14,17-20:5.
[4]*cis*9,12-18:2 + *cis*5,8,11,14-20:4 + *cis*6,9,12-18:3 + *cis*11,14-20:2 + *cis*8,11,14-20:3 + *cis*5,7,10,13,16-22:5.

CAPÍTULO III

(Normas: Journal of Dairy Science)

The Interaction of Monensin and Flaxseed Hulls on Ruminal and Milk Concentration of the Mammalian Lignan Enterolactone in Late-Lactating Dairy Cows

ABSTRACT

Four ruminally fistulated multiparous Holstein cows were assigned to a 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments to study the effects of monensin and flaxseed hulls supplementation on ruminal and milk concentration of the mammalian lignan enterolactone (EL) and ruminal and fecal activity of ß-glucuronidase. Treatments were: 1) control (no flaxseed hulls and monensin; CO), 2) diet containing (dry matter basis) 20% flaxseed hulls (FH), 3) diet with monensin (16 mg/kg of dry matter; MO), and 4) diet containing 20% (dry matter basis) flaxseed hulls and 16 mg/kg monensin (HM). Intake of dry matter was higher for CO and MO than for FH and HM and monensin had no effect. Milk production decreased in cows fed flaxseed hulls while monensin had no effect. Production of 4% fat-corrected milk and concentrations of milk fat, lactose, urea N, and total solids were similar among treatments. A combination of flaxseed hulls and monensin decreased milk production efficiency (kg of milk per kg of DMI). Monensin had no effect on milk production and milk composition. Although there was a decrease in ruminal activity of β-glucuronidase when feeding flaxseed hulls, the metabolism of plant into mammalian lignans may be increased as shown by enhanced concentrations of EL in the rumen and milk. Supplementation with flaxseed hulls then may contribute to change favourably milk composition for better human health by enhancing mammalian lignan EL concentration. Monensin had no effect on concentration of EL in milk.

Key words: Dairy cattle, flaxseed, lignans, milk production.

INTRODUCTION

In human nutrition, increased consumption of flaxseed is associated with lower incidence of cardiovascular disease, breast and prostate cancers, osteoporosis, and

postmenopausal symptoms (Murkies et al., 1998). The beneficial effects of flax are thought to be partly mediated by its high concentration of a mammalian lignan precursor secoisolariciresinol diglucoside (SDG). Upon ingestion, microbial enzymes convert SDG to mammalian lignans, mainly enterodiol (ED) and enterolactone (EL) (Setchell et al., 1980) under the action of colonic microflora, and ED and EL are subsequently absorbed into blood (Borrielo et al., 1985). In addition to their healthy effects, secoisolariciresinol diglucoside and its mammalian lignan metabolites have a high antioxidant activity (Kitts et al., 1999), which is an evidence of a great potential anticarcinogenic mechanism. Unfortunelately, mammalian lignan precursors are not yet commercially available on the market and flaxseed is not commonly eaten in North America. However, the intake of lignans can be achieved through the consumption of milk from dairy cows fed flaxseed products. Petit et al. (2005) have previously reported a linear increase in milk concentration of EL in cows fed greater amounts of flaxseed but no ED was detected. Lignans in grain are concentrated in the outer fibre-containing layers (Adlercreutz & Mazur, 1997), which would lead to higher concentration of SDG in hulls than in seeds. Recent *in vitro* results have shown that the main mammalian lignan metabolite produced from flaxseed hulls by ruminal microbiota was EL while fecal microbiota led mainly to the net production of ED (Côrtes et al., 2008). Feeding flaxseed hulls to dairy cows may then contribute to increase concentration of the mammalian lignan EL in milk.

Monensin, which is an ionophore, has been used extensively in the diet of dairy cows, and its effects on milk production and composition are well documented (Phipps et al., 2000; Duffield et al., 2003). In a previous experiment (Da Silva et al., 2007), monensin addition increased concentrations of *cis*9,*trans*11-18:2 CLA (rumenic acid) and decreased concentrations of saturated FA in milk fat. A combination of monensin with a source of n-3 FA such as flaxseed may contribute to modify milk composition for better human health. However, this has to be done without any detrimental effect on the production of EL from plant lignans in the rumen and the transfer of EL in milk. Monensin is known to decrease the growth of Gram positive bacteria and some strains of bacteria with ß-glucuronidase activity such as *Ruminococcus* and *Eubacterium* are Gram positive bacteria (Beaud et al., 2005). Supplementation with monensin may then affect ruminal ß-glucuronidase activity. Therefore, the main objective of the experiment was to determine the effect of feeding a

combination of monensin and flaxseed hulls on ruminal and milk concentration of EL and ruminal and fecal activity of ß-glucuronidase, which plays important roles in the metabolism and absorption of lignans (Jenab &Thompson, 1996). Milk oxidation was also determined.

MATERIALS AND METHODS

Cows and Diets

Four ruminally fistulated multiparous Holstein cows averaging 665 ± 21 kg of body weight and 190 ± 5 days in milk were assigned to a 4 × 4 Latin square design balanced for residual effect to determine the effects of monensin and flaxseed hulls supplementation on ruminal and milk concentration of the mammalian lignan EL and ruminal and fecal activity of ß-glucuronidase. The experimental diets (Table 1) consisted of four different total mixed diets with a 2 × 2 factorial arrangement of treatments: 1) control (no flaxseed hulls and no monensin; CO), 2) diet containing (dry matter basis) 20% flaxseed hulls (FH), 3) diet with monensin (16 mg/kg of dry matter; MO), and 4) diet containing 20% (dry matter basis) flaxseed hulls and 16 mg/kg monensin (HM). All diets provided equal amounts of crude protein and energy and were formulated to meet nutrient requirements of 615 kg cow producing 29 kg/d of milk containing 3.9 % of fat (NRC, 2001). Cows were housed in tie stalls, fed individually for ad libitum intake (10% refusals) twice a day (0830 and 1530 h), and milked twice daily at 0800 and 1900 h. Milk production was recorded at every milking. Yield of 4% fat-corrected milk (FCM) was calculated according to the equation of Tyrrell & Reid (1965). Cows were weighed on the first and last day of each experiment period. Cows were cared for in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1993).

Experimental Procedures

Each experimental period consisted of 28 d with 21 d of adaptation to the diets, one day of rumen and feces sampling, and 7 d of total milk collection. Feed intake and milk yield were measured daily. Samples of the total mixed diets were taken daily from d 22 to d 28 and pooled within period for each cow. All samples were frozen at -20^{o}C for subsequent

drying at 55°C. Milk samples were obtained from each cow for 14 consecutive milkings from d 22 to d 28 and pooled on a yield basis. One sample was kept frozen at -80°C without preservative for further analyses of milk fat and the mammalian lignan EL. Another sample was stored at + 4°C with a preservative (bronopol-B2) until analyzed for protein, urea N, lactose, total solids and somatic cell score.

On d 28, ruminal fluid (about 1 L) was collected from the anterior dorsal, anterior ventral, medium ventral, posterior dorsal, and posterior ventral locations within the rumen before feeding (0 h) and at 1, 2, 4, and 6 h after the am feeding. Ruminal fluid was strained through 2 layers of cheesecloth to separate the liquid and solid fractions. A 350-ml sample of strained ruminal fluid was immediately mixed with 26 g of ruminal solid contents and stored at -80°C for further determination of ß-glucuronidase activity. One sample of strained ruminal fluid was kept frozen at -80°C for determination of EL. On day 28, feces (250 g) were collected 2, 4, 6 and 8 h after the am feeding and pooled on a fresh basis for ß-glucuronidase activity analysis. All samples were frozen at -20°C until subsequently analyzed.

Chemical Analyses

Analytical dry matter of the diets was determined in a forced-air oven according to the procedure 934.01 (AOAC, 1990). Samples of the total mixed diets were ground to pass a 1-mm screen in a Wiley mill before chemical analyses. Total N content of total mixed diets was determined by thermal conductivity (LECO model FP-428 Nitrogen Determinator, LECO, St. Joseph, MI, USA) and crude protein was calculated as N × 6.25. The concentration of neutral detergent fibre (NDF) in diets was determined as described by Van Soest et al. (1991) without the use of sodium sulfite and with the inclusion of heat stable α-amylase. The acid detergent fibre (ADF) content in diets was determined according to AOAC (1990; Method 973.18). The NDF and ADF procedures were adapted for use in an ANKOM^{200} Fibre Analyzer (ANKOM Technology Corp., Fairport, NY, USA). Ether extraction in diets was conducted with Tecnal TE–044/1 (Piracicaba, São Paulo, Brazil) according to the method No. 7.060 (AOAC, 1990).

Milk fat concentration was determined by the method of Roese-Goettlib (AOAC, 1990). Protein, lactose, total solids, urea N concentrations and SCS in milk samples were

analyzed by infrared spectrophotometer (System 4000 Milkoscan; Foss Electric of Hillerod, Denmark). Total antioxidant capacity of milk (Ferric Reducing Antioxidant Power - FRAP) was determined according to Benzie & Strain (1999) adapted for milk after deproteinization with alcohol 100% before the assay. The FRAP method is based on a redox reaction in which an easily reduced oxidant (Fe^{3+}) is reduced to the ferrous (Fe^{2+}) form with an intense blue colour. The potential of antioxidants in milk to reduce Fe^{3+} to Fe^{2+} was expressed in µM of Fe^{2+}. It is assumed that the higher the measured FRAP value, the higher the content of antioxidants in milk which could reduce the ferric ion to the ferrous ion.

Extraction and analysis of plant secoisolariciresinol diglucoside in diets were performed according to the procedures described by Muir & Westcott (2000). Lignans in ruminal fluid and milk were hydrolysed and extracted according to the method of Frank & Custer (1996) with some modifications. Only EL was analyzed in milk and rumen fluid as other studies have shown that the mammalian lignan ED is below detection level in milk (Petit et al., 2005) and that EL is the main mammalian metabolite produced by ruminal microbiota (Côrtes et al., 2008). Freeze-dried samples of filtered ruminal fluid were resuspended in Milli-Q purified water (20 mg/0.5 ml) as described by Heinonen et al. (2001). Five hundred microliters of warmed (40°C) milk and resuspended ruminal samples were mixed with 5 µL of ß-glucuronidase/arylsulfatase from *Helix pomatia* (Roche-Diagnostics, Laval, QC, Canada). Milk samples were incubated for 1.5 h and ruminal samples were incubated overnight at 37°C in a shaking waterbath. After hydrolysis, all samples were acidified with 10 µL of 6 N HCl. Acidified milk samples were washed with 3 ml of hexane before extraction to remove lipids (Raffaelli et al., 2002). All samples were extracted with 2 mL of diethyl ether. The samples were vortex-mixed twice for 2 min. Organic layer was separated by freezing. The remaining liquid phase was submitted to a second extraction under the same conditions. The organic layers were pooled and evaporated by vacuum (Speed-Vac; Thermo Savant, Holbrook, NY, USA) at room temperature for 40 min. The dry extract was redissolved in 500 µL of EIA buffer and warmed at 37°C for EL analysis using an enzyme immunoassay (EIA; Cayman Chemical, Ann Arbor, MI, USA). Cayman Chemicals Enterolactone EIA kit is a competitive assay that utilizes a standard curve ranging from 15.6 to 2 000 pg/mL. The assay exhibits a limit

of quantification (defined as 80% B/B_0) of 70 pg/mL and an IC50 (50% B/B_0) of 240 pg/mL.

The determination of ß-glucuronidase activity was based on a modified method of Jenab & Thompson (1996). Briefly, fecal samples (5 g) were homogenized with a Polytron (Kinematica AG, Lucerne, Switzerland) in a final volume of 20 ml of cold KH_2PO_4 pH 6.8 for 15 sec while ruminal samples were homogenized using a stomacher (A. J. Seward & Co. Ltd, London, UK). Samples were then filtered through two layers of cheesecloth, sonicated on ice (two bursts, 1 min; Sonics and Materials Inc., Danbury, CT, USA) and centrifuged at 10 000 x *g* for 15 min at 4°C. The supernatant fraction was stored at -80°C until enzyme assay. Activity of the ß-glucuronidase was quantified by mixing 25 µL of extracted sample with 125 µL of 0·04 mol/L of KH_2PO_4 (pH 6·8), 50 µL of 0·5 mmol/l EDTA, and 50 µL of 5 mmol/L phenolphthalein diglucuronide (Sigma-Aldrich, Oakville, ON, Canada). The assay was done in quadruplicate and one duplicate was used as baseline and one was incubated at 37°C for 60 min. The reaction was stopped by adding 1.25 mL of glycine buffer (0.2 mol/L). After 10 min of incubation at room temperature, 200 µL of each replicate was transferred in 96-well flat-button plate and the plate was read on a Spectra Max 250 ELISA reader (Molecular Devices, Sunnyvale, CA, USA) at 540 nm. The absorbance values directly correlate to the amount of the phenolphthalein released based on a phenolphthalein standard curve. The specific activity of ß-glucuronidase was calculated by the formula:

$$= \frac{\text{(nmol phenolphthalein 60 min - nmol phenolphthalein 0 min)}}{\text{60 min * mg of protein}}$$

Protein was determined by bicinchoninic acid protein assay kit (Sigma-Aldrich) using bovine serum albumin as the standard.

Statistical Analysis

All results were analyzed using the MIXED procedure of SAS (2000) as a 4 × 4 Latin square design balanced for residual effect within a 2 × 2 factorial arrangement of treatments. Data collected on the last week of each experimental period for milk production, milk composition, and feed intake, were analyzed using with the following general model:

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

where: Y_{ijkl} = the dependent variable, μ = overall mean, C_i = random effect of cow (i = 1 to 4), P_j = fixed effect of period (k = 1 to 4), T_k = fixed effect of treatment (k = 1 to 4), and e_{ijk} = random residual error. Treatments were compared to provide factorial contrasts: 1) with vs. without monensin, 2) with vs. without flaxseed hulls, and 3) the interaction between monensin and flaxseed hulls. The residual effect was initially included in the model but was removed when it was not significant. Data on ruminal ß-glucuronidase activity and EL concentration were analyzed as repeated measurements. Results are reported as least squares means ± SEM. Data on EL concentration in milk were transformed (log) as previously performed by Côrtes et al. (2008) but results in the Figure are reported as the adjusted mean value (with confidence interval) on the original scale of measurements. Significance was declared at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$ unless otherwise stated.

RESULTS

There was no interaction ($P > 0.10$) between flaxseed hulls and monensin supplementation for dry matter intake, expressed in kilograms per day or as a percentage of body weight, initial and final body weight, and body weight change (Table 2). Intake of dry matter, expressed in kilograms per day or as a percentage of body weight, was higher (P= 0.04 and P=0.05, respectively) for treatments without flaxseed hulls (CO and MO) than for treatments with flaxseed hulls (FH and HM). Lower NSC content in diets with flaxseed hulls, which contains more structural carbohydrates, may likely limit feed intake by distension within the gastrointestinal tract (Allen, 2000). Intake of dry matter was similar (P>0.10) for cows supplemented or not with monensin. Initial body weight and body weight change were similar among treatments. Final body weight tended (P=0.07) to increase with monensin supplementation.

There was no interaction (P>0.10) between flaxseed hulls and monensin supplementation for milk production, 4% fat-corrected milk yield, milk composition and milk yield of components, and somatic cell score (Table 2) although protein concentration tended (P=0.10) to be lower for cows fed FH than for others treatments. Milk production

was decreased (P=0.03) for cows fed flaxseed hulls and the addition of monensin tended (P=0.09) to decrease it. Production of 4% fat-corrected milk and somatic cell score were similar (P>0.05) among treatments. There was an interaction between flaxseed hulls and monensin supplementation for the efficiency of milk production, expressed in kilogram of milk produced daily per kg of DMI, as a result of cows supplemented with flaxseed hulls and monensin having the lowest efficiency (1.24 *vs* 1.36 for the others treatments). Concentrations of milk fat, lactose, urea N, and total solids were not affected by treatments. Milk protein and lactose yields were significantly lower (P=0.04 and P=0.03, respectively) for cows fed flaxseed hulls and there was a trend (P=0.08) for lower yield of total solids. Milk fat yield was similar among treatments.

There was no interaction (P>0.10) between time and treatment for EL concentration in ruminal fluid and between flaxseed hulls and monensin supplementation for concentrations of EL in ruminal fluid and milk. Concentration of EL in ruminal fluid increased (P=0.001) postfeeding (Figure 1) and remained higher for cows fed flaxseed hulls (FH and HM) compared with those fed no flaxseed hulls (CO and MO). Monensin had no effect on EL ruminal concentration. Supplementation with flaxseed hulls increased (P=0.001) EL concentration in milk and monensin had no effect (Figure 2). Concentration of FRAP averaged 241 :mol/L of milk and was similar (P=0.10) among treatments.

There was no significant (P>0.10) interaction between hour and treatment for specific ß-glucuronidase activity in ruminal fluid but there was a difference (P=0.001) among hours. Ruminal ß-glucuronidase activity was lower before feeding and 1 h postfeeding before to increase and reach a plateau from 2 h postfeeding (Figure 3a). Cows fed flaxseed hulls had lower (P=0.002) ruminal ß-glucuronidase activity than those not supplemented with flaxseed hulls. Moreover, cows fed FH had lower (P=0.05) ß-glucuronidase activity in feces than those fed CO (Figure 3b) as shown by the interaction between flaxseed hulls and monensin supplementation. Fecal ß-glucuronidase activity tended (P=0.08) to be lower for cows fed HM compared to those fed CO. Monensin supplementation tended (P=0.09) to decrease ß-glucuronidase activity in ruminal fluid.

DISCUSSION

Milk production was decreased for cows fed flaxseed hulls likely as a result of lower dry matter intake. Diets with flaxseed hulls averaged 6.9% ether extract (dry matter basis), which may limit dry matter intake of late-lactating cows due to lower energy requirements compared to those of early-lactating cows. The effects of level and type of fat supplement on dry matter intake are negligible when total fat concentration is below 6% of the DM (Dhiman et al., 2000; Petit et al., 2002). Declines in dry matter intake with fat-supplemented diets appear to be related to ruminal effects of fats as no depression in dry matter intake occurred in studies where ruminal effects of fat were not observed (Petit et al., 2002). Benson et al. (2001) hypothesized that long chain FA are utilized differently in early compared with mid-lactation, suggesting that the negative effect of lipid supplementation on dry matter intake becomes more important as lactation progressed. As cows were in late lactation in the present experiment, they may have been negatively affected by the level of fat in the diet.

Monensin had no effect on intake of dry matter and milk production. Although the effects of including monensin as premix or controlled release capsule in dairy cattle rations have been extensively investigated, results have been variable in terms of dry matter intake. The addition of monensin did not influence (Ramanzin et al., 1997; Broderick, 2004) or decreased (Sauer et al., 1998) dry matter intake of lactating dairy cows. Moreover, feeding monensin at 24 and 22 mg/kg of DM, respectively, for 15- (Bell et al., 2006) and 35-d (Osborne et al., 2004) periods had no effect on dry matter intake and milk yield of dairy cows. Similarly, monensin supplementation had no effect on milk production (Ramanzin et al., 1997; Broderick, 2004). However, this disagrees with earlier trials establishing that the inclusion of 300 mg/d of monensin in dairy cow diets for the first 25 weeks of lactation would increase milk yield (Phipps et al., 2000). Discrepancies between studies could be related to factors such as stage of lactation, diet composition, and length of the trial.

Milk production efficiency was decreased with flaxseed hulls and monensin supplementation as a result of lower numerical values of DMI and milk production compared to cows fed the other treatments. The combination of flaxseed hulls and monensin in the diet would then be a negative strategy for milk production efficiency. Milk composition was similar (P>0.05) among treatments. In general, milk protein concentration

is little affected by monensin supplementation (Sauer et al., 1998; Bell et al., 2006) although it decreased (Phipps et al., 2000) or increased (Duffield et al., 2003) in some cases. In many of the studies where monensin reduced milk fat and protein concentrations, a parallel increase in milk production was observed, thus suggesting that a dilution effect was partly responsible for changes in milk composition (Phipps et al., 2000) although this was not the case in the current experiment.

Supplementation with flaxseed hulls increased EL concentration in ruminal fluid and milk, which agrees with recent in vitro results showing that EL is the main metabolite produced when flaxseed hulls are incubated with ruminal microbiota (Côrtes et al., 2008). Different studies have confirmed the presence of polyphenolic compounds such as equol, daidzein, and genistein (Bannwart et al., 1988) and mammalian lignan EL (Steinshamn et al., 2008; Petit et al., 2005) in milk. This may suggest that contrary to what has been previously reported for monogastric mammals such as humans (Heinonen et al., 2001) and pigs (Knudsen et al. 2003), absorption of EL may occur either in the rumen or in the small intestine of ruminants before further conversion of ED to EL by colon microbes. Similarly, it has been shown that other phytoestrogens of the isoflavones family such as formononetin are metabolized to equol by bovine ruminal microbiota and that equol is available for absorption from the gastrointestinal tract (Dickinson et al., 1988).

Concentration of FRAP in milk was similar among treatments. Although the FRAP assay is a test developed for measuring total antioxidant capacity in blood serum (Benzie & Strain, 1999), Smet et al. (2008) reported that FRAP can provide a good measurement of milk oxidation as the ferric reducing antioxidant power of the product. It is known that lignan metabolites have a great antioxidant activity (Kitts et al., 1999). Flaxseed hulls is a rich source of plant precursors to mammalian lignans (Côrtes et al., 2008) and Petit et al. (2005) already reported that the mammalian lignan EL can be transferred into milk when dairy cows were fed flaxseed. Although cows fed flaxseed hulls had higher EL concentration in milk than those fed no flaxseed hulls, the total antioxidant power FRAP test did not detect any difference between diets, thus suggesting that other techniques should be examined to determine the antioxidant status of milk enriched in the mammalian lignan EL.

Supplementation with flaxseed hulls decreased β-glucuronidase activity in both ruminal fluid and feces of dairy cows. Flaxseed hulls contain 53% n-3 FA, expressed as a percentage of total FA (data not shown), and results from a recent *in vitro* study (Maia et al., 2007) reported that growth of ruminal bacteria can be affected by polyunsaturated FA such as n-3 although sensitivity differs among species. The activity of β-glucuronidase has been attributed to bacteria belonging to the dominant human intestinal microbiota, such as *Ruminococcus*, *Bacteroides*, *Bifidobacterium*, and *Eubacterium* (Beaud et al., 2005). Henderson (1973) showed that the growth of some strains of some predominant rumen bacteria such as *Butyrivibrio, Ruminococcus* and *Methanobacterium* is strongly inhibited by the presence of long-chain FA, which may decrease the activity of β-glucuronidase in ruminal fluid. Although flaxseed hulls contain 1.15% secoisolariciresinol diglucoside in the dry matter, they are also rich in n-3 FA. The data of the present study may indicate that species of ruminal microbiota with β-glucuronidase activity are more sensitive to the presence of polyunsaturated FA than ruminal species involved in the conversion of plant secoisolariciresinol diglucoside into the mammalian lignan EL. Indeed, although there was a decrease in ruminal activity of β-glucuronidase when feeding flaxseed hulls, the metabolism of plant into mammalian lignans may be increased as shown by enhanced concentration of EL in the rumen.

The activity of ß-glucuronidase in ruminal fluid tended to decrease with monensin supplementation. Monensin is known to decrease the growth of Gram positive bacteria and some strains of bacteria with ß-glucuronidase activity such as *Ruminococcus* and *Eubacterium* are Gram positive bacteria. Therefore, supplementation with monensin may affect ruminal ß-glucuronidase activity.

CONCLUSIONS

In conclusion, feeding flaxseed hulls decreased milk production, but there was no effect on milk composition. A combination of flaxseed hulls and monensin affected negatively milk production efficiency. Although there was a decrease in ruminal activity of β-glucuronidase when feeding flaxseed hulls, the metabolism of plant into mammalian lignans may be increased as shown by enhanced concentration of EL in the rumen and milk. Monensin supplementation had no effect on milk production and milk composition.

Supplementation with flaxseed hulls then may contribute to change favourably milk composition for better human health by enhancing mammalian lignan EL concentration but monensin did not change EL concentration in milk.

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Table 1. Ingredient and chemical composition of total mixed diets of Holstein cows fed no flaxseed hulls and no monensin (CO), flaxseed hulls and no monensin (FH), no flaxseed hulls with monensin (MO) or a mixture of flaxseed hulls and monensin (HM).

	Treatment			
	CO	FH	MO	HM
Ingredient, % of DM				
Grass silage	33.1	30.1	33.2	30.1
Corn silage	27.0	24.3	27.0	24.3
Broken corn grain	18.2	8.8	18.2	8.8
Ground barley	9.4	9.1	9.4	9.1
Soybean meal	7.0	3.1	7.0	3.1
Monensin	0	0	0.0016	0.0016
Flaxseed hull	0	19.8	0	19.8
Top supplement[1]	3.2	3.1	3.2	3.1
Mineral[2]	2.1	1.7	2.1	1.7
Chemical analysis				
DM, %	43.6	46.7	43.5	46.7
CP, % of DM	15.1	15.2	14.7	15.3
Ether extract, % of DM	2.1	6.9	2.0	6.9
NDF, % of DM	32.5	30.6	34.3	32.0
ADF, % of DM	26.4	23.6	27.5	25.7
NSC, % of DM	44.9	41.6	43,2	39.6
NE_L, Mcal/kg of DM[3]	1.56	1.54	1.56	1.54
SDG[4], % of DM	0.06	0.33	0.06	0.33

[1]Contained 20% of canola meal, 30% of corn gluten meal, 20% of soybean meal, and 30% of brewer's corn. [2]Contained 9.02% Ca, 4.90% P, 4.89% Mg, 1.76% S, 14% Na, 1.43% K, 2068 mg/kg Fe, 2718 mg/kg Zn, 447 mg/kg Cu, 1814 mg Mn, 69 mg/kg I, 7 mg/kg Co, 20 mg/kg Se, 452,000 IU/kg of vitamin A, 58,000 UI/kg of vitamin D_3, and 2692,000 IU/kg of vitamin E. [3]Calculated using published values of feed ingredients (NRC, 2001). [4]Secoisolariciresinol diglucoside.

Table 2. Intake, initial and final body weight (BW), milk production and composition of Holstein cows fed total mixed diets containing no flaxseed hulls and no monensin (CO), flaxseed hulls and no monensin (FH), no flaxseed hulls with monensin (MO) or a mixture of flaxseed hulls and monensin (HM).

	Treatment					*P*-value		
	CO	FH	MO	HM	SE[1]	Flaxseed hulls	Monensin	Interaction
DMI, kg/d	20.1	19.0	20.0	18.6	0.4	0.04	0.56	0.72
DMI, % of BW	3.02	2.86	3.01	2.82	0.05	0.05	0.62	0.83
BW, kg								
Initial	662	659	659	663	2	0.90	0.81	0.20
Final	665	664	672	673	3	0.97	0.07	0.76
Change, kg/d	0.10	0.20	0.46	0.34	0.14	0.95	0.17	0.49
Milk production, kg/d	27.5	26.0	26.8	23.3	0.7	0.03	0.09	0.21
4% FCM, kg/d	23.4	24.3	23.9	20.9	0.9	0.31	0.19	0.12
Milk efficiency[2]	1.37	1.37	1.34	1.24	0.009	0.013	0.004	0.013
Milk composition, %								
Protein	3.67	3.45	3.65	3.65	0.05	0.11	0.18	0.10
Fat	2.99	3.55	3.25	3.27	0.06	0.15	0.94	0.17
Lactose	4.72	4.82	4.81	4.86	0.38	0.14	0.17	0.57
Total solids	12.4	12.8	12.7	12.8	0.13	0.14	0.36	0.26
Milk yield, kg/d								
Protein	1.01	0.90	0.98	0.85	0.03	0.04	0.28	0.75
Fat	0.83	0.93	0.88	0.77	0.05	0.91	0.33	0.13
Lactose	1.30	1.25	1.29	1.13	0.03	0.03	0.10	0.14
Total solids	3.40	3.33	3.40	2.97	0.10	0.08	0.16	0.17
Urea N, mg/dL	11.5	10.0	12.4	12.3	1.66	0.65	0.41	0.72
SCS[3]	1.06	1.41	1.12	1.53	0.16	0.10	0.60	0.87

[1]Least squares means with pooled standard error (SE).
[2]Milk (kg/d)/DMI (kg/d). [3]Somatic cell score = $\log_{10}$SCC.

FIGURES CAPTIONS

Figure 1. Concentration of enterolactone (nmol/L) in ruminal fluid of Holstein cows fed a total mixed diet containing no flaxseed hulls and no monensin (CO-♦), flaxseed hulls (FH-◊), a mixture of flaxseed hulls and monensin (HM-Δ), or monensin (MO-▲). Vertical bars represent the means ± SEM.

Figure 2. Concentration of enterolactone (nmol/L) in milk of Holstein cows fed a total mixed diet containing no flaxseed hulls and no monensin (CO), flaxseed hulls (FH), a mixture of flaxseed hulls and monensin (HM), or monensin (MO). Data are the mean values with confidence intervals represented by vertical bars.

Figure 3. Specific activity of ß-glucuronidase in ruminal fluid (a) and feces (b) of Holstein cows fed a total mixed diet containing no flaxseed hulls and no monensin (CO-♦), flaxseed hulls (FH-◊), a mixture of flaxseed hulls and monensin (HM-Δ), or monensin (MO-▲). Vertical bars represent the means ± SEM.

Figure 1.

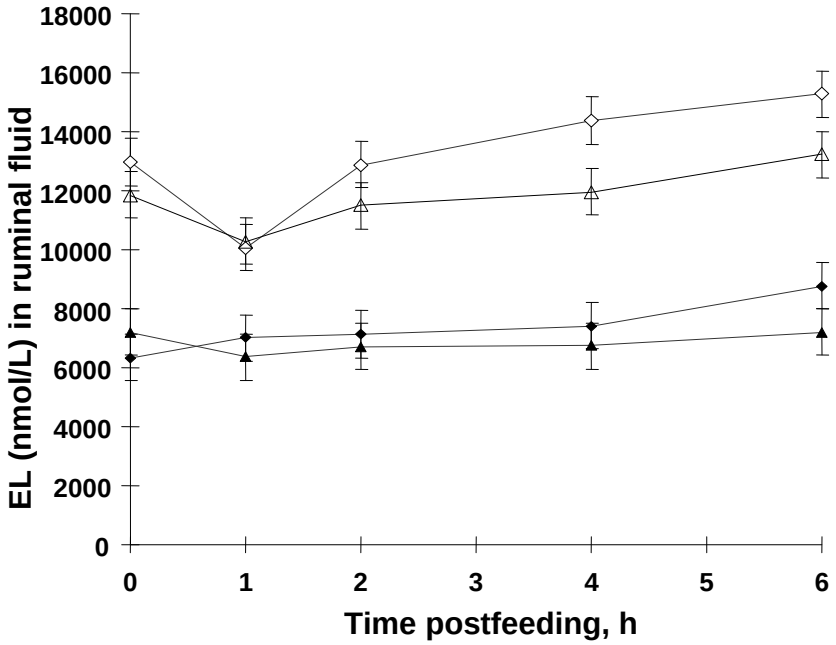


Figure 2

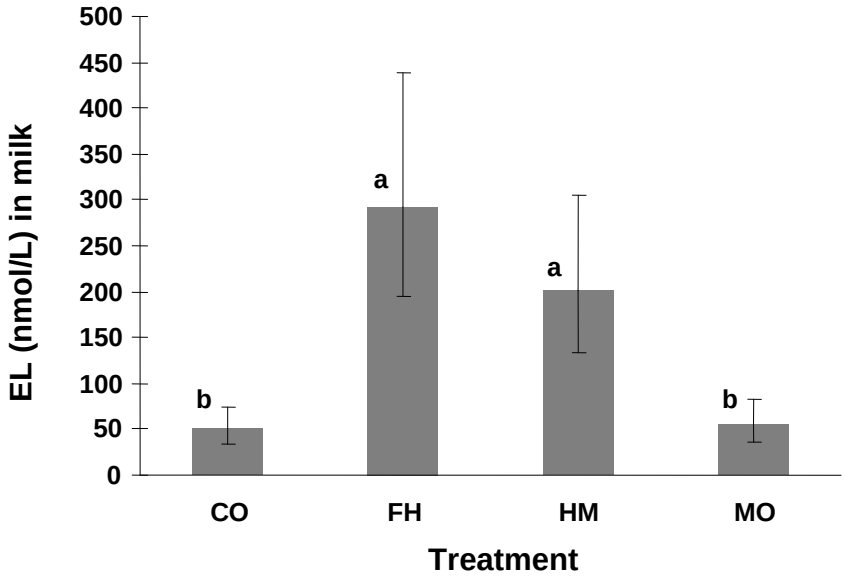


Figura 3a

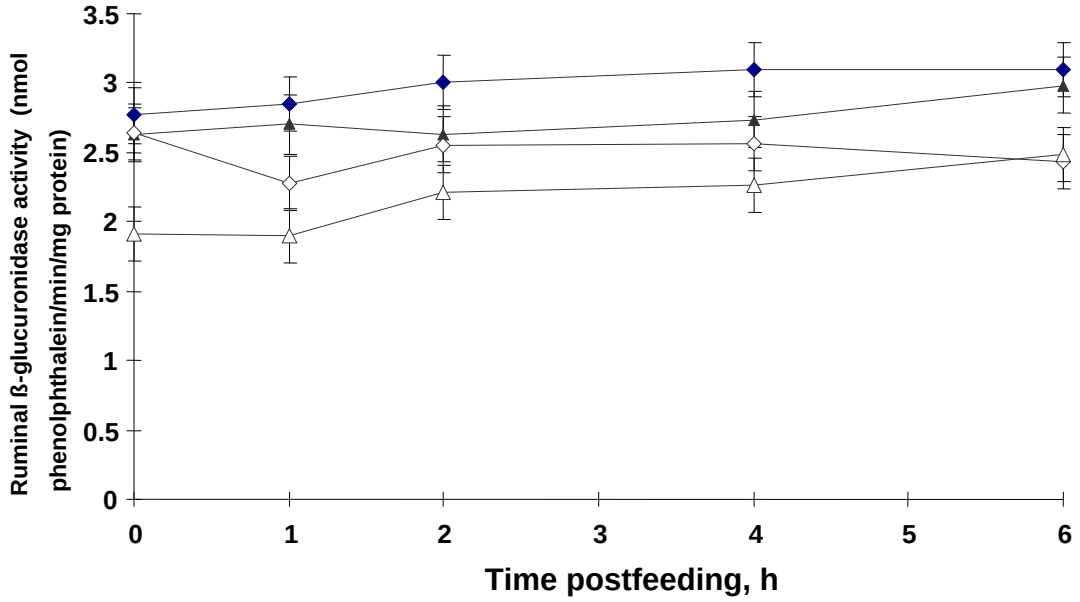
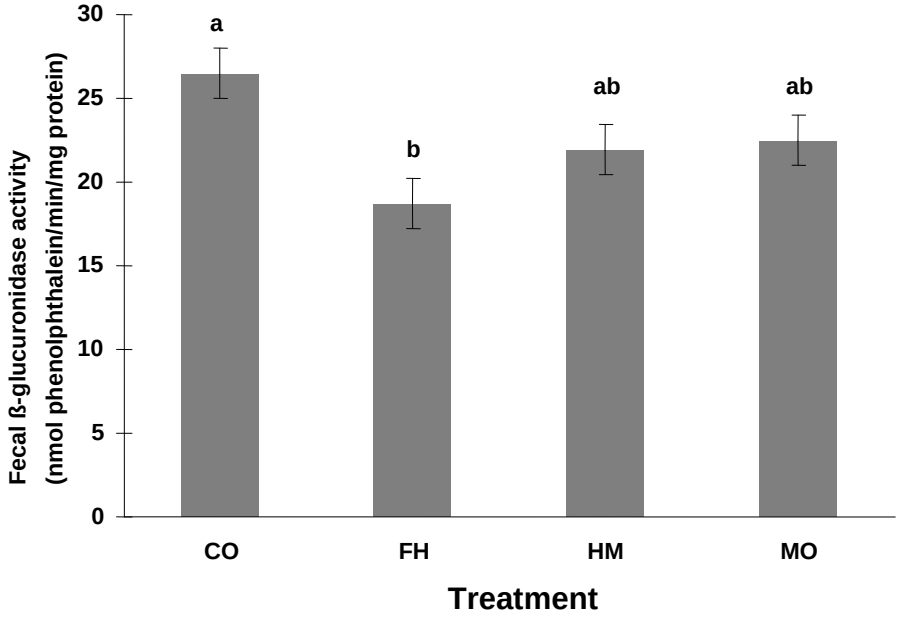


Figura 3b



CAPÍTULO IV

(Normas: Journal of Dairy Science)

Effect of Flaxseed and Calcium Salts of Flaxseed Oil on Intake, Digestibility and Milk Composition of Dairy Cows

ABSTRACT

Four lactating Holstein cows (BW = 602 kg; DIM = 64) fitted with ruminal cannulas were randomly assigned to a 4 x 4 Latin square design with experimental periods consisting of 21 d of diet adaptation and 7 d of data collection to determine the effects of feeding flaxseed and calcium salts of flaxseed oil on dry matter intake, digestibility, milk production and composition, concentration of enterolactone in milk and FA profile of milk. The treatments were: a control diet containing no flaxseed products (CO), 42 g/kg of DM of whole flaxseed (WF), 19 g/kg of DM of calcium salts of flaxseed oil (CF) or a mixture of 23 g/kg of DM of whole flaxseed and 8.3 g/kg of DM of calcium salts of flaxseed oil (MF). Dry matter intake, digestibility, milk production, 4% FCM, and milk production efficiency were not different among treatments. Milk composition also was similar among treatments except for milk fat percentage that was lower for cows fed CF compared to those fed CO and there was no difference between cows fed CO and those fed WF and MF. Milk yield of protein, fat, lactose and total solids and enterolactone concentration in milk were not affected by treatments. Concentrations of intermediates of FA biohydrogenation in milk were higher when calcium salts of flaxseed oil were fed, and rumenic FA proportion was increased when feeding CF as compared to when feeding WF. The alpha–linolenic FA in milk was higher for CF and MF than for CO, but it was similar to WF. Feeding whole flaxseed did not alter enterolactone concentration in milk compared to feeding calcium salts of flaxseed oil.

Key words: dairy cattle, enterolactone, fatty acids, lignan, milk quality

INTRODUCTION

Dietary polyunsaturated fatty acids (PUFA) are perceived to be healthier than saturated FA. As a result there has been a great deal of interest in manipulating the FA composition of milk fat to respond to the consumers' demand. Therefore, nutritionists began to feed PUFA protected against ruminal biohydrogenation by microbes to modify milk FA composition. For example, feeding PUFA such as flaxseed (rich in linolenic acid) has increased the proportion of n-3 FA in milk fat (Petit, 2002) to meet the guidelines for human health. Similarly, beneficial effects of feeding flaxseed oil on FA composition have been observed (Benchaar et al., 2006). Flaxseed is an excellent source of n-3 FA, which are known to be anticarcinogenic, to prevent cardiovascular diseases, and to increase visual activity (Wright et al., 1998). Unfortunately, the transfer of n-3 FA from flaxseed oil to milk is usually very low as a result of biohydrogenation of PUFA by rumen microbes. However, when infusing flaxseed oil in the small intestine and bypassing the rumen, we have shown that n 3 FA concentration increases up to 14% of total FA (Petit et al., 2002), which demonstrates that it is possible to increase the transfer of n-3 FA in milk. Ca-salts of fish oil have been found an effective way to increase n-3 FA in milk (Juchem et. al, 2008) but there is no information on the efficacy of Ca-salts of flaxseed oil to increase n 3 FA concentration in milk fat.

In human nutrition, increased consumption of flaxseed is associated with lower incidence of cardiovascular disease, breast and prostate cancers, osteoporosis, and postmenopausal symptoms. The beneficial effects of flax are thought to be partly mediated by its mammalian lignan precursor secoisolariciresinol diglucoside (SDG), which is very high in flax and is metabolized by bacteria in the colon into enterolactone (EL) and enterodiol (ED) (Setchell et al. 1980; Setchell et al., 1981). In addition to their healthy effects, SDG and its mammalian lignan metabolites have a great antioxidant activity (Kitts et al., 1999). This property is well known as an evidence of a great potential anticarcinogenic mechanism. A combination of greater concentrations of n-3 FA and antioxidants in milk may result in value-added dairy products. However, there is no information on the form of flaxseed oil on the production and transfer of EL in milk. Therefore, the objective of this experiment was to evaluate the source of flaxseed oil

(flaxseed versus calcium salts of flaxseed oil) on the transfer of polyunsaturated fatty acids in milk fat and mammalian lignans to milk.

MATERIALS AND METHODS

Four lactating cows fitted with rumen cannulas (BW = 602 kg; DIM = 64) were used in a 4 X 4 Latin square design and fed four different diets (Table 1): no flaxseed products in the concentrate (CO), whole flaxseed (WF), calcium salts of flaxseed oil (CF) and a mixture of whole flaxseed and calcium salts of flaxseed oil (MF). Each experimental period consisted of 21 d of adaptation to the diets and 7 d for daily data collection. Diets were offered in equal amounts twice daily (0800 and 1500 h) for ad libitum intake and feed consumption was recorded daily. Cows were milked twice daily at 0800 and 1900 h and milk yield was recorded at each milking. Yield of 4% fat-corrected milk (FCM) was calculated according to the equation of Tyrrell & Reid (1965). Body weight was determined at the beginning and the end of each experimental period after the am milking on two consecutive days.

Samples of diets were collected daily on the last week of each experiment period and pooled by period and within cow. On day 21 of each experimental period, cows were fitted with harnesses and tubes allowing the collection of feces with no urine contamination. For 7 consecutive days, total collection of feces was carried out. A 5%-subsample of feces was taken daily and pooled by period and within cow. Samples of diets, and feces were frozen at -20°C until subsequent chemical analyses. During the last week of each experimental period, milk samples were taken from each cow at each milking, pooled on a yield basis to obtain one composited milk sample per cow per period. One sample was kept frozen at -80^{o}C without preservative for further analyses of fat and EL. Another sample was stored at + 4°C with a preservative (bronopol-B2) until analyzed for protein, urea N, lactose, total solids and somatic cell score (SCS).

Chemical analysis

Analytical DM of the diets and feces was determined in a forced-air oven according to the procedure 934.01 (AOAC, 1990). Samples of the total mixed diets were ground to pass a 1-mm screen in a Wiley mill before chemical analyses. Total N content of total

mixed diets was determined by thermal conductivity (LECO model FP-428 Nitrogen Determinator, LECO, St. Joseph, MI, USA) and crude protein was calculated as N × 6.25. Concentration of neutral detergent fibre (NDF) in diets was determined as described by Van Soest et al. (1991) without the use of sodium sulfite and with the inclusion of heat stable α-amylase. The acid detergent fibre (ADF) content in diets was determined according to AOAC (1990; Method 973.18). The NDF and ADF procedures were adapted for use in an ANKOM^{200} Fibre Analyzer (ANKOM Technology Corp., Fairport, NY, USA). Ether extraction in diets was conducted with Tecnal TE–044/1 (Piracicaba, São Paulo, Brazil) according to the method No. 7.060 (AOAC, 1990). Milk fat concentration was determined by the method of Roese-Goettlib (AOAC, 1990). Protein, lactose, total solids, and urea N concentrations and SCS in milk samples were analyzed by infrared spectroscopy (System 4000 Milkoscan; Foss Electric of Hillerod, Denmark). Milk fat was extracted and FA were methylated according to the methods described by Chouinard et al. (1997b) while in situ transesterification was performed on diets according to Park and Goins (1994). Fatty acid methyl ester profiles were measured by GLC on a Hewlett-Packard 6890 chromatograph (Hewlett-Packard Lteé, Montreal, QC, Canada) with a G1315A auto sampler equipped with a flame ionization detector and a split-splitless injector as described by Delbecchi et al. (2001).

Lignans in milk were hydrolysed and extracted according to the method of Frank & Custer (1996) with some modifications. Only EL was analyzed in milk as other studies have shown that the mammalian lignan ED is below detection level in milk (Petit et al., 2009) and that EL is the main mammalian metabolite produced by ruminal microbiota (Côrtes et al. 2008). Five hundred microliters of warmed (40°C) milk samples were mixed with 5 µL of ß-glucuronidase/arylsulfatase from *Helix pomatia* (Roche-Diagnostics, Laval, QC, Canada). Milk samples were incubated for 1.5 h at 37°C in a shaking waterbath. After hydrolysis, all samples were acidified with 10 µL of 6 N HCl. Acidified milk samples were washed with 3 ml of hexane before extraction to remove lipids (Raffaelli et al. 2002). All samples were extracted with 2 mL of diethyl ether. The samples were vortex-mixed twice for 2 min. Organic layer was separated by freezing. The remaining liquid phase was submitted to a second extraction under the same conditions. The organic layers were pooled and evaporated by vacuum (Speed-Vac; Thermo Savant, Holbrook, NY, USA) at room

temperature for 40 min. The dry extract was redissolved in 500 µL of EIA buffer and warmed at 37°C for EL analysis using an enzyme immunoassay (EIA; Cayman Chemical, Ann Arbor, MI, USA). Cayman Chemicals Enterolactone EIA kit is a competitive assay that utilizes a standard curve ranging from 15.6 to 2 000 pg/mL. The assay exhibits a limit of quantification (defined as 80% B/B_0) of 70 pg/mL and an IC50 (50% B/B_0) of 240 pg/mL.

Statistical analysis

All results were analyzed using the GLM procedure of SAS (2000) Data were analyzed using a 4 X 4 Latin square design with the following general model:

$$Y_{ijkl} = \mu + C_j + P_k + T_l + e_{jkl}$$

Where: Y_{ijkl} = the dependent variable, μ = overall mean, C_j = random effect of cow (j = 1 to 4), P_k = fixed effect of period (k = 1 to 4), T_l = fixed effect of treatment (l = CO, WF, CF, MF), and e_{ijkl} = random residual error. Significance was declared at $P < 0.05$ and a trend at $P \# 0.10$, unless otherwise stated.

RESULTS

Dry matter intake (DMI) and apparent digestibility of DM (DMD) were not affected by treatments (Table 2).Milk production, 4% FCM production, and milk efficiency were similar among diets. Milk percentages of protein, lactose, urea N, and total solids also were similar among treatments. The highest and lowest milk fat concentrations were observed, respectively, for cows fed CO and CF and cows fed the other diets had milk fat concentrations similar to those fed CO and CF. Milk yield of protein, fat, lactose, and total solids did not differ among treatments. Concentration of EL in milk (nmol/L) was similar among diets and averaged 83.86 nmol/L.

In general, short and medium-chain FA were not affected by treatments, with the exception of 12:0 concentration that was lower (P=0.05) in milk fat when CF were added to the diet compared to when the CO diet was fed. Nevertheless, WF and MF did not differ from CO or CF for 12:0 concentration (Table 3). Milk fat concentration of 16:0 was higher (P=0.01) for cows fed CO compared to those fed the flaxseed products.

Concentration of *trans*9-18:1 increased (*P*=0.01) in milk fat when calcium salts were added to the diet (CF and MF diets) compared to when there were no calcium salts (CO and WF). Cows fed CF had higher concentration of *trans*11-18:1 (*P*=0.02) than those fed CO and WF, but there was no difference between cows fed CF and those fed MF. Moreover, cows fed MF, CO and WF had similar concentrations of *trans*11-18:1 in milk fat.

Milk fat concentrations of *cis*6-18:1 were different (*P*=0.001) for all diets and concentrations decreased significantly as followed: CF > MF>WF>CO. Concentration of rumenic acid (*cis*9,*trans*11-18:2) was higher (*P*=0.05) for cows fed CF than for those fed WF, and there was no difference between cows fed CF and those fed CO and MF. Moreover, cows fed CO, MF and WF had similar concentration of *cis*9,*trans*11-18:2 in milk fat. Concentration of alpha-linolenic acid (*cis*9,12,15-18:3) in milk fat was higher (*P*=0.02) for cows fed CF and MF compared to those fed CO, but it was similar for cows fed WF. Concentration of 19:0 in milk fat increased (*P*=0.01) when calcium salts were added to the diets (CF and MF diets) compared to the control diet.

DISCUSSION

The addition of whole flaxseed or calcium salts of flaxseed oil had no effect on feed intake and digestibility. In general, untreated whole flaxseed is readily accepted by dairy cows and feeding up to 15% of the total dry matter as flaxseed has no effect on DMI of mid- (Secchiari et al. 2003) and early-lactating (Petit, 2002) dairy cows. On the other hand, supplementation with calcium salts over 2% in the diet DM generally decreases DMI (Loften and Cornelius, 2004) as a result of negative effects of fat on gastro-intestinal motility, rumen function and palatability. Chouinard et al. (1997a) observed a linear decrease in DMI when calcium salts of canola oil were added at 0, 2 or 4% of the DM. Moreover, feeding approximately 3% Megalac® (calcium salts of long-chain fatty acids) in the DM of the diet decreased DMI from 27.3 to 24.1 kg/d compared to a control diet (Harvatine and Allen, 2006a) although no difference in DMD was observed (Harvatine and Allen, 2006c). Allen (2000) investigated several studies (28) involving the supply of calcium salts in the diet of dairy cows and concluded that for every 1% of added calcium salts over the control, a depression in the DMI of 2.5% occurred. In the present experiment,

the addition of calcium salts was below 1.9% of the DM, which may not have been high enough to cause negative effects on DMI and DMD as observed in previous studies.

Milk production and 4% FCM yield were not affected by treatments, which agree with the results reported by Juchem et al. (2008) where a mixture of calcium salts of fish oil and palm oil were offered at 1.9% of the DM compared to a diet containing 1.9% of the dry matter from tallow. However, when Megalac® was added at 3.0% of the dry matter compared to when a control diet was fed, yield of 3.5% FCM was decreased (Harvatine and Allen, 2006b).

Milk fat percentage decreased with calcium salts supplementation compared to the control diet. Similarly, feeding calcium salts (4% of DM) of canola, soybean or flaxseed oil to dairy cows decreased milk fat percentage compared to a control diet (Chouinard et al., 1998). Feeding highly unsaturated FA sources like fish oil can affect rumen fermentation and decrease milk fat concentration (Cant et al., 1997). According to Bauman and Griinari (2003), the milk fat depression associated with diets containing high concentrations of PUFA is a result of the FA intermediates formed in the rumen during biohydrogenation of unsaturated FA, which may suggest that the calcium salts of flaxseed oil fed in the present experiment were not totally inert in the rumen. This is corroborated with the fact that feeding calcium salts of flaxseed oil resulted in higher concentrations of the intermediates of ruminal biohydrogenation (*trans*9-18:1 and *trans*11-18:1) and *cis*9,*trans*11-18:2 in milk fat than when feeding whole flaxseed. Similarly, higher concentrations of *trans*9- and *trans*11-18:1 in milk fat when calcium salts of PUFA oils were fed compared to a control diet (Chouinard et al., 1998) and higher concentrations of *cis*9,*trans*11-18:2 when calcium salts of soybean oil (LAC® 100) were fed compared to whole flaxseed (Cavalieri et al., 2005) have been reported. According to Wu and Palmquist (1991), 58% of 18 carbon-chain FA in calcium salts are biohydrogenated *in vitro*. Moreover, Castañeda-Gutiérrez et al. (2007) reported that milk fat concentrations of EPA and DHA were 5 to 6 times higher when fish oil was infused in the abomasum compared to when calcium salts of fish oil were administered in the rumen, resulting in more than 85% of EPA and 75% of DHA of fish oil in the form of calcium salts being biohydrogenated in the rumen. In the present experiment, biohydrogenation of PUFA was more important when flaxseed oil was fed as calcium salts than as whole seed as shown by greater concentrations of biohydrogenation intermediates

of FA in milk fat of cows fed CF and MF diets. According to Chalupa et al. (1986), calcium salts dissociation occurs in the rumen with liberation of PUFA when the ruminal pH is below 6, however, in this experiment the pH was always up to 6 (data not showed). On the other hand, alpha-linolenic acid (*cis*9, 12, 15-18:3) concentration in milk fat was significantly higher for treatments with calcium salts compared to the control diet, thus demonstrating an effective transfer of *cis*9, 12, 15-18:3 from CF and MF diets to milk. Higher concentrations of alpha-linolenic acid has been observed in milk fat of cows fed ground compared to whole flaxseed (Da Silva et al., 2007), which may suggest that flaxseed processing increases the transfer of alpha-linolenic acid in milk fat.

Enterolactone in milk was not affected by the treatments. It was expected that whole flaxseed will enhance enterolactone concentration in milk since its plant precursor (SDG) is concentrated in hulls and not in the oil. Previous results have shown that feeding whole flaxseed and flaxseed meal increases concentration of enterolactone in milk compared to feeding a control diet (Petit et al., 2009). However, the amount of whole flaxseed offered in the present experiment was lower than the 10% fed in the experiment of Petit et al. (2009), which may be not high enough to affect concentration of enterolactone in milk. Nevertheless, numeric values were lower for treatments with calcium salts of flaxseed oil than for those with whole flaxseed.

CONCLUSIONS

Feeding a diet with no flaxseed product or a diet with 0.83% of calcium salts of flaxseed oil or 4.18% of whole flaxseed had no effect on dry matter intake, apparent digestibility of dry matter, and milk production of dairy cows. Calcium salts of flaxseed oil decreased milk fat concentration but resulted in similar concentrations of protein, lactose, urea N and total solids in milk compared to a control diet. Feeding 4.2% of whole flaxseed in the dry matter did not increase enterolactone concentration in milk. Supplementation with calcium salts of flaxseed oil compared to whole flaxseed enhanced milk fatty composition from a human health perspective.

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Table 1. Ingredient and chemical composition of the TMR from diets containing no flaxseed product (CO), whole flaxseed (WF), calcium salts of flaxseed oil (CF) or a mixture of whole flaxseed and calcium salts of flaxseed oil (MF).

	Treatment			
	CO	WF	CF	MF
Ingredient, % of DM				
Grass silage	27.67	27.67	30.81	28.70
Corn silage	27.83	27.71	30.77	28.70
Broken corn grain	21.64	19.65	14.42	18.02
Ground barley	7.31	7.24	7.31	7.31
Soybean meal	7.48	5.93	7.48	7.10
Calcium salts of flaxseed oil	0	0	1.87	0.83
Whole flaxseed	0	4.18	0	2.30
Top supplement[1]	4.59	4.16	4.59	4.20
Calcium carbonate	0.58	0.58	0.25	0.33
Mineral[2]	2.91	2.88	2.49	2.49
Chemical analysis				
DM, %	46.0	45.6	43.3	44.0
CP, % of DM	16.7	16.3	16.4	16.4
Ether extracts, % of DM	2.2	3.6	2.8	3.2
NDF, % of DM	28.2	29.7	31.6	30.3
ADF, % of DM	22.1	23.2	25.6	23.7
NE_L, Mcal/kg[3]	1.63	1.62	1.65	1.64
Fatty acids, % of total FA				
12:0	0.86	0.75	0.67	0.73
14:0	0.79	0.67	0.61	0.67
16:0	17.19	14.19	14.13	13.95
18:0	2.11	2.52	2.65	2.49
*cis*9,18:1	18.58	18.56	17.40	17.84
*Cis*11,18:1	1.14	1.12	1.10	1.10
*cis*9,12-18:2	47.08	37.47	36.42	36.97
20:0	0.58	0.22	0.32	0.33
*cis*9,12,15-18:3	7.81	21.83	23.50	22.57
22:0	0.44	0.20	0.33	0.35
24:0	0.35	0.27	0.30	0.30
Others	3.07	2.21	2.54	2.69
n6[4]:n3[5]	6.13	1.72	1.57	1.65

[1]Contained 20% of canola meal, 30% of corn gluten meal, 20% of soybean meal, and 30% of brewer's corn. [2]Contained 9.02% Ca, 4.90% P, 4.89% Mg, 1.76% S, 14% Na, 1.43% K, 2068 mg/kg Fe, 2718 mg/kg Zn, 447 mg/kg Cu, 1814 mg Mn, 69 mg/kg I, 7 mg/kg Co, 20 mg/kg Se, 452,000 IU/kg of vitamin A, 58,000 UI/kg of vitamin D_3, and 2692,000 IU/kg of vitamin E. [3]Calculated using published values of feed ingredients (NRC, 2001). [4]*cis*9,12-18:2. [5]*cis*9,12,15-18:3.

Table 2. DM intake (DMI) and digestibility (DMD), milk production, milk composition and concentration of enterolactone in milk of Holstein cows fed total mixed diets containing no flaxseed product (CO), whole flaxseed (WF), calcium salts of flaxseed oil (CF) or a mixture of whole flaxseed and calcium salts of flaxseed oil (MF).

	Treatment				SE[1]	*P*-value
	CO	WF	CF	MF		
DMI, kg/d	22.1	22.7	21.6	21.5	0.42	0.32
DMD, %	64.7	64.1	65.5	65.2	0.59	0.47
Milk production, kg/d	31.52	32.65	32.55	32.32	0.45	0.42
4% FCM, kg/d	24.70	25.35	21.93	24.07	0.71	0.13
Milk efficiency[2]	1.43	1.44	1.51	1.51	0.02	0.20
Milk composition, %						
Protein	3.52	3.51	3.53	3.68	0.14	0.83
Fat	2.57[a]	2.50[ab]	1.83[b]	2.36[ab]	0.11	0.05
Lactose	4.75	4.80	4.80	4.80	0.03	0.54
Urea N	12.66	12.12	12.20	13.00	0.66	0.76
Total solids	11.82	11.78	11.13	11.75	0.23	0.29
Milk yield, kg/d						
Protein	1.11	1.15	1.15	1.19	0.05	0.79
Fat	0.81	0.82	0.59	0.74	0.04	0.07
Lactose	1.50	1.57	1.56	1.55	0.02	0.14
Total solids	3.72	3.85	3.62	3.80	0.09	0.47
SCS[3]	2.17	1.74	0.97	1.20	0.44	0.38
Enterolactone, nmol/L	102.96	88.73	62.97	80.77	8.80	0.16

[a.b.c]Means within a row with no common superscript differ ($P < 0.05$).
[1]Least squares means with pooled standard error (SE).
[2]Milk production (kg/d)/DMI (kg/d).
[3]Somatic cell score = $\log_{10}$ (somatic cell count/mL).

Table 3. Fatty acids in milk (% of total fatty acids) of Holstein cows fed a total mixed diets containing no flaxseed product (CO), whole flaxseed (WF), calcium salts of flaxseed oil (CF) or a mixture of whole flaxseed and calcium salts of flaxseed oil (MF).

Fatty acid	Treatment				SE[1]	*P*-value
	CO	WF	CF	MF		
4:0	5.13	5.15	5.79	5.78	0.32	0.42
5:0	0.07	0.07	0.06	0.06	0.004	0.19
6:0	2.63	2.61	2.73	2.76	0.11	0.76
7:0	0.12	0.13	0.08	0.12	0.02	0.34
8:0	1.57	1.55	1.50	1.57	0.03	0.34
9:0	0.14	0.14	0.10	0.14	0.02	0.47
10:0	3.59	3.40	2.99	3.24	0.11	0.10
11:0	0.19	0.17	0.11	0.15	0.02	0.25
12:0	4.35^{a}	4.07^{ab}	3.37^{b}	3.79^{ab}	0.13	0.05
*cis*11-12:1	0.23	0.23	0.17	0.24	0.03	0.52
13:0	0.22	0.20	0.13	0.17	0.03	0.26
14:0	12.6	12.1	11.5	11.8	0.30	0.26
*cis*9-14:1	1.19	1.12	1.01	1.08	0.05	0.22
15:0	1.49	1.28	1.03	1.07	0.13	0.24
16:0	32.1^{a}	29.0^{b}	26.4^{b}	27.4^{b}	0.43	0.01
*trans*9-16:1	0.08	0.09	0.10	0.11	0.02	0.72
*cis*9-16:1	1.73	1.51	1.36	1.47	0.10	0.24
17:0	0.69	0.66	0.58	0.65	0.04	0.43
18:0	8.89	11.01	12.20	11.52	0.53	0.07
*trans*9-18:1	0.28^{b}	0.33^{b}	0.41^{a}	0.37^{a}	0.01	0.01
*trans*11-18:1	0.91^{b}	0.94^{b}	1.65^{a}	1.26^{ab}	0.08	0.02
*cis*6-18:1	0.73^{d}	1.08^{c}	1.98^{a}	1.44^{b}	0.04	0.001
*cis*9-18:1	14.78^{b}	16.70^{a}	17.60^{a}	16.70^{a}	0.14	0.003
*cis*11-18:1	0.68	0.58	0.56	0.54	0.04	0.27
*trans*9,12-18:2	0.10	0.10	0.13	0.17	0.05	0.76
*cis*9,12-18:2	2.38	2.31	2.41	2.30	0.07	0.68

*cis*9,*trans*11-18:2	0.43[ab]	0.42[b]	0.67[a]	0.53[ab]	0.04	0.05
*trans*10,*cis*12-18:2	0.02	0.01	0.02	0.02	0.01	0.69
*cis*6,9,12-18:3	0.07	0.07	0.06	0.08	0.01	0.65
*cis*9,12,15-18:3	0.59[b]	0.84[ab]	1.03[a]	0.95[a]	0.05	0.02
19:0	0.21[c]	0.30[bc]	0.51[a]	0.40[ab]	0.02	0.01
*cis*7-19:1	0.09	0.05	0.09	0.09	0.02	0.51
20:0	0.30	0.27	0.32	0.35	0.05	0.74
*cis*8-20:1	0.20	0.28	0.20	0.25	0.03	0.70
*cis*11-20:1	0.13	0.14	0.13	0.17	0.03	0.71
*cis*11,14-20:2	0.13	0.15	0.13	0.18	0.03	0.63
*cis*8,11,14-20:3	0.18	0.18	0.15	0.18	0.01	0.40
*cis*5,8,11,14-20:4	0.22	0.20	0.17	0.20	0.01	0.22
*cis*5,8,11,14,17-20:5	0.10	0.10	0.10	0.12	0.02	0.71
22:0	0.12	0.14	0.12	0.15	0.01	0.31

[a.b.c]Means within a row with no common superscript differ ($P < 0.05$).
[1]Least squares means with pooled standard error (SE).

CONSIDERAÇÕES FINAIS

Ambas, microflora ruminal e fecal são capazes de transformar lignana vegetal (SDG) em lignanas mamíferas (EL e ED) a partir de semente ou casca de linhaça incubada *in vitro*, colaborando para um melhor entendimento da via metabólica de lignanas mamíferas em vacas em lactação e direcionando estudos para a manipulação da concentração no leite.

O fornecimento de casca de linhaça para vacas em lactação é efetivo em melhorar a qualidade do leite em relação à composição em ácidos graxos e, além disso, transfere lignana mamífera (EL) para o leite. Desta forma, é possível produzir um leite enriquecido com substâncias desejáveis à saúde humana. Porém, a incorporação de monensina sódica em dietas com ou sem casca de linhaça não traz mudanças na composição do leite, ou seja, a sua suplementação não é eficiente em incorporar ácidos graxos poli-insaturados e lignanas mamíferas.

Sais de cálcio de óleo de linhaça também alteram, em menor proporção, a composição em ácido graxo do leite para melhor. Por outro lado, fornecer grãos de linhaça inteiros (com casca possuindo lignanas vegetais), não aumenta a transferência de enterolactona para o leite se comparado a uma dieta sem linhaça ou somente com uma fonte de óleo.