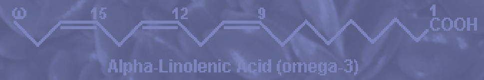
Ruminal fatty acid metabolism

Altering rumen biohydrogenation to improve milk fatty acid profile of dairy cows



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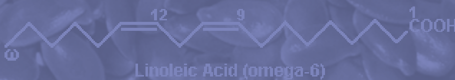

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This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences (WIAS).

Ruminal fatty acid metabolism

Altering rumen biohydrogenation to improve milk fatty acid profile of dairy cows

Attje-Rieke Sterk

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. dr. M.J. Kropff
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 12 October 2011
at 4 p.m. in the Aula.

Sterk, A. Ruminal fatty acid metabolism. Altering rumen biohydrogenation to improve milk fatty acid profile of dairy cows.

Thesis, Wageningen University, the Netherlands (2011)
With references, with summaries in English, Dutch, and Frisian

ISBN 978-94-6173-020-6

Abstract

Nutritional guidelines promote a reduced intake of saturated fatty acids (FA) and increased intake of unsaturated FA by humans. Milk and dairy products contain a high proportion of saturated FA caused by extensive alterations of dietary lipids in the rumen through the processes of lipolysis and biohydrogenation. Therefore, marked differences exist between the FA profile in the diet (mostly unsaturated FA) and the FA profile of lipids leaving the rumen (mostly saturated FA). The objective of the research described in this thesis is therefore to improve the milk FA profile of dairy cows by altering diet composition and ruminal FA metabolism, thereby increasing ruminal outflow of unsaturated FA and consequently the secretion of unsaturated FA into milk fat. In the first study, a meta-analysis, it was shown that various fat sources, their technological form (oil, seed, protected, or addition of fish oil), and their inclusion to diets differing in forage type could significantly alter the FA profile of milk fat. In addition, the technological form of the fat source and the forage type in the basal diet affect the relationship between the dietary nutrient composition (FA and NDF content) and the milk FA profile. In the second study, various technologically and chemically treated linseed products were evaluated in vitro and it was shown that only formaldehyde treated crushed linseed and extruded whole linseed were able to decrease the extent of biohydrogenation of C18:3*n*3, whereas the addition of docosahexaenoic acid (DHA) to linseed oil inhibited the complete biohydrogenation to C18:0. In the third study, FA intake, omasal FA flows and plasma and milk FA profiles were measured from cows fed crushed linseed, formaldehyde treated linseed oil, extruded whole linseed, or linseed oil combined with DHA. The extent of biohydrogenation of C18:3*n*3 was lower for cows fed the extruded whole linseed treatment as shown by the higher omasal C18:3*n*3 flow compared with the other treatments. However, fat digestibility of this product was lower, resulting in no effects on plasma and milk C18:3*n*3 proportions. Cows fed formaldehyde treated linseed oil did show higher plasma and milk C18:3*n*3 proportions compared with the other treatments, but unsaturated FA content of milk fat did not differ between treatments. The cows fed linseed oil in combination with DHA showed increased omasal flows and plasma and milk fat proportions of biohydrogenation intermediates. In the final study, the milk FA profile of high producing dairy cows was evaluated after feeding an increasing proportion of crushed linseed in combination with varying forage type (grass versus maize silage) and forage to concentrate ratio. It was shown that the milk FA profile of cows fed an increasing proportion of crushed linseed depends on the forage type and forage to concentrate ratio of the diet. In conclusion, the results described in this thesis indicate that the FA profile of bovine milk fat can be altered by manipulation of the ration composition. Changes in ration composition affect ruminal FA metabolism, the profile of absorbed FA, and eventually the proportions of FA secreted in milk fat.

Voorwoord

Tijdens de afgelopen vier en een half jaar van mijn promotieonderzoek heb ik een super fijne en leerzame tijd gehad en deze tijd wordt nu afgesloten met het afronden van dit proefschrift. Veel mensen hebben geholpen om mijn promotieonderzoek tot een succes te maken en ik wil dan ook graag van deze gelegenheid gebruik maken om een woord van dank uit te spreken.

In de herfst van 2006 is het eerste contact tussen de projectpartners, Royal FrieslandCampina, Agrifirm Feed, Agrifirm Innovation Center, Barenbrug Holland B.V. en de leerstoelgroep Diervoeding van Wageningen University, gelegd om gedetailleerd onderzoek te doen naar het beïnvloeden van de vetzuursamenstelling van melk. Al snel werd een groot onderzoeksvoorstel geschreven, uiteindelijk gefinancierd door het Food & Nutrition Delta programma van SenterNovem en de projectpartners, met daarin plaats voor een promovendus die onderzoek zou doen naar het aanpassen van de melkvetzuursamenstelling vanuit het rantsoen van de koe en met specifieke aandacht voor het vetzuur metabolisme in de pens. Toen Ruud Tijssens mij eind 2006 aanbood om als parttime promovenda dit project te gaan uitvoeren, heb ik deze kans dan ook met beide handen aangegrepen. Hierbij wil ik dan ook een bijzonder woord van dank uitspreken aan Ruud Tijssens voor de mogelijkheid om dit promotieonderzoek uit te voeren. Daarnaast wil ik mijn promotor, prof. Wouter Hendriks en co-promotoren, Jan Dijkstra en Ad van Vuuren, bedanken voor het in mij gestelde vertrouwen om dit onderzoek te mogen uitvoeren. Wouter, bedankt voor de vrijheid die je mij hebt gegeven om het promotieonderzoek tot een goed einde te brengen en bedankt voor de laatste kritische check. Jan en Ad, bedankt voor alle adviezen, opmerkingen en commentaar tijdens onze regelmatige besprekingen. Daarnaast ben ik jullie heel veel dank verschuldigd voor het kritisch doornemen van alle manuscripten, zeker in de laatste weken, waarin we een strak schema hadden om alles op tijd klaar te krijgen en te kunnen indienen. Jan, extra bedankt voor jouw inzet om ook de meta-analyse nog op tijd af te ronden. Door al jullie opmerkingen en adviezen zijn al twee papers gepubliceerd en geaccepteerd en zullen de andere papers hopelijk snel geaccepteerd worden voor publicatie. Ook wil ik graag mijn collega bij AIC, Hassan Taweel, bedanken voor de vrijheid die ik heb gekregen om dit promotieonderzoek uit te voeren en op tijd af te ronden. Hassan, bedankt voor je betrokkenheid bij mijn onderzoek, je adviezen en je flexibiliteit en ik hoop dat we met het rundvee-team een goede tijd tegemoet gaan. Verder wil ik graag de leden van de stuurgroep bedanken voor hun inbreng en interesse tijdens de regelmatige project-bijeenkomsten.

Om de proeven beschreven in dit proefschrift uit te voeren, ben ik veel dank verschuldigd aan vele collega's van het WFPC, het ANU lab en de proefbedrijven: de Ossekampen (Wageningen) en Nötcenter Viken (Fälkoping, Zweden). Tamme en Sven, heel erg bedankt voor de hulp bij de eerste proeven waarbij we lijnzaad op diverse manieren hebben behandeld, zodat ik vervolgens kon testen of er verschillen in biohydrogenatie optraden. Ronald, Leen, Ries, Willem en Andre wil ik heel hartelijk bedanken voor alle hulp tijdens de proef op de Ossekampen, waarbij we met een nieuwe methode vanuit de boekmaag monsters konden nemen om de stroom aan vetzuren te bepalen. Ook Ronald Zom, van harte bedankt voor het aanleren van de boekmaag bemonsteringstechniek. Het was best lastig om bij de Holstein koeien de boekmaag te vinden, maar het is gelukt! A special word of thanks

is also reserved for the people of trial farm Nötcenter Viken in Sweden, who helped in taking a lot of feed, milk and blood samples. The help of Björn Johansson was essential in conducting the trial. Björn, I would like to thank you very much for your help and hospitality every time I came over to Sweden. I would like to thank also Michael Murphy for the critical remarks in designing, analysing and discussing the results of the trial. Bij alle proeven zijn heel wat monsters verzameld en met behulp van Saskia, Jane-Martine, Truus, Xuan Huong, Leon, Dick, Robert, Michel, Niels en Adriaan zijn alle monsters geanalyseerd. Bedankt voor alle uitleg, hulp en uitvoering van analyses! Saskia, de afgelopen jaren kon ik altijd bij je terecht met vragen, problemen, verzoeken en natuurlijk gezellige praatjes. Daarom vind ik het erg fijn dat jij mij tijdens de verdediging bij wilt staan als paranimf. Robert, een speciaal woord van dank voor jouw hulp bij het opzetten van de vetzuuranalyses. Ik ben erg blij dat je nu bij humane voeding op je plaats zit en dat je toch steeds de tijd kon nemen om ons met de lastige analyses te helpen. Michel, ook een speciaal woord van dank voor jouw hulp bij de uitvoering van de vetzuuranalyses. Zonder jouw hulp was ik nu nog GC-plaatjes aan het kijken! Aanvullend wil ik graag Bruno Vlaeminck bedanken voor de adviezen over de vetzuuranalyses en proefopzetten en voor het kritische commentaar op de manuscripten. Ook de hulp van MSc-studenten Gerrit Kingma, Steven van der Weerd en Ard van Veen bij de uitvoering van de proeven was onontbeerlijk, bedankt hiervoor! For the meta-analysis, I would like to thank Jennifer St. Pierre for the advice to find the correct method for the statistical analysis of the dataset. En voor het regelen van alle ondersteunende zaken tijdens mijn promotieonderzoek wil ik graag Betty Looijen en Yvonne van Holland van harte bedanken.

Nazir Khan and Antoon Jacobs, my roommates for the past four and a half years at Zodiac. We had a good time with the three of us and it was a good working place with three persons working all on the same subject, fatty acids. Thanks for the time we spent together and I wish you both all the best in your future carriers. ANU heeft mij een zeer fijne werkomgeving geboden tijdens mijn promotieonderzoek en ik wil alle collega's van ANU daarvoor van harte bedanken. Het was altijd gezellig tijdens de pauzes, BBQ's, playbackshows, nieuwjaarsborrels, enz. en ik zal de goede sfeer zeker missen. Naast mijn tijd bij ANU heb ik de afgelopen jaren met veel plezier in het rundveevoedingsonderzoek voor AIC gewerkt. Mijn collega's van AIC en Agrifirm Feed wil ik daarom van harte bedanken voor de goede werksfeer en flexibiliteit van de afgelopen jaren.

Naast het werk was er ook zeker tijd voor de nodige ontspanning. Het was altijd gezellig tijdens de spelavondjes, ski-vakanties, volleybalwedstrijden, -trainingen en het paardrijden, enz. Lieve familie en vrienden, bedankt voor alle interesse en mentale ondersteuning, zeker tijdens de laatste weken voor de deadline. Heit en Mem, graag wil ik jullie speciaal bedanken voor alle kansen en steun die jullie mij hebben gegeven en waardoor ik nu hier voor deze belangrijke gebeurtenis sta. En bedankt dat jullie de samenvatting hebben willen vertalen in het Frysk, daar ben ik trots op. Pieteke en Abel, ik ben heel blij dat jullie weer thuis zijn na anderhalf jaar avontuur en Pieteke, ik vind het super dat jij als paranimf naast mij op het podium wilt staan. Ik ben benieuwd of het lukt om "biohydrogenation" goed uit te spreken als het gevraagd wordt. Lieve Sake, het proefschrift is klaar! Bedankt voor je interesse, geduld, liefde en gezelligheid. Ik ben heel benieuwd wat de tijd voor ons zal brengen en heel gelukkig dat we de toekomst met z'n tweeën tegemoet gaan!

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Chapter 1

General Introduction

Milk and dairy products make up a substantial proportion of the daily fat intake in the Western diet (Caroll et al., 2006). Dairy milk fat consists of approximately 70% saturated fatty acids (SFA), 25% mono-unsaturated fatty acids (MUFA), and 5% poly-unsaturated fatty acids (PUFA). Due to its relatively high proportion of SFA, mainly myristic (C14:0) and palmitic (C16:0) acid, dairy milk fat has been associated with human cardiovascular health problems (Elwood et al., 2010; Astrup et al., 2011). However, recent reviews have reported no association or even a positive effect between the intake of milk and dairy products with variables (e.g. reduced blood pressure) related to the risk of cardio vascular health problems (Elwood et al., 2010; Bauman and Lock, 2010). Several bioactive fatty acids (FA) found in milk fat have potential benefits for health maintenance and prevention of chronic diseases (Bauman and Lock, 2010). Methods to manipulate the FA composition of milk fat are therefore receiving increased attention. Omega-3 FA (*n*3) are essential for growth and development and when consumed have shown several beneficial effects for human health and prevention of chronic diseases such as cardiovascular diseases, inflammatory diseases, and neurological disorders (Yashodhara et al., 2009). The *n*3 proportion in dairy milk fat is generally low and mainly consists of linolenic acid (*cis*-9,*cis*-12,*cis*-15-C18:3; C18:3*n*3; 0.5 g/100 g FA; Heck et al., 2009). Therefore, increasing specific unsaturated fatty acids (UFA) such as conjugated linoleic acid (CLA), linoleic acid (*cis*-9,*cis*-12-C18:2; C18:2*n*6), and C18:3*n*3 in milk fat, would increase consumer interest and acceptance of milk due to health benefits associated with these FA (Bauman and Lock, 2010).

Milk FA are derived from two sources, viz. de novo synthesis and uptake of preformed FA. Substrates for de novo synthesis are mainly acetate and ß-hydroxybutyrate derived from rumen organic matter fermentation (Lock and Bauman, 2004). They are used by the mammary epithelial cells to synthesize short- and medium-chain fatty acids (C4:0 to C14:0) plus a portion of the 16-carbon FA. The second source of FA in milk is the mammary uptake of circulating long-chain FA. This source provides a portion of the 16-carbon and all of the long-chain FA (≥ C18:0), and represents FA that originate from the intestinal absorption of dietary and microbial lipids and from the mobilization of body fat reserves (Bauman and Griinari, 2003; Lock and Bauman, 2004). Under normal dietary and physiological conditions, about one-half of the FA in milk originate from de novo synthesis in the mammary gland, while the other half originate from the uptake of preformed FA. In this situation the mobilization of body fat reserves accounts for less than 10 % of the FA in milk fat. However, when cows are in a negative energy balance, the contribution from mobilized FA increases in direct proportion to the extent of the energy deficit (Van Knegsel et al., 2007a).

Lipid metabolism in the rumen

Dietary FA composition can significantly affect the FA profile of milk fat (Grummer, 1991). Dairy diets are normally composed of a mix of fresh forages, conserved forages and concentrates, all of which contain lipids. These lipids can be characterized as structural or polar lipids (glycolipids, phospholipids), free fatty acids (FFA), triacylglycerides (TAG) and sterol esters (Yang and Fujita, 1997). In forages and grains, structural lipids predominate, whereas the main components in oil seeds and oils are TAG (Pokorný and Schmidt, 2003). Diets consumed by lactating dairy cows are normally

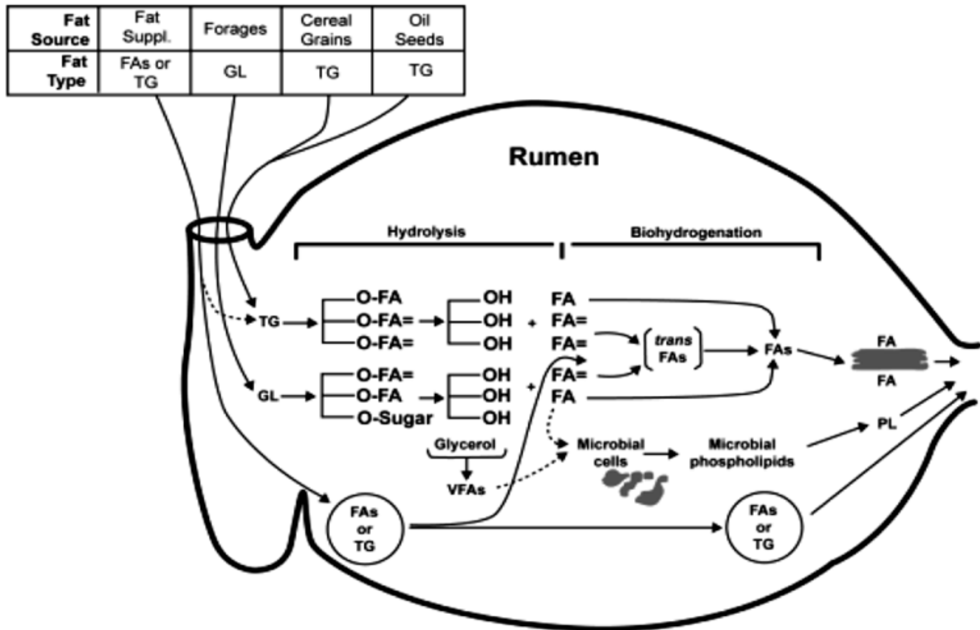

Figure 1. Lipid metabolism in the rumen including the predominant fat types in common feedstuffs (TG = TAG = triacylglycerides, GL = glycolipids and FA = fatty acids; Bauman and Lock, 2006).

low in fat content, generally containing only about 40 to 50 g/kg DM total fat. The predominant PUFA in ruminant diets are C18:2*n*6 and C18:3*n*3 with C18:2*n*6 being a major component of maize silage, oilseeds, and grains, whereas C18:3*n*3 is a major component of grass products and linseed (Lock and Bauman, 2004).

When dietary lipids enter the rumen, the initial step in lipid metabolism is the hydrolysis of the ester linkages found in TAG, phospholipids and glycolipids, and this is primarily carried out by hydrolases produced by rumen bacteria (Figure 1; Jenkins et al., 2008). The extent of hydrolysis is generally high (>85 %), and a number of factors that affect the rate and extent of hydrolysis have been identified. For example, the extent of hydrolysis might be reduced as the dietary level of fat is increased or when a low rumen pH inhibits the activity and growth of bacteria (Lock and Bauman, 2004).

Biohydrogenation of UFA is the second major transformation that dietary lipids can undergo in the rumen requiring a FFA for propagation. As a consequence, rates are always lower than those for hydrolysis, and factors that affect hydrolysis also affect biohydrogenation. In addition, the rate of rumen biohydrogenation of FA typically increases as the extent of unsaturation in the FA increases (Bauman and Lock, 2006). Several micro-organisms in the rumen are responsible for biohydrogenation of PUFA which form a protective mechanism against toxic effects of PUFA (Jenkins et al., 2008). Classical pathways of biohydrogenation are established using pure cultures of rumen organisms (Figure 2; Harfoot and Hazlewood, 1997). The initial step in rumen biohydrogenation typically involves an isomerization of the *cis*-12 double bond to a *trans*-11 configuration resulting in a conjugated

di- or trienoic FA. The next step is a hydrogenation reaction, which results in the conversion of an unsaturated double bond to a saturated single bond. In the case of C18:2*n*6 and C18:3*n*3 this is a reduction of the *cis*-9 double bond resulting in a *trans*-11 FA. The final step is a further hydrogenation of the *trans*-11 double bond producing C18:0 (C18:2*n*6 and C18:3*n*3 pathways) or *cis*-15 or *trans*-15-C18:1 (C18:3*n*3 pathway).

Rumen biohydrogenation is extensive and for most diets hydrogenation of C18:2*n*6 and C18:3*n*3 ranges between 70 to 95 % and 85 to 100 %, respectively (Doreau and Ferlay, 1994; Doreau and Chilliard, 1997; Harfoot and Hazlewood, 1997; Chilliard et al., 2007). Jenkins et al. (2008) evaluated the quantitative significance of different bacterial species in the biohydrogenation of PUFA. Eleven of 26 predominant bacterial species in the rumen were able to metabolize PUFA to a substantial extent. Three strains of *Butyrivibrio* and 2 strains of *Clostridium proteoclasticum* produced *trans*-11-C18:1, whereas only *C. proteoclasticum* produced C18:0 (Jenkins et al., 2008). Wallace et al. (2006) screened four hundred random sheep rumen isolates and found that the bacteria that produced substantial quantities of *cis*-9,*trans*-11-C18:2 and *trans*-11-C18:1 were butyrate producers. It was concluded that C18:0 producers clustered on a branch with *C. proteoclasticum* (Jenkins et al., 2008). Rumen protozoal lipids contain proportionally more UFA than the bacterial fraction (Harfoot and Hazlewood, 1997). However, it seems that the presence of protozoa was not necessary for biohydrogenation to occur, but they might have a role in the rumen outflow of UFA in the dairy cow (Jenkins et al., 2008).

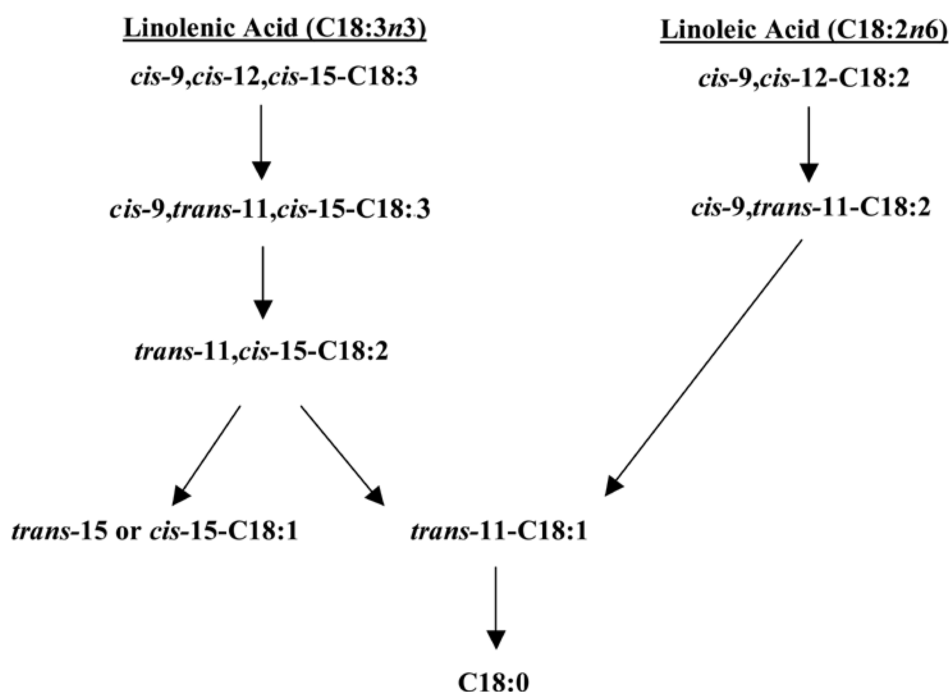

Figure 2. Classical biochemical pathways for the biohydrogenation of C18:2*n*6 and C18:3*n*3 in the rumen (Harfoot and Hazlewood, 1997).

Anaerobic fungi form a minor part of the ruminal micro-organisms and they seems to make only a small contribution to overall biohydrogenation of PUFA compared with the rumen bacteria (Jenkins et al., 2008).

Lipid metabolism in the mammary gland

As a consequence of the extensive hydrolysis and biohydrogenation occurring in the rumen, the FA that reach the small intestine are mainly saturated FFA. However, some biohydrogenation intermediates can also escape from the rumen (Lock and Bauman, 2004). Besides the processes in the rumen, the FA profile of milk fat is also influenced by processes in the mammary gland of dairy cows (Figure 3). Whilst in the rumen the dietary UFA will be transformed to SFA and some biohydrogenation intermediates, in the mammary gland the opposite transformations take place under influence of enzyme activity (Stearoyl Co-enzyme A Desaturase; SCD) in a process that is called desaturation (Harvatine et al., 2009; Jacobs et al., 2011). In this process part of the SFA will be transformed in MUFA, and part of the MUFA in PUFA (e.g. two-thirds of the C18:0 taken up by the mammary gland is converted to *cis*-9-C18:1; Bauman and Lock, 2010).

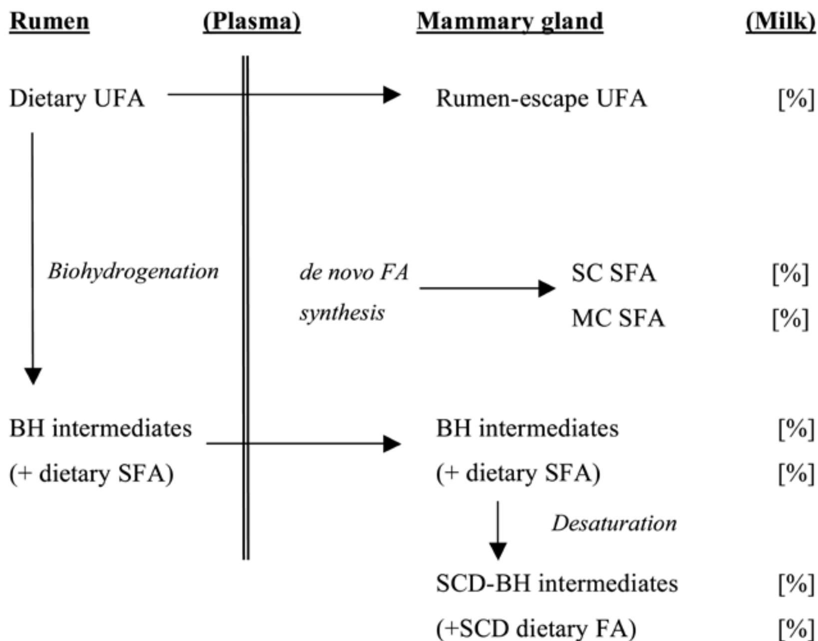

Figure 3. Schematic relationships between ruminal biohydrogenation (BH) and milk fatty acid (FA) profile. UFA, unsaturated FA; SC SFA, short-chain saturated FA; MC SFA, medium-chain saturated FA; SCD, *cis*-9-desaturated; [%] changes in milk FA proportions (g/100 g of total FA), as a result of changes in the flows of the different FA (Chilliard et al., 2007).

Opportunities to alter milk fatty acid profile

Changing the dietary composition of ruminants provides a natural way for farmers to alter milk FA profile towards a more desirable profile. Responses in milk FA profile from lipid supplementation are largely influenced by the characteristics of the lipid (source, technological form, and inclusion rate) and by the characteristics of the basal diet (forage type, nutrient composition; Chilliard et al., 2007). Different fat sources that are available can change the FA composition of the diet and the FA in these sources can be protected against the activity of the ruminal microbial population. Several rumen lipid protection technologies have been developed that involve either encapsulation of UFA inside a microbial-resistant shell, or alterations of FA structure to resist the action of microbial enzymes (Jenkins, 2006). From existing literature it is suggested that the available rumen protection technologies only partially protect UFA from biohydrogenation and provide only moderate increases in rumen outflow of desirable UFA or in proportions of UFA in milk fat compared with unprotected FA. In addition, most studies reported different levels of supplementation of different fat sources to achieve a specific increase in the proportion of UFA in milk, which makes it difficult to compare between fat sources and technological forms. In addition, characteristics of the basal diet can have a significant effect on ruminal FA metabolism, such as the amount of readily available UFA, amount of fibre, and amount of starch (Palmquist et al., 2005). These characteristics can regulate the extent of biohydrogenation and the formation of biohydrogenation intermediates that are formed. Readily available UFA and biohydrogenation intermediates can have a toxic effect on the micro-organisms in the rumen and by this means fermentation of carbohydrates could be inhibited resulting in loss of nutrients. An important research area is therefore the optimisation of the basal diet, e.g. the roughage composition or the forage to concentrate ratio, in combination with optimisation of the level and form of oilseed supplementation to avoid ruminal disturbances.

Objective and outline of this thesis

The objective of the research described in this thesis is to improve the milk FA profile of dairy cows. The main focus is on altering the diet composition and ruminal FA metabolism resulting in increases in desirable FA, such as C18:3*n*3, in rumen outflow and milk fat.

Chapter 2 describes the results of a meta-analysis carried out to determine the effects of different fat sources, their technological forms, addition of fish oil, and inclusion rate in combination with characteristics of the basal diet (main forage type, forage to concentrate ratio, NDF content) on milk FA profile.

Chapter 3 describes an in vitro study evaluating the effects of several chemically or technologically treated forms of linseed and linseed oil in combination with the addition of docosahexaenoic acid (DHA, C22:6*n*3) on rumen biohydrogenation kinetics of C18:3*n*3.

Chapter 4 and 5 describe an in vivo study with ruminally cannulated lactating dairy cows to evaluate the effects of different linseed sources and linseed oil in combination with DHA addition on FA intake, omasal FA flows, extent of rumen C18:3*n*3 biohydrogenation (**Chapter 4**), and plasma,

and milk FA profiles (**Chapter 5**).

Chapter 6 describes a 3-factor multivariate study in which the effects of an increasing proportion of crushed linseed in combination with varying forage type (grass or maize silage), and forage to concentrate ratio on milk FA profile of high producing dairy cows was evaluated.

Chapter 7 discusses the importance of milk fat and the opportunities to alter milk FA profile through changes in intake, ruminal FA metabolism, and mammary gland metabolism. The second part of the discussion focuses on effects of diets containing more UFA on animal metabolism and methane production. Finally, the general conclusions of the thesis are provided.

Chapter 2

Effects of different fat sources, technological forms and characteristics of the basal diet on milk fatty acid profile in lactating dairy cows – A meta-analysis

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Abstract

A meta-analysis was conducted to study milk fatty acid (FA) profile in dairy cows in response to changes in dietary nutrient composition in relation to supplementation of fat sources, their technological form, addition of fish oil, and main forage type in the basal diet. Data comprised 151 treatment means from 50 experiments, which were included in the database when diet composition, nutrient composition, FA composition, DMI, milk yield, milk composition, and milk FA profile were reported. Mixed model regression analysis including a random experiment effect and unequal variances among experiments was used. Least squares means were obtained for the different fat sources (unsupplemented, canola, soybean + sunflower, linseed, or fish oil), technological form including addition of fish oil (oil, seed, protected, added fish oil), and main forage type (alfalfa silage, barley silage, maize silage, grass silage, maize silage combined with haylage, or haylage) in the basal diet. Results showed that the technological form of supplemental canola, soybean, sunflower, or linseed significantly influenced the effect of dietary nutrient composition on milk FA profile resulting in significant differences between technological forms within the different fat sources. Protected canola and linseed increased C18:2*n*6 and C18:3*n*3 proportions in milk fat, respectively, whereas soybean and sunflower seed increased transfer efficiencies for C18:2*n*6 and C18:3*n*3 and their proportions in milk fat. Soybean, sunflower, or linseed supplied as oil increased *trans*-11-C18:1 proportions in milk fat, whereas the addition of fish oil to a diet containing soybean or sunflower decreased C18:0 and *cis*-9-C18:1 proportions in milk fat. Main forage type in the diet also significantly influenced the effect of dietary nutrient composition on milk FA profile resulting in significant differences between main forage types in the diet within the different fat sources. Maize silage as the main forage type increased *trans*-11-C18:1 in unsupplemented diets or diets supplemented with a source of soybean or sunflower. For canola supplemented diets, barley silage increased transfer efficiency and milk fat proportion of C18:2*n*6, whereas grass silage increased proportion of C18:3*n*3 in milk fat. For soybean or sunflower supplemented diets, haylage increased proportions of SFA, *cis*-9-C18:1, and C18:2*n*6, whereas the combination of maize silage and haylage increased transfer efficiency and milk fat proportion of C18:3*n*3. For linseed supplemented diets grass silage as the main forage type resulted in the highest C18:3*n*3 proportion, whereas *cis*-9-C18:1 proportion was comparable for grass silage, alfalfa silage, and maize silage as the main forage type. This meta-analysis confirmed that the effect of dietary nutrient composition on several milk FA proportions, depends on the type and form of fat supplementation, addition of fish oil, and main forage type in the basal diet.

Introduction

Changing the milk fatty acid (FA) profile of dairy cows towards an increased proportion of unsaturated fatty acids (UFA) is considered an improvement of the dietary value of bovine milk (Jenkins and Bridges, 2007). Milk FA are derived from two sources, viz. de novo synthesis from acetate and ß-hydroxybutyrate originating from ruminal fermentation and mammary uptake of FA available from absorption of dietary and microbial FA and FA from fat mobilization (Lock and Bauman, 2004). Fatty acids in the diet of dairy cows are mainly C18 FA from forages, cereals, and oil seeds (Chilliard et al., 2007). Oilseeds are used in diets of dairy cows to increase energy intake, increase efficiency of milk fat synthesis (Jones et al., 2001), and alter the FA profile of milk fat. Feeding whole untreated sunflower seeds increases the proportion of UFA in milk fat up to 40 % (Petit et al., 2004), although extensive biohydrogenation normally occurs in the rumen (Harfoot and Hazlewood, 1997). A reduction of this extensive biohydrogenation of UFA is required to increase the delivery of these UFA to the duodenum for absorption. The extent of biohydrogenation is affected by the technological form of the fat source (oil, seed, or protected; Chilliard et al., 2007) and the characteristics of the basal diet (such as forage type, and forage to concentrate ratio; Dewhurst et al., 2006).

Fat sources for dairy cows differ in their FA profile and hence can result in changes in the profile of FA absorbed and secreted as part of the milk fat. Canola sources contain oleic acid (*cis*-9-C18:1) as the most abundant FA, whereas soybean and sunflower sources are rich in linoleic acid (*cis*-9,*cis*-12-C18:2, C18:2*n*6), and linseed sources contain mainly linolenic acid (*cis*-9,*cis*-12,*cis*-15-C18:3, C18:3*n*3). Unprotected fat sources have only a limited use in dairy diets because they tend to upset cellulolytic activity and fibre digestion in the rumen (Harfoot and Hazlewood, 1997). A number of studies and several reviews have been published on responses of milk FA profile to these fat sources when included in diets for dairy cows (Dewhurst et al., 2006; Jenkins and Bridges, 2007; Glasser et al., 2008). Glasser et al. (2008) studied the responses of milk FA to several fat supplements and focussed on the response to increasing amounts of the supplemental fat sources. The effects of interfering dietary (e.g. technological form) or animal factors (e.g. lactation stage) were difficult to assess from their available dataset. In addition, Glasser et al. (2008) excluded diets supplemented with fish oil or marine algae or combinations of fat sources including fish oil from the analysis. Due to the specific effects of fish oil on biohydrogenation routes (Shingfield et al., 2005; Fievez et al., 2007), the effects of addition of fish oil to diets supplemented with a fat source, such as canola or linseed, are of interest. Consequently, the objective of this meta-analysis was to study milk FA profile in response to changes in dietary FA composition in relation to different fat sources, their technological form and/or addition of fish oil, and characteristics of the basal diet (forage type, NDF content).

Material and Methods

Data collection

A database was built from studies investigating the effects on milk FA profile in lactating dairy cows in response to different fat sources, with or without rumen lipid protection technology, with or

without addition of fish oil, and supplied to different basal rations. Data were obtained from scientific publications published between 1995 and 2009. A prerequisite for inclusion of an experiment in the database was that proportions of all the major feedstuffs in the diet, dietary ether extract (EE) or total FA content (g/kg DM), dietary NDF content (g/kg DM), dietary FA composition (g/kg DM), DMI (kg/d), milk yield (kg/d), milk protein and milk fat yield (g/d), and FA profile of milk fat (g/100 g FA) were reported. Publications reporting several experiments were given a specific code for each experiment. This resulted in a database of 47 publications reporting 50 experiments with in total 151 treatments (Appendix 1). The experiments contained on average 4 experimental treatments (range: 2 to 8) and each observation included in the dataset corresponded to the mean of a treatment group.

Animals, feeding and housing

All studies in the database used lactating dairy cows and each treatment group consisted of on average 9 cows (± 5 cows; mean ± SD), which were on average 110 days in milk (± 54 days). Most experiments were conducted as Latin square designs with 21 to 28 day experimental periods. Duration of experiments set-up as complete block designs was at least 6 weeks. Cows used in the experiments were Holstein cows, however, in the experiments of Franklin et al. (1999), Whitlock et al. (2002; 2006), and AbuGhazaleh et al. (2004), Holsteins and Brown-Swiss cows were used. Cows in most experiments were multiparous or a mixture of primiparous and multiparous cows. Jones et al. (2001) and AbuGhazaleh et al. (2002; 2003) used only primiparous cows in their experiments. Cows were housed individually in tie-stalls or housed in free-stall barns with Calan Broadbent feeding doors (American Calan, Inc., Northwood, NH). Cows were fed individually either a TMR or a ration with haylage and concentrates separately (Loor et al., 2005).

Grouping of experimental factors

In most experiments, one treatment group received a control diet, and the other treatment groups received the control diet plus a substantial amount of a fat source. The fat sources in the dataset were classed as: unsupplemented, canola, soybean + sunflower, linseed, and fish. Within fat sources, technological form of the fat source, addition of fish oil, and main forage type were distinguished. However, for fish oil as the main fat source, the number of treatment means was too low to be used in the analysis per fat source. For canola, technological form was grouped as: oil (all oil types included), seed (sources fed as whole seed, ground seed, heat treated seed, or extruded seed), and protected (sources fed as FA amides, or Ca-salts of FA). For soybean + sunflower sources, technological form was grouped as: oil (all oil types included), seed (sources fed as whole seed, ground seed, heat treated seed, extruded seed, or micronized seed), protected (sources fed as FA amides or Ca-salts of FA), and added fish oil (additional supply of fish oil to a diet containing soybean or sunflower). For linseed, technological form was grouped as: oil (all oil types included), seed (sources fed as whole seed, ground seed, extruded seed, and micronized seed), protected (formaldehyde treated), and added fish oil. Main forage type in the diets was encoded as: alfalfa silage, barley silage, maize silage, a combination of maize silage and haylage (maize/haylage), grass silage, and haylage. Unsupplemented diets contained barley silage, maize silage, maize/haylage, grass silage, or haylage as main forage type. For canola

sources, diets contained alfalfa silage, barley silage, maize silage, or grass silage as main forage type. For soybean + sunflower sources, diets contained maize silage, maize/haylage, grass silage, or haylage as main forage type. For linseed sources, diets contained alfalfa silage, maize silage, grass silage, or haylage as main forage type.

FA analysis methodology

Different FA analysis methods were used across the 47 publications. Differences among these methods would contribute to the experiment effect in the regression models developed. For many FA the precise isomer description was not reported. Fatty acids that were only described by C18:1 were assumed to be *cis*-9-C18:1, *trans*-C18:1 was assumed to be *trans*-11-C18:1, C18:2 was assumed to be C18:2*n*6 and C18:3 was assumed to be C18:3*n*3. Identification of specific C18:1 isomers was limited to publications reporting several *cis*- and *trans*-C18:1 isomers.

Statistics

Principle component analysis (PCA) was carried out using SPSS software (version 17; SPSS Inc, Chicago IL) to evaluate within fat sources the relationships between milk FA profile [saturated FA (SFA), UFA, C18:0, *cis*-9-C18:1, *trans*-11-C18:1, C18:2*n*6, C18:3*n*3], transfer efficiency for C18:2*n*6 and C18:3*n*3 from feed to milk, and diet characteristics [technological form of the fat source, main forage type, forage to concentrate ratio (F/C ratio), and dietary contents of NDF, total FA, UFA, *cis*-9-C18:1, C18:2*n*6, and C18:3*n*3 (DM basis)]. Results of the PCA were represented graphically in two-dimensional plots, showing relationships among these variables. An example of the plot for the soybean and sunflower supplemented diets for principal component 1 versus 2 is presented in Figure 1. With the results of the PCA a selection of independent variables that showed negative or positive relationships with the dependent variables was made and with these variables multiple regression models were fitted.

The statistical methods used to adjust the data for the random effect of experiment and unequal variance among experiments have been described by St-Pierre (2001). Dependent variables included milk FA profile (SFA, UFA, C18:0, *cis*-9-C18:1, *trans*-11-C18:1, C18:2*n*6, C18:3*n*3) and transfer efficiencies for C18:2*n*6 and C18:3*n*3 from feed to milk. Independent continuous variables included the dietary contents of NDF, total FA, UFA, *cis*-9-C18:1, C18:2*n*6, and C18:3*n*3. Independent class variables included fat source, technological form, and forage type. Multiple regression models were fitted using PROC MIXED (SAS Inst. Inc., Cary, NC) with inclusion of both class variables and continuous variables within a mixed model analysis as described by Firkins et al. (2001) and St-Pierre (2001). Variables were included in the models when $P < 0.10$. In the first models, fat source was used as fixed-effect class variable, while the dietary FA contents were used as the fixed-effect continuous variables, and experiment was the random-effect variable. The regression models were weighted for the unequal variance among studies using the milk C18:0 SEM reported in the experiments. In the second analysis, within fat source classes, technological form and main forage type were used as the fixed-effect class variables in the model. Non-significant ($P > 0.10$) main effects remained in the model when they were contained in an interaction effect.

Table 1. Mean, standard deviation and number of treatments for animal characteristics, diet characteristics, and performance for the total dataset (50 studies with 151 treatment means)

Parameter	Fat source														
	Unsupplemented			Canola			Soybean+Sunflower			Linseed			Fish		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Animal characteristics															
Cows, #	9	4.2	43	8	3.2	20	9	6.6	46	9	5.1	28	9	4.1	14
DIM, d	109	45.6	43	114	60.2	20	97	52.0	46	127	62.1	28	115	59.3	14
BW, kg	627	43.2	28	624	48.6	9	628	70.1	15	596	46.1	17	667	68.6	4
Diet characteristics, g/kg DM															
Forage %	51.7	11.40	43	57.9	10.60	20	49.8	4.11	46	53.6	7.35	28	46.1	6.87	14
CP	167	18.3	43	162	21.2	20	173	12.6	44	169	12.8	28	173	10.7	14
NDF	336	59.9	43	352	75.2	20	320	37.3	42	377	44.7	28	311	33.8	14
Total fatty acids	27.3	7.3	43	44.6	12.2	20	49.5	9.3	46	54.1	10.9	28	41.1	10.1	14
cis-9-C18:1	5.5	2.40	43	18.1	7.87	20	10.1	2.91	46	10.2	3.14	28	7.6	2.85	14
cis-9,*cis*-12-C18:2	10.8	4.12	43	11.3	3.94	20	22.3	7.40	46	12.4	3.44	28	11.8	3.68	14
cis-9,*cis*-12,*cis*-15-C18:3	2.5	1.80	43	4.9	1.98	20	3.5	1.63	46	21.1	9.11	28	2.6	0.95	14
Unsaturated fatty acids	18.8	5.96	43	34.9	11.44	20	36.1	9.92	46	44.3	11.35	28	24.2	6.45	14
Performance															
DMI, kg/d	22.7	3.14	43	21.9	3.41	20	22.0	3.46	46	19.1	2.88	28	22.7	3.96	14
Milk yield, kg/d	31.3	5.37	43	32.2	6.70	20	33.1	5.87	46	27.0	6.89	28	30.9	4.72	14
Protein %	3.25	0.26	43	3.14	0.34	20	3.07	0.29	46	3.17	0.21	28	3.17	0.17	14
Fat %	3.78	0.40	43	3.41	0.40	20	3.25	0.38	46	3.76	0.57	28	2.89	0.54	14
Lactose %	4.79	0.14	35	4.87	0.09	13	4.75	0.21	37	4.69	0.20	24	4.86	0.11	10

Table 2. Mean, standard deviation and number of treatments for milk fatty acid (FA) profile and transfer efficiency for *cis*-9,*cis*-12-C18:2 and *cis*-9,*cis*-12,*cis*-15-C18:3 for the total dataset (50 studies with 151 treatment means)

Parameter	Fat source														
	Unsupplemented			Canola			Soybean+Sunflower			Linseed			Fish		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Milk fatty acid profile, g/100 g FA															
C4:0	3.51	1.04	23	4.12	1.41	13	3.34	0.94	32	2.93	1.05	16	2.56	0.64	12
C6:0	2.46	0.78	28	2.57	1.05	16	1.90	0.59	35	1.97	0.89	22	1.62	0.46	14
C8:0	1.42	0.60	34	1.57	1.03	16	1.09	0.31	38	1.15	0.39	24	1.06	0.38	14
C10:0	3.44	0.83	36	2.80	0.98	16	2.44	0.69	45	2.52	0.81	28	2.43	0.64	14
C12:0	4.13	0.77	38	3.21	0.95	20	2.76	0.71	45	2.85	0.72	28	3.00	0.69	14
C13:0	0.14	0.03	12				0.08	0.03	12	0.11	0.03	13	0.13	0.04	5
C14:0	12.65	1.85	43	11.42	2.67	20	9.77	1.91	46	10.10	1.78	28	10.61	1.31	14
cis-9-C14:1	1.29	0.36	26	1.15	0.57	5	0.87	0.36	40	0.90	0.20	24	1.32	0.46	11
C15:0	1.30	0.23	33	1.09	0.18	5	0.85	0.25	37	0.99	0.22	19	1.09	0.13	8
C16:0	32.43	3.08	43	24.23	3.47	20	24.50	4.35	46	24.93	3.64	28	28.50	2.65	14
cis-9-C16:1	1.87	0.53	36	1.55	0.22	10	1.53	0.78	46	1.40	0.49	28	2.41	0.90	14
C17:0	0.68	0.16	31	0.68	0.06	4	0.47	0.10	29	0.58	0.08	19	0.60	0.07	6
C18:0	9.81	2.21	43	13.66	2.15	20	11.85	2.95	46	14.56	3.93	28	6.55	3.08	14
cis-9-C18:1	20.08	3.87	43	27.24	4.68	20	23.48	4.11	46	26.09	4.15	28	15.64	5.56	14
cis-11-C18:1	0.58	0.20	17				0.69	0.15	17	0.48	0.19	13	0.98	0.46	9
cis-12-C18:1	0.31	0.08	6				0.79	0.45	3	0.50	0.34	2			
cis-13-C18:1	0.08	0.02	6				0.12	0.02	3	0.19	0.06	2			
cis-15-C18:1	0.11	0.01	6				0.17	0.05	3	0.83	0.41	4			
trans-6+7+8-C18:1	0.21	0.06	14				0.52	0.23	15	0.47	0.23	6	0.40	0.21	9
trans-9-C18:1	0.21	0.05	15				0.50	0.12	15	0.45	0.12	6	0.49	0.31	9
trans-10-C18:1	0.79	0.78	11	2.23	2.39	5	2.36	2.28	11	0.94	0.38	6	3.22	2.57	4
trans-11-C18:1	1.68	1.02	40	2.38	0.94	20	4.47	2.55	30	2.86	1.52	26	4.18	2.33	14
trans-12-C18:1	0.38	0.04	6				0.75	0.31	3	0.81	0.13	4			
trans-13+14-C18:1	0.34	0.05	2				1.17	0.69	3	2.36	0.56	4			
trans-16-C18:1	0.29	0.02	4				0.42	0.02	2	0.83	0.12	3			
cis-9,*cis*-12-C18:2	2.69	0.83	43	2.39	0.66	20	3.57	0.86	46	2.14	0.56	28	2.67	0.86	14
cis-9,*trans*-11-C18:2	0.54	0.18	26	0.97	0.38	10	1.15	0.46	31	1.16	0.63	20	1.44	0.54	14

Table 2. Continued.

Parameter	Fat source														
	Unsupplemented			Canola			Soybean+Sunflower			Linseed			Fish		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
trans-10,*cis*-12-C18:2	0.03	0.04	11				0.05	0.04	20	0.05	0.03	6	0.07	0.06	7
cis-9,*cis*-12,*cis*-15-C18:3	0.46	0.19	42	0.54	0.20	20	0.58	0.21	44	1.03	0.30	28	0.46	0.16	14
C20:0	0.17	0.12	12				0.27	0.21	11	0.24	0.14	9	0.24	0.11	5
C20:1	0.12	0.09	14				0.15	0.06	14	0.14	0.02	8	0.34	0.16	9
C20:4*n*6	0.21	0.09	12	0.10	0.00	4	0.20	0.21	15	0.17	0.15	13	0.20	0.08	7
C20:5*n*3	0.07	0.04	14				0.09	0.05	20	0.12	0.11	14	0.23	0.12	13
C22:5	0.08	0.03	12				0.11	0.04	18	0.09	0.05	7	0.23	0.13	10
C22:6*n*3	0.04	0.04	13				0.16	0.34	20	0.10	0.06	8	0.14	0.08	13
Other	3.77	3.26	15	7.72	0.36	4	6.23	3.56	33	5.14	5.66	4	10.21	5.18	8
Summary															
SFA[1]	67.61	5.27	43	61.17	6.77	20	56.94	4.63	46	60.34	5.17	28	56.96	4.76	14
MUFA[2]	24.91	3.83	43	32.06	5.88	20	30.30	5.03	46	32.52	3.62	28	27.65	4.18	14
PUFA[3]	3.81	1.05	43	3.44	0.54	20	5.38	1.36	46	4.63	1.68	28	5.70	1.28	14
UFA[4]	28.72	4.30	43	35.50	6.10	20	35.68	5.14	46	37.15	4.47	28	33.36	5.04	14
Transfer efficiency, %															
cis-9,*cis*-12-C18:2	12.89	4.12	43	11.25	4.99	20	8.03	3.08	46	8.80	2.63	28	8.62	3.00	14
cis-9,*cis*-12,*cis*-15-C18:3	13.43	8.09	40	6.68	4.93	20	8.10	2.89	44	3.09	2.14	28	7.39	3.64	14

[1]Saturated fatty acids: ∑ (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0).

[2]Mono unsaturated fatty acids: ∑ (*cis*-9-C14:1, *cis*-9-C16:1, *cis*-9-C18:1, *cis*-11-C18:1, *cis*-12-C18:1, *cis*-13-C18:1, *cis*-15-C18:1, *trans*-6+7+8-C18:1, *trans*-9-C18:1, *trans*-10-C18:1, *trans*-11-C18:1, *trans*-12-C18:1, *trans*-13+14-C18:1, *trans*-16-C18:1, C20:1).

[3]Poly unsaturated fatty acids: ∑ (*cis*-9,*cis*-12-C18:2, *cis*-9,*trans*-11-C18:2, *trans*-10,*cis*-12-C18:2, *cis*-9,*cis*-12,*cis*-15-C18:3, C20:4*n*6, C20:5*n*3, C22:5, C22:6*n*3).

[4]Unsaturated fatty acids: ∑ (MUFA, PUFA).

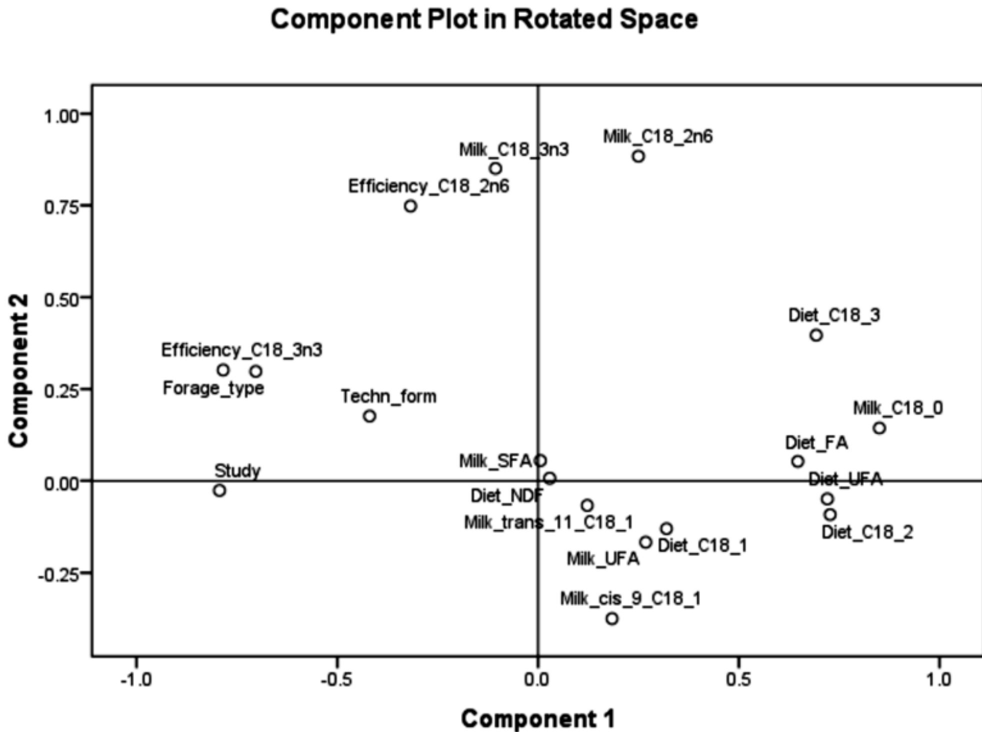

Figure 1. Principal component analysis describing relationships among dietary variables and milk FA profile. The plot is based on the first two principal components (component 1: 44.0%, component 2: 16.3%).

Assessment of the best fit model was conducted by calculation of the root mean square prediction error (RMSPE; Bibby and Toutenburg, 1977). Expressed as a percentage of the observed mean, the RMSPE was used as a measure for accuracy of prediction. The MSPE was decomposed into error due to overall bias of prediction, error due to deviation of the prediction line from unity, and error due to disturbance (random error; Bibby and Toutenburg, 1977). All developed models showed the random error to be the most important source of error and therefore MSPE decomposition was not presented in the tables. The concordance correlation coefficient (CCC) was calculated to evaluate the precision and accuracy of predicted values (Lin, 1989). The best fit model was chosen based on the lowest RMSPE, highest CCC value, and biologically logical intercepts and coefficients. The presented models per fat source contain seed as technological form or maize silage as main forage type when technological form or main forage type, respectively, were used as class variables in the regression models. Regression intercepts and slopes were adjusted for other technological forms or forage types, respectively, when the effects of these class variables (main effect: adjustment of intercept; interaction: adjustment of slope) were significant ($P < 0.10$). Least squares means for different fat sources, technological form including added fish oil, and main forage type in the diet were calculated from the best fit models and were adjusted for the random experiment effect and the means of all continuous variables in the final models. Pairwise differences were tested using the Tukey adjustment.

Results and Discussion

Meta-analysis approach

The database is summarized per fat source in Tables 1 and 2. The animal and diet characteristics and performance parameters for the dataset are presented in Table 1, whereas the milk FA profile including transfer efficiencies for C18:2*n*6 and C18:3*n*3 is presented in Table 2. Although a large number of studies evaluating the response of milk FA to several fat sources were published, it was difficult to obtain a large and solid database with results for different fat sources, technological forms, and diet compositions. To conduct a meta-analysis with these factors, a database containing diet characteristics as well as FA intake and specified milk FA profiles is required. Glasser et al. (2008) had to pool several forms of fat supplementation to obtain sufficient data to quantify relationships on milk FA profile. In the current meta-analysis, the number of publications that met the selection criteria was limited and therefore it was also necessary to pool technological form to the four classes used (oil, seed, protected, and added fish oil) and main forage type to the six classes used (alfalfa silage, barley silage, maize silage, grass silage, maize silage combined with haylage, and haylage). Using the multiple regression technique, taking into account the random effect of experiment and unequal variances among experiments as applied previously for other research questions (Firkins et al., 2001), it was possible to obtain models that upon application result in least squares means for technological form or main forage type within each fat source.

Effect of different fat sources on milk FA profile

In Table 3 the final models for the total dataset are presented with the presented models for diets not supplemented with a fat source. The effect of fat source was significant for the selected milk FA proportions and efficiencies except for the proportion of UFA in milk fat. The intercepts in the final models therefore need to be adjusted for the different fat sources. In addition, the regression slope should also be adjusted for the different fat sources for the proportion of C18:3*n*3 in milk fat and the transfer efficiencies for C18:2*n*6 and C18:3*n*3. The proportion of UFA in milk fat showed a quadratic response to increasing dietary UFA content, which was not affected by fat source. The relationship between the observed and predicted UFA proportion in milk fat and the residuals (observed – predicted UFA) are presented in Figure 2. Least squares means for milk FA are presented in Table 4 and show the differences between fat sources for SFA, C18:0, *cis*-9-C18:1, *trans*-11-C18:1, C18:2*n*6, and C18:3*n*3 proportions in milk fat.

The response in milk FA profile to lipid supplements integrates both rumen metabolism of substrates and post-absorptive metabolism of nutrients within the cow. In the current meta-analysis, higher least squares means of C18:0 were reported for diets supplemented with a source of canola, soybean, sunflower, and linseed. An increased proportion of C18:0 in milk fat can originate either from an increased dietary C18:0 intake, from increased body fat mobilization, or from the dietary supplementation of *cis*-9-C18:1, C18:2*n*6, or C18:3*n*3, resulting in an increased rumen outflow of C18:0 due to complete biohydrogenation to C18:0 (Chilliard et al., 2007). Diets supplemented with fish oil showed the lowest C18:0 and highest *trans*-11-C18:1 proportions in milk fat in the

Table 3. Regression models to predict proportions of milk saturated fatty acids (SFA), unsaturated fatty acids (UFA), C18:0, *cis*-9-C18:1, *trans*-11-C18:1, *cis*-9,*cis*-12-C18:2 (C18:2*n6*), and *cis*-9,*cis*-12,*cis*-15-C18:3 (C18:3*n3*; all in g/100 g) and transfer efficiencies from intake to milk for C18:2*n6* and C18:3*n3* (%) from dietary fatty acid (FA) contents (%) and responses to fat source standardized to diets not supplemented with a fat source[1]

Parameter	Intercept	SE	P-value fat source	Variable	Coefficient	SE	P-value coefficient	RMSPE %[2]	CCC
SFA	74.0	1.2	<0.001	FA	-0.249	0.032	<0.001	7.46	0.712
UFA	17.8	1.4	ns	UFA	0.720	0.088	<0.001	8.74	0.859
				UFA*UFA	-0.006	0.001	<0.001		
C18:0	8.77	0.68	<0.001	FA	0.046	0.021	0.032	19.99	0.694
cis-9-C18:1	18.21	0.85	<0.001	*cis*-9-C18:1	0.303	0.080	<0.001	15.25	0.720
trans-11-C18:1	0.15	0.44	<0.001	UFA	0.078	0.017	<0.001	27.14	0.893
C18:2*n6*	2.07	0.14	<0.001	C18:2*n6*	0.042	0.015	0.005	20.41	0.758
C18:3*n3*	0.37	0.06	<0.001	C18:3*n3*	0.025	0.018	0.074[3]	25.85	0.827
Efficiency C18:2*n6*	25.7	1.5	<0.001	C18:2*n6*	-1.578	0.213	<0.001[4]	19.57	0.873
				C18:2*n6** C18:2*n6*	0.028	0.008	<0.001		
Efficiency C18:3*n3*	21.4	1.2	<0.001	C18:3*n3*	-3.494	0.441	<0.001[5]	38.21	0.846

[1]Data are adjusted for the random effect of experiment and weighted for unequal variance. The equations are standardized for diets not supplemented with a fat source. Both intercept and coefficient would be adjusted for fat source. Class and continuous variables are included when $P < 0.10$.

[2]RMSPE %: root mean square prediction error as a percentage of the observed mean, CCC: concordance correlation coefficient.

[3]The interaction between fat source * C18:3*n3* was $P < 0.01$; estimates for canola: intercept = 0.43, slope = 0.029; estimates for soybean+sunflower: intercept = 0.40, slope = 0.056; estimates for linseed: intercept = 1.15, slope = -0.0033; estimates for fish: intercept = 0.43, slope = 0.0010.

[4]The interaction between fat source * C18:2*n6* was $P = 0.01$; estimates for canola: intercept = 26.4, slope = -1.829; estimates for soybean+sunflower: intercept = 29.0, slope = -1.617; estimates for linseed: intercept = 17.9, slope = -1.070; estimates for fish: intercept = 21.4, slope = -1.303.

[5]The interaction between fat source * C18:3*n3* was $P < 0.001$; estimates for canola: intercept = 10.2, slope = -1.294; estimates for soybean+sunflower: intercept = 13.0, slope = -1.009; estimates for linseed: intercept = 8.9, slope = -0.261; estimates for fish: intercept = 16.4, slope = -2.301.

Table 4. Least squares means of proportions of milk saturated fatty acids (SFA), unsaturated fatty acids (UFA), C18:0, *cis*-9-C18:1, *trans*-11-C18:1, *cis*-9,*cis*-12-C18:2 (C18:2*n*6), and *cis*-9,*cis*-12,*cis*-15-C18:3 (C18:3*n*3; all in g/100 g) for Holstein cows fed unsupplemented diets or diet supplemented with different fat sources[1]

Fat source		SFA		UFA		C18:0		*cis*-9-C18:1		*trans*-11-C18:1		C18:2*n*6		C18:3*n*3	
	n[2]	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Unsupplemented	43	63.27[a]	0.89	ns[3]	ns	10.74[bc]	0.54	21.12[b]	0.71	2.59[b]	0.31	2.69[bc]	0.13	0.55[b]	0.09
Canola	20	60.30[bc]	0.88	ns	ns	13.08[a]	0.56	23.71[a]	0.92	2.32[b]	0.32	2.62[bc]	0.15	0.64[b]	0.07
Soybean + sunflower	46	59.00[c]	0.82	ns	ns	12.11[ab]	0.51	24.07[a]	0.69	3.87[a]	0.31	3.45[a]	0.15	0.79[b]	0.07
Linseed	28	61.99[ab]	0.96	ns	ns	13.38[a]	0.59	25.40[a]	0.79	1.71[bc]	0.39	2.42[c]	0.15	1.13[a]	0.07
Fish	14	60.81[bc]	0.95	ns	ns	9.76[c]	0.57	20.22[b]	0.84	3.89[a]	0.37	2.78[b]	0.15	0.44[b]	0.22

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

[1]All least squares means are adjusted for the random effect of experiment and for the mean of the continuous variables in the final models (see Table 2).

[2]Number of treatment means.

[3]Effect of fat source was not significant in the model ($P > 0.10$).

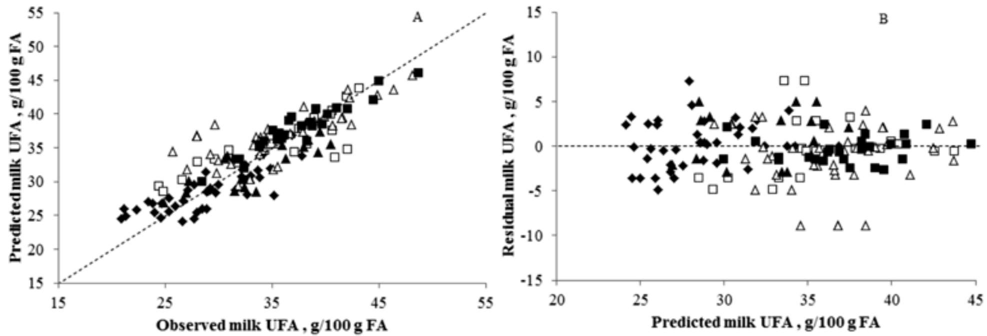

Figure 2. Observed and predicted milk UFA proportion (A), and residuals (i.e. observed – predicted; B) for milk UFA proportion, adjusted for the random effect of experiment and weighted for unequal variance. Predicted milk UFA proportion (g/100 g FA): 17.8 + 0.720 x UFA - 0.006 x UFA^2 (RMSPE: 8.74 % of observed mean, CCC: 0.859). Data are for unsupplemented (◆), canola (□), soybean + sunflower (Δ), linseed (■), and fish (▲) sources.

current meta-analysis. When fish oil or marine algae were included in the diet, a notable reduction in the conversion of *trans*-11-C18:1 to C18:0 in the rumen is shown in vitro (Boeckaert et al., 2007; Vlaeminck et al., 2008; Sterk et al., 2010) or in vivo (Boeckaert et al., 2008b), and milk fat proportions of C18:0 and *trans*-11-C18:1 markedly decreased and increased, respectively (Boeckaert et al. 2008a). Several studies suggested that docosahexaenoic acid (C22:6n3; DHA) was responsible for the inhibitory effects on ruminal FA biohydrogenation (AbuGhazaleh and Jenkins, 2004; Boeckaert et al., 2007), which were modulated through changes in the rumen microbial population (Boeckaert et al., 2008b).

In the current meta-analysis higher proportions of *cis*-9-C18:1 were found after supplementation of canola, soybean or sunflower, and linseed. This was in agreement with the origin of *cis*-9-C18:1 in milk fat coming either directly from an increased intake of *cis*-9-C18:1 that escapes rumen biohydrogenation or from complete rumen biohydrogenation to C18:0 followed by mammary desaturation to *cis*-9-C18:1 (Chilliard et al., 2007). Due to the decreased rumen outflow of C18:0 in diets containing fish oil (Shingfield et al., 2003) or marine algae (Boeckaert et al., 2008b), the substrate for mammary desaturation to *cis*-9-C18:1 decreased, which in the current analysis resulted in a lower proportion of *cis*-9-C18:1 in milk fat. The proportion of C18:2*n*6 generally varies between 2.0 and 3.0 g/100 g FA (Chilliard et al., 2007) and was significantly higher when diets were supplemented with a source of soybean or sunflower containing high proportions of C18:2*n*6 compared with unsupplemented diets or diets supplemented with a source of canola, linseed, or fish oil. The proportion of C18:3*n*3 in milk fat for unsupplemented diets is generally 0.5 g/100 g FA (Heck et al., 2009) and can increase to around 1.2 g/100 g FA when unprotected linseed is supplemented to the diet (Glasser et al., 2008). In the current meta-analysis, the unsupplemented diet showed a least squares mean of 0.55 g C18:3*n*3/100 g FA, whereas the linseed supplemented diets showed a least squares mean of 1.13 g C18:3*n*3/100 g FA. Least squares means for transfer efficiencies for C18:2*n*6 and C18:3*n*3 could

not be determined, because they were calculated using the means of the continuous variables in the model according to Firkins et al. (2001). This resulted in negative transfer efficiencies for C18:2*n*6 and C18:3*n*3 due to the difference in dietary C18:2*n*6 and C18:3*n*3 contents among the unsupplemented diets and diets supplemented with the fat sources. Using the means of dietary C18:2*n*6 and C18:3*n*3 for each fat source from Table 1, calculated transfer efficiencies were highest for unsupplemented diets. Diets supplemented with soybean or sunflower resulted in the lowest transfer efficiency for C18:2*n*6, because these diets had the highest dietary C18:2*n*6 content. Diets supplemented with linseed had the highest dietary C18:3*n*3 content and therefore the lowest transfer efficiency for C18:3*n*3.

Effect of nutrients on changes in milk FA profile

In general, the proportion of forage in the diet is an important factor regulating the extent of ruminal biohydrogenation (Dewhurst et al., 2006). In addition, incomplete biohydrogenation associated with the accumulation of several biohydrogenation intermediates, arises when diets contain high amounts of readily available UFA, low amounts of fibre, or high levels of starch, causing a low ruminal pH (Palmquist et al., 2005). In the current meta-analysis, the variation in F/C ratio was small, with only a few treatments (n = 10) with a proportion of concentrates higher than 60%. Chilliard et al. (2007) concluded that the effect of increasing the proportion of concentrates in the diet is dependent on the range of increase, with a strong effect when the proportion of concentrates in the diet is increased above 60%. In the current meta-analysis dietary NDF content (339 ± 56 g/kg DM) rather than dietary forage proportion was used as the independent variable representing the availability of fibre in the diets.

The extent of the changes in milk FA profile following changes in dietary nutrient composition may depend on the basal forage type (Dewhurst et al., 2006). Zebeli et al. (2008) conducted a meta-analysis to assess the adequacy of dietary fibre in high yielding dairy cows. The dietary content of physically effective fibre required to stabilize rumen pH and maintain milk fat content depended on various other factors, including degradability of non-fibre carbohydrates (notably starch). Since rumen pH is an important factor in biohydrogenation processes in the rumen, such findings indicate that the effect of level of fibre may depend on the type of forage and the levels of easily degradable carbohydrates in the forage. Final models for unsupplemented diets are shown in Table 5. Regression intercepts and slopes are adjusted for main forage type to calculate the least squares means for milk FA and transfer efficiencies per main forage type (Table 6).

The UFA proportion in milk fat was affected by the UFA and NDF content in the diet, whereas the effect of the NDF content depends on the main forage type in the diet. When the diet contained haylage (more pronounced) or barley silage there is a negative effect of dietary NDF content on the UFA proportion in milk fat, whereas when the diet contained maize silage, maize silage in combination with haylage, or grass silage as the main forage type the effect of NDF content on UFA proportion in milk fat is positive. However, no significant differences in the least squares means between the different main forage types were detected. The difference in effect of fibre on milk UFA proportion when the diet contains different forages might be related to the presence of C18:1 isomers in the UFA proportion. A lower fibre content is related to more incomplete biohydrogenation (Palmquist et al., 2005), which explains a higher UFA proportion in milk fat. However, the positive effect of NDF

Table 5. Regression models to predict proportions of milk saturated fatty acids (SFA), unsaturated fatty acids (UFA), C18:0, *cis*-9-C18:1, *trans*-11-C18:1, *cis*-9,*cis*-12-C18:2 (C18:2*n*6), and *cis*-9,*cis*-12,*cis*-15-C18:3 (C18:3*n*3; all in g/100 g) and transfer efficiencies from intake to milk for C18:2*n*6 and C18:3*n*3 (%) from diet characteristics and responses to forage type standardized to diets containing maize silage as main forage type for diets not supplemented with a fat source[1]

Parameter	Intercept	SE	P-value forage type	Variable	Coefficient	SE	P-value coefficient	RMSPE %[2]	CCC
SFA	78.9	2.5	0.020	FA	-0.385	0.082	<0.001	3.60	0.856
UFA	17.2	5.4	0.051	UFA	0.541	0.076	<0.001	4.95	0.928
				NDF	0.0008	0.015	0.341[3]		
C18:0	-7.55	6.12	0.033	FA	0.457	0.196	0.026	11.89	0.809
				NDF	0.048	0.020	0.021		
				FA*NDF	-0.0012	0.0006	0.069		
cis-9-C18:1	8.11	3.31	0.004	*cis*-9-C18:1	1.036	0.115	<0.001	3.55	0.983
				NDF	0.017	0.009	0.513[4]		
trans-11-C18:1	-0.20	1.31	0.097	UFA	0.095	0.019	<0.001	11.67	0.980
				NDF	0.0005	0.003	0.169[5]		
C18:2*n*6	-4.41	2.29	<0.001	C18:2*n*6	0.657	0.183	0.001[6a]	9.53	0.945
				NDF	0.015	0.006	0.782[6b]		
				C18:2*n*6*NDF	-0.0014	0.0005	0.008		
C18:3*n*3	0.40	0.10	0.383	C18:3*n*3	0.032	0.026	0.076[7]	27.04	0.710
Efficiency C18:2*n*6	21.6	1.5	ns	C18:2*n*6	-0.809	0.131	<0.001	19.08	0.762
Efficiency C18:3*n*3	-0.6	7.4	0.001	C18:3*n*3	-2.861	0.817	<0.001[8]	23.33	0.919
				NDF	0.057	0.021	0.012		

[1]Data are adjusted for the random effect of experiment and weighted for unequal variance. The equations are standardized for diets containing maize silage as the main forage type. Both intercept and coefficient would be adjusted for different forage types. Class and continuous variables are included when P < 0.10.
[2]RMSPE %: root mean square prediction error as a percentage of the observed mean, CCC: concordance correlation coefficient.
[3]The interaction between forage type * NDF was P = 0.06; estimates for barley silage: intercept = 25.7, slope = -0.0027; estimates for maize silage/haylage: intercept = 13.6, slope = 0.018; estimates for grass silage: intercept = 3.7, slope = 0.039; estimates for haylage: intercept = 56.4, slope = -0.121.
[4]The interaction between forage type * NDF was P < 0.01; estimates for barley silage: intercept = 19.42, slope = -0.010; estimates for maize silage/haylage: intercept = 15.31, slope = -0.009; estimates for grass silage: intercept = 0.75, slope = 0.040; estimates for haylage: intercept = 41.70, slope = -0.085.
[5]The interaction between forage type * NDF was P = 0.06; estimates for barley silage: intercept = 0.29, slope = -0.0014; estimates for maize silage/haylage: intercept = 4.50, slope = -0.016; estimates for grass silage: intercept = 0.33, slope = -0.0019; estimates for haylage: intercept = 0.29, slope = -0.0014.
[6a]The interaction between forage type * C18:2*n*6 was P < 0.001; estimates for barley silage: intercept = 13.78, slope = -0.128; estimates for maize silage/haylage: intercept = 3.35, slope = 0.388; estimates for grass silage: intercept = -4.50, slope = 0.704; estimates for haylage: intercept = 4.30, slope = 0.834.
[6b]The interaction between forage type * NDF was P < 0.01; estimates for barley silage: intercept = 13.78, slope = -0.018; estimates for maize silage/haylage: intercept = 3.35, slope = -0.002; estimates for grass silage: intercept = -4.50, slope = -0.013; estimates for haylage: intercept = 4.30, slope = -0.018.
[7]The interaction between forage type * C18:3*n*3 was P < 0.10; estimates for barley silage: intercept = 0.73, slope = -0.194; estimates for maize silage/haylage: intercept = 0.69, slope = -0.113; estimates for grass silage: intercept = 0.51, slope = -0.007; estimates for haylage: intercept = 0.68, slope = -0.033.
[8]The interaction between forage type * C18:3*n*3 was P < 0.01; estimates for barley silage: intercept = 13.1, slope = -11.033; estimates for maize silage/haylage: intercept = 14.6, slope = -8.602; estimates for grass silage: intercept = -6.0, slope = -1.849; estimates for haylage: intercept = 21.6, slope = -11.033.

Table 6. Least squares means of proportions of milk saturated fatty acids (SFA), unsaturated fatty acids (UFA), C18:0, *cis*-9-C18:1, *trans*-11-C18:1, *cis*-9,*cis*-12-C18:2 (C18:2*n6*), and *cis*-9,*cis*-12,*cis*-15-C18:3 (C18:3*n3*; all in g/100 g) and transfer efficiencies from intake to milk for C18:2*n6* and C18:3*n3* (%) for Holstein cows fed different fat sources with different technological forms, addition of fish oil, or main forage type in the diet[1]

Fat source		SFA		UFA		C18:0		*cis*-9-C18:1		*trans*-11-C18:1		C18:2*n6*		C18:3*n3*		Eff. C18:2*n6*		Eff. C18:3*n3*	
	n[2]	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Unsupplemented																			
Barley silage	3	64.69[ab]	2,34	35.06	3.02	11.32[ab]	1.09	28.25[a]	1.90	ne[3]	ne	ne	ne	0.23	0.12	ns[4]	ns	ne	ne
Maize silage	16	68.20[ab]	1.28	27.73	0.89	10.34[ab]	0.64	19.59[b]	0.53	1.78[a]	0.19	2.71[a]	0.16	0.48	0.06	ns	ns	11.1	1.7
Maize/haylage	11	64.79[b]	1.18	30.02	1.12	9.30[b]	0.61	17.75[b]	0.64	0.88[b]	0.25	1.93[b]	0.22	0.40	0.06	ns	ns	11.8	2.6
Grass silage	5	67.42[ab]	2.36	27.00	1.58	9.13[ab]	0.97	19.81[b]	1.05	1.50[ab]	0.42	2.54[ab]	0.27	0.49	0.13	ns	ns	8.2	4.0
Haylage	4	72.06[a]	1.87	26.15	1.57	12.58[a]	0.87	18.60[b]	0.88	1.61[ab]	0.37	2.54[ab]	0.65	0.59	0.10	ns	ns	12.7	2.8
Canola																			
Oil	3	ns	ns	ns	ns	9.06	3.04	ns	ns	ns	ns	1.81[ab]	2.00	ns	ns	4.6	8.5	ns	ns
Seed	12	ns	ns	ns	ns	15.28	1.48	ns	ns	ns	ns	1.97[b]	0.22	ns	ns	10.4	0.6	ns	ns
Protected	3	ns	ns	ns	ns	13.82	1.59	ns	ns	ns	ns	2.66[a]	0.22	ns	ns	11.8	0.9	ns	ns
Alfalfa silage	2	69.34	4.76	27.58	4.79	14.15	2.72	ns	ns	ns	ns	2.36[b]	0.45	0.68[ab]	0.14	11.1[a]	1.2	ns	ns
Barley silage	4	58.21	3.47	41.47	3.58	ne	ne	ns	ns	ns	ns	5.30[a]	0.60	0.25[b]	0.10	15.7[ab]	3.7	ns	ns
Maize silage	10	60.37	2.44	35.51	2.49	14.05	1.38	ns	ns	ns	ns	2.11[b]	0.23	0.59[ab]	0.07	12.2[a]	0.6	ns	ns
Grass silage	2	65.71	3.20	30.01	3.98	17.14	5.12	ns	ns	ns	ns	1.67[b]	0.31	0.80[a]	0.11	4.5[b]	1.5	ns	ns
Soybean + sunflower																			
Oil	6	ns	ns	28.39	5.48	13.90[a]	1.20	22.34[ab]	1.51	8.25[ab]	2.14	3.28[ab]	0.42	0.42[ab]	0.08	8.4[ab]	1.5	5.8[bc]	1.0
Seed	16	ns	ns	35.40	1.29	13.56[a]	0.40	24.39[a]	0.69	1.73[b]	0.76	4.16[a]	0.23	0.72[a]	0.05	9.6[a]	0.7	9.0[a]	0.5
Protected	4	ns	ns	44.46	3.41	11.79[ab]	0.98	23.27[ab]	1.56	6.52[a]	0.98	3.08[ab]	0.47	0.33[b]	0.09	3.3[b]	1.9	3.8[c]	1.9
Added fishoil	11	ns	ns	37.77	2.22	9.95[b]	0.47	21.65[b]	0.84	4.54[a]	0.75	3.46[b]	0.26	0.66[a]	0.05	7.3[b]	0.8	8.4[ab]	0.6
Maize silage	12	53.93[b]	1.80	ns	ns	12.40[ab]	0.70	25.01[a]	0.76	6.36[a]	0.67	3.35[b]	0.25	0.45[b]	0.06	7.1	0.9	5.6[b]	0.7
Maize/haylage	13	54.16[ab]	1.89	ns	ns	9.46[b]	0.60	18.40[b]	1.09	3.57[ab]	0.70	3.46[b]	0.20	0.76[a]	0.06	7.1	0.9	9.4[a]	0.6
Grass silage	7	61.87[ab]	2.49	ns	ns	15.41[a]	0.97	16.98[ab]	3.24	1.91[b]	1.33	4.17[ab]	0.37	0.57[ab]	0.10	10.5	1.4	7.5[ab]	1.0
Haylage	5	62.89[a]	2.47	ns	ns	13.03[a]	1.06	25.35[a]	0.94	6.01[ab]	1.11	5.21[a]	0.38	0.64[a]	0.09	10.8	1.4	7.9[ab]	0.9

Table 6. Continued.

Fat source		SFA		UFA		C18:0		*cis*-9-C18:1		*trans*-11-C18:1		C18:2*n6*		C18:3*n3*		Eff. C18:2*n6*		Eff. C18:3*n3*	
	n[2]	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Linseed																			
Oil	3	48.53[b]	3.89	ns	ns	11.78	2.22	18.24[c]	2.30	7.73[a]	0.77	ns	ns	0.81[ab]	0.41	ns	ns	ns	ns
Seed	18	60.66[a]	0.61	ns	ns	15.95	0.72	26.81[ab]	0.87	2.45[b]	0.20	ns	ns	1.01[b]	0.04	ns	ns	ns	ns
Protected	2	62.14[ab]	4.29	ns	ns	17.31	1.92	27.01[b]	1.62	ne	ne	ns	ns	1.74[a]	0.18	ns	ns	ns	ns
Added fish oil	3	57.71[ab]	1.23	ns	ns	14.60	1.75	37.36[a]	4.30	2.67[b]	0.51	ns	ns	1.40[ab]	0.23	ns	ns	ns	ns
Alfalfa silage	2	57.81[b]	1.39	ns	ns	11.27	2.08	27.20[ab]	2.10	2.99[ab]	0.89	ns	ns	1.09	0.17	ns	ns	2.4	0.6
Maize silage	8	59.95[b]	0.74	ns	ns	15.37	1.14	24.84[ab]	1.35	2.38[b]	0.56	ns	ns	0.99	0.07	ns	ns	2.2	0.2
Grass silage	12	63.77[a]	1.15	ns	ns	15.31	0.93	27.62[a]	1.11	2.16[b]	0.52	ns	ns	1.39	0.12	ns	ns	3.2	0.4
Haylage	4	54.29[b]	2.32	ns	ns	10.11	1.76	20.25[b]	2.00	5.64[a]	0.79	ns	ns	0.91	0.17	ns	ns	2.0	0.6

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

[1]All least squares means are adjusted for the random effect of experiment and for the mean of all continuous variables remaining in the final models (see Tables 5, 7, 8, 9).

[2]Number of treatment means.

[3]Not estimated in the model because of a limited number of treatment means.

[4]Effect of technological form or forage type was not significant in the model ($P > 0.10$).

content on UFA proportion for diets containing maize silage, maize silage combined with haylage, or grass silage remains difficult to explain.

The proportion of C18:0 in milk fat was positively affected by total FA and NDF content in the diet and slightly negative by the interaction between the total FA and NDF content. Forage type significantly affected the C18:0 proportion in milk fat with the highest proportion achieved when the diet contained haylage compared with a combination of maize silage and haylage as the main forage type. However, Palmquist et al. (2005) concluded that complete biohydrogenation to C18:0 is most extensive when animals are fed diets containing high amounts of ensiled forages, which was therefore not confirmed in this meta-analysis. Proportions of *trans*-11-C18:1 and C18:2*n*6 were higher for diets containing maize silage as the main forage type compared with diets containing a combination of maize silage and haylage. The *trans*-11-C18:1 and C18:2*n*6 proportions in milk fat were differently affected by NDF and C18:2*n*6 content when the main forage type in the diet changed. The proportion of C18:3*n*3 in milk fat was also differently affected by dietary C18:3*n*3 content when the main forage type in the diet changed, but no significant differences in the least squares means for the C18:3*n*3 proportion in milk fat could be determined. Kliem et al. (2008) showed increased proportions of many *trans* isomers and C18:2*n*6 and a decreased proportion of C18:3*n*3 in milk fat in diets with increasing maize silage at the expense of grass silage. In contrast to the results of Kliem et al. (2008), the current study showed only numerically increased proportions of *trans*-11-C18:1 and C18:2*n*6 and no differences in the proportion of C18:3*n*3 in milk fat for diets containing maize silage compared with grass silage as the main forage type. In general, cows on hay based diets can have a higher proportion of C18:3*n*3 in milk fat compared with grass silage based diets because of a higher transfer efficiency from diet to milk (Chilliard et al., 2007). In this respect, Boufaïed et al. (2003) showed a higher ruminal bypass of C18:3*n*3 for timothy hay compared to silage. In the current meta-analysis only a numerical increase in the transfer efficiency for C18:3*n*3 and proportion of C18:3*n*3 in milk fat for diets containing haylage as the main forage type compared with silages was found. Transfer efficiency for C18:3*n*3 decreased with increasing dietary C18:3*n*3 content influenced by the main type of forage in the diet and increased with increasing NDF content in the diet. Transfer efficiency for C18:2*n*6 was negatively affected by the dietary C18:2*n*6 content and showed no differences when main forage type differed.

Changes in milk FA profile for diets supplemented with canola

Final models for diets supplemented with canola are shown in Table 7, whereas least squares means are shown in Table 6. Besides effects of the nutrient composition of the diet, the technological form of fat supplementation is known to have an effect on rumen metabolism and milk FA profile. However, rumen protected fats currently provide inconsistent and limited rumen protection responses (Jenkins et al., 2007). Differences in response of milk FA to dietary FA and NDF contents when fat sources are supplied as different technological forms, may help to explain the inconsistent responses between experiments.

The proportion of UFA in milk fat was significantly increased by dietary UFA content, whereas technological form of canola did not affect the milk UFA proportion. Protected canola showed a

numerically higher transfer efficiency for C18:2*n*6 and a significantly higher proportion of C18:2*n*6 in milk fat. The proportion of C18:2*n*6 was increased when dietary C18:2*n*6 content increased, whereas technological form interacted with NDF content, resulting in a positive relationship with NDF for canola seed and negative relationships with NDF for canola oil and protected canola. Two of the protected canola treatments were oleamides and Loor et al. (2002) concluded that oleamides showed a lower extent of biohydrogenation of *cis*-9-C18:1, but in the present meta-analysis none of the independent variables significantly affected *cis*-9-C18:1. The proportion of C18:0 did not differ between the different technological forms. However, the effect of dietary total FA content on C18:0 proportion was influenced by technological form with a stronger negative relationship when canola was supplied as seed or as a protected source. Canola sheaths appear to have a less protective effect than soybean or sunflower sheaths (Chilliard and Ferlay, 2004), which was confirmed by the numerically higher C18:0 content of canola fed as seeds compared with oil. Proportions of SFA and C18:3*n*3 in milk fat and transfer efficiency for C18:3*n*3 were not affected by form of canola supply.

The regression equations for transfer efficiency for C18:2*n*6 and milk fat proportion of C18:2*n*6 were significantly affected by main forage type in the diet. This resulted in a higher transfer efficiency for C18:2*n*6 when the diet contained barley silage ($P = 0.07$), alfalfa silage, or maize silage compared with grass silage, and a higher C18:2*n*6 proportion in milk fat for barley silage compared with alfalfa silage, maize silage, or grass silage. In addition, the C18:3*n*3 proportion in milk fat was lower for diets containing barley silage as the main forage type compared with grass silage. The higher transfer efficiency for C18:2*n*6, higher milk fat proportion of C18:2*n*6, and lower milk fat proportion of C18:3*n*3 probably reflect the difference in FA composition of these forages with higher C18:2*n*6 proportions in barley silage and higher C18:3*n*3 proportions in grass silage. The relationship between C18:0 proportion in milk fat and dietary total FA content was significantly affected by main forage type, but this did not result in significant differences in milk fat C18:0 proportion between the main forage types in a diet supplemented with a source of canola.

Changes in milk FA profile for diets supplemented with soybean and sunflower

Final models for diets supplemented with a source of soybean or sunflower are shown in Table 8 with the least squares means in Table 6. The proportion of UFA in milk fat was significantly increased with dietary UFA content, with a more pronounced effect when soybean or sunflower oil was used compared with a protected source. In addition, the dietary NDF content also affected milk UFA proportion showing different effects when technological form changed. When soybean or sunflower were fed in the most accessible form, oil, the effect of dietary NDF content on milk UFA proportion was most negative. In contrast, when soybean or sunflower were fed in a protected form, there was a positive effect of dietary NDF content on milk UFA proportion. Fibre stimulates the rumen biohydrogenation of free UFA (Harfoot and Hazlewood, 1997) which explains the negative effect of NDF when fed as oil, but the positive effect of NDF when protected sources are fed remains unclear. However, the effects of technological form including addition of fish oil on the relationships with dietary UFA and NDF contents did not result in significant differences in milk UFA proportion.

The supply of soybeans and sunflower as seed resulted in the highest transfer efficiencies and milk

Table 7. Regression models to predict proportions of milk saturated fatty acids (SFA), unsaturated fatty acids (UFA), C18:0, *cis*-9-C18:1, *trans*-11-C18:1, *cis*-9,*cis*-12-C18:2 (C18:2*n6*), and *cis*-9,*cis*-12,*cis*-15-C18:3 (C18:3*n3*; all in g/100 g) and transfer efficiencies from intake to milk for C18:2*n6* and C18:3*n3* (%) from diet characteristics and responses to technological form standardized to seed, and forage type standardized to diets containing maize silage as main forage type for diets supplemented with canola fat[1]

Parameter	Intercept	SE	P-value tech. form	P-value forage type	Variable	Coefficient	SE	P-value coefficient	RMSPE %[2]	CCC
SFA	124.2	20.2	ns	-	UFA	-1.589	0.574	0.015	3.18	0.949
					NDF	-0.129	0.051	0.024		
					UFA*NDF	0.0031	0.0015	0.057		
SFA	69.7	3.8	-	0.083	UFA	-0.282	0.081	0.004	5.97	0.791
UFA	28.8	4.6	ns	-	UFA	0.206	0.124	0.117	12.81	0.616
UFA	28.3	4.8	-	0.090	UFA	0.218	0.115	0.081	8.09	0.861
C18:0	66.3	27.4	0.034	-	FA	-0.931	0.482	0.129[5a]	12.06	0.544
					NDF	-0.122	0.066	0.093		
					FA*NDF	0.0022	0.0012	0.093		
C18:0	15.3	2.0	-	0.067	FA	-0.028	0.031	0.832[5b]	11.54	0.484
cis-9-C18:1[3]										
trans-11-C18:1[4]										
C18:2*n6*	-2.91	1.30	0.067	-	C18:2*n6*	0.049	0.024	0.065	7.88	0.954
					NDF	0.012	0.003	0.829[6a]		
C18:2*n6*	1.79	0.26	-	0.028	C18:2*n6*	0.030	0.013	0.003[6b]	14.97	0.833
C18:3*n3*	0.89	0.31	ns	-	C18:3*n3*	0.050	0.014	0.003	39.39	0.355
					NDF	-0.0017	0.0008	0.061		
C18:3*n3*	0.59	0.07	-	0.013	-	-	-	-	18.69	0.848
Efficiency C18:2*n6*	-3.2	10.8	0.070	-	C18:2*n6*	0.556	0.812	0.510	0.01	1.000
					NDF	0.073	0.028	0.618[7a]		
					C18:2*n6**NDF	-0.0048	0.0022	0.057		
Efficiency C18:2*n6*	26.6	1.0	-	0.018	C18:2*n6*	-1.333	0.075	0.993[7b]	5.64	0.991
Efficiency C18:3*n3*	-37.1	12.1	ns	-	C18:3*n3*	8.540	2.095	0.001	1.72	1.000
					NDF	0.148	0.031	<0.001		
					C18:3*n3**NDF	-0.030	0.005	<0.001		
Efficiency C18:3*n3*	-20.0	15.3	-	0.049	C18:3*n3*	6.526	3.206	0.029[8]	0.02	1.000
					NDF	0.115	0.039	0.018		
					C18:3*n3**NDF	-0.028	0.008	0.009		

Table 7. Continued.

Parameter	Intercept	SE	P-value tech. form	P-value forage type	Variable	Coefficient	SE	P-value coefficient	RMSPE %[2]	CCC

[1]Data are adjusted for the random effect of experiment and weighted for unequal variance. The equations are standardized for diets containing canola as seed or containing maize silage as the main forage type. Both intercept and coefficient would be adjusted for different technological forms or forage types. Class and continuous variables are included when P < 0.10.

[2]RMSPE %: root mean square prediction error as a percentage of the observed mean, CCC: concordance correlation coefficient.

[3]No significant model could be fitted.

[4]No significant model could be fitted.

[5a]The interaction between technological form * FA was P = 0.03; estimates for oil: intercept = 34.84, slope = -0.342; estimates for protected: intercept = 70.50, slope = -1.063.

[5b]The interaction between forage type * FA was P = 0.04; estimates for alfalfa silage: intercept = 1.30, slope = 0.300; estimates for barley silage: intercept = 19.32, slope = -0.175; estimates for grass silage: intercept = 24.64, slope = -0.175.

[6a]The interaction between technological form * NDF was P = 0.08; estimates for oil: intercept = 3.78, slope = -0.007; estimates for protected: intercept = 2.16, slope = -0.00005.

[6b]The interaction between forage type * C18:2*n*6 was P = 0.02; estimates for alfalfa silage: intercept = 1.89, slope = 0.044; estimates for barley silage: intercept = -17.68, slope = 2.138; estimates for grass silage: intercept = 0.22, slope = 0.135.

[7a]The interaction between technological form * NDF was P = 0.06; estimates for oil: intercept = 27.6, slope = -0.032; estimates for protected: intercept = 17.3, slope = 0.018.

[7b]The interaction between forage type * C18:2*n*6 was P = 0.04; estimates for alfalfa silage: intercept = 18.9, slope = -0.725; estimates for barley silage: intercept = -5.7, slope = 1.993; estimates for grass silage: intercept = 3.3, slope = 0.103.

[8]The interaction between forage type * diet C18:3*n*3 content was P = 0.02; estimates for alfalfa silage: intercept = -19.1, slope = 6.550; estimates for barley silage: intercept = -101.2, slope = 21.366; estimates for grass silage: intercept = -84.1, slope = 14.620.

Table 8. Regression models to predict proportions of milk saturated fatty acids (SFA), unsaturated fatty acids (UFA), C18:0, *cis*-9-C18:1, *trans*-11-C18:1, *cis*-9,*cis*-12-C18:2 (C18:2*n6*), and *cis*-9,*cis*-12,*cis*-15-C18:3 (C18:3*n3*; all in g/100 g) and transfer efficiencies from intake to milk for C18:2*n6* and C18:3*n3* (%) from diet characteristics and responses to technological form standardized to seed, and forage type standardized to diets containing maize silage as main forage type for diets supplemented with soybean or sunflower fat[1]

Parameter	Intercept	SE	P-value tech. form	P-value forage type	Variable	Coefficient	SE	P-value coefficient	RMSPE %[2]	CCC
SFA	139.1	47.8	ns	-	FA	-1.697	0.810	0.044	6.11	0.641
					NDF	-0.237	0.156	0.138		
					FA * NDF	0.0049	0.0026	0.067		
SFA	161.8	45.9	-	0.014	FA	-1.811	0.779	0.027	3.83	0.883
					NDF	-0.310	0.143	0.038		
					FA * NDF	0.0051	0.0024	0.041		
UFA	41.0	16.4	0.142	-	UFA	0.153	0.158	<0.001[3a]	7.59	0.825
					NDF	-0.036	0.058	0.610[3b]		
UFA	25.3	3.1	-	ns	UFA	0.303	0.084	<0.001	11.34	0.593
C18:0	-2.24	2.48	0.038	-	FA	0.315	0.053	0.002[4]	5.98	0.958
C18:0	12.40	0.70	-	<0.001	-	-	-	-	10.28	0.875
cis-9-C18:1	17.23	1.86	0.033	-	*cis*-9-C18:1	0.719	0.191	<0.001	5.71	0.930
cis-9-C18:1	-110.6	32.1	-	<0.001	*cis*-9-C18:1	10.572	2.730	<0.001	0.03	1.000
					NDF	0.424	0.109	0.007[5]		
					cis-9-C18:1*NDF	-0.032	0.009	0.002		
trans-11-C18:1	-9.16	5.34	0.135	-	UFA	-0.058	0.082	0.116[6]	9.42	0.987
					NDF	0.042	0.019	0.045		
trans-11-C18:1	2.71	2.47	-	0.005	UFA	0.103	0.060	0.098	0.03	1.000
C18:2*n6*	4.16	0.23	0.022	-	-	-	-	-	16.77	0.634
C18:2*n6*	3.35	0.25	-	<0.001	-	-	-	-	6.34	0.957
C18:3*n3*	0.72	0.05	0.004	-	-	-	-	-	25.10	0.379
C18:3*n3*	-0.71	0.69	-	0.004	C18:3*n3*	0.434	0.147	0.006	12.19	0.930
					NDF	0.0026	0.0022	0.235		
					C18:3*n3**NDF	-0.0011	0.0004	0.018		
Efficiency C18:2*n6*	18.4	9.6	0.085	-	C18:2*n6*	-0.381	0.103	<0.001	15.66	0.911
					NDF	-0.0002	0.0321	0.795[7]		
Efficiency C18:2*n6*	14.7	2.3	-	0.051	C18:2*n6*	-0.340	0.087	<0.001	14.05	0.929

Table 8. Condinued.

Parameter	Intercept	SE	P-value tech. form	P-value forage type	Variable	Coefficient	SE	P-value coefficient	RMSPE %[2]	CCC
Efficiency C18:3*n*3	12.6	1.2	0.002	-	C18:3*n*3	-0.941	0.260	<0.001	17.20	0.857
Efficiency C18:3n3	14.0	4.5	-	0.003	NDF	-0.027	0.014	0.076	9.14	0.961

[1]Data are adjusted for the random effect of experiment and weighted for unequal variance. The equations are standardized for diets containing soybean and sunflower as seed or containing maize silage as the main forage type. Both intercept and coefficient would be adjusted for different technological forms or forage types. Class and continuous variables are included when P < 0.10.

[2]RMSPE %: root mean square prediction error as a percentage of the observed mean, CCC: concordance correlation coefficient.

[3a]The interaction between technological form * UFA was P = 0.09; estimates for oil: intercept = 23.2, slope = 1.133; estimates for protected: intercept = -59.2, slope = 1.004; estimated for added fish oil: intercept = 21.1, slope = 0.272.

[3b]The interaction between technological form * NDF was P = 0.06; estimates for oil: intercept = 23.2, slope = -0.117; estimates for protected: intercept = -59.2, slope = 0.212; estimated for added fish oil: intercept = 21.1, slope = 0.021.

[4]The interaction between technological form * FA was P = 0.01; estimates for oil: intercept = 13.57, slope = 0.007; estimates for protected: intercept = 6.99, slope = 0.096; estimated for added fish oil: intercept = 3.68, slope = 0.125.

[5]The interaction between forage type * NDF was P < 0.001; estimates for maize silage/haylage: intercept = -48.58, slope = 0.207; estimates for grass silage: intercept = -149.16, slope = 0.521; estimates for haylage: intercept = -80.55, slope = 0.329.

[6]The interaction between technological form * NDF was P = 0.05; estimates for oil: intercept = 1.84, slope = -0.185; estimates for protected: intercept = -25.43, slope = 0.538; estimated for added fish oil: intercept = -11.63, slope = 0.091.

[7]The interaction between technological form * NDF was P = 0.09; estimates for oil: intercept = 16.1, slope = 0.0031; estimates for protected: intercept = 36.2, slope = -0.077; estimated for added fish oil: intercept = -13.4, slope = 0.094.

fat proportions of C18:2*n*6 and C18:3*n*3, whereas the addition of fish oil to a diet containing soybean or sunflower had a similar transfer efficiency and milk fat proportion of C18:3*n*3 as soybean or sunflower supplied as seed. Protected soybean and sunflower sources did not increase C18:2*n*6 proportion compared with the other supplement forms. The results originated mainly from the study of Lundy et al. (2004), in which only a slightly decreased extent of C18:2*n*6 biohydrogenation was found for the amides and Ca-salts compared with the soybean oil. However, the milk fat proportion of C18:2*n*6 in this study did not differ between the protected form and oil. When soybean and sunflower sources were provided as seed they were able to increase the proportion of C18:2*n*6 in milk fat, confirming the protective effects of the seed coat restricting bacterial access to the FA (Chilliard et al., 2007).

The addition of fish oil to a diet containing soybean or sunflower resulted in the lowest proportions of C18:0 and *cis*-9-C18:1, and a higher proportion of *trans*-11-C18:1 in milk fat compared with supplementation as seed. These results confirm the inhibiting effect of fish oil on the last step of biohydrogenation (Shingfield et al., 2003) and consequently the lower supply of C18:0 available for desaturation to *cis*-9-C18:1. The proportion of C18:0 is increased with dietary total FA content and this effect was most pronounced when soybean or sunflower were supplied as seed. Proportion of *trans*-11-C18:1 was affected by dietary UFA and NDF contents, whereas the form of supply influenced the effect of dietary UFA with increased proportions when soybean or sunflower were supplied as oil (more pronounced) or seed and decreased proportions when soybean or sunflower were supplied as a protected source (more pronounced) or when fish oil was added to the diet. Biohydrogenation seems to be most extensive in the oil form, due to the easy accessibility of the FA in oil compared with whole or processed seeds or protected sources (Chilliard et al., 2007).

Milk UFA proportion was not affected by the main forage type in the diet, whereas the proportion of SFA was highest when the diet contained haylage as the main forage type. Haylage as the main forage type also resulted in the highest milk fat proportions of *cis*-9-C18:1 and C18:2*n*6. When the diet contained maize silage combined with haylage as the main forage type transfer efficiency for C18:3*n*3 and milk fat proportion of C18:3*n*3 were highest. Maize silage as the main forage type showed a higher proportion of *trans*-11-C18:1 in milk fat compared with grass silage, which was in agreement with Chilliard et al. (2007) who concluded that rumen biohydrogenation appears to be less complete when adding linseed or sunflower oil to a diet containing maize silage compared with grass silage. This may be related to the higher level of fibre required to stabilize rumen pH when a higher amount of degradable starch is present (Zebeli et al., 2008) and the effects of rumen pH on the rate of biohydrogenation.

Changes in milk FA profile for diets supplemented with linseed

Final models for diets supplemented with linseed are shown in Table 9 with the least squares means in Table 6. Milk fat proportion of UFA was affected by the UFA content in the diet, whereas the form of linseed supply did not affect the UFA proportion. Transfer efficiency for C18:3*n*3 decreased with increasing dietary C18:3*n*3 content and decreasing NDF content and was not affected by form of linseed supply or addition of fish oil. However, proportion of C18:3*n*3 in milk fat was higher for protected linseed compared with linseed supplied as seed. Form of linseed supply or addition of

fish oil affected the relationship between dietary C18:3*n*3 content and C18:3*n*3 proportion in milk fat with a more pronounced effect for protected linseed (higher intercept and more negative slope). However, this effect was not confirmed in results of Petit et al. (2002a) and Petit (2003) who fed formaldehyde treated whole linseed. Formaldehyde treatment though is known to be able to decrease biohydrogenation of C18:3*n*3 when the oilseed is pre-treated before applying formaldehyde treatment (Sterk et al., 2010). Increasing dietary NDF content decreased C18:3*n*3 proportion and in addition there was a positive interaction between dietary C18:3*n*3 and NDF content.

The proportion of *trans*-11-C18:1 in milk fat was higher when linseed was supplied as oil compared with linseed fed as seed or linseed including an additional supply of fish oil. For the proportion of *trans*-11-C18:1 in milk fat the form of linseed supply affected the relationship between dietary UFA content and *trans*-11-C18:1 proportion in milk fat with a higher intercept and more negative slope when linseed was supplied as oil. Increasing NDF content decreased *trans*-11-C18:1 proportion and a positive interaction between dietary UFA and NDF content was found. Because of the easy accessibility of the FA in the oil form compared with whole or processed seeds or protected sources, biohydrogenation is most extensive (Chilliard et al., 2007), which in the current meta-analysis seems to be mainly incomplete biohydrogenation as shown by the highest proportion of *trans*-11-C18:1 in milk fat. The proportion of C18:0 in milk fat was not different between the linseed forms, whereas the relationship between dietary total FA content and C18:0 proportion was affected with a more pronounced effect for linseed supplied with fish oil (negative intercept, more positive slope). The addition of fish oil did not result in a significantly lower proportion of C18:0 in the current analysis, which was caused by the inclusion of a linseed supplemented diet with added fish meal containing a relatively low amount of oil (Ward et al., 2002). However, fish oil added to a diet containing formaldehyde treated linseed showed a significantly lower proportion of C18:0 in milk fat (Petit et al., 2002a). The lower proportion of *trans*-11-C18:1 for cows fed linseed in combination with added fish oil compared with linseed fed as oil, was not expected. However, in combination with increased proportions of *trans*-11-C18:1 the proportion of *trans*-10-C18:1 is often increased as rumen micro-organisms shift their biohydrogenation pathway accordingly (Shingfield et al., 2003; 2006). The effect on *trans*-10-C18:1 could not be determined in the current meta-analysis, due to the low number of studies reporting this biohydrogenation intermediate.

Proportion of UFA in milk fat was not affected by the main forage type in the diet, whereas proportions of SFA, *cis*-9-C18:1, and C18:3*n*3 (P = 0.06) were highest when grass silage was the main forage type in the diet. Proportions of C18:0 and *cis*-9-C18:1 were affected by dietary total FA and NDF content and their interaction, and dietary *cis*-9-C18:1 content, respectively. The effect of NDF on proportion of C18:0 in milk fat was affected by the main forage type in the diet; effects were more pronounced when grass silage or haylage were the main forage type. For the proportion of *cis*-9-C18:1 in milk fat the main forage type in the diet affected the relation with dietary *cis*-9-C18:1 content. When the diet contained alfalfa silage or haylage as the main forage type, a higher intercept and a negative relation with dietary *cis*-9-C18:1 content was shown, whereas when the diet contained maize silage or grass silage as the main forage type a lower intercept and a positive relation with dietary *cis*-9-C18:1 content was found. Glasser et al. (2008) reported for linseed supplemented diets an effect

Table 9. Regression models to predict proportions of milk saturated fatty acids (SFA), unsaturated fatty acids (UFA), C18:0, *cis*-9-C18:1, *trans*-11-C18:1, *cis*-9,*cis*-12-C18:2 (C18:2*n6*), and *cis*-9,*cis*-12,*cis*-15-C18:3 (C18:3*n3*; all in g/100 g) and transfer efficiencies from intake to milk for C18:2*n6* and C18:3*n3* (%) from diet characteristics and responses to technological form standardized to seed, and forage type standardized to diets containing maize silage as main forage type for diets supplemented with linseed fat[1]

Parameter	Intercept	SE	P-value tech. form	P-value forage type	Variable	Coefficient	SE	P-value coefficient	RMSPE %[2]	CCC
SFA	67.2	6.0	0.010	-	FA	-0.118	0.112	0.790[3]	0.00	1.000
SFA	71.9	4.7	-	0.003	FA	-0.216	0.087	0.022	0.00	1.000
UFA	28.0	2.8	ns	ns	UFA	0.205	0.060	0.002	0.01	1.000
C18:0	12.23	5.41	0.011	-	FA	0.067	0.097	0.007[4a]	11.45	0.858
C18:0	-151.1	41.8	-	0.027	FA	3.347	0.847	0.001	8.15	0.938
					NDF	0.425	0.110	0.002[4b]		
					FA*NDF	-0.0085	0.0022	0.001		
cis-9-C18:1	26.22	7.52	0.025	-	*cis*-9-C18:1	0.759	0.301	0.022	7.81	0.835
					NDF	-0.019	0.020	0.017[5a]		
cis-9-C18:1	12.37	5.76	-	0.084	*cis*-9-C18:1	1.208	0.504	0.373[5b]	7.25	0.867
trans-11-C18:1	42.43	10.10	<0.001	-	UFA	-0.914	0.234	<0.001[6]	7.55	0.989
					NDF	-0.109	0.026	<0.001		
					UFA*NDF	0.0024	0.0006	0.001		
trans-11-C18:1	31.50	13.02	-	0.011	UFA	-0.678	0.302	0.039	22.21	0.900
					NDF	-0.086	0.035	0.024		
					UFA*NDF	0.0020	0.0008	0.023		
C18:2*n6*	4.42	0.90	ns	ns	C18:2*n6*	0.068	0.029	0.026	19.02	0.656
					NDF	-0.0085	0.0024	0.002		
C18:3*n3*	3.12	0.91	0.009	-	C18:3*n3*	-0.132	0.053	0.011[7a]	7.99	0.960
					NDF	-0.0056	0.0025	0.038		
					C18:3*n3* * NDF	0.0003	0.0001	0.024		
C18:3*n3*	3.88	1.12	-	0.036	C18:3*n3*	-0.187	0.068	0.009[7b]	0.13	1.000
					NDF	-0.0076	0.0031	0.026		
					C18:3*n3* * NDF	0.0005	0.0002	0.014		
Efficiency C18:2*n6*	-34.1	17.1	ns	ns	C18:2*n6*	3.857	1.277	0.006	23.57	0.630
					NDF	0.124	0.046	0.014		
					C18:2*n6* * NDF	-0.011	0.003	0.004		

Table 9. Continued.

Parameter	Intercept	SE	P-value tech. form	P-value forage type	Variable	Coefficient	SE	P-value coefficient	RMSPE %[2]	CCC
Efficiency C18:3*n*3	3.8	2.0	ns	-	C18:3*n*3	-0.254	0.036	<0.001	12.26	0.984
					NDF	0.012	0.006	0.052		
Efficiency C18:3*n*3	5.7	1.0	-	0.063	C18:3*n*3	-0.376	0.037	<0.001[8]	0.04	1.000
					NDF	0.013	0.003	0.001		

[1]Data are adjusted for the random effect of experiment and weighted for unequal variance. The equations are standardized for diets containing linseed as seed or containing maize silage as the main forage type. Both intercept and coefficient would be adjusted for different technological forms or forage types. Class and continuous variables are included when P < 0.10.

[2]RMSPE %: root mean square prediction error as a percentage of the observed mean, CCC: concordance correlation coefficient.

[3]The interaction between technological form * FA was P = 0.01; estimates for oil: intercept = 3.2, slope = 0.820; estimates for protected: intercept = 90.7, slope = -0.515; estimated for added fish oil: intercept = 75.4, slope = -0.319.

[4a]The interaction between technological form * FA was P = 0.06; estimates for oil: intercept = 0.84, slope = 0.198; estimates for protected: intercept = 4.90, slope = 0.224; estimated for added fish oil: intercept = -14.29, slope = 0.522.

[4b]The interaction between forage type * NDF was P = 0.03; estimates for alfalfa silage: intercept = -153.65, slope = 0.421; estimates for grass silage: intercept = -199.97, slope = 0.554; estimated for haylage: intercept = -199.20, slope = 0.538.

[5a]The interaction between technological form * NDF was P = 0.03; estimates for oil: intercept = -53.19, slope = 0.177; estimates for protected: intercept = 31.50, slope = -0.032; estimated for added fish oil: intercept = -45.65, slope = 0.198.

[5b]The interaction between forage type * *cis*-9-C18:1 was P = 0.03; estimates for alfalfa silage: intercept = 57.44, slope = -0.293; estimates for grass silage: intercept = 20.93, slope = 0.065; estimated for haylage: intercept = 30.63, slope = -0.101.

[6]The interaction between technological form * UFA was P < 0.001; estimates for oil: intercept = 62.96, slope = -1.234; estimates for protected: intercept = 43.14, slope = -0.914; estimated for added fish oil: intercept = 36.79, slope = -0.790.

[7a]The interaction between technological form * C18:3*n*3 was P = 0.02; estimates for oil: intercept = 2.74, slope = -0.124; estimates for protected: intercept = 7.03, slope = -0.276; estimated for added fish oil: intercept = 3.24, slope = -0.109.

[7b]The interaction between forage type * C18:3*n*3 was P = 0.09; estimates for alfalfa silage: intercept = 5.11, slope = -0.238; estimates for grass silage: intercept = 5.40, slope = -0.238; estimated for haylage: intercept = -3.71, slope = -0.183.

[8]The interaction between forage type * C18:3*n*3 was P = 0.03; estimates for alfalfa silage: intercept = 2.7, slope = -0.230; estimates for grass silage: intercept = 2.9, slope = -0.207; estimated for haylage: intercept = -0.319, slope = -0.111.

of forage type for milk fat *cis*-9-C18:1 proportion only, with the greatest increase for alfalfa-based diets, followed by maize silage, grass hay, and grass silage based diets. In the current meta-analysis, the *cis*-9-C18:1 proportion was indeed high for linseed supplemented diets with alfalfa silage as the main forage type, however, the proportion was comparable to the *cis*-9-C18:1 proportion achieved on grass silage based diets and maize silage based diets, whereas haylage based diets showed a lower *cis*-9-C18:1 proportion in milk fat.

The proportion of C18:3*n*3 was significantly affected by dietary contents of C18:3*n*3, NDF, and their interaction, with an interaction between main forage type and dietary C18:3*n*3 content. Alfalfa silage or grass silage as the main forage type showed higher intercepts and more negative relationships with dietary C18:3*n*3 content compared with maize silage and haylage. The proportion of *trans*-11-C18:1 showed negative regression slopes for dietary UFA and NDF contents and a positive regression slope for their interaction. The intercept was adjusted for main forage type in the diet, resulting in a higher *trans*-11-C18:1 proportion in milk fat when haylage was the main forage type compared with maize silage or grass silage. The conclusion by Palmquist et al. (2005) that complete biohydrogenation to C18:0 is most extensive when high amounts of ensilaged forages are fed, seems to be confirmed for linseed supplemented diets. However, feeding haylage as the main forage type did not result in a higher transfer efficiency for C18:3*n*3 and C18:3*n*3 proportion in milk fat compared with grass silages.

Conclusions

Different technological forms in which FA are provided to dairy cows from canola, soybean, sunflower, or linseed significantly affected the relationships between the dietary nutrient composition (FA and NDF contents) and milk FA profile. This resulted in significant differences in several milk FA for different technological forms within fat sources. The effect of the main forage type in the ration significantly influenced the effect of dietary FA and NDF contents on milk FA profile, which resulted in significant differences in several milk FA for different main forage types within unsupplemented diets or diets supplemented with FA from canola, soybean, sunflower, or linseed. This meta-analysis showed that the effect of dietary nutrient composition on several milk FA proportions, is dependent on the type and form of fat supplementation, addition of fish oil, and main forage type in the basal diet.

Acknowledgments

Jennifer Ellis (Animal Nutrition Group, Wageningen University) and Hassan Taweel (Agrifirm Innovation Center) are gratefully acknowledged for their contribution to the statistical analysis. The financial support of the Food and Nutrition Delta program of Senter Novem (Den Haag, the Netherlands), Royal FrieslandCampina (Amersfoort, The Netherlands), Agrifirm Group (Apeldoorn, The Netherlands), Agrifirm Innovation Center (Apeldoorn, The Netherlands), Barenbrug Holland B.V. (Oosterhout, The Netherlands), and the Animal Nutrition Group of Wageningen University (Wageningen, The Netherlands) is gratefully acknowledged.

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Chapter 3

Effects of chemically or technologically treated linseed products and docosahexaenoic acid addition to linseed oil on biohydrogenation of C18:*3n*3 in vitro

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Journal of Dairy Science 93: 5286-5299

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Abstract

Rumen biohydrogenation kinetics of C18:3*n*3 from several chemically or technologically treated linseed products and docosahexaenoic acid (DHA; C22:6*n*3) addition to linseed oil were evaluated in vitro. Linseed products evaluated were linseed oil, crushed linseed, formaldehyde treated crushed linseed, sodium hydroxide/formaldehyde treated crushed linseed, extruded whole linseed (two processing variants), extruded crushed linseed (two processing variants), micronized crushed linseed, commercially available extruded linseed, lipid encapsulated linseed oil and DHA addition to linseed oil. Each product was incubated with rumen liquid using equal amounts of supplemented C18:3*n*3 and fermentable substrate (freeze-dried total mixed ration) for 0, 0.5, 1, 2, 4, 6, 12, and 24 h using a batch culture technique. Disappearance of C18:3*n*3 was measured to estimate the fractional biohydrogenation rate and lag time according to an exponential model and to calculate effective biohydrogenation of C18:3*n*3, assuming a fractional passage rate of 0.060/h. Treatments showed no differences in rumen fermentation parameters, including gas production rate and volatile fatty acid concentration. Technological pretreatment (crushing) followed by chemical treatment applied as formaldehyde of linseed resulted in effective protection of C18:3*n*3 against biohydrogenation. Additional chemical pretreatment (sodium hydroxide) before applying formaldehyde treatment did not further improve the effectiveness of protection. Extrusion of whole linseed compared to extrusion of crushed linseed was effective in reducing C18:3*n*3 biohydrogenation, whereas the processing variants were not different in C18:3*n*3 biohydrogenation. Crushed linseed, micronized crushed linseed, lipid encapsulated linseed oil, and DHA addition to linseed oil did not reduce C18:3*n*3 biohydrogenation. Compared with the other treatments, docosahexaenoic acid addition to linseed oil resulted in a comparable *trans*-11,*cis*-15-C18:2 biohydrogenation but a lesser *trans*-10+11-C18:1 biohydrogenation. This suggests that addition of DHA in combination with linseed oil was effective only in inhibiting the last step of biohydrogenation from *trans*-10+11-C18:1 to C18:0.

Introduction

Changing the fatty acid (FA) profile of bovine milk fat towards a nutritionally more beneficial profile has received increasing attention. Increasing the proportion of unsaturated FA (UFA) in milk fat through the diet of dairy cows is considered an improvement of the dietary value of milk (Jenkins and Bridges, 2007). Dietary UFA, however, are subject to extensive biohydrogenation by ruminal micro-organisms, yielding *trans*-FA intermediates and saturated FA as end products (Harfoot and Hazlewood, 1997). The extent of lipolysis and biohydrogenation is determined by several factors, including the nature of dietary FA, the retention time in the rumen, and the composition of the microbial population (Jenkins et al., 2008). Fats in the ruminant diet are mainly derived from forages, grains and oil supplements, especially vegetable oils. The latter, however, have only a limited use in dairy diets because they tend to reduce fibrolytic activity and fibre digestion in the rumen (Harfoot and Hazlewood, 1997). The rumen environment, therefore, has to be protected against adverse effects of oil supplements and oil supplements have to be protected against ruminal biohydrogenation to increase postruminal UFA flow. Three main protection technologies can be distinguished: 1) chemical protection (e.g., formaldehyde treatment of oilseeds); 2) alterations of FA structure through formation of calcium salts and amides of FA; and 3) technological treatments of oilseeds (e.g., extrusion, cracking; Fievez et al., 2007). Several in vitro and in vivo studies evaluated the potential of these protection technologies to increase post-ruminal UFA flow. Sinclair et al. (2005) showed that formaldehyde treatment reduced biohydrogenation of C18:3*n*3 from linseed only when it was preceded by chemical pre-treatment (sodium hydroxide or formic acid) to induce permeability of the seed coat. Technological pretreatment, such as crushing, might be able to induce the same permeability of the seed coat and result in effective UFA protection after formaldehyde treatment. Technological treatments, such as extrusion, increased the proportions of biohydrogenation intermediates in vitro (Enjalbert et al., 2003) and in milk fat (Bayourthe et al., 2000; Chouinard et al., 2001; Akraim et al., 2007). The effect of extrusion conditions, such as temperature, were evaluated in these studies, but oilseeds were always ground before extrusion. Therefore, extrusion of whole linseed versus crushed linseed in combination with different processing conditions, such as steam and water percentage, might result in differences in postruminal UFA flow. Protection of UFA in a sphere of FA with a high melting point was hypothesized as a possible protection technology (Jenkins and Bridges, 2007). However, to our knowledge no studies have been reported in which this technology was applied to protect seed oil. Besides protecting oilseeds against biohydrogenation, complete biohydrogenation towards stearic acid can be inhibited by the addition of docosahexaenoic acid (DHA; C22:6*n*3). Recent research suggests that DHA provokes accumulation of various *trans*-FA, including *trans*-11-C18:1, in vitro (Vlaeminck et al., 2008) and in vivo (Boeckaert et al., 2008a) through changes in the rumen microbial population. The effect of DHA addition on the biohydrogenation of C18:3*n*3 from linseed oil, however, was not studied.

Metabolism of UFA in the rumen may be studied by time series of in vitro incubations to allow estimation of kinetic parameters such as fractional rate of biohydrogenation and lag time (Fievez et al., 2007). Such kinetic parameters and assumptions on fractional passage rate enable calculation of

effective biohydrogenation of UFA. Furthermore, fermentation parameters such as gas production, VFA concentration and OM degradability can be measured to examine the effects of UFA supplementation on ruminal fermentation.

To our knowledge, the effectiveness of protection of UFA by several protection technologies for linseed and changes in biohydrogenation of linseed oil following DHA addition have not been compared under identical experimental conditions. The objective of the current study was to investigate, by means of in vitro incubations, whether several chemically or technologically treated linseed products and addition of DHA to linseed oil can effectively change rumen biohydrogenation kinetics of C18:3*n*3.

Material and Methods

Animals and diet

The experiment was approved by the Institutional Animal Care and Use Committee of Wageningen University (Wageningen, The Netherlands). Ruminal fluid was collected just before the morning feeding from four lactating Holstein-Friesian dairy cows (lactation stage: 176 ± 105 DIM; fat- and protein-corrected milk: 31.2 ± 11.0 kg/d; BW: 657 ± 60 kg), each fitted with a ruminal cannula and fed ad libitum a TMR diet. The TMR contained (fresh weight basis) 35.9% ryegrass silage, 54.4% maize silage, 1.1% straw, 0.4% minerals, and 8.2% concentrate (containing 32.7% soybean meal, 32.7% wheat, 32.7% rapeseed meal, and 2.0% cane molasses). A freeze-dried and ground (1 mm) sample of this TMR was used as the basal incubation substrate. After collection, rumen fluid was immediately transferred into prewarmed and CO_2-flushed thermos flasks.

Linseed products

The linseed products tested in vitro were as follows: 1) pure linseed oil (LO); 2) linseed crushed in a roller mill (CL; 0.25 mm; Ipswich Turner, Christy Turner Ltd, Ipswich, UK); 3) formaldehyde treated crushed linseed (FCL; 4.5 g/kg formaldehyde applied as formalin, according to Sinclair et al., 2005); 4) sodium hydroxide/formaldehyde treated crushed linseed (SFCL; 3.0 g/kg sodium hydroxide applied as a 50:50 vol/vol solution, followed by 4.5 g/kg formaldehyde applied as formalin, according to Sinclair et al., 2005); 5 an 6) extruded mixture of whole linseed and wheatbran [70:30 vol/vol linseed:wheatbran, prepared in a small-scale single-screw extruder line of Almex AL150 (Almex, Zutphen, the Netherlands) equipped with a pellet press of Robinson/Heesen V2/30 (Heesen, Boxtel, the Netherlands) and cooler unit; EL1: 6% steam and 2% water, 127˚C for 20-30 s; EL2: 2% steam and 6% water, 130˚C for 20-30 s]; 7 and 8) extruded mixture of crushed linseed and wheat bran (70:30 vol/vol crushed linseed:wheatbran, extruder line and pellet press as described previously; ECL1: 6% steam and 2% water, 115˚C for 20-30 s; ECL2: 2% steam and 6% water, 118˚C for 20-30 s); 9) micronized crushed linseed (MCL; heated with infrared gas generators to 115-120˚C for 90 s; gas-heated infrared irradiation belt; HOAF/WU-design, Oldenzaal, the Netherlands); 10) commercially available extruded linseed product (CEL; containing 56.0% crushed linseed, 21.0% wheat, 15.0% sunflower cake, 4.5% field beans, 2.0% butylated hydroxytoluene (BHT), 1.0% linseed oil, and 0.5%

salt; Nutex Compact, Dumoulin, Seilles, Belgium; Van et al., 2008); 11) lipid encapsulated linseed oil (LELO; canola meal with 12% linseed oil and 2% pork fat prepared in a twin-shaft paddle mixer-vacuum coater; Dinissen, Pegasus PG-10lab, Sevenum, the Netherlands); and 12) linseed oil + DHA addition (LO+DHA; 10 mg DHA/g incubation substrate; DHA Gold Martek Biosciences Corp., Columbia, MD; Vlaeminck et al., 2008). All linseed and linseed oil treatments were made from one batch of linseed and linseed oil, respectively. The FA composition of the TMR, linseed products and DHA Gold is given in Table 1.

In vitro incubations

The rumen fluid of the four cows was mixed and strained through a double layer of cheese cloth continuously flushed with CO_2, diluted with a phosphate buffer (per litre distilled water: 28.8 g $Na_2HPO_4.12H_2O$, 6.1 g $NaH_2PO_4.H_2O$, and 1.4 g NH_4Cl, adjusted to pH 6.8 by adding NaOH solution), and placed on a stirrer to ensure complete mixing of the rumen fluid/phosphate buffer mixture (1:4). Accurately weighed treatment products (~1 g) and 50 mL of the rumen fluid-phosphate buffer mixture were added to gastight incubation flasks (150 mL) under anaerobic conditions. Each sample was incubated in duplicate in 2 separate runs on separate days. To provide equal amounts of supplemented C18:3*n*3 and fermentable substrate (Table 2), treatment products comprised 1.00 g TMR with 0.06 g LO; 0.89 g TMR with 0.17 g CL, MCL, FCL, or SFCL; 0.82 g TMR with 0.24 g EL1, EL2, ECL1, ECL2, or CEL; 0.50 g TMR with 0.57 g LELO; and 1.00 g TMR with 0.06 g LO + 50.6 mg of DHA Gold. Flasks were flushed with CO_2 before incubation started in a shaking water bath at 39°C for 0, 0.5, 1, 2, 4, 6, 12 and 24 h. At the end of the incubation periods, flasks were removed sequentially and immediately placed on ice. Then, flasks were opened and pH was measured. The incubation residue was collected, stored at -20˚C and freeze-dried before FA analysis was carried out. In a second duplicated set of 24-h flasks, cumulative gas production (Cone et al., 1996), fluid VFA content and OM disappearance were measured. In these 24-h flasks, first a subsample of 0.75 mL of the incubation fluid was taken for VFA analysis. These samples were stored with 85% H_3PO_4 (1:1 vol/vol) and kept in a freezer at -20ºC pending VFA analysis. The incubation residue of the 24-h flasks was filtered and analysed for ash content to determine OM degradability.

Analysis

The FA in the TMR (375 mg of freeze-dried material), treatment products (375 mg of material), and incubation residues (375 mg of freeze-dried material) were extracted with 15 mL chloroform-methanol (2:1 vol/vol) and 375 µL of distilled water (Folch et al., 1957). The homogenized extracts were filtered and centrifuged at 800 x *g* after adding 2.2 mL distilled water for a clear separation. The upper phase was removed thoroughly, using repeated washing with wash solution (30 mL of chloroform, 480 mL of methanol, and 470 mL of NaCl solution (7.3 g/L of water)).

Table 1. Fatty acid composition (g/100 g of fatty acids) and total fatty acid content (mg/g DM) of TMR and linseed products[1]

Fatty acid	TMR	LO	CL	MCL	ECL1	ECL2	CEL	EL1	EL2	LELO	DHA Gold[2]
C14:0	0.66	0.05	0.05	0.05	0.08	0.07	0.08	0.10	0.08	0.25	13.78
C16:0	16.74	5.54	5.46	5.55	6.14	6.05	6.43	6.58	6.18	7.95	32.22
Cis-9-C16:1	0.44	0.06	0.07	0.07	0.07	0.07	0.06	0.09	0.07	0.54	0.22
C18:0	2.81	3.70	3.58	3.58	3.46	3.51	3.34	3.39	3.53	3.77	0.64
Cis-9-C18:1	13.60	19.32	18.29	18.59	18.47	18.54	17.77	19.15	18.17	27.08	0.09
Cis-11-C18:1	1.80	0.73	0.72	0.72	0.74	0.75	0.72	0.79	0.76	2.28	0.37
C18:2*n*6	26.54	16.16	15.94	15.82	17.06	16.98	19.09	17.57	17.32	18.01	0.08
C18:3*n*3	25.08	53.41	54.70	54.59	52.87	52.96	51.36	51.13	52.75	38.48	0.26
C20:0	0.73	0.13	0.13	0.13	0.12	0.12	0.13	0.17	0.15	0.19	0.11
C22:0	1.10	0.13	0.13	0.13	0.12	0.12	0.13	0.16	0.14	0.12	0.03
C20:5*n*3	0.19	ND	ND	ND	ND	ND	0.03	ND	ND	0.03	1.67
C22:5*n*3	ND[3]	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.47
C22:6*n*3	ND	ND	ND	ND	ND	ND	0.05	ND	ND	ND	42.59
Other	10.31	0.76	0.94	0.76	0.87	0.83	0.79	0.88	0.83	1.31	7.46
Total fatty acids	17.6	950.0[4]	292.3	298.9	191.7	192.6	179.3	NT[5]	NT	118.3	NT

[1]LO: linseed oil; CL: crushed linseed; EL1: extruded linseed 1; EL2: extruded linseed 2; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; LELO: lipid encapsulated linseed oil.

[2]Martek Biosciences Corp., Columbia, MD.

[3]ND: not detected.

[4]CVB, 2007.

[5]NT: no total fatty acid content determined due to incomplete fatty acid extraction.

Table 2. Calculated amounts of C18:3*n*3 (mg per flask containing 50 mL of incubation fluid) and fermentable OM (mg per flask containing 50 mL of incubation fluid) for the control treatment (CON) and linseed treatments[1] before incubation

Parameter	Treatments												
	CON	LO	CL	FCL	SFCL	MCL	ECL1	ECL2	CEL	EL1	EL2	LELO	LO+DHA
C18:3n3	4.41	36.48	31.59	30.82	30.99	31.65	28.07	28.16	25.86	28.21	28.02	28.21	36.46
Fermentable OM	510	512	502	501	501	501	499	499	500	499	498	471	511

[1]LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL: sodium hydroxide pretreated formaldehyde treated crushed linseed; EL1: extruded linseed 1; EL2: extruded linseed 2; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

3

Approximately 3 mL of the lower phase, containing lipids, was collected and solvents were evaporated by vacuum centrifugation. The residual lipids were collected and FA were methylated with 0.5 mL of 0.5 *N* NaOH methanolate (10 min at 80ºC) followed by 0.5 mL of 14% boron trifluoride (2 min at 80ºC). Fatty acid methyl esters were collected in 1 mL of hexane. For a clear separation of the hexane layer a saturated salt solution (400 g of NaCl/L of water) was added and tubes were centrifuged at 800 x *g* for 5 min. Residues were dissolved in 1 mL of hexane and transferred to GC vials. Fatty acid methyl esters were quantified using gas chromatography (Trace GC Ultra, Thermo Fisher Scientific, Waltham, MA) with a fused silica capillary column (100 m x 0.250 mm and 0.2 μm film thickness; SP2560, Supelco, St. Louis, MO) using helium as a carrier gas at a constant flow of 1.5 mL/min. The flame ionization detector was set at 280ºC. The time-temperature program used, started with an initial temperature of 140ºC for 4 min, increased 4ºC per min to a final temperature of 240ºC, and held at this temperature for 20 min. Fatty acid methyl esters were identified using external standards (S37, Supelco). Separation of the isomers *trans*-10-C18:1 and *trans*-11-C18:1 was not possible in all samples and therefore *trans*-10+11-C18:1 were reported together.

The pH was measured using an electronic pH meter (pH electrode HI 1230, Hanna Instruments B.V., IJsselstein, the Netherlands). The concentration of VFA was determined using gas chromatography (GC type Fisons HRGC MEGA2, Fisons Instruments, Milano, Italy) as described by Taweel et al. (2005). Ash was determined by combustion at 550ºC (ISO 5984; ISO, 2002).

Calculations and Statistics

All statistical analyses were carried out in SAS version 9.1 (SAS Institute, Cary, NC). Total C18 FA remained constant over the 24-h period and therefore individual C18 FA were calculated as proportions of total C18 FA. Disappearance of C18:3*n*3 from the incubation flasks at each sampling time was calculated relative to the 0-h time point. This disappearance of C18:3*n*3 was then used to estimate the fractional biohydrogenation rate and lag time according to an exponential model with the NLIN procedure of SAS. Lag time was constrained to be positive. Effective C18:3*n*3 biohydrogenation was calculated according to Dhanoa et al. (1999) assuming a fractional passage rate of 0.060/h.

The individual FA and pH measured at the different sampling times were analysed using the MIXED procedure of SAS. The statistical model included the fixed effects of incubation run, treatment, time, and the interaction between treatment and time. Posthoc analyses were carried out using the Tukey test to test pairwise comparisons. Least square means are reported, and significance was declared at $P < 0.05$.

Gas production profiles obtained with the automated system were fitted by iteration for individual incubation flasks to a generalized Michaelis-Menten model without lag time (France et al., 2000) with the NLIN procedure. The gas production profiles were characterized by the cumulative gas production (OMCV; mL/g incubated OM), the estimated asymptotic gas production (parameter A; mL/g incubated OM), a constant that determines the sharpness of the switching characteristic of the profile (parameter B), the time after incubation at which half of the asymptotic gas production has been reached (parameter C; h), the maximum rate of gas production (mL/h), and the time to reach the maximum rate of gas production (Tmax; h). Parameters B and C were constrained to be positive.

Biohydrogenation kinetic parameters, gas production kinetic parameters, VFA concentration, and OM degradability were analysed with the MIXED procedure, with incubation run and treatment as the fixed variables.

Results

Rumen fermentation pattern

Fluid pH decreased significantly ($P < 0.001$) with time for all treatments (results not shown) from an average of 6.61 at 0 h to 6.18 at 24 h of incubation. An effect of treatment on fluid pH ($P < 0.001$) was found; however, after 24 h of incubation the vessel fluid pH was > 6.10 for all treatments (results not shown). The concentration of total VFA; the molar proportions of acetate, butyrate and propionate; and the ratio of nonglucogenic to glucogenic VFA were not influenced by treatment ($P > 0.05$; Table 3). However, the molar proportions of isobutyrate, valerate and isovalerate were increased ($P < 0.001$) in LELO compared with the other treatments, although valerate proportion was similar for LELO and CEL. Organic matter degradability was influenced ($P = 0.016$) by linseed treatment; CEL showed a greater OM degradability than FCL and the other treatments showed intermediate results (Table 3).

The effects of the various linseed products on gas production parameters are shown in Table 4. No effects ($P > 0.05$) of the linseed treatments were found on OMCV. A tendency ($P < 0.10$) was found toward a greater OMCV for the LELO treatment compared with the FCL treatment. The LO and FCL treatments showed a significantly greater value for parameter A compared to the ECL2 and EL2 treatments. The value for parameter B was significantly greater in the CL, ECL2, and CEL treatments compared with the LO, FCL, and MCL treatments, and the value for parameter C was greatest in the FCL treatment compared with the other treatments. The maximum rate of gas production was greater in the ECL1, ECL2, CEL, and LELO treatments compared with the FCL, SFCL, and MCL treatments, and the Tmax was greatest in the SFCL treatment, although Tmax was similar for SFCL and CL, FCL, and LO+DHA.

FA composition

Figures 1, 2, 3 and 4 show the changes in vessel proportions of C18:3*n*3, *trans*-11,*cis*-15-C18:2, *trans*-10+11-C18:1, and C18:0, respectively, during the 24 h of incubation. Proportion of C18:3*n*3 decreased with time ($P < 0.001$), and this decrease was influenced by treatment (interaction treatment x time; $P < 0.001$). After 24 h of incubation the proportion of C18:3*n*3 relative to 0 h was greater in the EL1, EL2, FCL, and SFCL treatments compared with the CEL and LELO treatments (Table 5), with intermediate values for the other treatments. Biohydrogenation intermediate *trans*-11,*cis*-15-C18:2 reached a peak value after 12 h of incubation for the LO, CEL, and LELO treatments. Proportion of *trans*-11,*cis*-15-C18:2 continued to increase until 24 h of incubation for the other linseed treatments. The CL, ECL1, ECL2, MCL, and CEL treatments resulted in greater proportions of *trans*-11,*cis*-15-C18:2 after 24 h of incubation compared with the LO, EL1, and EL2 treatments ($P < 0.05$). Proportion of *trans*-10+11-C18:1 increased during the 24 h of incubation for all treatments,

Table 3. Effect of control treatment (CON) and linseed treatments[1] on OM degradability (%) and concentration of total VFA (mmol/L), and VFA molar proportions (mmol/mol) after 24 h of incubation

Parameter	Treatments													SED[2]	Significance[3]
	CON	LO	CL	FCL	SFCL	MCL	ECL1	ECL2	CEL	EL1	EL2	LELO	LO+DHA		
OM-degradability	8.4	64.5^{ab}	65.2^{ab}	59.8^{b}	62.3^{ab}	65.6^{ab}	63.1^{ab}	65.4^{ab}	66.0^{a}	60.7^{ab}	60.7^{ab}	65.4^{ab}	64.6^{ab}	1.6	0.016
Total VFA	95	100	98	94	96	100	101	104	98	97	99	96	103	4.9	0.688
Acetate	605	605	591	600	598	596	590	592	582	604	603	585	594	8.7	0.202
Propionate	278	278	282	282	281	282	288	287	287	277	278	277	286	4.5	0.145
Butyrate	83	81	85	85	84	82	84	83	89	84	84	85	84	3.0	0.669
Isobutyrate	5	5^{cd}	7^{bc}	5^{d}	6^{bcd}	7^{b}	6^{bcd}	6^{bcd}	7^{bc}	6^{bcd}	6^{bcd}	10^{a}	6^{bcd}	0.5	< 0.001
Valerate	19	19^{b}	20^{b}	19^{b}	19^{b}	20^{b}	19^{b}	19^{b}	21^{ab}	19^{b}	18^{b}	25^{a}	19^{b}	1.2	<0.001
Isovalerate	11	11^{bcd}	14^{b}	10^{d}	11^{bcd}	13^{bc}	12^{bcd}	12^{bcd}	14^{b}	11^{cd}	11^{bcd}	18^{a}	11^{bcd}	1.0	< 0.001
NGR[4]	2.65	2.63	2.57	2.62	2.61	2.57	2.52	2.54	2.52	2.67	2.65	2.56	2.55	0.05	0.112

$^{a–d}$Means within a row with different superscripts differ ($P < 0.05$).

[1]LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL: sodium hydroxide pretreated formaldehyde treated crushed linseed; EL1: extruded linseed 1; EL2: extruded linseed 2; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

[2]SED: standard error of difference.

[3]Significance of treatment.

[4]NGR = nonglucogenic to glucogenic VFA ratio calculated as [(acetate + 2 * (butyrate + isobutyrate) + valerate + isovalerate)]/(propionate + valerate + isovalerate).

Table 4. Effect of control treatment (CON) and linseed treatments[1] on gas production kinetics[2] during 24 h of incubation

Parameter	Treatments													SED[3]	Significance[4]
	CON	LO	CL	FCL	SFCL	MCL	ECL1	ECL2	CEL	EL1	EL2	LELO	LO+DHA		
OMCV	126	119	120	107	114	113	116	115	119	117	110	127	110	5.34	0.059
A	169	162^{a}	151^{abc}	162^{a}	154^{abc}	154^{abc}	141^{abc}	137^{bc}	138^{abc}	150^{abc}	136^{c}	161^{ab}	144^{abc}	6.86	0.002
B	1.49	1.44^{bc}	1.61^{a}	1.41^{c}	1.55^{abc}	1.44^{bc}	1.55^{ab}	1.61^{a}	1.62^{a}	1.46^{abc}	1.54^{abc}	1.52^{abc}	1.53^{abc}	0.04	< 0.001
C	11.51	11.42^{bc}	10.13^{bcd}	14.61^{a}	11.96^{b}	11.61^{bc}	8.85^{d}	8.76^{d}	8.32^{d}	9.90^{bcd}	9.57^{cd}	9.82^{bcd}	10.80^{bcd}	0.61	< 0.001
Tmax	3.67	3.47^{bcd}	4.07^{ab}	3.92^{abc}	4.40^{a}	3.53^{bcd}	3.28^{cd}	3.49^{bcd}	3.34^{bcd}	3.14^{d}	3.46^{bcd}	3.42^{bcd}	3.76^{abcd}	0.21	< 0.001
Rmax	9.29	8.88^{abcd}	9.20^{abcd}	7.16^{e}	7.90^{de}	8.21^{cde}	9.84^{a}	9.71^{ab}	10.40^{a}	9.40^{abc}	8.78^{abcd}	10.06^{a}	8.37^{bcde}	0.40	< 0.001

[a–e]Means within a row with different superscripts differ ($P < 0.05$).

[1]LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL sodium hydroxide pretreated formaldehyde treated crushed linseed; EL1: extruded linseed 1; EL2: extruded linseed 2; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

[2]OMCV: cumulative gas production (ml per g incubated OM); A: estimate asymptotic gas production (mL per g incubated OM); B: constant that determines the sharpness of the switching characteristic of the gas production profile; C: time after incubation at which half of the asymptotic gas production has been reached (h), Tmax: time to reach the maximum rate of gas production (h); Rmax: maximum rate of gas production (mL per h).

[3]SED: standard error of difference.

[4]Significance of treatment.

Table 5. Proportion of C18:3*n*3 after 24 h of incubation and estimated biohydrogenation kinetic parameters for C18:3*n*3 for control treatment (CON) and linseed treatments[1]

Parameter	Treatments													SED[2]	Significance[3]
	CON	LO	CL	FCL	SFCL	MCL	ECL1	ECL2	CEL	EL1	EL2	LELO	LO+DHA		
C18:3*n*3, %[4]	0.21	0.37[abcd]	0.40[abcd]	0.59[abc]	0.54[abc]	0.35[abcd]	0.30[cd]	0.31[bcd]	0.22[d]	0.66[a]	0.63[ab]	0.16[d]	0.44[abcd]	0.08	<0.001
kh, %/h[5]	14.04	6.65[b]	4.13[b]	2.47[b]	2.61[b]	4.53[b]	5.93[b]	5.69[b]	8.04[ab]	1.79[b]	2.23[b]	13.10[a]	4.42[b]	1.45	<0.001
Lag time, h	0	1.49	2.21	2.99	0.06	2.01	2.00	2.12	2.00	0.25	1.49	3.43	1.37	0.99	0.149
Eff. bh, %[6]	70.0	43.5[abc]	35.6[bcd]	24.3[d]	30.1[cd]	38.1[bcd]	43.7[abc]	42.9[abc]	50.8[ab]	22.7[d]	25.0[d]	55.5[a]	38.3[abcd]	4.30	<0.001

[a–d]Means within a row with different superscripts differ ($P < 0.05$).

[1] LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL sodium hydroxide pretreated formaldehyde treated crushed linseed; EL1: extruded linseed 1; EL2: extruded linseed 2; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

[2] SED: standard error of difference.

[3]Significance of treatment.

[4]C18:3*n*3: proportion of C18:3*n*3 of total C18 FA after 24 h of incubation relative to 0 h sample.

[5]kh: fractional rate of biohydrogenation.

[6]Eff. bh: calculated effective biohydrogenation assuming a fractional passage rate of 0.06/h

except for LO, where a maximum proportion of 11.1 g *trans*-10+11-C18:1/100 g total C18 FA was reached after 12 h of incubation. Proportion of *trans*-10+11-C18:1 at 24 h was greater for the CEL, ECL1, ECL2, LELO, and LO+DHA treatments compared with the FCL, SFCL, EL1, and EL2 treatments ($P < 0.05$). Compared with the LO treatment, all linseed treatments except CEL and LELO showed a lesser C18:0 proportion after 24 h of incubation ($P < 0.05$). This decrease was more important for the LO+DHA treatment, which showed only a slight increase in C18:0 proportion during the 24 h of incubation.

The biohydrogenation kinetic parameters for C18:3*n*3 are presented in Table 5. The fractional biohydrogenation rate was greater ($P < 0.001$) for the LELO treatment compared to the other treatments; only CEL showed intermediate results. The lag time was not different between the various linseed treatments ($P > 0.05$). The calculated effective biohydrogenation was significantly influenced by chemical and technological treatment of linseed. The FCL, EL1, and EL2 treatments resulted in a lesser effective biohydrogenation of C18:3*n*3 compared with the LO, ECL1, ECL2, CEL, and LELO treatments.

Discussion

Modelling biohydrogenation kinetic parameters

The results of our experiment show a large variation in the rate and extent of biohydrogenation of C18:3*n*3 across substrates. Fractional biohydrogenation rate of C18:3*n*3 was significantly affected by treatment, but lag time was not affected. Enjalbert et al. (2003) reported a negative lag time in their in vitro experiment to evaluate biohydrogenation kinetics of UFA. However, such results are physiologically not acceptable and can be indicative of an inappropriate model or data that are not compatible with the requirements of the model (Dhanoa et al., 1996). Sinclair et al. (2005) evaluated the biohydrogenation of protected linseed sources in vitro and reported fractional biohydrogenation rates without accounting for a lag time. Lag time is related to the time needed for lipolysis, is dependent on fat source, and might be associated with DM digestibility and time for the microbes to adapt to the substrate and incubation conditions (Ribeiro et al., 2007). In our experiment, the model with a lag time constrained to be positive resulted in a better model fit than without a lag time, and estimated lag time differed significantly from zero in 13 of the 24 replicates (results not shown). Unesterified FA at 0 h of incubation may contribute to the differences in observed lag times (Ribeiro et al., 2007). To account for the reported biohydrogenation rates with or without lag time, effective biohydrogenation was calculated according to Dhanoa et al. (1999) and used to compare the effectiveness of protection.

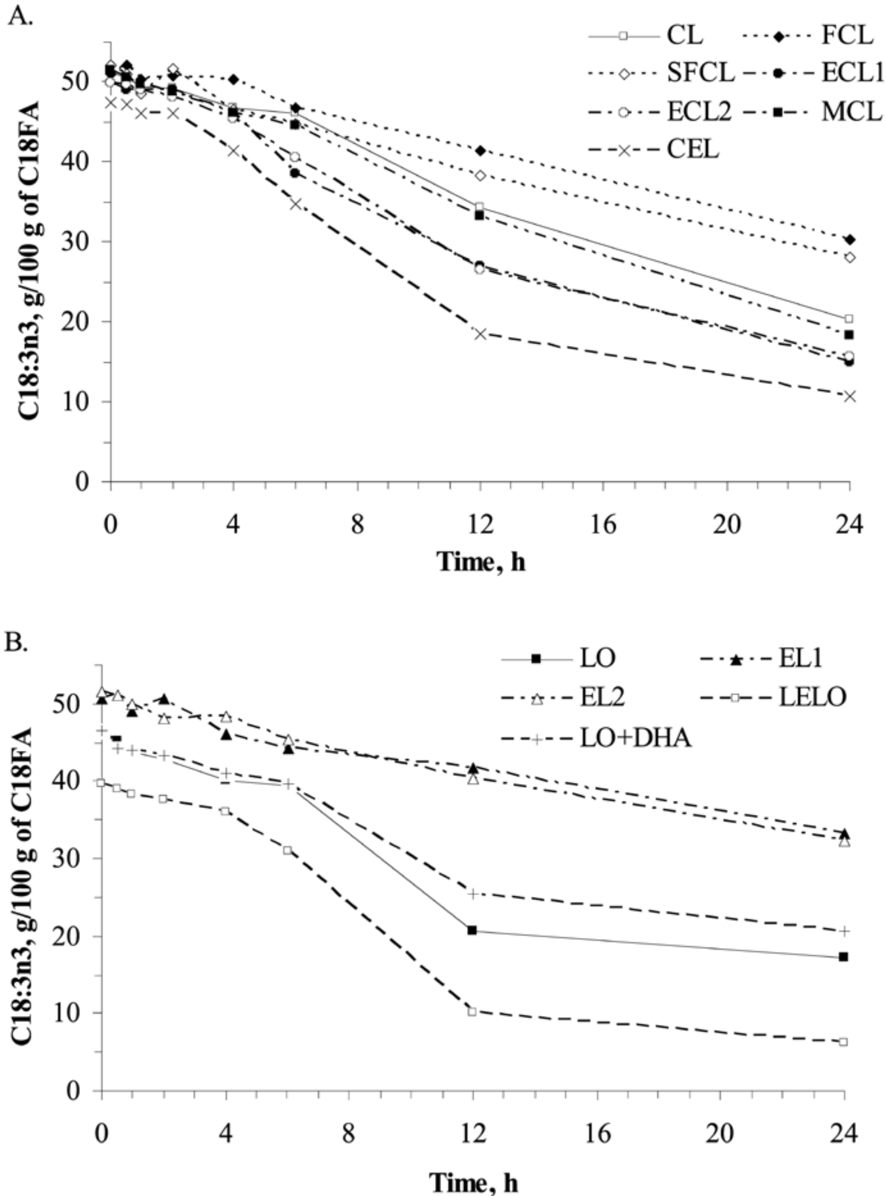

Figure 1. Changes in proportion of C18:3*n*3 for linseed treatments during 24 h of incubation (treatment: $P < 0.001$; time: $P < 0.001$; treatment x time: $P < 0.001$; SED: 1.88). LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL sodium hydroxide pretreated formaldehyde treated crushed linseed; EL1: extruded linseed 1; EL2: extruded linseed 2; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

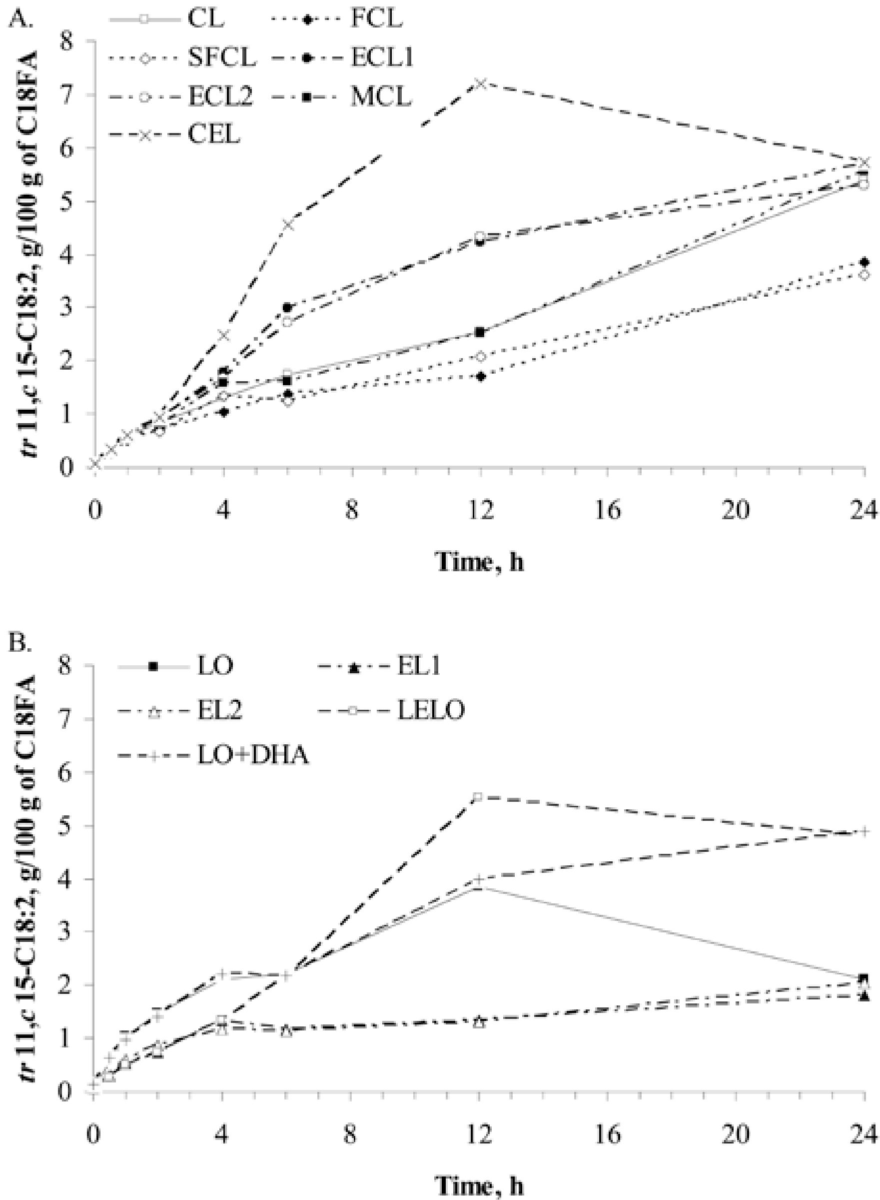

Figure 2. Changes in proportion of *trans*-11,*cis*-15-C18:2 for linseed treatments during 24 h of incubation (treatment: $P < 0.001$; time: $P < 0.001$; treatment x time: $P < 0.001$; SED: 0.48). LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL sodium hydroxide pretreated formaldehyde treated crushed linseed; EL1: extruded linseed 1; EL2: extruded linseed 2; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

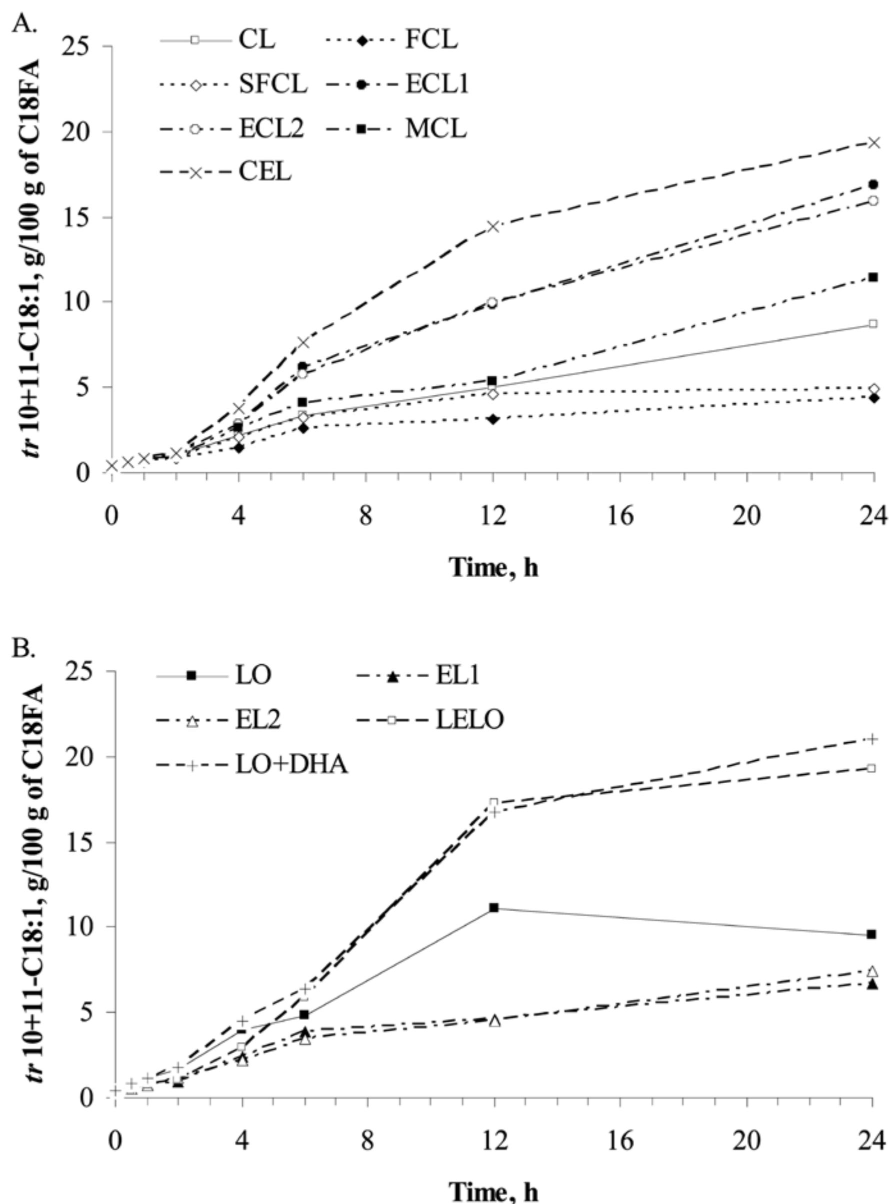

Figure 3. Changes in proportion of *trans*-10+11-C18:1 for linseed treatments during 24 h of incubation (treatment: $P < 0.001$; time: $P < 0.001$; treatment x time: $P < 0.001$; SED: 1.39). LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL sodium hydroxide pretreated formaldehyde treated crushed linseed; EL1: extruded linseed 1; EL2: extruded linseed 2; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

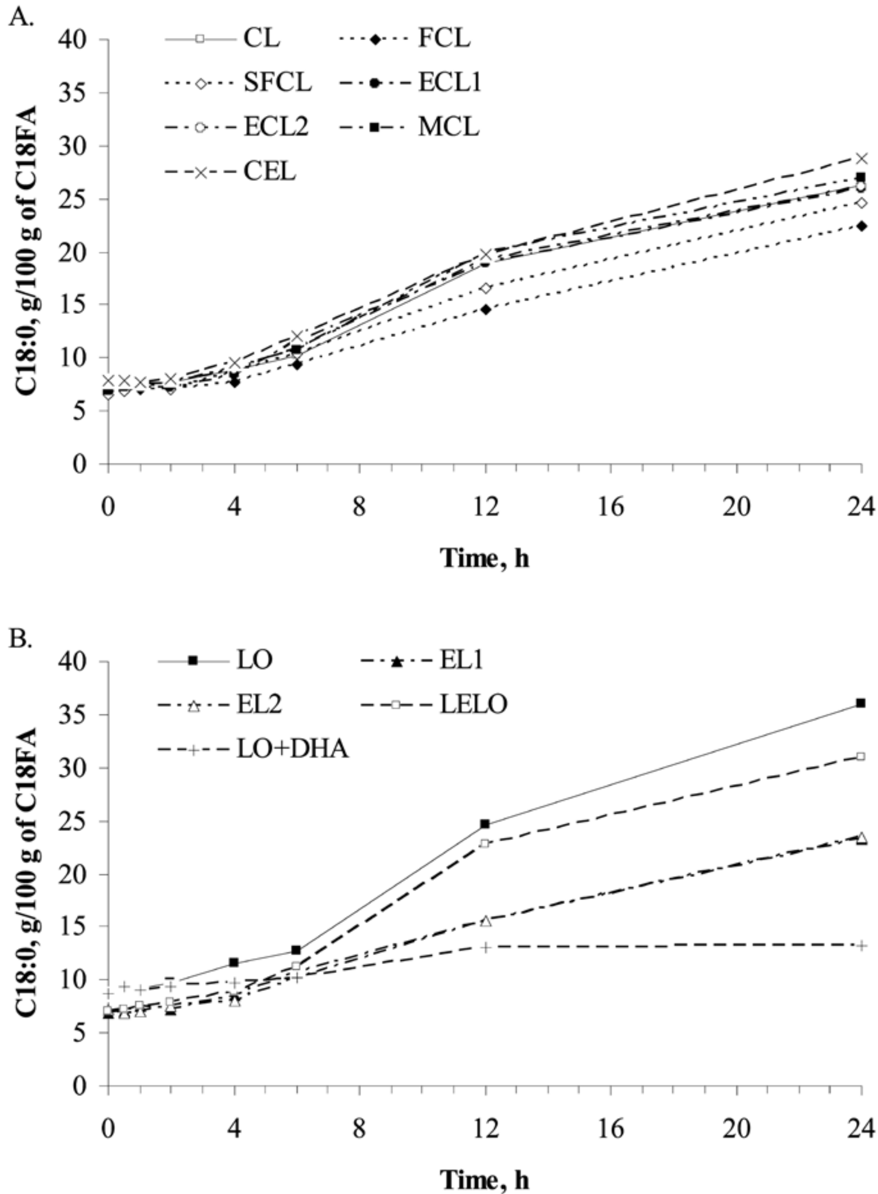

Figure 4. Changes in proportion of C18:0 for linseed treatments during 24 h of incubation (treatment: $P < 0.001$; time: $P < 0.001$; treatment x time: $P < 0.001$; SED: 1.88). LO: linseed oil; CL: crushed linseed; FCL: formaldehyde treated crushed linseed; SFCL sodium hydroxide pretreated formaldehyde treated crushed linseed; EL1: extruded linseed 1; EL2: extruded linseed 2; ECL1: extruded crushed linseed 1; ECL2: extruded crushed linseed 2; MCL: micronized crushed linseed; CEL: commercial extruded linseed product; LELO: lipid encapsulated linseed oil; LO+DHA: linseed oil and DHA Gold (Martek Biosciences Corp., Columbia, MD).

Rumen fermentation pattern

Troegeler-Meynadier et al. (2003) examined the effect of pH on biohydrogenation of C18:2*n*6 and C18:3*n*3 and concluded that biohydrogenation of C18:2*n*6 and C18:3*n*3 was inhibited when pH was below 6.0 compared with above 6.5. In the present experiment, fluid pH showed a decrease with time, but incubation flasks were buffered sufficiently to maintain a minimal pH above 6.1 after 24 h of incubations for all treatments. Therefore, it is expected that biohydrogenation of C18:3*n*3 was not influenced by the pH change in time. Total VFA concentration, acetate, propionate, butyrate proportions, and the ratio of nonglucogenic to glucogenic VFA were not different between the treatments, indicating no differences in fermentation pattern. Ribeiro et al. (2005) concluded that changes in fermentation pattern likely reflect shifts in the bacterial population in response to changes in fermentable substrates. The results of the present trial therefore indicate that no shift occurred in bacterial population for the different linseed products. The proportions of valerate and branched-chain VFA did differ between the treatments, with the greatest proportions found in the lipid encapsulated linseed oil treatment compared with the other treatments except the commercial extruded linseed product for the valerate proportion. Valerate and branched-chain VFA result mainly from fermentation of protein (Bannink et al., 2006); therefore, these changes are probably related to the canola meal, which was used as the carrier product for the linseed oil.

Overall cumulative gas production did not differ between the treatments, whereas some of the gas production curve parameters did differ between treatments. However, no clear effect was found of one of the treatments on these parameters. Sinclair et al. (2005) did not observe differences in gas production profiles when different treatments rich in C18:3*n*3 were incubated in vitro for 48 h. Organic matter degradability was greater in the CEL treatment compared with the FCL treatment. The CEL treatment contained 56% crushed linseed and 44% other products including wheat and sunflower cake, which might be responsible for the greater OM degradability. In view of the moderate decrease in pH and the absence of differences in total gas production and in the major VFA, it appears that no differences in rumen fermentation exist between tested linseed products.

C18:3*n*3 biohydrogenation

Gonthier et al. (2005) hypothesized that chemical treatments such as formaldehyde treatment could be more effective than heat treatment in the protection of UFA from ruminal biohydrogenation. Sinclair et al. (2005) observed in vitro that pretreatment of linseed with sodium hydroxide or formic acid followed by treatment with formaldehyde resulted in effective protection of C18:3*n*3. This observation was confirmed by Fievez et al. (2007), who concluded that oilseed pretreatment, either chemically or through emulsification, is essential for the formation of the inert formaldehyde-protein matrix. In the present experiment, we hypothesized that technological pretreatment (crushing) would be as effective as chemical pretreatment (sodium hydroxide) in inducing permeability of the seed coat and thereby as effective in forming the inert formaldehyde-protein matrix resulting in protection against biohydrogenation. Indeed, formaldehyde treatment of crushed linseed showed to be effective in reducing biohydrogenation of C18:3*n*3 compared with linseed oil. Additional pretreatment with sodium hydroxide before applying formaldehyde treatment did not improve the effectiveness of

protection any further; numerically, additional pretreatment with sodium hydroxide resulted in lesser effective protection compared with crushing only as pretreatment. These results confirm that crushing makes the protein of the oilseed accessible to formaldehyde to form the inert formaldehyde-protein matrix. Indeed, Petit (2003) did not show an effective protection of C18:3*n*3 from linseed that was protected by spraying the formalin (37% formaldehyde) on the whole seed, whereas Goodridge et al. (2001) found an increased protection of C18:3*n*3 from ground linseed protected by encapsulation in a matrix of aldehyde treated protein.

Because of the size of whole linseed, it is expected that the seed coat will not be crushed completely during chewing and ruminating, resulting in less oil being released and available for biohydrogenation (Petit, 2003). However, the seed coat will also limit digestion postruminally, thus preventing complete release and absorption of the desired internal PUFA (Jenkins, 2006). The physical crushing of linseed may therefore contribute to an increased availability of UFA for absorption and hence, biohydrogenation, and possible transfer into milk fat (da Silva et al., 2007). Feeding ground linseed indeed increased the proportions of C18:3*n*3 and *trans*-FA in milk fat compared with feeding whole linseed (da Silva et al., 2007). Compared with linseed oil, crushed linseed may result in lesser biohydrogenation because of the localization of the oil in the seed or meal (Chilliard et al., 2000). In the current experiment, crushed linseed and linseed oil did not differ significantly in effective biohydrogenation of C18:3*n*3. Sinclair et al. (2005) evaluated the differences in biohydrogenation of C18:3*n*3 between linseed oil and ground linseed that was preground for 5 s in a coffee grinder and also did not observe differences in C18:3*n*3 biohydrogenation.

Extrusion of oilseeds may contribute to an increased availability of UFA for absorption by rupturing the seed to liberate the oil from the seed cells. This process might influence the production of intermediates and end products of biohydrogenation (Dhiman et al., 1999; Neves et al., 2007). It was hypothesized that extrusion of whole linseed versus extrusion of crushed linseed under different processing conditions (steam and water percentage) might result in differences in postruminal UFA flow. The extruded whole linseed treatments resulted in lesser calculated effective biohydrogenation of C18:3*n*3 compared with the LO, extruded crushed linseed treatments, CEL, and LELO treatments. The extrusion process possibly ruptured the whole seeds only to a certain extent, leaving the seed coat intact in part of the linseeds. Because the intact seed coat provides an effective barrier against biohydrogenation (Jenkins, 2006), the C18:3*n*3 inside the whole seeds was protected against biohydrogenation. Overprotection by the seed coat, however, might prevent the release and absorption of the C18:3*n*3 postruminally, which was not measured in this study. Different conditions during extrusion (6% steam and 2% water vs. 2% steam and 6% water) did not result in differences in biohydrogenation for both the extruded whole linseed treatments and the extruded crushed linseed treatments. Chouinard et al. (2001) studied the effect of ground soybeans extruded at 120°C, 130°C, and 140°C on milk FA composition and found no differences in C18:3*n*3 proportion in milk fat. In the present study, extrusion temperature was 127°C for the extruded whole linseed product with 6% steam and 2% water (EL1), 130°C for the extruded whole linseed product with 2% steam and 6% water (EL2), 115°C for the extruded crushed linseed product with 6% steam and 2% water (ECL1), and 118°C for the extruded crushed linseed product with 2% steam and 6% water (ECL2). The

processing parameters resulted in a very small temperature range, which did not induce differences in biohydrogenation kinetics between the products.

Micronisation is a heat treatment in which the seed is rapidly heated internally accompanied by a rise in water vapour pressure. The micronisation process will cook the seed from inside out and the seed will expand to the point of eversion (Wang et al., 1997). Petit et al. (2002b) showed that micronisation at different temperatures (130°C, 140°C, 150°C, and 160°C) resulted in a similar loss of C18:3*n*3 after incubation, except for the linseed micronized at 160°C for 0.5 h, which reduced C18:3*n*3 proportion because of FA oxidation. These researchers suggested that micronisation temperature should not exceed 130°C to protect linseed. In the present study, a micronisation temperature of 115 to 120°C was used for 90 s; however, no difference was found in effective biohydrogenation of C18:3*n*3 between the MCL treatment and the LO treatment. This finding was in agreement with the results of Mustafa et al. (2003), who showed that micronized linseed (115°C for 1.5 min) was extensively biohydrogenated in the rumen.

Encapsulation of UFA in a sphere of high melting point saturated FA was hypothesized as a possible way of protecting UFA against biohydrogenation (Jenkins and Bridges, 2007). Perfield et al. (2004) showed that a lipid encapsulated conjugated linoleic acid (Balchem Encapsulates, New Hampton, NY), a stable powder at room temperature, decreased milk fat to the same extent as an amide protected conjugated linoleic acid, suggesting an equal amount of protection in the rumen and an equal postruminal availability. In the present experiment, linseed oil was incorporated into canola meal and coated with pork fat. This product, however, resulted in the greatest fractional biohydrogenation rate compared to all other linseed products and therefore clearly showed no protection against biohydrogenation.

Microbial population characteristics are associated with the extent of biohydrogenation in the rumen. Recent in vitro (Vlaeminck et al., 2008) and in vivo (Boeckaert et al., 2008a) research showed that DHA addition results in a significant reduction of the conversion of *trans*-11-C18:1 to C18:0 through changes in the rumen microbial population. In the current experiment, DHA (DHA Gold) was added to a linseed oil treatment. The calculated effective biohydrogenation of C18:3*n*3 did not differ from that of the other treatments. The proportion of *trans*-10+11-C18:1, however, was significantly greater and the proportion of C18:0 was significantly lesser after the 24-h incubation period. Vlaeminck et al. (2008) showed an increased proportion of *trans*-11-C18:1 and *trans*-11,*cis*-15-C18:2 when DHA was added to rumen fluid. The present study also showed an increased proportion of *trans*-11,*cis*-15-C18:2 after 24 h of incubation. This increased *trans*-11,*cis*-15-C18:2 proportion, however, was comparable to the increase in *trans*-11,*cis*-15-C18:2 proportion in the other treatments. This comparable increase of *trans*-11,*cis*-15-C18:2 in all treatments suggests that, unlike the rate of biohydrogenation of *trans*-10+11-C18:1, the rate of biohydrogenation of *trans*-11,*cis*-15-C18:2 was not different compared with the other treatments. It was therefore suggested that the addition of DHA in combination with linseed oil was effective only in inhibiting the last step of biohydrogenation from *trans*-10+11-C18:1 to C18:0.

Conclusions

Technological pretreatment (crushing) followed by chemical treatment applied as formaldehyde of linseed was effective in protecting C18:3*n*3 against biohydrogenation in vitro. Additional chemical pretreatment (sodium hydroxide) before formaldehyde treatment did not further improve the effectiveness of protection. Extrusion of whole linseed compared with extrusion of crushed linseed was effective in reducing C18:3*n*3 biohydrogenation, whereas steam and water percentage did not influence C18:3*n*3 biohydrogenation. Crushing linseed, micronizing crushed linseed, incorporating linseed oil into canola meal and coating with pork fat, and adding DHA in combination with linseed oil did not reduce C18:3*n*3 biohydrogenation. Addition of DHA in combination with linseed oil resulted in a comparable *trans*-11,*cis*-15-C18:2 biohydrogenation and a lesser *trans*-10+11-C18:1 biohydrogenation compared with the other treatments. This suggests that addition of DHA in combination with linseed oil was effective only in inhibiting the last step of biohydrogenation from *trans*-10+11-C18:1 to C18:0. Regarding all evaluated linseed products, only FCL and extruded whole linseed show a potential use in the ruminant diet to increase post-ruminal C18:3*n*3 flow.

Acknowledgements

The authors would like to thank the laboratory staff of the Animal Nutrition Group, Wageningen University (the Netherlands), for their help in chemical analysis and Tamme Zandstra and Sven Alferink (both of Animal Nutrition Group, Wageningen University, Wageningen, the Netherlands) for their assistance in preparing the linseed products. The financial support of the Food and Nutrition Delta program of Senter Novem (Den Haag, the Netherlands), Royal FrieslandCampina (Amersfoort, The Netherlands), Agrifirm Group (Apeldoorn, The Netherlands), Agrifirm Innovation Center (Apeldoorn, The Netherlands), and Barenbrug Holland B.V. (Oosterhout, The Netherlands) is gratefully acknowledged. Bruno Vlaeminck is a Postdoctoral Fellow of the Fund for Scientific Research-Flanders (Belgium).

Chapter 4

Effects of feeding different linseed sources on omasal fatty acid flows and C18:*3n3* biohydrogenation in lactating dairy cows

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Submitted

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Abstract

The aim of this experiment was to study the effects of feeding different linseed sources on omasal fatty acid flows and C18:3*n*3 biohydrogenation in dairy cows. In a 4 × 4 Latin square design, four ruminally cannulated lactating Holstein Friesian cows were assigned to four dietary treatments, consisting of crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed oil (FL) and linseed oil in combination with marine algae rich in docosahexaenoic acid (DHA; DL). Each period in the Latin square design was 21 d with the first 16 d for adaptation. Omasal flow was estimated using Cr-EDTA, Yb-acetate and ADL as digesta flow markers. Average feed intake was 20.6 ± 2.5 kg DM/d and average C18:3*n*3 intake was 341 ± 51 g/d. Omasal flows of OM (8.50 ± 1.40 kg/d), NDF (2.88 ± 0.78 kg/d), and CP (3.06 ± 0.51 kg/d) did not differ between treatments. Rumen digestibilities of DM (37.0 ± 5.0%), OM (55.9 ± 2.7%) and NDF (61.3 ± 7.0%) were similar for the linseed treatments. Whole tract digestibility of crude fat was lower for the EL treatment (64.8%) compared with the CL treatment (71.3%) and both the EL and CL treatment were lower than the FL (78.5%) and DL (80.4%) treatments. Omasal flow of C18:3*n*3 was higher for the EL treatment (33.8 g/d) compared with the CL (21.8 g/d) and FL (15.5 g/d) treatments, which were higher compared with the DL treatment (4.6 g/d). This resulted in a lower C18:3*n*3 biohydrogenation for the EL treatment (90.9%) compared with the CL (94.0%) and FL (95.4%) treatments. The DL treatment resulted in the highest extent of C18:3*n*3 biohydrogenation (98.5%). Flows of total *trans*-C18:1 isomers were higher in the DL treatment (357.2 g/d) compared with the CL (98.7 g/d), EL (76.6 g/d) and FL (82.8 g/d) treatments, while flow of C18:0 was lower for the DL treatment (148.0 g/d) compared with the CL (368.5 g/d), EL (342.6 g/d) and FL (331.6 g/d) treatments. The results indicate that feeding extruded whole linseed results in a higher omasal C18:3*n*3 flow and consequently a lower extent of C18:3*n*3 biohydrogenation, while total tract digestibility of crude fat is decreased. Feeding formaldehyde-treated linseed oil does not increase omasal flow of C18:3*n*3 compared with the unprotected crushed linseed. Adding linseed oil in combination with DHA results in a low omasal C18:3*n*3 flow and a high extent of C18:3*n*3 biohydrogenation, whereas omasal flow of C18:0 is lower and flows of biohydrogenation intermediates are markedly increased.

Introduction

Increasing the level of polyunsaturated long-chain fatty acids (FA), including linolenic acid (*cis*-9,*cis*-12,*cis*-15-C18:3; C18:3*n*3), at the expense of saturated fatty acids, is considered an attractive way to modify milk fat composition. Several roughages, especially fresh and ensiled grass, have a high proportion of C18:3*n*3 in the total content of FA. C18:3*n*3 is also found in non-roughage feedstuffs, with linseed being an oilseed that contains a high proportion of C18:3*n*3 (> 50 % of FA; Chilliard et al., 2000). However, apparent transfer efficiency of C18:3*n*3 from feed to milk is low (Glasser et al., 2008) and is related to the extensive biohydrogenation of C18:3*n*3 by ruminal bacteria (Harfoot and Hazlewood, 1997). Several technologies have been developed to prevent lipolysis and biohydrogenation of FA in the rumen (Fievez et al., 2007). Several studies reported postruminal flows of FA in lactating cows fed diets with different vegetable oils or oilseeds (Gonthier et al., 2004; Loor et al., 2005b; Shingfield et al., 2008). Duodenal C18:3*n*3 flow increased when diets were supplemented with linseed (Gonthier et al., 2004) or linseed oil (Loor et al., 2005b). In addition, Gonthier et al. (2004) showed that feeding micronized linseed results in a higher C18:3*n*3 flow compared with feeding extruded linseed. However, biohydrogenation of C18:3*n*3 was high in all treatments, varying between 92.9% for the micronized linseed and 96.6% for the extruded linseed. Addition of docosahexaenoic acid (C22:6*n*3; DHA) to a diet including linseed oil can effectively change biohydrogenation of C18:3*n*3 by inhibition of complete biohydrogenation to C18:0 as shown in vitro (Sterk et al., 2010). However, no in vivo studies have been reported that determined omasal FA flows when DHA was added to a diet containing linseed oil.

In a previous in vitro study, several chemically or technologically treated linseed products were evaluated in order to decrease ruminal biohydrogenation of C18:3*n*3, with the most promising treatments being formaldehyde-treated crushed linseed and extruded whole linseed (Sterk et al., 2010). However, in vitro procedures tend to overestimate the extent of rumen by-pass C18:3*n*3 (Fievez et al, 2007) and the true extent of rumen inertness of these linseed products should be determined in vivo. To our knowledge, a comparison of flows of C18:3*n*3 from the rumen with crushed linseed, extruded whole linseed, formaldehyde-treated linseed oil and linseed oil in combination with DHA has not been reported. Therefore, the objective of this study was to evaluate the effects of crushed linseed, extruded whole linseed, formaldehyde-treated linseed oil, and linseed oil in combination with marine algae rich in DHA on FA flows through the gastro intestinal tract of lactating dairy cows. Results on production performance and plasma and milk FA profiles are reported in a companion paper (Chapter 5).

Materials and Methods

Experimental design, animals and housing

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Wageningen University and carried out under the Dutch Law on Animal Experimentation. Four lactating multiparous Holstein-Friesian dairy cows (625 ± 69 kg BW; 52 ± 22 DIM; values expressed

as means ± SD) fitted with a ruminal cannula (10 cm i.d.; Bar Diamond Inc., Parma, ID) were fed different linseed diets according to a 4 × 4 Latin square design. Each period in the Latin square design lasted 21 d with the first 16 d for adaptation. Cows were housed in individual tie-stalls with continuous access to water and milked twice daily at 0630 and 1700h.

Diets

Dietary treatments consisted of a basal diet with the addition of 1) crushed linseed (CL), 2) extruded whole linseed (EL), 3) formaldehyde-treated linseed oil (FL), and 4) DHA in combination with linseed oil (DL). The ingredient and chemical composition of the four diets are shown in Table 1 and 2, respectively. The diets were designed to provide equal amounts of C18:3*n*3. Crushed linseed was prepared in a roller mill (0.25 mm; Ipswich Turner, Christy Turner Ltd, Ipswich, UK). Extruded whole linseed was prepared as a mixture of whole linseed and wheat bran (70:30 vol/vol linseed:wheat bran) in a small scale single screw extruder line of Almex AL150 (Almex, Zutphen, the Netherlands) equipped with a pellet press of Robinson/Heesen V2/30 (Heesen, Boxtel, the Netherlands) and cooler unit (6% steam and 2% water, 127°C for 20-30 s). Formaldehyde-treated linseed oil was prepared by homogenizing Na-casein and linseed oil (35:65 vol/vol Na-casein:linseed oil) and spray drying the emulsion in a conventional spray dryer with a nozzle atomizer (Spray dryer P12.5, Gea Niro, Soeborg, Denmark) and an external fluid bed. The spray dried emulsion was then treated with 0.65% formalin (37% formaldehyde). The DHA was supplemented as a concentrate containing 11% DHA (product basis; DHA Gold; Martek Biosciences Corp., Columbia, MD) and the linseed oil was provided as such. To prevent variation in feed intake and C18:3*n*3 intake, diets were offered at 95% of ad libitum intake, which was measured during the first 7 days of the first experimental period. The diets were offered as two equal meals at 0615 and 1645h. The concentrates and linseed products were thoroughly mixed with the basal diet just before feeding.

Measurements and sampling

Feed intake measurements determined from day 15 to day 20 of each experimental period were used to calculate average nutrient intake per cow per period. All ration ingredients were sampled weekly and pooled per period. The pooled samples of grass and maize silage were stored at -20°C, freeze-dried and ground to pass through a 1 mm sieve before analysis. The pooled samples of the concentrates and linseed products were ground to pass through a 1 mm sieve and stored at 4°C pending analysis.

The digesta flow into the omasum was assessed by the triple marker method (France and Siddons, 1986) using Cr-EDTA, Yb-acetate, and acid detergent lignin (ADL) as indigestible markers for liquid, small particle, and large particle phases, respectively. Cr-EDTA was prepared using standard procedures (Binnerts et al., 1968) and Yb-acetate was obtained from a commercial source (Dasico A/S, Birkerød, Denmark). ADL was used as an internal marker naturally present in the diet (Table 2). Starting at day 15, Cr-EDTA (3.0 g Cr/d) and Yb-acetate (1.7 g Yb/d) were dissolved in 2 litres distilled water and infused via separate lines into the rumen at a constant rate (83 mL/h) using a peristaltic pump (Isco WIZ Peristaltic Pump Diluter Dispenser, ISCO Inc., Lincoln, NE). Infusions

Table 1. Ingredient composition of the diets supplemented with crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed oil (FL) and docosahexaenoic acid addition to linseed oil (DL; g/kg DM; DM basis)

Ingredient	Dietary treatment			
	CL	EL	FL	DL
Grass silage[1]	312	310	312	312
Maize silage[2]	294	292	294	294
Wheat	68	64	73	77
Rapeseed meal	65	61	69	69
Soybean meal	49	47	50	49
Maize	52	45	60	54
Palm kernel expeller	22	19	25	22
Soybean hulls	21	18	24	22
Rapeseed expeller	16	14	18	16
Beet pulp	11	9	12	22
Molasses	13	11	15	16
Rapeseed meal, formaldehyde-treated	8	7	9	8
Maize gluten feed	-	-	-	4
Toasted soybean expeller	-	-	-	2
Soybean meal, formaldehyde-treated	-	-	-	2
Premix[3]	2	1	2	2
Limestone	2	1	2	2
Salt	1	1	1	1
Palm oil	1	1	1	1
Crushed linseed	65	-	-	-
Extruded linseed	-	99	-	-
Formaldehyde-treated linseed oil	-	-	33	-
Linseed oil	-	-	-	21
DHA Gold[4]	-	-	-	4

[1]Grassilage, g/kg DM: 439 DM (fresh weight basis), 89 crude ash, 188 CP, 33 crude fat, 530 NDF, 34 sugar, 75 DVE, 6.07 MJ NE_L, 18.45 total fatty acids, 0.20 C12:0, 0.15 C14:0, 3.40 C16:0, 0.50 C16:1, 0.25 C18:0, 0.59 *cis*-9-C18:1, 3.25 *cis*-9,*cis*-12-C18:2, 10.11 *cis*-9,*cis*-12,*cis*-15-C18:3.
[2]Maize silage, g/kg DM: 328 DM (fresh weight basis), 46 crude ash, 64 CP, 29 crude fat, 399 NDF, 340 starch, 45 DVE, 6.69 MJ NE_L, 19.26 total fatty acids, 0.03 C12:0, 0.03 C14:0, 3.08 C16:0, 0.48 C18:0, 4.23 *cis*-9-C18:1, 10.45 *cis*-9,*cis*-12-C18:2, 0.94 *cis*-9,*cis*-12,*cis*-15-C18:3.
[3]Contained per kg of mix: 93 g of Ca, 400 g of Mg, 5 mg of S, 4 g of Cu, 3.3 g of Mg, 322 mg of I, 97 mg of Co, 80 mg of Se, 2600000 IU of vitamin A, 580000 IU of vitamin E (Premix 2033, PreMervo, Utrecht, The Netherlands).
[4]Martek Biosciences Corp., Columbia, MD.

of Cr-EDTA and Yb-acetate started with primer doses of 4.5 and 2.6 g Cr and Yb, respectively. These primer doses were used to reach a rapid equilibrium of the ruminal marker concentrations. Marker infusions were stopped on day 19 when the last digesta sample was taken.

Samples (775 g) of digesta flowing into the omasal canal were collected three times daily at 4-h intervals on day 18 and 19 using the omasal sampling device (Huhtanen et al., 1997) with modifications (Ahvenjärvi et al., 2000). In addition, the sampling device was adjusted by adding a rugby ball shaped device with 13 openings (8 mm i.d.) to the tube orifice to prevent the tube from being blocked by coarse

Table 2. Chemical and fatty acid composition of the diets supplemented with crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed oil (FL) and docosahexaenoic acid addition to linseed oil (DL)

Composition	Dietary treatment			
	CL	EL	FL	DL
Nutrients, g/kg DM				
DM, g/kg	583	589	583	582
CP	170	171	171	163
Crude fat	62	65	55	56
NDF	360	361	354	354
ADF	216	215	213	212
ADL	32	32	31	31
Starch	148	147	152	153
Sugar	38	38	39	40
Crude ash	68	67	69	69
DVE[1]	84	83	90	87
NE_L, MJ/kg DM[2]	7.14	7.09	7.20	7.23
Fatty acids, g/kg DM				
Total fatty acids	45.44	46.35	44.48	44.34
C12:0	1.15	1.01	1.51	1.21
C14:0	0.43	0.38	0.56	0.71
C16:0	4.52	4.63	4.66	5.06
C16:1	0.19	0.19	0.20	0.20
C18:0	1.32	1.36	0.95	1.21
Cis-9-C18:1	8.79	8.92	8.99	8.53
Cis-9,*cis*-12-C18:2	11.78	11.98	11.92	11.60
Cis-9,*cis*-12,*cis*-15-C18:3	17.26	17.88	15.70	15.22
C22:6*n*3	ND[4]	ND	ND	0.59
UFA[3]	38.02	38.97	36.80	36.14

[1]Intestinal digestible protein (Tamminga et al., 1994).
[2]Net energy for lactation calculated with VEM system (Van Es, 1975).
[3]Unsaturated fatty acids: ∑ (*cis*-9-C18:1, *cis*-9,*cis*-12-C18:2, *cis*-9,*cis*-12,*cis*-15-C18:3, C22:6*n*3).
[4]ND: not detectable.

digesta. The sampling device was installed in the omasum at d 15 at the same time when marker infusions were started. At some sampling points the openings of the sampling device needed to be manually unblocked as described by Brito et al. (2007). Over the two sampling days, a total of six samples per cow were taken with the first sample at 1200h and the last sample at 2200h, thereby covering a 12-h period which was considered representative for an entire feeding cycle. Sub samples for VFA and ammonia-N analysis were immediately taken from each sample and stabilized with phosphoric acid (VFA analysis) and trichloroacetic acid (ammonia-N sample) as described by Taweel et al. (2005). Samples were immediately stored at -20°C after sampling pending analysis. After the collection period, digesta samples were thawed at room temperature, pooled, and separated into large particle, small particle, and liquid fractions by filtration and centrifugation (Ahvenjärvi et al., 2000). Each fraction was freeze-dried and stored at 4°C pending analysis. The large particle fraction was ground to pass a 1 mm sieve before analysis. The relative proportions of the fluid, small particle, and large particle fractions in true digesta were reconstituted using the marker concentrations in the different fractions (France and Siddons, 1986).

Faeces were quantitatively collected for a total of 60 h, starting at 1200h on day 16 and finishing at 0000h on day 19. Every 8 h, the excreted faeces were weighed, thoroughly mixed, and sampled (5%, w/w). Samples were stored at -20°C, then freeze-dried, ground (1 mm), pooled, and stored at 4°C before analysis.

Analytical procedures

The composite samples of the silages, concentrates, and linseed products were analysed for DM, ash, nitrogen (N), crude fat, starch, sugars, NDF, ADF, ADL, and FA methyl esters (FAME). Liquid, small particle and large particle fractions were analysed for DM, ash, N, crude fat, FAME, and marker concentrations (Cr, Yb). Additionally, in the small and large particle fractions NDF and ADL were measured, whereas in the liquid fraction, VFA and ammonia-N were determined. Faecal samples were analysed for DM, crude fat, and marker concentrations (Cr, Yb, and ADL).

Dry matter, ash, N, crude fat, starch, sugars, NDF, ADF, and ADL were analysed as described by Abrahamse et al. (2008a, b). Chromium was determined by carbonization at 550°C followed by combustion at 550°C. The Cr_2O_3 is then solubilized by oxidizing Cr(III) to Cr(VI) by potassium bromate in a phosphoric acid manganese solution. After dilution, Cr(VI) was measured by ICP-AES (Perkin Elmer Optima 3300 DV ICP, Groningen, The Netherlands). Ytterbium was determined by carbonization at 550°C followed by combustion at 550°C. The ash was then destructed in diluted nitric acid and subsequently Yb measured by ICP-AES. The concentration of VFA was determined using gas chromatography (GC type Fisons HRGC MEGA2, Fisons Instruments, Milano, Italy) as described by Taweel et al. (2005). Ammonia-N was determined by spectrophotometry using the Berthelot reaction as described by Taweel et al. (2005).

Fatty acids in feed and omasal samples were determined as described by Khan et al. (2009). Briefly, FA in 375 mg feed and omasal samples were extracted with 15 mL chloroform-methanol (2:1 vol/vol) according to Folch et al. (1957). Internal standard (C13:0) was added with the chloroform-methanol mixture (3 mg C13:0/20 mL of chloroform-methanol). Fatty acids were methylated with 0.5 *N* of NaOH methanolate followed by 1.25 *N* of HCL in methanol and collected in hexane. Hexane was then evaporated and the FAME were resuspended in 1 mL of hexane and transferred to GC vials. The FAME were quantified using gas chromatography (Trace GC Ultra™, Thermo Fisher Scientific, Waltham MA, USA) with a fused silica capillary column (100 m x 0.250 mm and 0.2 µm film thickness; Supelco; SP2560, Bellefonte PA, USA) using helium as a carrier gas at a constant flow of 1.5 mL/min. The flame ionization detector was set at 280ºC. The time-temperature program used, started with an initial temperature of 70 ºC for 4 min, increased with 1 ºC/min to 165 ºC for 20 min, increased with 2 ºC/min to 170 ºC for 10 min, and increased with 4 ºC/min to a final temperature of 215 ºC for 20 min. Fatty acid methyl esters were identified using external standards (S37, Supelco, Bellefonte PA, USA; odd and branched chain fatty acids, *trans*-11-C18:1, *cis*-9,*trans*-11-C18:2, *trans*-10,*cis*-12-C18:2, Larodan Fine Chemicals AB, Malmö, Sweden). The FA *trans*-6+7+8-C18:1, *trans*-10-C18:1, *trans*-12-C18:1, *trans*-13+14-C18:1, *cis*-12-C18:1, *cis*-13-C18:1, *cis*-14+*trans*-16-C18:1, *cis*-15-C18:1, *trans*-11,*cis*-15-C18:2 were identified according to the elution sequence reported by Loor et al. (2004) and Shingfield et al. (2006).

Statistics

Nutrient intake, fermentation characteristics of omasal fluid (averaged per cow per period), nutrient flow into the omasum, and apparent rumen digestibility, rumen biohydrogenation, and faecal digestibility data were analysed as a Latin square design using the MIXED procedure of SAS version 9.2 (SAS Institute, Cary, NC, USA) according to:

$Y_{ijkl} = \mu + T_i + P_j + C_k + \epsilon_{ijkl}$

where Y_{ijkl} is the individual observation, μ the overall mean, T_i the effect of dietary treatment, P_j the effect of experimental period, C_k the effect of cow, and ϵ_{ijkl} the residual error. The effect of cow was treated as a random effect. Significance of treatment effects was declared at $P \leq 0.05$ and trends at $P \leq 0.10$. Post-hoc analyses were carried out using the Tukey test to test pairwise comparisons. Data are reported as least square means ± SEM.

Results

Nutrient intake

The experimental diets were formulated to provide equal amounts of C18:3*n*3. However, the dietary C18:3*n*3 content of the FL and DL diets was slightly lower than for the CL and EL diets (Table 2). Nutrient intake for the different dietary treatments is presented in Table 3. The difference in dietary C18:3*n*3 content was reflected in the intake of C18:3*n*3, which was slightly lower for the FL and DL treatments compared with the CL and EL treatments. Intake of C22:6*n*3 for the DL treatment was 11.6 g/d.

Fermentation characteristics

The dietary treatments had no effect on ammonia-N and total VFA concentration in digesta flowing into the omasal canal (Table 4). Fermentation pattern shifted towards propionate in the DL treatment compared with the FL treatment, concomitant with the opposite shift in acetate.

Nutrient flow into the omasum

Flows of DM, OM, CP, NDF, and crude fat into the omasum were not affected by the different dietary treatments (Table 5). Similarly, total FA flow was not affected by the different treatments. However, the individual FA flows were significantly affected by the linseed treatments. The C18:3*n*3 flow was higher for the EL treatment compared with the CL, FL, and DL treatments, while the C18:3*n*3 flow for the CL and FL treatments was also higher compared with the DL treatment. Total non-conjugated C18:2 flow did not differ between the linseed treatments. However, *cis*-9,*cis*-12-C18:2 flow was higher for the EL treatment compared with the DL treatment, while *trans*-9,*trans*-12-C18:2 flow was higher for the DL treatment compared with the other treatments. The DL treatment had a lower total saturated FA flow compared with the CL treatment, mainly due to the lower C18:0 flow for the DL treatment compared with the other treatments. In contrast, omasal flow of individual *trans*-C18:1 isomers was markedly higher for the DL treatment compared with the other linseed treatments. Total and individual *cis*-C18:1 flows were not affected by the treatments. Flow of *trans*-

Table 3. Nutrient intake (kg/d) and fatty acid (g/d) intake of cows fed diets supplemented with crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed (FL) and docosahexaenoic acid addition to linseed oil (DL)

Parameter	Dietary treatment			
	CL	EL	FL	DL
Nutrient intake (kg/d)				
DMI	20.37	20.75	21.57	19.81
OM	19.00	19.36	20.09	18.43
CP	3.47	3.54	3.70	3.23
Crude fat	1.28	1.34	1.19	1.12
NDF	7.32	7.48	7.63	7.02
ADF	4.39	4.45	4.59	4.21
ADL	0.65	0.66	0.67	0.61
Starch	3.04	3.07	3.28	3.03
Sugar	0.74	0.77	0.84	0.80
Fatty acid (g/d)				
Total fatty acids	929.0	963.5	960.3	877.3
C12:0	23.5	21.0	32.7	24.0
C14:0	8.7	7.8	12.0	14.2
C16:0	92.4	96.1	100.7	100.1
C16:1	3.8	3.8	4.3	3.9
C18:0	27.1	28.4	20.8	23.9
Cis-9-C18:1	180.4	185.8	193.8	168.8
Cis-9,*cis*-12-C18:2	241.4	249.0	257.5	229.4
Cis-9,*cis*-12,*cis*-15-C18:3	351.7	371.6	338.5	301.4
C22:6*n*3	ND[2]	ND	ND	11.6
UFA[1]	777.3	810.2	794.0	715.1

[1]Unsaturated fatty acids: ∑ (*cis*-9-C18:1, *cis*-9,*cis*-12-C18:2, *cis*-9,*cis*-12,*cis*-15-C18:3, C22:6*n*3).
[2]ND: not detectable.

10,*cis*-12-C18:2 was higher in the DL treatment compared with the CL and EL treatments, while flow of *cis*-9,*trans*-11-C18:2 was not affected. The concentration of C22:6*n*3 was below detection limit in the CL, EL, and FL treatments. The DL treatment showed a small C22:6*n*3 flow (1.00 g/d).

Digestibility and biohydrogenation

Rumen digestibility of DM, OM, and NDF was not affected by the linseed treatments (Table 6). Whole tract apparent digestibility of DM was higher for the DL treatment compared with the EL treatment. Whole tract crude fat digestibility was higher for the FL and DL treatments compared with the EL and CL treatments, while the CL treatment also showed a higher crude fat digestibility compared with the EL treatment. Whole tract apparent digestibility of NDF was not affected by the linseed treatments.

The extent of biohydrogenation of *cis*-9-C18:1 was not affected by the linseed treatments (Table 7). The EL treatment showed a lower extent of biohydrogenation of *cis*-9,*cis*-12-C18:2 compared with the DL treatment, while CL and FL treatments showed intermediate results. The extent of biohydrogenation of C18:3*n*3 was lower for the EL treatment compared with the CL, FL, and DL

Table 4. Concentration of ammonia-N (mg/l), total VFA (m*M*), and VFA molar proportions (mmol/mol) in omasal samples of cows fed diets supplemented with crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed (FL) and docosahexaenoic acid addition to linseed oil (DL)

Parameter	Dietary treatment				SEM[1]	P-value
	CL	EL	FL	DL		
Ammonia-N	114	106	129	112	18.6	0.426
Total VFA	99	101	100	103	3.2	0.761
Acetate	632[ab]	635[ab]	640[a]	614[b]	15.8	0.049
Propionate	216[ab]	217[ab]	198[b]	236[a]	17.8	0.028
Butyrate	111	110	122	115	4.0	0.092
Isobutyrate	10	9	9	8	0.6	0.214
Valerate	14	14	14	13	1.0	0.553
Isovalerate	17[a]	15[ab]	17[a]	14[b]	0.5	0.013

[a,b]Means within a row with different superscripts differ ($P < 0.05$).
[1]SEM: standard error of mean.

Table 5. Nutrient (kg/d) and fatty acid (g/d) flows into the omasum of cows fed diets supplemented with crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed (FL) and docosahexaenoic acid addition to linseed oil (DL)

Parameter	Dietary treatment				SEM[1]	P-value
	CL	EL	FL	DL		
Nutrients						
DM	13.40	13.23	13.15	12.47	1.282	0.667
OM	8.65	8.56	8.61	8.18	0.751	0.806
CP	3.06	3.10	3.19	2.89	0.267	0.678
NDF	3.00	2.95	2.80	2.78	0.428	0.659
Crude fat	1.31	1.27	1.15	1.19	0.094	0.491
Fatty acids						
C12:0	7.66[ab]	6.29[b]	10.76[a]	8.55[ab]	1.247	0.014
Iso-C13:0	0.58	0.54	0.54	0.67	0.062	0.282
Iso-C14:0	1.02	1.17	1.18	0.84	0.179	0.532
C14:0	10.87[b]	9.65[b]	11.74[b]	17.38[a]	1.834	0.004
C14:1	1.01	0.92	0.55	0.80	0.240	0.544
Iso-C15:0	3.46	2.72	2.97	3.50	0.342	0.194
Anteiso-C15:0	6.64	6.35	6.05	6.95	0.898	0.611
C15:0	5.29	5.00	5.36	6.13	0.673	0.484
Iso-C16:0	4.15	3.34	3.83	3.83	0.651	0.790
C16:0	95.55	91.03	100.37	123.74	13.713	0.097
C16:1	1.11[b]	0.90[b]	0.86[b]	2.62[a]	0.284	0.007
Iso-C17:0	1.47[ab]	1.20[b]	1.47[ab]	2.61[a]	0.270	0.038
Anteiso-C17:0	2.47	1.55	1.75	1.58	0.490	0.521
C17:0	2.46	2.29	2.33	2.85	0.345	0.389
C18:0	368.45[a]	342.62[a]	331.58[a]	147.98[b]	32.199	0.007
Total *trans*-C18:1[2]	98.69[b]	76.63[b]	82.75[b]	357.23[a]	41.165	0.002
Trans-4-C18:1	0.59[b]	0.56[b]	0.52[b]	1.45[a]	0.199	0.016
Trans-5-C18:1	0.40[b]	0.34[b]	0.37[b]	1.45[a]	0.204	0.007
Trans-6+7+8-C18:1	5.38[b]	4.23[b]	4.44[b]	13.29[a]	1.729	0.018

Table 5. Continued.

Parameter	Dietary treatment				SEM[1]	P-value
	CL	EL	FL	DL		
Trans-9-C18:1	3.38[b]	2.92[b]	2.97[b]	9.13[a]	0.999	0.005
Trans-10-C18:1	7.62[b]	6.24[b]	5.42[b]	149.59[a]	20.758	0.005
Trans-11-C18:1	35.62[ab]	25.97[b]	32.56[ab]	92.22[a]	14.717	0.034
Trans-12-C18:1	6.74[ab]	5.08[b]	5.49[b]	13.71[a]	1.624	0.021
Trans-13+14-C18:1	28.53	22.39	22.23	52.43	7.383	0.060
Trans-15+*cis*-11-C18:1	5.99[ab]	5.28[b]	5.89[ab]	12.21[a]	1.956	0.030
Trans-16+*cis*-14-C18:1	4.45[ab]	3.63[ab]	2.86[b]	11.75[a]	2.021	0.043
Total *cis*-C18:1[3]	60.45	55.71	67.56	76.00	12.164	0.423
Cis-9-C18:1	42.50	40.98	51.96	57.43	8.927	0.217
Cis-12-C18:1	6.79	5.09	6.23	2.68	1.397	0.179
Cis-13-C18:1	9.61	8.55	8.06	11.13	1.946	0.703
Cis-15-C18:1	1.55	1.09	1.31	4.76	1.004	0.091
Total non-conjugated C18:2[4]	34.58	33.68	39.22	74.34	13.387	0.154
Trans-9,*trans*-12-C18:2	0.16[b]	0.16[b]	0.20[b]	7.03[a]	1.172	0.012
Trans-11,*cis*-15-C18:2	11.62	8.52	17.34	45.40	9.280	0.100
Cis-9,*cis*-12-C18:2	17.69[ab]	20.15[a]	16.29[ab]	10.69[b]	2.353	0.025
Total conjugated C18:2[5]	6.95	5.35	11.66	6.89	1.998	0.197
Cis-9,*trans*-11-C18:2	4.74	3.47	8.69	3.99	1.524	0.153
Trans-10,*cis*-12-C18:2	0.02[b]	0.11[b]	0.24[ab]	0.59[a]	0.094	0.014
Cis-9,*cis*-12,*cis*-15-C18:3	21.81[b]	33.83[a]	15.47[b]	4.59[c]	2.594	<0.001
Total ≥ C20:0[6]	12.68	11.93	14.98	14.69	1.781	0.335
C20:0	3.50	3.20	3.62	3.43	0.406	0.602
C22:0	1.87	1.77	1.86	2.24	0.273	0.391
C22:5*n*6	3.99	3.70	4.41	3.07	0.654	0.564
C22:6*n*3	ND[13]	ND	ND	1.00		
C24:0	1.58	1.46	1.63	1.82	0.233	0.418
Unidentified	4.58[b]	2.58[b]	14.63[a]	2.34[b]	2.073	0.014
Summary						
Total FA[7]	751.92	695.40	727.60	866.11	103.21	0.373
SFA[8]	497.23[a]	463.33[ab]	469.24[ab]	314.11[b]	43.784	0.025
OBCFA[9]	27.54	24.16	25.47	28.94	3.227	0.572
MUFA[10]	161.82[b]	134.58[b]	153.71[b]	438.19[a]	52.414	0.004
PUFA[11]	68.51	78.04	72.23	91.50	17.229	0.717
UFA[12]	230.32[b]	212.62[b]	225.94[b]	529.69[a]	69.091	0.015

[a,b,c]Means within a row with different superscripts differ ($P < 0.05$).
[1]SEM: standard error of mean.
[2]Total *trans*-C18:1: ∑ (*trans*-4-C18:1, *trans*-5-C18:1, *trans*-6+7+8-C18:1, *trans*-9-C18:1, *trans*-10-C18:1, *trans*-11-C18:1, *trans*-12-C18:1, *trans*-13+14-C18:1, *trans*-15+*cis*-11-C18:1, *trans*-16+*cis*-14-C18:1).
[3]Total *cis*-C18:1: ∑ (*cis*-9-C18:1, *cis*-12-C18:1, *cis*-13-C18:1, *cis*-15-C18:1).
[4]Total non-conjugated C18:2: ∑ (*trans*-9,*trans*-12-C18:2, *cis*-9,*trans*-13-C18:2, *trans*-8,*cis*-13-C18:2, *cis*-9,*trans*-12-C18:2, *trans*-9,*cis*-12-C18:2, *trans*-11,*cis*-15-C18:2, *cis*-9,*cis*-12-C18:2, *cis*-9,*cis*-15-C18:2).
[5]Total conjugated C18:2: ∑ (*cis*-9,*trans*-11-C18:2, *trans*-10,*cis*-12-C18:2, *cis*-9,*cis*-11-C18:2+*trans*-11,*cis*-13-C18:2, *trans*-11,*trans*-13-C18:2, *trans*-9,*trans*-11-C18:2+*trans*-10,*trans*-12-C18:2).
[6]Total ≥ C20:0: ∑ (C20:0, C20:1, C20:2, C20:3*n*3, C20:4*n*6, C22:0, *cis*-13-C22:1, C22:5, C22:6, C24:0)
[7]Total fatty acids.
[8]Saturated fatty acids: ∑ (C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, C24:0).
[9]Odd and branched chain fatty acids: ∑ (*iso*-C13:0, *iso*-C14:0, *iso*-C15:0, *anteiso*-C15:0, C15:0, *iso*-C16:0, *iso*-C17:0, *anteiso*-C17:0, C17:0).
[10]Mono-unsaturated fatty acids: ∑ (C14:1, C16:1, Total *cis*-C18:1, Total *trans*-C18:1, C20:1, *cis*-13-C22:1).
[11]Poly-unsaturated fatty acids: ∑ (Total non-conjugated C18:2, Total conjugated C18:2, C18:3*n*6, C18:3*n*3, C20:2, C20:3*n*3, C20:4*n*6, C22:5, C22:6).
[12]Unsaturated fatty acids: ∑ (MUFA, PUFA).
[13]ND: not detectable.

Table 6. Apparent rumen digestibility (%) and apparent whole tract digestibility (%) of cows fed diets supplemented with crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed (FL) and docosahexaenoic acid addition to linseed oil (DL)

Parameter	Dietary treatment				SEM[1]	P-value
	CL	EL	FL	DL		
Rumen apparent digestibility						
DM	34.9	36.6	39.2	37.2	2.75	0.370
OM	54.9	55.9	57.2	55.7	1.51	0.412
NDF	60.1	61.0	63.6	60.6	3.78	0.439
Whole tract apparent digestibility						
DM	73.3[ab]	72.7[b]	74.5[ab]	74.7[a]	0.93	0.025
Crude fat	71.3[b]	64.8[c]	78.5[a]	80.4[a]	1.06	<0.001
NDF	67.3	68.1	68.6	68.9	2.09	0.462

[a,b,c]Means within a row with different superscripts differ ($P < 0.05$).
[1]SEM: standard error of mean.

treatments. In addition, the CL and FL treatments had a lower C18:3*n*3 biohydrogenation compared with the DL treatment.

Discussion

Nutrient digestibility

The aim of the present study was to determine the effects of different linseed treatments on FA flows through the gastro intestinal tract of lactating dairy cows. The FA proportion in the diet, feed intake, and microbial activity in the rumen may affect the omasal flow of FA. The different linseed treatments did not affect omasal DM, OM, and NDF flows, rumen DM, OM, and NDF digestibilities, and whole tract apparent NDF digestibility in the present study. This absence of a treatment effect on digestibility is in agreement with results of Doreau et al. (2009a), who reported no differences in total tract and forestomach OM and fibre digestibility between rolled linseed, extruded linseed, and linseed oil combined with linseed meal. In another study, Martin et al. (2008) also found no differences in OM and NDF digestibility for linseed oil versus crude linseed or extruded linseed. It was suggested that the effect of linseed on ruminal digestion depends on the level of linseed supply, whereas the

Table 7. Apparent ruminal biohydrogenation (%) of cows fed diets supplemented with crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed (FL) and docosahexaenoic acid addition to linseed oil (DL)

Parameter	Dietary treatment				SEM[1]	P-value
	CL	EL	FL	DL		
Cis-9-C18:1	77.4	78.0	73.5	65.9	3.825	0.080
Cis-9,*cis*-12-C18:2	92.9[ab]	91.9[b]	93.7[ab]	95.3[a]	0.600	0.015
Cis-9,*cis*-12,*cis*-15-C18:3	94.0[b]	90.9[c]	95.4[b]	98.5[a]	0.494	<0.001

[a,b,c]Means within a row with different superscripts differ ($P < 0.05$).
[1]SEM: standard error of mean.

form of the linseed is not important (Doreau et al., 2009a). Average OM digestibility in the present study was slightly higher compared with digestibilities (47.2 ± 4.7%) found in the evaluation of omasal sampling studies by Huhtanen et al. (2010), but comparable with other studies (Ahvenjärvi et al., 2000; Owens et al., 2008) and within the biological limits described by Titgemeyer (1997). Digestibility of NDF (61.3 ± 7.0%) in the present study was within the range of NDF digestibilities reported by Huhtanen et al. (55.1 ± 12.5%; 2010) and Owens et al. (37.9-71.6%; 2008), but slightly lower compared with Shingfield et al. (65.5-66.9%; 2003).

Whole tract crude fat digestibility was lower for the extruded whole linseed diet in the present experiment. Doreau et al. (2009b) found lower digestibilities of total FA, *cis*-9-C18:1, *cis*-9,*cis*-12-C18:2, and C18:3*n*3 for rolled linseed compared with extruded linseed or linseed oil. The differences among the linseed variants in crude fat in the present experiment and FA digestibilities in the experiment of Doreau et al. (2009b) can probably be explained by the differences in accessibility of the oil inside the seed coat.

Fermentation characteristics

Inclusion of treated linseed or linseed oil did not affect the rumen fermentation pattern (Doreau et al., 2009a). Indeed, in the present study no differences in fermentation pattern measured in omasal samples was found between the CL, EL, and FL treatments, whereas addition of DHA to linseed oil resulted in a higher propionate and a lower acetate proportion compared with the FL treatment. Similar effects of DHA on rumen fermentation pattern were reported before (Fievez et al., 2003, 2007; Vlaeminck et al., 2008). In these studies, supplementing DHA resulted in a decreased rumen concentration of VFA (Fievez et al., 2003, 2007; Boeckaert et al., 2008a; Vlaeminck et al., 2008), an effect which is related to the amount of DHA supplemented (Fievez et al., 2003, 2007). This could explain the absence of a decrease in the omasal concentration of VFA in the current study as DHA intake (11.6 g/d) was lower compared with previous studies (73.1 and 43.7 g/d; Boeckaert et al., 2008a). Various unsaturated FA have a negative effect on degradation of NDF in the rumen, and fibre degradation is associated with a relatively large acetate to propionate ratio (Bannink et al., 2008). However, in the present experiment rumen NDF degradation did not differ between treatments and cannot explain the change in VFA profile observed.

Omasal FA flow and C18:3*n*3 biohydrogenation

Schmidely et al. (2008) reported a relationship between duodenal FA flow and FA intake, in which proportionally 75% of ingested FA were recovered in duodenal FA flow. The cases in which FA intake was higher than duodenal FA flow, were related to diets containing more than 4% FA in the DM (Schmidely et al., 2008). Indeed, Jenkins (1993) reported that lipid disappearance from the rumen was more common for diets with added fat than for control diets. However, it was not possible to associate a lower recovery of duodenal FA to the characteristics of the fat sources in the diet, including the rumen inertness of the fat source (Doreau and Ferlay, 1994). In the current study, the recovery of omasal FA was 72.2% for the EL diet, 75.8% for the FL diet, 80.9% for the CL diet, and 98.7% for the DL diet. Possible reasons for the lower duodenal or omasal FA flows compared

with FA intake are absorption in the rumen, degradation to shorter chains, and/or underestimation of the flow (Wu et al., 1991). Shingfield et al. (2008) indicated a net synthesis of FA in the rumen on diets with incremental levels of sunflower oil. This finding was in agreement with other studies where sunflower oil (Lock and Garnsworthy, 2002; Kalscheur et al., 1997) or soybean oil (Lundy et al., 2004) was fed. Shingfield et al. (2008) concluded that these differences between studies reflect the differences in experimental techniques used to estimate postruminal DM flow and the FA content of feed ingredients and digesta.

Heat treatment of linseed showed no effect on the duodenal flow of long chain FA compared with raw linseed (Gonthier et al., 2004). However, feeding extruded linseed compared with micronized linseed resulted in a lower C18:3*n*3 flow, suggesting a higher exposure of the extruded linseed to ruminal bacteria resulting in a higher ruminal biohydrogenation (Gonthier et al., 2004). In the present study, the omasal flow of C18:3*n*3 was higher for the extruded whole linseed treatment compared with the other treatments. A numerically higher duodenal flow of C18:3*n*3 for extruded compared with rolled linseed was also found by Doreau et al. (2009b). The latter authors concluded that the higher C18:3*n*3 flow could be explained by the rapid release of the lipids in the extruded linseed leading to a higher passage rate. A decrease in protein degradability following extrusion is reported to decrease C18:3*n*3 biohydrogenation (Gonthier et al., 2004), but Doreau et al. (2009a) reported no difference in protein digestibility between the rolled and extruded linseed diets. In the present study there was also no difference in omasal CP flow between the various linseed treatments, which confirms that the higher C18:3*n*3 flow in the EL treatments is probably not caused by a decrease in protein degradability. The whole tract apparent digestibility of crude fat was lower for the EL treatment compared with the other treatments in the present study. Sterk et al. (2010) hypothesized that overprotection by the seed coat prevented the C18:3*n*3 to be released and absorbed. The lower whole tract crude fat digestibility indeed suggests that the FA were still captured in the seed coat and might therefore not be absorbed.

Formaldehyde treatment of crushed linseed resulted in a lower calculated effective biohydrogenation in earlier in vitro research (Sterk et al., 2010). However, in the present study omasal C18:3*n*3 flow was lower for the FL treatment compared with the EL treatment suggesting formaldehyde-treatment was ineffective in protecting linseed oil from rumen biohydrogenation. The protein of the oilseed should be accessible to formaldehyde to form the inert formaldehyde-protein matrix, resulting in the effective protection of the FA (Fievez et al., 2007). In the current study, linseed oil was emulsified and encapsulated in a formaldehyde-treated casein, which is known to be able to provide an effective protection against biohydrogenation (Ashes et al., 1992). When digesta is sampled from the omasal canal, it is possible that particles with different functional specific gravities segregate as they travel through the sample tube (Ipharraguerre et al., 2007). This could result in an underestimation of the flow of particles of high specific gravity (e.g. maize kernels) (Ipharraguerre et al., 2007). The C18:3*n*3 flow for the FL treatment might therefore be underestimated due to the specific appearance of the product.

Boeckaert et al. (2008b) observed increased proportions of biohydrogenation intermediates *cis*-9,*trans*-11,*cis*-15-C18:3, *trans*-11,*cis*-15-C18:2, *cis*-9,*trans*-11-C18:2, *trans*-10,*cis*-12-C18:2, *cis*-

9,*cis*-11-C18:2+*trans*-11,*cis*-13-C18:2, and all *trans*-C18:1 isomers in the ruminal digesta of dairy cows receiving 43.7 g DHA/d in their diet. The increased flow of biohydrogenation intermediates and decreased flow of C18:0 is a clear indication of the inhibitory effect of DHA on rumen biohydrogenation (Boeckaert et al., 2008b). In the present study, omasal flows of *trans*-11,*cis*-15-C18:2 tended to be higher and *trans*-10,*cis*-12-C18:2 and total *trans*-C18:1 were higher for the DL treatment compared with the other linseed treatments. In the present study a lower level of DHA (11.6 g/d) was fed in combination with linseed oil (420 g/d), which confirms the marked effects of DHA on the rumen biohydrogenation pathways. *Butyrivibrio* species have an active role in the partial or complete biohydrogenation of unsaturated C18 FA (Jenkins et al., 2008). Boeckaert et al. (2008b), using denaturing gradient gel electrophoresis techniques, concluded that the increase in various *trans*-C18:1 intermediates upon DHA supplementation was associated with changes in the *Butyrivibrio* community without affecting the total amount of *Butyrivibrio* bacteria. In agreement with earlier in vitro research (Sterk et al., 2010) the extent of biohydrogenation of C18:3*n*3 was high, which confirms that the first step of the biohydrogenation pathway is not influenced by the DHA addition.

Conclusions

Feeding extruded whole linseed resulted in a higher omasal C18:3*n*3 flow and therefore lower ruminal C18:3*n*3 biohydrogenation compared with unprotected, crushed linseed, formaldehyde-treated linseed oil and linseed oil with DHA. However, whole tract crude fat digestibility was lower for the extruded whole linseed compared with the other linseed sources. Feeding linseed oil in combination with marine algae rich in DHA resulted in an inhibition of the complete C18:3*n*3 biohydrogenation towards C18:0, as shown by a low omasal C18:0 flow and high omasal flows of biohydrogenation intermediates.

Acknowledgments

The authors would like to thank the staff of trial farm The Ossekampen, Wageningen University for their assistance in animal care, feeding and sampling; the laboratory staff of the Animal Nutrition Group, Wageningen University, for their help in chemical analysis; and Tamme Zandstra and Sven Alferink for their assistance in preparing the linseed treatments. MSc students Steven van der Weerd and Ard van Veen are gratefully acknowledged for their contribution in the experiment. The financial support of the Food and Nutrition Delta program of Senter Novem (Den Haag, the Netherlands), Royal FrieslandCampina (Amersfoort, The Netherlands), Agrifirm Group (Apeldoorn, The Netherlands), Agrifirm Innovation Center (Apeldoorn, The Netherlands), and Barenbrug Holland B.V. (Oosterhout, The Netherlands) is gratefully acknowledged. Bruno Vlaeminck is a postdoctoral fellow of the Fund for Scientific Research-Flanders (Belgium).

Chapter 5

Effects of feeding different linseed sources on fatty acid profiles of plasma and milk fat in lactating dairy cows

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Abstract

The aim of this experiment was to study the effect of physical form of linseed or linseed oil in combination with docosahexaenoic acid (DHA) addition on plasma and milk fatty acid (FA) profiles in dairy cows. Four ruminally cannulated lactating Holstein Friesian cows were assigned to four dietary treatments in a 4 × 4 Latin square design. Dietary treatment consisted of crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed oil (FL), and linseed oil in combination with marine algae rich in DHA (DL). Each period in the Latin square design lasted 21 d, with the first 16 d for adaptation. Diets contained on average 16.5 g C18:3*n*3 per kg DM. Milk yield did not differ between treatments and averaged 32.0 kg/d with milk fat yield being lower for the DL treatment (0.96 kg/d) compared with the other linseed treatments (CL, 1.36 kg/d; EL, 1.49 kg/d; FL, 1.54 kg/d). Proportions of C18:0 in plasma triacylglycerols and milk fat were lower and proportions of biohydrogenation intermediates, especially *trans*-C18:1 isomers, were higher for the DL treatment compared with the other linseed treatments. Proportion of *trans*-10-C18:1 was negatively related to milk fat yield. Proportion of C18:3*n*3 in plasma triacylglycerols tended to be higher for the FL treatment compared with the other linseed treatments (FL, 3.60 g/100 g FA; CL, 1.22 g/100 g FA; EL, 1.35 g/100 g FA; DL, 1.12 g/100 g FA) and proportion of C18:3*n*3 in milk fat was higher for the FL treatment compared with the other treatments (FL, 3.19 g/100 g FA; CL, 0.87 g/100 g FA; EL, 0.83 g/100 g FA; DL, 0.46 g/100 g FA). Transfer efficiency from C18:3*n*3 intake to C18:3*n*3 yield in milk was higher for the FL treatment (13.1%) compared with the other linseed treatments (CL: 3.2%; EL: 3.0%; DL: 1.3%). The results indicate that feeding formaldehyde-treated linseed oil results in less rumen biohydrogenation and consequently higher C18:3*n*3 proportions in plasma triacylglycerols and milk fat. Feeding linseed oil in combination with DHA inhibited the biohydrogenation steps from *trans*-11,*cis*-15-C18:2 to *trans*-11-C18:1 to C18:0, shown by the increased proportions of these biohydrogenation intermediates in plasma triacylglycerols and milk fat.

Introduction

The fatty acid (FA) composition of milk fat is largely dependent on FA intake, FA metabolism in the rumen (Jenkins et al., 2008), lipid mobilization, and FA metabolism in the mammary gland (Chilliard et al., 2007). Major dietary sources of linolenic acid (*cis*-9,*cis*-12,*cis*-15-C18:3; C18:3*n*3) include grass (> 60% of FA) and linseed (> 50% of FA), and diets that contain these sources have a relatively high proportion of C18:3*n*3. The proportion of C18:3*n*3 in milk fat, however, is generally low (< 1% of FA; Heck et al., 2009), because in the rumen, dietary lipids undergo extensive transformations by ruminal micro-organisms in two major processes; lipolysis and biohydrogenation. Consequently, marked differences exist between the FA profile in the diet (mainly unsaturated FA) and the FA profile leaving the rumen (mainly saturated FA; Jenkins et al., 2008). To overcome these ruminal transformations, protection technologies have been developed, which aim to prevent ruminal FA metabolism or ensure the accumulation of specific biohydrogenation intermediates (Fievez et al., 2007). Protection of linseed with formaldehyde treatment can increase the proportion of C18:3*n*3 in milk fat up to 6.4% of total FA (Goodridge et al., 2001). However, effective formaldehyde treatment requires pretreatment of linseed to allow the formation of cross-links between formaldehyde and protein (Fievez et al., 2007). When C18:3*n*3 was directly infused in the abomasum, the proportion of C18:3*n*3 in milk fat increased up to 13.9% of total FA (Petit et al., 2002a). Duodenal infusion of 160 g/d of free C18:3*n*3 increased the proportion of C18:3*n*3 in milk fat even up to 25.4% of total FA (Khas-Erdene et al., 2010). Extrusion of whole linseed showed a reduction of C18:3*n*3 biohydrogenation in vitro (Sterk et al., 2010). Postruminal C18:3*n*3 digestibility from extruded whole linseed, however, was not determined and could be low due to the presence of intact seed hulls protecting the seed contents in the extruded product. The accumulation of biohydrogenation intermediates from C18:3*n*3 biohydrogenation of linseed oil can be influenced by the addition of docosahexaenoic acid (C22:6*n*3; DHA) as shown in vitro (Sterk et al., 2010), although no in vivo studies have been conducted to determine effects on plasma and milk FA profiles of DHA added to diets containing linseed or linseed oil.

A previous in vitro study evaluating several chemically or technologically treated linseed products showed that formaldehyde-treated crushed linseed and extruded whole linseed were able to decrease ruminal biohydrogenation of C18:3*n*3 (Sterk et al., 2010). However, because of a possible overestimation of protected C18:3*n*3 in vitro (Fievez et al., 2007), the true rumen inertness and transfer efficiency from feed to milk should be determined in vivo. The objective of this study was therefore to determine the effects of feeding crushed linseed, extruded whole linseed, formaldehyde-treated linseed oil, and linseed oil in combination with DHA addition on plasma and milk FA profiles of lactating dairy cows. Crushed linseed was included in the study to be able to compare the effects of the different treated linseed sources with an unprotected linseed source. Nutrient digestibility, FA intake and FA flows into the omasal canal were reported in a companion paper (Chapter 4).

Materials and Methods

Experimental design, animals and housing

The Institutional Animal Care and Use Committee of Wageningen University approved all experimental procedures, which were carried out under the Dutch Law on Animal Experimentation. Four multiparous Holstein Friesian cows (625 ± 69 kg BW; 52 ± 22 DIM; values expressed as means ± SD) fitted with a ruminal cannula to enable omasal sampling (10 cm i.d.; Bar Diamond Inc., Parma, ID) were used in a 4 × 4 Latin square design. Cows were fed four different linseed treatments during 21-d experimental periods, with the first 16 d for adaptation. Animals were housed in individual tie-stalls and daily rations were offered as equal meals at 0615 and 1645h. Cows had continuous access to water and were milked twice daily at 0630 and 1700h.

Diets

Dietary treatments consisted of a basal mixed diet with 1) crushed linseed (CL), 2) extruded whole linseed (EL), 3) formaldehyde-treated linseed oil (FL), or 4) DHA in combination with linseed oil (DL). The basal diet (DM basis) consisted of 31% grass silage, 29% maize silage, and 40% concentrate. For the different treatments, part of the concentrate was replaced to supply in the total diet 6.5% CL, 9.9% EL, 3.3% FL, or 0.4% DHA together with 2.1% linseed oil. The diets were designed to provide equal amounts of C18:3*n*3. The FA composition of the diets is presented in Table 1. Details of the ingredient composition and the chemical analysis of the diets were reported in the companion paper (Chapter 4). Just before feeding, the silages were thoroughly mixed with the concentrate and linseed products. Diets were offered at 95 % of ad libitum intake, measured during the first 7 days of the experiment.

Measurements and sampling

Milk yield was recorded from day 15 to day 20 of each experimental period. Milk samples were collected from each cow over two consecutive milkings (d17 p.m. and d18 a.m.) during each experimental period. Pooled milk samples (equal volume) per cow per period were stored pending analysis for fat, protein, lactose, MUN, and SCC. A second set of milk samples was taken on the same days during each experimental period and immediately stored at -20°C pending FA analysis. These samples were pooled per cow per period (equal volume) during the FA analysis.

Blood samples from the tail vein were obtained with heparinized Vacutainer® tubes (Becton Dickinson, Breda, the Netherlands) at 0900h on d17 of each experimental period. Blood was centrifuged at 3,000 x *g* for 15 min and plasma was collected and stored at -80°C until analysis of FA in the triacylglycerol (TAG) fraction.

Analytical procedures

Analysis of fat, protein, lactose, MUN, and SCC in milk samples was carried out as described by Van Zijderveld et al. (2011a). For milk FA analysis, total lipids were extracted with diethyl ether and petroleum ether according to the Rose-Gottlieb method (AOAC, 1990). Fatty acids from milk

Table 1. Fatty acid composition (g/kg DM) of the diets supplemented with crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed oil (FL) and docosahexaenoic acid addition to linseed oil (DL)

Fatty acid	Treatments			
	CL	EL	FL	DL
C12:0	1.15	1.01	1.51	1.21
C14:0	0.43	0.38	0.56	0.71
C16:0	4.52	4.63	4.66	5.06
C16:1	0.19	0.19	0.20	0.20
C18:0	1.32	1.36	0.95	1.21
Cis-9-C18:1	8.79	8.92	8.99	8.53
Cis-9,*cis*-12-C18:2	11.78	11.98	11.92	11.60
Cis-9,*cis*-12,*cis*-15-C18:3	17.26	17.88	15.70	15.22
C22:6*n*3	ND[2]	ND	ND	0.59
UFA[1]	38.02	38.97	36.80	36.14

[1]Unsaturated fatty acids: ∑ (*cis*-9-C18:1, *cis*-9,*cis*-12-C18:2, *cis*-9,*cis*-12,*cis*-15-C18:3, C22:6*n*3).
[2]ND: not detectable.

lipids were methylated with 2.0 *N* of methanolic $NaOCH_3$, neutralized with $NaHSO_4$ and dried with Na_2SO_4. Fatty acid methyl esters were recovered in 1 mL of hexane.

Plasma lipids were extracted with n-octane. The TAG fraction was separated with silica columns (Bond Elut SI, 500 mg, 3 mL; Varian Inc., Walnut Creek, CA, USA) rinsed with a mixture of hexane with methyl-t-butyl-ether (96:4 vol/vol). The solvent of the TAG fraction was evaporated and FA were methylated with 0.4 mL 0.5 *N* of methanolic $NaOCH_3$ (80˚C for 10 min), followed by 0.5 mL of 14% boron trifluoride (80˚C for 2 min). Fatty acid methyl esters were recovered in 100 μL hexane containing 25 μg butyl-hydroxy-toluene to prevent oxidation.

Fatty acid methyl esters from milk and plasma TAG samples were quantified using gas chromatography (Trace GC Ultra™, Thermo Fisher Scientific, Waltham MA, USA) with a fused silica capillary column (100 m x 0.250 mm and 0.2 μm film thickness; Supelco; SP2560, Bellefonte PA, USA). For milk samples, the carrier gas was helium at a constant flow of 1.5 mL/min. For plasma TAG samples, hydrogen was used as the carrier gas at a constant flow of 1.5 mL/min. The flame ionization detector was set at 280ºC. The time-temperature program used, started with an initial temperature of 70ºC for 4 min, increased with 1ºC/min to 165ºC for 20 min, increased with 2ºC/min to 170ºC for 10 min, and increased with 4ºC/min to a final temperature of 215ºC for 20 min. In addition, for the plasma TAG samples, a second time-temperature program was used to separate the C18:1 isomers; initial temperature of 70ºC for 1 min, increased with 5ºC/min to 100ºC for 2 min, increased with 10ºC/min to 175ºC for 40 min, and increased with 10ºC/min to a final temperature of 215ºC for 20 min. Identification of FA methyl esters was described in the companion paper (Chapter 4).

Statistics

Milk yield, milk composition, milk FA profile, plasma TAG FA profile and transfer efficiencies of C18:3*n*3 were analysed as a Latin square design using the MIXED procedure of SAS version 9.2 (SAS Institute, Cary, NC, USA) according to:

$Y_{ijkl} = \mu + T_i + P_j + C_k + \epsilon_{ijkl}$

where Y_{ijkl} is the individual observation, μ the overall mean, T_i the effect of dietary treatment, P_j the effect of experimental period, C_k the effect of cow, and ϵ_{ijkl} the residual error. The effect of cow was treated as a random effect. Treatment effects were considered significant at a probability of $P \le 0.05$ and as a trend at a probability of $0.05 < P \le 0.10$. Posthoc analyses were carried out using the Tukey test to test pair wise comparisons. Data are reported as least squares means ± SEM.

Results

Milk yield and composition

Milk production was not affected by the different linseed treatments (Table 2). Milk fat concentration and yield were lower for the DL treatment compared with the other treatments. Milk protein concentration was not affected by the linseed treatments; however, milk protein yield tended

Table 2. Milk yield and composition of cows fed diets supplemented with crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed oil (FL) and docosahexaenoic acid addition to linseed oil (DL)

Parameter	Dietary treatment				SEM[1]	P-value
	CL	EL	FL	DL		
Milk yield, kg/d	33.1	31.4	33.7	29.7	2.41	0.402
Milk lactose						
%	4.62	4.52	4.64	4.42	0.076	0.145
kg/d	1.53	1.42	1.57	1.31	0.113	0.301
Milk fat						
%	4.30[a]	4.75[a]	4.67[a]	3.27[b]	0.570	0.002
kg/d	1.36[a]	1.49[a]	1.54[a]	0.96[b]	0.140	<0.001
Milk protein						
%	3.18	3.27	3.26	3.09	0.117	0.552
kg/d	1.05	1.03	1.09	0.91	0.066	0.054
MUN, mg/dl	13.0	12.9	13.0	12.5	0.85	0.973
SCC, x 1,000 cells/ml	134	352	173	559	136.7	0.123

[a,b]Means within a row with different superscripts differ ($P < 0.05$).
[1]SEM: standard error of mean.

Table 3. Fatty acid profile (g/100 g fatty acids) in plasma triacylglycerol of cows fed diets supplemented with crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed oil (FL) and docosahexaenoic acid addition to linseed oil (DL)

Parameter	Dietary treatment				SEM[1]	P-value
	CL	EL	FL	DL		
C14:0	1.24	1.12	0.98	1.43	0.256	0.546
C15:0	0.60	0.75	0.65	0.48	0.130	0.509
C16:0	12.20	12.72	11.40	13.13	0.891	0.212
Cis-9-C16:1	8.13	6.96	9.11	8.30	0.606	0.142
C17:0	0.66	0.76	0.62	1.01	0.129	0.202
C18:0	43.06[a]	42.04[a]	37.11[a]	17.88[b]	3.156	0.001

Table 3. Continued.

Parameter	Dietary treatment				SEM[1]	P-value
	CL	EL	FL	DL		
Total *trans*-C18:1[2]	6.54[b]	6.53[b]	6.42[b]	27.18[a]	2.299	<0.001
Trans-6+7+8-C18:1	0.47[b]	0.45[b]	0.49[b]	1.82[a]	0.166	0.002
Trans-9-C18:1	0.35	0.30	0.33	0.71	0.124	0.162
Trans-10-C18:1	0.65[b]	0.63[b]	0.58[b]	11.50[a]	1.673	0.007
Trans-11-C18:1	1.84[b]	1.78[b]	2.11[b]	6.72[a]	0.640	0.001
Trans-12-C18:1	0.80[b]	0.69[b]	0.59[b]	1.61[a]	0.111	<0.001
Trans-13+14-C18:1	2.25[b]	2.25[b]	1.93[b]	4.31[a]	0.299	0.003
Trans-16+*cis*-14-C18:1	0.19	0.32	0.24	0.36	0.098	0.626
Total *cis*-C18:1[3]	9.94	9.78	10.15	10.09	0.794	0.978
Cis-9+*trans*-15-C18:1	6.85	6.76	6.80	5.97	0.828	0.578
Cis-11-C18:1	0.61	0.63	0.71	1.04	0.113	0.057
Cis-12-C18:1c12	0.79	0.74	0.81	1.30	0.134	0.058
Cis-13-C18:1c13	0.65[a]	0.49[ab]	0.78[a]	0.33[b]	0.080	0.009
Cis-15-C18:1c15	1.05	1.16	1.05	1.46	0.129	0.148
Total non-conjugated C18:2[4]	4.60[c]	5.41[bc]	7.18[b]	9.91[a]	0.756	0.001
Trans-11,*cis*-15-C18:2	0.51[b]	0.54[b]	0.66[b]	2.02[a]	0.278	0.015
Cis-9,*cis*-12-C18:2	1.84	2.05	3.20	2.73	0.627	0.458
Total conjugated C18:2[5]	0.04	0.10	0.17	0.16	0.075	0.512
Cis-9,*trans*-11-C18:2	0.04	0.06	0.15	0.13	0.060	0.485
Trans-10,*cis*-12-C18:2	0.00	0.03	0.02	0.03	0.028	0.803
Cis-9,*cis*-12,*cis*-15-C18:3	1.22	1.35	3.60	1.12	0.526	0.043
Total ≥ C20:0[6]	1.23	1.06	0.82	1.41	0.268	0.371
C22:0	0.52	0.43	0.19	0.27	0.118	0.083
C22:2	0.38	0.24	0.24	0.68	0.207	0.454
C24:0	0.33[b]	0.39[ab]	0.39[ab]	0.46[a]	0.020	0.020
Unidentified	10.54	11.40	11.79	7.89	1.311	0.220
Summary						
SFA[7]	58.62[a]	58.21[a]	51.33[a]	34.66[b]	3.449	0.002
OBCFA[8]	1.26	1.51	1.27	1.49	0.167	0.344
MUFA[9]	24.61[b]	23.28[b]	25.69[b]	45.57[a]	2.513	<0.001
PUFA[10]	6.23[c]	7.10[bc]	11.19[ab]	11.88[a]	1.190	0.013
UFA[11]	30.84[b]	30.38[b]	36.88[b]	57.44[a]	3.211	<0.001

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

[1]SEM: standard error of mean.

[2]Total *trans*-C18:1: ∑ (*trans*-4-C18:1, *trans*-5-C18:1, *trans*-6+7+8-C18:1, *trans*-9-C18:1, *trans*-10-C18:1, *trans*-11-C18:1, *trans*-12-C18:1, *trans*-13+14-C18:1, *trans*-16+*cis*-14-C18:1).

[3]Total *cis*-C18:1: ∑ (*cis*-9+*trans*-15-C18:1, *cis*-11-C18:1, *cis*-12-C18:1, *cis*-13-C18:1, *cis*-15-C18:1).

[4]Total non-conjugated C18:2: ∑ (*trans*-9,*trans*-12-C18:2, *cis*-9,*trans*-13-C18:2, *trans*-8,*cis*-13-C18:2, *cis*-9,*trans*-12-C18:2, *trans*-9,*cis*-12-C18:2, *trans*-11,*cis*-15-C18:2, *cis*-9,*cis*-12-C18:2, *cis*-9,*cis*-15-C18:2).

[5]Total conjugated C18:2: ∑ (*cis*-9,*trans*-11-C18:2, *trans*-10,*cis*-12-C18:2).

[6]Total > C20:0: ∑ (C20:0, C22:0, C22:2, C24:0).

[7]Saturated fatty acids: ∑ (C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, C24:0).

[8]Odd and branched chain fatty acids: ∑ (C15:0, C17:0).

[9]Mono-unsaturated fatty acids: ∑ (*cis*-9-C16:1, Total *cis*-C18:1, Total *trans*-C18:1).

[10]Poly-unsaturated fatty acids: ∑ (Total non-conjugated C18:2, Total conjugated C18:2, C18:3*n*3, C22:2).

[11]Unsaturated fatty acids: ∑ (MUFA, PUFA).

to be lower for the DL treatment compared with the FL treatment. Milk lactose concentration and yield, MUN and SCC were not affected by the linseed treatments.

Plasma TAG FA composition

The FL treatment tended to have a higher proportion of C18:3*n*3 in plasma TAG compared with the other treatments (Table 3). The DL treatment had lower saturated FA and higher mono-unsaturated FA compared with the other treatments. The DL treatment had a lower proportion of C18:0, while proportions of *trans*-6+7+8-C18:1, *trans*-10-C18:1, *trans*-11-C18:1, *trans*-12-C18:1, and *trans*-13+14-C18:1 isomers were markedly higher compared with the other treatments. The DL treatment had a higher proportion of poly-unsaturated FA compared with the FL and EL treatments and the FL treatment had a higher proportion of poly-unsaturated FA compared with the CL treatment. This difference was partly caused by the proportion of total non-conjugated C18:2; the DL treatment had a higher proportion of *trans*-11,*cis*-15-C18:2 compared with the other treatments, while the proportion of *cis*-9,*cis*-12-C18:2 was not affected by the different linseed treatments.

Milk FA composition

The FL treatment resulted in higher C18:3*n*3 and *cis*-9,*cis*-12-C18:2 proportions in milk fat compared with the other treatments (Table 4). The DL treatment had a lower C18:0 proportion in milk fat, whereas the proportions of total *trans*-C18:1, *trans*-9,*trans*-12-C18:2, *trans*-11,*cis*-15-C18:2, and *cis*-9,*trans*-11-C18:2 were clearly higher compared with the other treatments. The higher proportion of total *trans*-C18:1 was caused by the higher proportions of *trans*-6+7+8-C18:1, *trans*-9-C18:1, *trans*-10-C18:1, *trans*-11-C18:1, *trans*-12-C18:1, and *trans*-13+14-C18:1 isomers for the DL treatment compared with the other treatments. In addition, the DL treatment had higher proportions of *cis*-13-C18:1 and *cis*-15-C18:1 isomers compared with the FL treatment, while proportions of *cis*-9-C18:1 and *cis*-12-C18:1 isomers were lower for the DL treatments compared with the other treatments, respectively the CL treatment.

Transfer efficiency of C18:3n3

Transfer efficiency of C18:3*n*3 from intake to milk was higher for the FL treatment compared with the other treatments (Table 5). The FL treatment also resulted in a higher efficiency from omasal C18:3*n*3 flow to milk C18:3*n*3 yield compared with the other treatments; however, the efficiency was calculated to be 288%.

Table 4. Milk fatty acid profile (g/100 g fatty acids) of cows fed diets supplemented with crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed oil (FL) and docosahexaenoic acid addition to linseed oil (DL)

Fatty acid	Dietary treatment				SEM[1]	P-value
	CL	EL	FL	DL		
C4:0	1.89	2.06	1.72	1.64	0.200	0.510
C6:0	2.09	1.99	2.19	1.74	0.210	0.294
C8:0	1.50	1.33	1.64	1.20	0.181	0.170
C10:0	3.30	2.66	3.54	2.68	0.499	0.219
C11:0	0.34	0.30	0.36	0.24	0.048	0.073
C12:0	3.70	3.08	4.09	3.39	0.520	0.197
Iso-C13:0	0.03^{b}	0.03^{b}	0.03^{ab}	0.04^{a}	0.004	0.031
Anteiso-C13:0	0.08	0.07	0.09	0.10	0.015	0.041
C13:0	0.16	0.13	0.17	0.16	0.028	0.219
Iso-C14:0	0.08	0.08	0.08	0.07	0.005	0.592
Anteiso-C14:0	0.02	0.01	0.02	0.02	0.010	0.547
C14:0	11.00	10.27	11.10	11.99	0.701	0.174
Cis-9-C14:1	0.72^{b}	0.86^{ab}	0.71^{b}	1.32^{a}	0.180	0.037
Iso-C15:0	0.19	0.18	0.18	0.21	0.018	0.347
Anteiso-C15:0	0.40	0.37	0.35	0.41	0.054	0.257
C15:0	0.81^{ab}	0.73^{b}	0.78^{b}	0.94^{a}	0.103	0.018
Anteiso-C16:0	0.19	0.20	0.19	0.15	0.030	0.708
C16:0	23.6^{4b}	25.02^{b}	25.51^{b}	28.58^{a}	1.293	0.003
Cis-9-C16:1	1.47	1.71	1.27	2.28	0.363	0.198
C17:0	0.54	0.53	0.53	0.48	0.021	0.312
Cis-9-C17:1	0.17	0.19	0.14	0.17	0.039	0.747
C18:0	14.25^{a}	14.94^{a}	13.49^{a}	6.57^{b}	1.312	0.002
Total *trans*-C18:1[2]	5.43^{b}	4.18^{b}	4.36^{b}	17.18^{a}	1.212	<0.001
Trans-6+7+8-C18:1	0.34^{b}	0.28^{b}	0.26^{b}	0.83^{a}	0.071	0.002
Trans-9-C18:1	0.26^{b}	0.21^{b}	0.20^{b}	0.56^{a}	0.058	0.014
Trans-10-C18:1	0.43^{b}	0.57^{b}	0.33^{b}	7.47^{a}	1.095	0.006
Trans-11-C18:1	1.31^{b}	0.63^{b}	1.06^{b}	3.20^{a}	0.323	0.006
Trans-12-C18:1	0.44^{b}	0.34^{b}	0.45^{b}	1.00^{a}	0.071	<0.001
Trans-13+14-C18:1	1.47^{b}	1.13^{b}	1.18^{b}	2.59^{a}	0.222	0.005
Trans-15-C18:1	0.56	0.47	0.40	0.77	0.092	0.123
Trans-16+*Cis*-14-C18:1	0.64	0.54	0.47	0.76	0.102	0.292
Total *cis*-C18:1[3]	22.83^{a}	24.40^{a}	19.55^{a}	11.90^{b}	1.808	0.001
Cis-9-C18:1	21.68^{a}	23.33^{a}	18.60^{a}	10.32^{b}	1.807	<0.001
Cis-11-C18:1	0.46	0.48	0.43	0.80	0.127	0.169
Cis-12-C18:1	0.34^{a}	0.26^{ab}	0.30^{ab}	0.14^{b}	0.048	0.039
Cis-13-C18:1	0.08^{ab}	0.09^{ab}	0.05^{b}	0.19^{a}	0.024	0.023
Cis-15-C18:1	0.28^{ab}	0.25^{ab}	0.16^{b}	0.44^{a}	0.070	0.048
Total non-conjugated C18:2[4]	2.45	2.32	2.99	3.31	0.364	0.062
Trans-9,*trans*-12-C18:2	0.45^{ab}	0.41^{b}	0.32^{b}	0.69^{a}	0.093	0.011
Trans-11,*cis*-15-C18:2	0.31^{b}	0.23^{b}	0.27^{b}	0.98^{a}	0.135	0.015
Cis-9,*cis*-12-C18:2	1.30^{b}	1.29^{b}	2.12^{a}	1.14^{b}	0.158	0.003
Total conjugated C18:2[5]	0.57^{b}	0.35^{b}	0.45^{b}	1.45^{a}	0.199	0.007
Cis-9,*trans*-11-C18:2	0.56^{b}	0.35^{b}	0.43^{b}	1.45^{a}	0.199	0.007
Trans-10,cis-12-C18:2	0.01	0.00	0.01	0.00	0.007	0.404
Cis-9,*cis*-12,*cis*-15-C18:3	0.87^{b}	0.83^{b}	3.19^{a}	0.46^{b}	0.253	<0.001

Table 4. Continued.

Fatty acid	Dietary treatment				SEM[1]	P-value
	CL	EL	FL	DL		
Total ≥ C20:0[6]	0.58	0.53	0.71	0.44	0.061	0.076
C20:0	0.13	0.11	0.12	0.10	0.013	0.332
C20:1	0.07[ab]	0.09[a]	0.06[ab]	0.01[b]	0.007	0.034
C20:2	0.09[ab]	0.07[b]	0.18[a]	0.03[b]	0.027	0.010
C20:4*n*6	0.04	0.03	0.04	0.02	0.010	0.371
C21:0	0.02[b]	0.00[b]	0.02[b]	0.05[a]	0.008	0.004
C22:0	0.05	0.04	0.04	0.05	0.008	0.783
C22:1*n*9	0.05	0.06	0.05	0.04	0.005	0.203
C22:5*n*6	0.08	0.07	0.10	0.07	0.010	0.120
C22:6*n*3	ND[12]	ND	ND	ND		
C23:0	0.06[ab]	0.06[a]	0.08[a]	0.03[b]	0.008	0.009
Unidentified	0.67[b]	0.67[b]	0.59[b]	0.85[a]	0.063	0.007
Summary						
SFA[7]	63.47	63.24	65.37	59.83	2.536	0.108
OBCFA[8]	3.04	2.82	2.93	3.03	0.246	0.096
MUFA[9]	31.23[ab]	31.95[ab]	26.58[b]	33.62[a]	2.288	0.037
PUFA[10]	4.11[b]	3.67[b]	6.96[a]	5.35[ab]	0.707	0.008
UFA[11]	35.34	35.62	33.54	38.97	2.504	0.110

[a,b]Means within a row with different superscripts differ ($P < 0.05$).
[1]SEM: standard error of mean.
[2]Total *trans*-C18:1: ∑ (*trans*-4-C18:1, *trans*-5-C18:1, *trans*-6+7+8-C18:1, *trans*-9-C18:1, *trans*-10-C18:1, *trans*-11-C18:1, *trans*-12-C18:1, *trans*-13+14-C18:1, *trans*-15-C18:1, *trans*-16+*cis*-14-C18:1).
[3]Total *cis*-C18:1: ∑ (*cis*-9-C18:1, *cis*-11-C18:1, *cis*-12-C18:1, *cis*-13-C18:1, *cis*-15-C18:1).
[4]Total non-conjugated C18:2: ∑ (*trans*-9,*trans*-12-C18:2, *cis*-9,*trans*-13-C18:2, *trans*-8,*cis*-13-C18:2, *cis*-9,*trans*-12-C18:2, *trans*-9,*cis*-12-C18:2, *trans*-11,*cis*-15-C18:2, *cis*-9,*cis*-12-C18:2, *cis*-9,*cis*-15-C18:2).
[5]Total conjugated C18:2: ∑ (*cis*-9,*trans*-11-C18:2, *trans*-10,*cis*-12-C18:2).
[6]Total > C20:0: ∑ (C20:0, C20:1, C20:2, C20:3*n*6, C20:3*n*3, C20:4*n*6, C21:0, C22:0, C22:1*n*9, C22:2, C22:5*n*6, C22:6*n*3, C23:0, C24:0).
[7]Saturated fatty acids: ∑ (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0, C24:0).
[8]Odd and branched chain fatty acids: ∑ (C11:0, *iso*-C13:0, *anteiso*-C13:0, C13:0, *iso*-C14:0, *anteiso*-C14:0, *iso*-C15:0, *anteiso*-C15:0, C15:0, *cis*-9-C15:1, *iso*-C16:0, *anteiso*-C16:0, *iso*-C17:0, *anteiso*-C17:0, C17:0, unknown C17, *cis*-9-C17:1).
[9]Mono-unsaturated fatty acids: ∑ (*cis*-9-C14:1, *cis*-9-C16:1, 3 unknown C16:1, Total *cis*-C18:1, Total *trans*-C18:1, C20:1, C22:1*n*9).
[10]Poly-unsaturated fatty acids: ∑ (Total non-conjugated C18:2, Total conjugated C18:2, C18:3*n*6, C18:3*n*3, C20:2, C20:3*n*6, C20:3*n*3, C20:4*n*6, C22:2, C22:5*n*6, C22:6*n*3).
[11]Unsaturated fatty acids: ∑ (MUFA, PUFA).
[12]ND: not detectable.

Discussion

Milk yield and composition

The aim of the current study was to determine the effects of various linseed treatments on FA profiles in blood plasma and milk fat. Petit (2010) reviewed the literature with regard to the effects of feeding linseed on production performance of dairy cows and reported that the physical breakdown of linseed before feeding generally results in an increased milk production. In the current experiment all linseed sources were treated, either physically by crushing, technologically by extruding, chemically by emulsifying and formaldehyde treating, or just feeding as linseed oil. Combined with the restricted DMI and similar nutrient flows (DM, OM, CP, NDF, and crude fat) and digestibility coefficients (DM, OM, and NDF; Chapter 4), this resulted in an absence of effect of the linseed treatments on milk production. Cows fed linseed oil in combination with 11.6 g/d DHA produced 34% less milk fat compared with cows fed the CL, EL, and FL treatments. Boeckaert et al. (2008a) reported a decrease of 59% in milk fat yield when feeding 43.7 g/d DHA to dairy cows. The reduced milk fat secretion is generally related to the inhibition of *de novo* FA synthesis in the mammary gland due to increased proportions of *trans*-10,*cis*-12-C18:2. The proportion of *trans*-10,*cis*-12-C18:2 was significantly increased for the DL treatment in the omasal flow (Chapter 4), whereas differences in plasma TAG and milk fat were not detected due to the low proportions found. An intermediate of the *trans*-10,*cis*-12-C18:2 pathway is *trans*-10-C18:1, which is strongly related to milk fat depression in several studies, whereas no regulatory role was demonstrated (Lock et al., 2007). Proportion of *trans*-10-C18:1 in the present study was significantly higher for the DL treatment in omasal flow, plasma TAG and milk fat. Processing of linseed shows little effect on milk protein proportion and yield, and milk lactose proportion and yield (Petit, 2010), which is in agreement with the results of the present study, where no differences were found between the different linseed treatments.

Plasma and milk fatty acid composition

Plasma FA are present in different lipid fractions, including cholesterol esters, phospholipids, TAG and nonesterified FA (Loor et al., 2002b) and FA are preferentially incorporated into these lipid fractions. Addition of unprotected rapeseed oil and linseed oil resulted in higher total blood plasma proportions of *cis*-9-C18:1 and C18:3*n*3, respectively, but addition of unprotected soybean oil did not significantly increase the proportion of *cis*-9,*cis*-12-C18:2 in blood plasma (Jacobs et al., 2011). This is probably because the latter is the most abundant FA in blood plasma and preferentially incorporated into phospholipids and plasma cholesterol esters. This was also shown by Loor et al. (2002b) who found that cows fed mechanically extracted soybean meal had a higher *cis*-9,*cis*-12-C18:2 proportion in phospholipids, cholesterol esters and triglycerides, whereas *trans*-11-C18:1 was only increased in the TAG fraction. However, the mammary gland primarily extracts FA from TAG and nonesterified FA fractions (Loor et al., 2002b) and therefore, FA profile of the TAG fraction was reported in the present study.

C18:3*n*3 proportion was higher in both plasma TAG and milk fat for the FL treatment compared with the other treatments. Without protection, average C18:3*n*3 proportion in milk fat maximally

increases to 1.2% of total FA (Glasser et al., 2008), whereas unsupplemented diets generally contain 0.4 to 0.6% of total FA (Heck et al., 2009). C18:3*n*3 proportion in the FL treatment reached 3.19% of total FA and it is therefore concluded that part of the C18:3*n*3 from the FL treatment was protected against biohydrogenation. This is in agreement with the in vitro results of Sterk et al. (2010), who found that pretreatment followed by formaldehyde-treatment of linseed provides an effective protection against biohydrogenation. However, omasal flow of C18:3*n*3 for the FL treatment was similar compared with the CL treatment and lower compared with the EL treatment (Chapter 4). Earlier results (Sterk, unpublished) confirm the high C18:3*n*3 proportion in milk fat after feeding a formaldehyde-treated combination of linseed oil and soybean oil. In the companion paper it was suggested that the flow of C18:3*n*3 could have been underestimated due to the specific functional gravity of the product (Chapter 4).

In an earlier in vitro study, extruded whole linseed showed a lower calculated effective biohydrogenation compared with crushed linseed (Sterk et al., 2010), but in the current study a similar C18:3*n*3 proportion in milk fat was found for the EL treatment compared with the CL treatment. Chilliard et al. (2009) compared whole linseed with extruded linseed and reported a higher C18:3*n*3 proportion in milk fat for the cows that received extruded linseed. The researchers suggest that extrusion increases the rate of oil release from the seeds resulting in some protection of the C18:3*n*3 against biohydrogenation (Chilliard et al., 2009), or increases the rate of passage to the duodenum (Doreau et al., 2009b). In the present study, whole linseed was extruded but the extrusion process did likely not lead to complete rupture of the seed coat. This was confirmed by the higher omasal C18:3*n*3 flow, but lower whole tract crude fat digestibility compared with the other treatments (Chapter 4).

C18:3*n*3 proportion in both milk fat and plasma TAG for the DL treatment was similar compared with the CL and EL treatments. This is in agreement with earlier in vitro results, which showed that calculated effective C18:3*n*3 biohydrogenation from linseed oil was not influenced by DHA addition (Sterk et al., 2010). Proportion of C18:3*n*3 was therefore not influenced, whereas complete biohydrogenation to C18:0 is inhibited resulting in the marked increase in biohydrogenation intermediates. Boeckaert et al. (2008a) reported a DHA proportion in milk fat of 0.28 g/100 g of FA after feeding 43.7 g DHA/d, but in the current study DHA could not be detected in plasma TAG or milk fat after feeding 11.6 g DHA/d, due to the lower DHA supply and the high extent of biohydrogenation of DHA (Fievez et al., 2007). In the present study, the lower C18:0 and higher *trans*-FA proportions in both plasma TAG and milk fat for the DL treatment reflect the inhibition of the biohydrogenation steps from *trans*-11,*cis*-15-C18:2 to *trans*-11-C18:1 and further to C18:0. The proportion of *cis*-9,*trans*-11-C18:2 was only higher in milk fat. This FA is not an intermediate in the biohydrogenation of C18:3*n*3, but is mainly produced in the mammary gland from *trans*-11-C18:1. Increased milk fat proportions of *trans*-10-C18:1, *trans*-11-C18:1, *trans*-11,*cis*-15-C18:2, and *cis*-9,*trans*-11-C18:2 after algae supplementation were also found in the study of Boeckaert et al. (2008a). Both Shingfield et al. (2006) and Boeckaert et al. (2008a) observed a shift from the production of *trans*-11-C18:1 to a relatively greater production of *trans*-10-C18:1. In the present study also a large increase in the proportion of *trans*-10-C18:1 was observed. This might be related to the inclusion of

Table 5. Transfer efficiency (%) of C18:3*n*3 from feed to milk and from omasal flow to milk of cows fed diets supplemented with crushed linseed (CL), extruded whole linseed (EL), formaldehyde-treated linseed oil (FL) and docosahexaenoic acid addition to linseed oil (DL)

Parameter	Dietary treatment				SEM[1]	P-value
	CL	EL	FL	DL		
C18:3*n*3 efficiency from intake to milk	3.2[b]	3.0[b]	13.1[a]	1.3[b]	0.63	<0.001
C18:3*n*3 efficiency from omasal flow to milk	59.2[b]	33.5[b]	287.8[a]	89.1[b]	16.33	<0.001

[a,b]Means within a row with different superscripts differ ($P < 0.05$).
[1]SEM: standard error of mean.

DHA in combination with the linseed oil, resulting in various biohydrogenation intermediates from C18:3*n*3 through the inhibition of the last step of biohydrogenation to C18:0.

Transfer efficiency

Transfer efficiency from C18:3*n*3 intake to C18:3*n*3 yield in milk varied between 1.3% for the DL treatment, 3.0% for the EL treatment, 3.2% for the CL treatment, and up to 13.1% for the FL treatment. Efficiencies for the DL, EL, and CL treatments were largely in line with reported

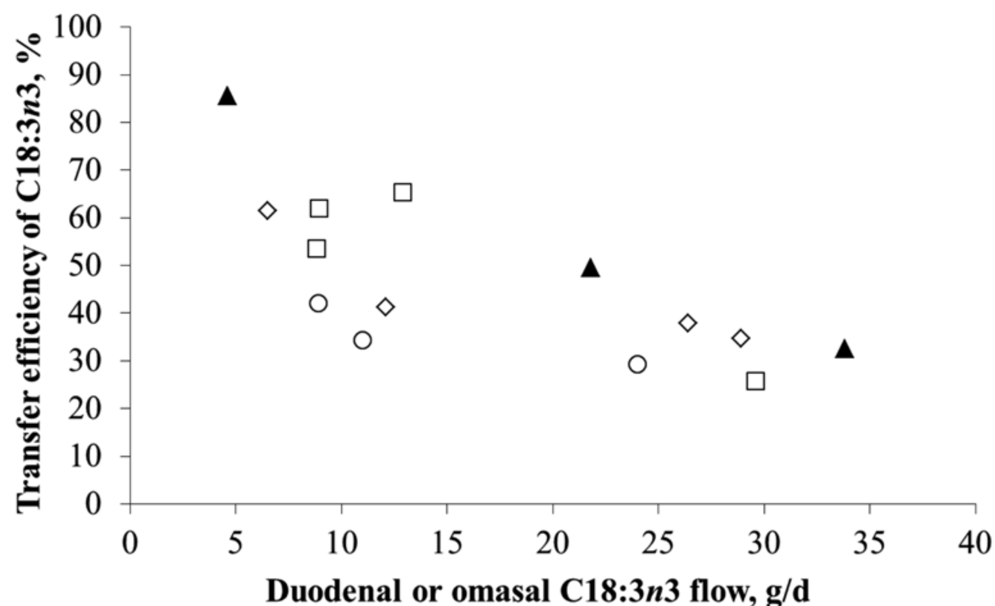

Figure 1. Transfer efficiency from C18:3*n*3 flow in the duodenum or omasum to C18:3*n*3 in milk. (◊) Gonthier et al., 2004; 2005; (□) Loor et al., 2004; 2005b; (○) Loor et al., 2005c; 2005d; (▲) current study (excluding treatment FL). Transfer efficiency = 67.9 (± 6.6) - 1.2 (± 0.3) x C18:3*n*3 flow; $R^2 = 0.53$; $P = 0.003$.

transfer efficiencies for raw linseed (2.0%), and extruded linseed (2.2%; Gonthier et al., 2005), and crude linseed (1.4%), extruded linseed (1.9%), and linseed oil (0.5%; Chilliard et al., 2009). Transfer efficiency for the FL treatment in the current study was significantly higher, confirming the effective protection of C18:3*n*3 in this treatment. Chilliard et al. (2000) reported transfer efficiencies from C18:3*n*3 infused into the intestine to C18:3*n*3 secreted in milk to range from 35 to 70%. Figure 1 shows the transfer efficiency from duodenal or omasal flow of C18:3*n*3 to milk yield of C18:3*n*3 for the current study and the studies of Gonthier et al. (2004; 2005) and Loor et al. (2004; 2005b, c, d). Transfer efficiency ranged from 26 to 86% and was negatively correlated with the flow of C18:3*n*3 in duodenum or omasum ($R^2 = 0.53$; $P < 0.01$; excluding FL treatment). Due to the low omasal flow of C18:3*n*3 and the high C18:3*n*3 yield in milk for the FL treatment, the calculated transfer efficiency for this treatment was physiologically impossible (288%), indicating omasal flow of C18:3*n*3 to be underestimated as discussed previously (Chapter 4).

Conclusions

Feeding formaldehyde-treated linseed oil, but not extruded whole linseed or linseed oil with DHA, resulted in higher C18:3*n*3 proportions in plasma TAG and milk fat compared with unprotected, crushed linseed. Transfer efficiency from C18:3*n*3 in feed to C18:3*n*3 in milk was much higher for the cows receiving formaldehyde-treated linseed oil. Feeding DHA in combination with linseed oil resulted in an inhibition of the complete biohydrogenation of C18:3*n*3 to C18:0, increased proportions of biohydrogenation intermediates in plasma and milk fat and decreased milk fat secretion.

Acknowledgments

The authors would like to thank the staff of trial farm The Ossekampen, Wageningen University for their assistance in animal care, feeding and sampling; the laboratory staff of the Animal Nutrition Group, Wageningen University, for their help in chemical analysis; and Tamme Zandstra and Sven Alferink for their assistance in preparing the linseed treatments. MSc students Steven van der Weerd and Ard van Veen are gratefully acknowledged for their contribution in the experiment. The financial support of the Food and Nutrition Delta program of Senter Novem (Den Haag, the Netherlands), Royal FrieslandCampina (Amersfoort, The Netherlands), Agrifirm Group (Apeldoorn, The Netherlands), Agrifirm Innovation Center (Apeldoorn, The Netherlands), and Barenbrug Holland B.V. (Oosterhout, The Netherlands) is gratefully acknowledged.

Chapter 6

Effects of forage type, forage to concentrate ratio, and crushed linseed supplementation on milk fatty acid profile in lactating dairy cows

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Journal of Dairy Science: In Press

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Abstract

The effects of an increasing proportion of crushed linseed (CL) in combination with varying forage type (grass or maize silage), forage to concentrate ratio (F/C ratio), and their interactions on milk fatty acid (FA) profile of high producing dairy cows was studied using a 3-factor Box-Behnken design. Sixteen Holstein and twenty Swedish Red cows were blocked according to breed, parity, and milk yield, and randomly assigned to four groups. Groups were fed different treatment diets formulated from combinations of the three main factors each containing three levels. Forage type (fraction of total forage DM) included 20%, 50%, and 80% grass silage, with the remainder being maize silage. Forage to concentrate ratio (DM basis) was 35:65, 50:50, and 65:35 and CL was supplied at 1%, 3%, and 5% of diet DM. Starch and NDF content (DM basis) of the treatment diets ranged from 117 to 209 g/kg and 311 to 388 g/kg, respectively. Thirteen treatment diets were formulated according to the Box-Behnken design. During four experimental periods of 21 d each, all treatment diets were fed including a repetition of the centre point treatment (50% grass silage, 50:50 F/C ratio, 3% CL) during every period. Intake, production performance and milk FA profile were measured and response surface equations were derived for these variables. Shifting from 80% grass silage to 80% maize silage in the diet linearly increased DMI, NE_L intake, *cis*-9,*cis*-12-C18:2 (C18:2*n*6) intake, and milk yield, and linearly decreased *cis*-9,*cis*-12,*cis*-15-C18:3 (C18:3*n*3) intake and milk fat content. Shifting from a high-forage diet to a high-concentrate diet linearly increased DMI, NE_L intake, C18:2*n*6 intake, and milk yield, and decreased milk fat content. Supplementation of CL linearly increased C18:3*n*3 intake, but had no effect on DMI, NE_L intake, milk yield, and milk fat content. Shifting from 80% grass silage to 80% maize silage linearly increased proportions of *trans*-10-C18:1 and C18:2*n*6 in milk fat, whereas proportions of *trans*-11,*cis*-15-C18:2 and C18:3*n*3 linearly decreased. Significant interactions between CL supplementation and F/C ratio were found for proportions of *trans*-10-C18:1, *trans*-15-C18:1, *cis*-15-C18:1, *trans*-11,*cis*-15-C18:2, and C18:3*n*3 in milk fat, with the highest levels achieved when the diet contained 5% CL and a 35:65 F/C ratio. This study showed that the effect of supplementing CL on several milk FA proportions, including C18:2*n*6 and C18:3*n*3, depends significantly on the F/C ratio and forage type in the basal diet.

Introduction

Due to its relatively large proportion of saturated FA, dairy milk fat has been associated with human cardiovascular health problems (Elwood et al., 2010; Bauman and Lock, 2010). On the contrary, mono-unsaturated FA such as oleic acid (*cis*-9-C18:1), long chain omega-3 FA, and conjugated linoleic acid in milk fat have been associated with potential benefits for human health (Bauman and Lock, 2010). Because of these effects of milk FA profile on human health, the dietary manipulation of milk FA profile has been the subject of extensive research in the last years. The fatty acid (FA) profile of milk fat is largely dependent on FA intake and FA metabolism in the rumen (Jenkins et al., 2008), and on lipid mobilization and FA metabolism in the mammary gland (Chilliard et al., 2007). Dietary FA are extensively metabolized and hydrogenated in the rumen, resulting in a wide range of ruminal biohydrogenation intermediates (Chilliard et al., 2007). Ruminal biohydrogenation of *cis*-9,*cis*-12-C18:2 (C18:2*n*6) and *cis*-9,*cis*-12,*cis*-15-C18:3 (C18:3*n*3) results in the secretion of various *trans*-C18:1, *cis*-C18:1, and non-conjugated and conjugated C18:2 and C18:3 isomers in milk fat. Chilliard et al. (2007) reported that the main factor in the variation of biohydrogenation is the forage to concentrate ratio (F/C ratio) in the diet. After adding linseed oil to a high concentrate diet, major biohydrogenation intermediates secreted in milk fat were *trans*-11-C18:1, *trans*-13+14-C18:1, *cis*-9,*trans*-13-C18:2, and *trans*-11,*cis*-15-C18:2 (Loor et al., 2005b), whereas *trans*-15-C18:1 and *cis*-15-C18:1 were increased in duodenal flow (Loor et al., 2004). Compared with grass silage, inclusion of maize silage in a diet supplemented with fish oil and sunflower oil resulted in higher proportions of *trans*-C18:1 and lower proportions of C18:0 and *trans*-C18:2 in milk fat (Shingfield et al., 2005). There appears to be a pronounced impact of the basal diet on ruminal metabolism of FA from supplemental fat sources (Shingfield et al., 2005, Soita et al., 2005), which might be related to shifts in rumen pH and microbial populations. Feeding a high starch diet markedly affects the ratio of cellulolytic to propionogenic, lactogenic, and amylolytic bacteria, which in turn affects ruminal biohydrogenation (Latham et al., 1972, Loor et al., 2004). Thus, interactions between level of lipid supplementation and other dietary changes are likely to occur.

Few direct comparisons exist between the different characteristics of the basal diet, such as type of forage and F/C ratio, and lipid supplements. In addition, a large diversity of diets exists and quantifying interactions is of great importance. To our knowledge, the effects of adding crushed linseed (CL) to diets that vary in F/C ratio and in proportion of grass silage versus maize silage and their interactions on milk FA profile within a single experiment have not been reported. Designing an experiment in which multiple factors are considered simultaneously allows quantification of the curvature in relationships as well as interactions among factors (St-Pierre and Weiss, 2009). The Box-Behnken design (Box and Behnken, 1960) is a multifactor experimental model specifically designed for the exploration of response surfaces and it involves a lower number of experimental points compared with a full-factorial design. The objective of this study was therefore to evaluate the effects of CL supplementation, and varying forage type and F/C ratio, and their mutual interactions, on intake, production performance, and milk FA profile. The study was carried out by varying grass silage at the expense of maize silage, F/C ratio, and level of CL supplementation in a 3-factor multivariate Box-Behnken design.

Materials and Methods

Experimental design and diets

The experimental design was a 3-factor Box-Behnken design with forage type (grass silage or maize silage), F/C ratio, and proportion of CL supplementation as the main factors. Forage type included 20%, 50%, and 80% grass silage (DM basis), with the remainder being maize silage. Forage to concentrate ratio was 35:65, 50:50, and 65:35 (DM basis) and CL was supplied at 1%, 3%, and 5% of diet (DM basis). Thirteen treatment diets with varying levels of grass silage, maize silage, F/C ratio, and CL were formulated according to the Box-Behnken design, including the centre point treatment (50% grass silage, 50:50 F/C ratio, and 3% CL). The experiment consisted of four experimental periods of 21 d each, with four treatments evaluated, including the centre point treatment, during each period. As such the centre point treatment was repeated four times (Table 1). To formulate the treatment diets, three commercial concentrate mixtures were used and the treatment diets were balanced for crude protein content. Contents of starch and NDF were allowed to differ for the different treatment diets because of the varying forage type and F/C ratio. Starch and NDF content (DM basis) in the treatment diets ranged from 117 to 209 g/kg and 311 to 388 g/kg, respectively. The treatment diets met or exceeded the requirement for NE_L (Dutch NE_L system; Van Es, 1975) and intestinal digestible protein (DVE; Tamminga et al., 1994). All treatment diets were offered as TMR diets. The CL was obtained from Vegolia (Falkenberg, Sweden). The specified ingredient and chemical composition of the diets are shown in Tables 2 and 3, respectively. Increasing grass silage % mainly decreased starch and C18:2*n*6 contents, whereas C18:3*n*3 content increased (Table 3). Increasing forage proportion mainly increased NDF and forage NDF contents, whereas starch, NE_L, DVE, C12:0, C14:0, C16:0, *cis*-9-C18:1, C18:2*n*6, and C18:3*n*3 contents decreased. Increasing CL proportion mainly increased C18:3*n*3 content in the diets.

Table 1. Experimental design for the different cow groups, periods and treatment combinations with varying forage type (20, 50, and 80% grass silage; DM basis), forage to concentrate ratio (35, 50, and 65% forage; DM basis), and proportion of crushed linseed (1, 3, and 5% CL; DM basis)

Cow group	Period	Forage type (% grass silage)	Forage to concentrate ratio	Crushed linseed (%)
1	1	80	35:65	3
1	2	50	50:50	3
1	3	80	65:35	3
1	4	50	65:35	5
2	1	20	50:50	5
2	2	20	65:35	3
2	3	50	50:50	3
2	4	50	35:65	1
3	1	50	35:65	5
3	2	20	50:50	1
3	3	80	50:50	5
3	4	50	50:50	3
4	1	50	50:50	3
4	2	20	35:65	3
4	3	50	65:35	1
4	4	80	50:50	1

Table 2. Ingredient composition (DM basis; g/kg DM) for diets with varying forage type (20, 50, and 80% grass silage; DM basis), forage to concentrate ratio (F/C ratio; 35, 50, and 65% forage; DM basis), and proportion of crushed linseed (1, 3, and 5% CL; DM basis)

Ingredient	Forage type (grass silage %)												
	20				50					80			
	F/C ratio (forage %)												
	35	50		65	35		50	65		35	50		65
	Crushed linseed (%)												
	3	1	5	3	1	5	3	1	5	3	1	5	3
Grass silage[1]	70	100	100	130	175	175	250	325	325	280	400	400	520
Maize silage[2]	280	400	400	520	175	175	250	325	325	70	100	100	130
Crushed linseed[3]	30	10	50	30	10	50	30	10	50	30	10	50	30
Wheat	131	94	87	48	142	135	105	66	57	148	123	114	75
Oats	120	86	79	43	130	123	95	60	52	135	112	104	68
Rapeseed meal, heat treated	92	63	58	29	99	94	66	41	35	101	76	70	46
Soybean meal	41	60	55	75	29	24	41	52	47	13	22	18	24
Soypass[4]	53	48	44	39	53	50	47	38	33	51	48	44	33
Sugar beet pulp	63	39	36	14	67	63	34	19	17	65	36	34	22
Rapeseed meal	22	33	30	41	16	13	22	28	26	7	12	10	13
Rapeseed, crushed	3	4	4	5	2	2	3	4	3	1	2	1	2
Oat bran	25	13	12	2	26	25	8	3	3	24	6	6	4
Wheat bran meal	9	4	4	-	9	8	1	-	-	8	-	-	-
Triticale	7	3	3	-	7	7	1	-	-	6	-	-	-
Palm expeller	3	3	3	3	4	4	5	4	3	5	7	6	4
DDGS[5]	5	5	4	4	5	5	7	5	4	6	9	8	5

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Table 2. Continued

Ingredient	Forage type (grass silage %)												
	20				50					80			
	F/C ratio (forage %)												
	35	50		65	35		50	65		35	50		65
	Crushed linseed (%)												
	3	1	5	3	1	5	3	1	5	3	1	5	3
Other[6]	44	32	30	17	47	45	33	22	19	48	37	34	23
Premix[7]	1	1	1	1	1	1	1	1	1	1	1	1	1

[1]Grass silage, g/kg DM: 252 DM (g/kg), 89 crude ash, 184 CP, 36 crude fat, 486 NDF, 15 sugar, 64 DVE [Intestinal digestible protein (Tamminga et al., 1994)], 5.82 MJ NE_L [Net energy for lactation calculated with VEM system (Van Es, 1975)], 18.0 total fatty acids, 0.1 C12:0, 0.1 C14:0, 3.8 C16:0, 0.3 cis-9-C16:1, 0.3 C18:0, 0.4 cis-9-C18:1, 3.3 cis-9,cis-12-C18:2, 9.6 cis-9,cis-12,cis-15-C18:3.

[2]Maize silage, g/kg DM: 271 DM (g/kg), 38 crude ash, 86 CP, 19 crude fat, 483 NDF, 210 starch, 45 DVE, 6.05 MJ NE_L, 13.2 total fatty acids, 0.0 C12:0, 0.1 C14:0, 3.4 C16:0, 0.3 C18:0, 2.3 cis-9-C18:1, 6.3 cis-9,cis-12-C18:2, 0.6 cis-9,cis-12,cis-15-C18:3.

[3]Crushed linseed, g/kg DM: 932 DM (g/kg), 42 crude ash, 198 CP, 433 crude fat, 293 NDF, 9 starch, 22 sugar, 70 DVE, 12.11 MJ NE_L, 335.6 total fatty acids, 0.0 C12:0, 0.2 C14:0, 19.9 C16:0, 7.5 C18:0, 53.9 cis-9-C18:1, 50.1 cis-9,cis-12-C18:2, 203.6 cis-9,cis-12,cis-15-C18:3 (Vegolia, Falkenberg, Sweden).

[4]Soypass, heat, xylose, and lignosulfate treated soybeanmeal (Cargill, Amsterdam, The Netherlands).

[5]Dried distillers grains and solubles from wheat.

[6]Containing: blendmeal ruminants, (Lantmännen, Lidköping, Sweden), magnesium oxide, monocalcium phosphate, salt, limestone, Akofeed 45 (AarhusKarlshamn, Karlshamn, Sweden), Lipitec Bovi 85 (Lipitec, Vantinge, Denmark), Lignobond DD (Borregaard Lignotechn, Sarpsborg, Norway).

[7]Contained per kg of mix: 58 g of Ca, 416 g of Mg, 1 g of S, 5 g of Cu, 10 g of Mn, 350 mg of I, 90 mg of Co, 200 mg of Se, 2000000 IU of vitamin A, 1000000 IU of vitamin D, 20000 mg of vitamin E (all-rac tocopherol acetate; Premix KO, Lantmännen, Sweden).

Table 3. Chemical composition of diets with varying forage type (20, 50, and 80% grass silage; DM basis), forage to concentrate ratio (F/C ratio; 35, 50, and 65% forage; DM basis), and proportion of crushed linseed (1, 3, and 5% CL; DM basis)

Composition, g/kg DM	Forage type (grass silage %)												
	20				50					80			
	F/C ratio (forage %)												
	35	50		65	35		50	65		35	50		65
	Crushed linseed (%)												
	3	1	5	3	1	5	3	1	5	3	1	5	3
DM, g/kg	504	428	425	373	474	510	416	352	344	518	389	412	356
CP	177	176	178	172	183	182	185	180	177	187	189	189	187
Crude fat	60	45	60	45	57	69	56	42	59	64	54	65	53
NDF	311	344	346	382	314	317	340	369	388	317	345	326	365
Forage NDF	172	245	244	319	171	171	245	318	318	171	244	244	317
ADF	176	202	200	229	193	185	211	232	242	193	223	210	240
ADL	29	29	29	29	35	31	32	31	32	33	34	33	34
Starch	209	187	184	155	204	197	170	151	134	189	154	151	117
Sugar	53	50	49	44	43	51	46	41	36	50	37	44	38
Crude ash	57	56	57	54	62	64	63	64	59	70	66	71	74
NE_L, MJ/kg DM[1]	7.55	7.12	7.28	6.85	7.45	7.60	7.15	6.73	6.89	7.50	7.04	7.20	6.78
DVE[2]	98	96	93	89	98	96	94	89	86	96	94	91	85
Fatty acids													
C12:0	1.0	0.6	0.6	0.2	1.1	1.0	0.4	0.2	0.2	1.0	0.2	0.3	0.2
C14:0	0.6	0.4	0.4	0.2	0.7	0.6	0.4	0.2	0.2	0.6	0.4	0.3	0.2
C16:0	12.8	10.4	10.2	8.1	14.8	12.9	11.1	8.5	9.6	13.1	13.2	11.2	8.8
C18:0	3.0	2.3	2.3	1.6	2.1	3.1	2.5	2.2	1.6	3.1	2.2	3.5	2.4
Cis-9-C18:1	10.7	8.0	9.6	7.0	11.9	11.5	9.0	5.8	8.5	10.6	9.2	9.0	6.3
Cis-9,*cis*-12-C18:2	11.4	9.3	10.6	8.8	10.3	11.6	9.5	7.7	9.5	10.5	8.7	9.7	7.8
Cis-9,*cis*-12,*cis*-15-C18:3	7.9	3.9	11.3	8.3	4.7	12.0	9.3	6.0	14.2	9.1	6.7	14.8	11.9
Total fatty acids[3]	47.7	35.1	45.2	34.6	45.8	52.9	42.4	30.9	44.3	48.4	40.9	49.1	38.1

[1]NE_L: Net energy for lactation calculated with VEM system (Van Es, 1975).
[2]DVE: Intestinal digestible protein (Tamminga et al., 1994).
[3]Total fatty acids: ∑ (C12:0, C14:0, C16:0, C18:0, *cis*-9-C18:1, *cis*-9,*cis*-12-C18:2, *cis*-9,*cis*-12,*cis*-15-C18:3, C20:0).

Animals and housing

The experiment was approved and carried out under the Swedish Law on Animal Experimentation. Sixteen Holstein and twenty Swedish Red cows (620 ± 50 kg BW; 2.1 ± 0.9 parity; 72 ± 17 DIM; 48.1 ± 5.3 kg/d milk; values expressed as means ± SD) were blocked according to breed, parity, and milk yield, and randomly assigned to four groups. Groups were fed the different treatment diets during the four experimental periods. Cows were housed in a free stall with slatted floor and boxes bedded daily with sawdust on top of rubber mattresses. Individual feed intake was continuously monitored using automated feed bins with weighing equipment (BioControl A/S, Rakkestad, Norway). Each group of nine cows had access to five automated feed bins. Cows were fitted with transponders to enable individual feed intake recording from the automated feed bins. Weight changes of the bins (accuracy 0.1 kg) were recorded and the bins were calibrated at the start of the experiment. Cows had free access to water and were milked thrice daily at 0600, 1300, and 2100h.

Measurements and sampling

The DMI and milk production were recorded daily during each experimental period. Milk samples were collected over 9 consecutive milkings during the last 3 days of each period, pooled per day (equal volume), and stored at 4ºC using sodium azide bronopol as preservative pending analysis for fat, protein, lactose, and MUN. A second set of milk samples was taken on the same 9 consecutive milkings and immediately stored at -20ºC pending FA analysis. These samples were pooled (equal volume) per cow per period during the first step in the FA analysis. Samples of all individual feed components were taken on the last 3 days of each period, pooled per period and stored at -20ºC pending analysis.

Analytical procedures

Contents of fat, protein, lactose, and MUN in milk samples were analysed by a Milkoscan FT 6000 (A/S N., Foss Electric, Hillerød, Denmark) at Steins Laboratory (Jönköping, Sweden). Milk samples for FA analysis were heated to 40ºC and 3 mL of each individual cow milk sample was taken and pooled to form a representative milk sample of 27 mL per cow per period. These samples were then subjected to the same procedure as described by Jacobs et al. (2011). The composite samples of the individual feed components were analysed for DM, ash, nitrogen (N), crude fat, starch, sugars, NDF, ADF, and acid detergent lignin (ADL), as described by Abrahamse et al. (2008a, b). Preparation of feed samples for FA analysis was carried out as described by Khan et al. (2009).

Fatty acid methyl esters from milk and feed samples were quantified using gas chromatography (Trace GC Ultra™, Thermo Fisher Scientific, Waltham MA, USA) with a fused silica capillary column (100 m x 0.25 mm and 0.2 µm film thickness; Restek; Rt®-2560, Bellefonte PA, USA). The carrier gas was hydrogen at a constant flow of 0.9 mL/min and the flame ionization detector was set at 280ºC. For milk samples, a time-temperature program was employed starting with an initial temperature of 70ºC and held for 4 min, increasing at 1ºC/min to 165ºC and then held for 20 min, increasing with 2ºC/min to 170ºC and then held for 10 min, and increasing with 4ºC/min to a final temperature of 215ºC and held for 20 min. In addition, a second time-temperature program was employed to separate the C18:1 isomers; initial temperature of 70ºC and held for 1 min, increasing with 5ºC/min

to 100°C and then held for 2 min, increasing with 10°C/min to 175°C and then held for 40 min, and increasing with 10°C/min to a final temperature of 215°C and held for 20 min. For feed samples, a shorter time-temperature program starting with an initial temperature of 140°C and held for 4 min, and increasing with 4°C/min to a final temperature of 240°C and held for 20 min was employed. Fatty acid methyl esters were identified using external standards (S37, Supelco, Bellefonte PA, USA; odd and branched chain fatty acids, *trans*-11-C18:1, *cis*-9,*trans*-11-C18:2, *trans*-10,*cis*-12-C18:2, Larodan Fine Chemicals AB, Malmö, Sweden). The fatty acids *trans*-4-C18:1, *trans*-5-C18:1, *trans*-6+7+8-C18:1, *trans*-10-C18:1, *trans*-12-C18:1, *trans*-13+14-C18:1, *trans*-15-C18:1, *cis*-12-C18:1, *cis*-13-C18:1, *cis*-14+trans-16-C18:1, *cis*-15-C18:1, *trans*-11,*cis*-15-C18:2 were identified according to the elution sequence reported by Loor et al. (2004) and Shingfield et al. (2006).

Statistics

Intake, milk yield, and milk composition were averaged within cow and period for the 3-d collection periods. Results were analysed using the MIXED procedure of SAS (SAS version 9.2; SAS Institute Inc., Cary NC, USA) according to the model described by St-Pierre and Weiss (2009). Using this model, response surface equations were derived for intake, milk yield, milk composition, and selected milk FA (main milk FA: C4:0 to C14:0 saturated FA (C4-C14), C14:0, C16:0, C18:0, *cis*-9-C18:1, C18:2*n*6, C18:3*n*3, and main biohydrogenation intermediates: trans-10-C18:1, trans-11-C18:1, *trans*-13+14-C18:1, *trans*-15-C18:1, *cis*-15-C18:1, *trans*-11,*cis*-15-C18:2, and *cis*-9,*trans*-11-C18:2). The model included linear and quadratic main effects (forage type, F/C ratio, and CL) and all 2-way interactions as fixed effects. Random effects included cow group, period within cow group, and cow nested within cow group. Non-significant fixed effects ($P > 0.10$) were removed from the model. Non significant ($P > 0.10$) linear effects remained in the model when they were included in a quadratic effect or an interaction effect. Linear changes in parameters for a main factor were described at the medium levels of the other main factors.

Results

Intake and performance

Individual treatment means for DMI, NE_L intake, FA intake, milk yield, and milk composition are shown in Table 4. Equations for response surfaces were derived for DMI, NE_L intake, C18:2*n*6 intake, C18:3*n*3 intake, milk yield, and milk composition (Table 5). Dry matter intake, NE_L intake, C18:2*n*6 intake, and C18:3*n*3 intake averaged 23.0 ± 3.6 kg/d, 166 ± 24 MJ/d, 179 ± 199 g/d, and 172 ± 107 g/d, respectively. Shifting from 80% grass silage to 80% maize silage in the diet linearly increased DMI ($P = 0.038$), NE_L intake ($P = 0.030$), and C18:2*n*6 intake ($P = 0.007$) by 2.7 kg/d, 20 MJ/d, and 43 g/d, respectively, and decreased C18:3*n*3 intake ($P = 0.003$) by 42 g/d. Shifting from a high forage (65:35 F/C ratio) to a high concentrate (35:65 F/C ratio) diet linearly increased DMI ($P < 0.001$), NE_L intake ($P < 0.001$), and C18:2*n*6 intake ($P < 0.001$) by 5.3 kg/d, 54 MJ/d, and 109 g/d, respectively. Increasing CL proportion in the diet linearly increased ($P < 0.001$) C18:3*n*3 intake by 180 g/d.

Table 4. Treatment means for DMI, NE_L intake, fatty acid intake, milk yield, and composition for cows fed diets with varying forage type (20, 50, and 80% grass silage; DM basis), forage to concentrate ratio (F/C ratio; 35, 50, and 65% forage, DM basis), and proportion of crushed linseed (1, 3, and 5% CL; DM basis)

Parameter	Forage type (grass silage %)												
	20				50					80			
	F/C ratio (forage %)												
	35	50		65	35		50	65		35	50		65
	Crushed linseed (%)												
	3	1	5	3	1	5	3	1	5	3	1	5	3
DMI, kg/d	26.5	24.2	24.8	23.0	24.8	25.7	22.7	20.1	21.2	24.9	22.5	22.3	17.8
NE_L intake, MJ/d[1]	200	173	180	158	185	195	163	135	146	187	159	160	121
Fatty acid intake, g/d													
C16:0	339	252	252	187	368	331	250	171	202	326	297	248	157
C18:0	80	55	56	38	51	79	59	43	36	78	49	79	44
Cis-9-C18:1	284	194	239	161	294	294	203	117	176	265	207	200	113
Cis-9,*cis*-12-C18:2	302	225	263	203	255	298	215	154	198	262	196	216	139
Cis-9,*cis*-12,*cis*-15-C18:3	209	94	279	191	117	308	210	120	296	228	152	331	212
Milk yield, kg/d	46.9	42.4	43.9	39.7	41.6	44.5	41.6	37.1	37.0	48.0	38.9	38.6	35.1
FPCM yield, kg/d[2]	42.1	40.7	41.4	38.9	40.1	39.5	40.7	36.7	36.5	42.7	38.4	38.6	35.1
Milk lactose													
%	4.86	4.81	4.86	4.74	4.74	4.82	4.79	4.99	4.78	4.80	4.80	4.71	5.08
kg/d	2.29	2.04	2.13	1.89	1.97	2.15	1.99	1.85	1.77	2.31	1.87	1.82	17.9
Milk fat													
%	3.35	3.81	3.54	3.90	3.70	3.18	3.95	4.12	4.02	3.18	3.96	4.18	4.18
kg/d	1.49	1.59	1.55	1.54	1.52	1.39	1.63	1.53	1.49	1.52	1.54	1.61	1.47
Protein													
%	3.21	3.16	3.29	3.26	3.38	3.13	3.13	2.92	3.06	3.10	3.22	2.99	2.96
kg/d	1.49	1.33	1.43	1.29	1.40	1.38	1.30	1.08	1.13	1.49	1.25	1.15	1.04
MUN, mg/dl	14.1	14.5	14.9	15.7	15.4	12.0	15.5	14.7	17.2	14.6	16.5	14.3	14.4

[1]NE_L: Net energy for lactation calculated with VEM system (Van Es, 1975).

[2]FPCM: fat- and protein-corrected milk; (0.337 + 0.116 * fat % + 0.06 * protein %) * milk yield (kg/d).

Table 5. Effects of varying forage type (20, 50, and 80% grass silage; DM basis), forage to concentrate ratio (35, 50, and 65% forage; DM basis), and proportion of crushed linseed (1, 3, and 5% CL; DM basis) on DMI (kg/d), NE_L intake (MJ/d), *cis*-9,*cis*-12-C18:2 intake, *cis*-9,*cis*-12,*cis*-15-C18:3 intake (g/d), and milk yield (kg/d) and composition[1]

Dependent variable	Intercept	Grass silage %	Forage %	CL %	Forage % x Forage %	CL % x CL %	Grass silage % x CL %	Forage % x CL %	Grass silage % x Forage %	RMSE[2]
DMI	34.0	-0.0444	-0.1770	ns	ns	ns	ns	ns	ns	2.2065
	(2.21)	(0.0196)	(0.0383)							
NE_L intake	272.5	-0.3394	-1.8150	ns	ns	ns	ns	ns	ns	211.87
	(16.05)	(0.1421)	(0.2777)							
Cis-9,*cis*-12-C18:2 intake	438.8	-0.7194	-3.6089	ns	ns	ns	ns	ns	ns	851.13
	(27.01)	(0.2357)	(0.4704)							
Cis-9,*cis*-12,*cis*-15-C18:3 intake	40.6	0.6984	ns	44.9203	ns	ns	ns	ns	ns	2184.30
	(14.24)	(0.1964)		(2.9052)						
Milk yield	57.94	-0.0567	-0.2746	ns	ns	ns	ns	ns	ns	4.9760
	(3.601)	(0.0308)	(0.0589)							
FPCM yield[3]	7.46	0.2117	1.2693	ns	-0.0116	ns	ns	ns	-0.0047	2.3592
	(10.138)	(0.0954)	(0.0385)		(0.0037)				(0.0019)	
Fat %	2.30	0.0049	0.0251	ns	ns	ns	ns	ns	ns	0.0409
	(0.314)	(0.0028)	(0.0053)							
Protein %	3.73	0.0037	-0.0141	-0.0788	ns	ns	-0.0019	0.0030	ns	0.0056
	(0.246)	(0.0024)	(0.0044)	(0.0749)			(0.0008)	(0.0014)		

[1]The full model included linear and quadratic effects of forage type (20, 50, and 80% grass silage; DM basis), forage to concentrate ratio (35, 50, and 65% forage), crushed linseed (1, 3, and 5% CL; DM basis) and all 2-way interactions. The final models include significant effects ($P < 0.10$). The SE of the coefficient is given within parenthesis.
[2]RMSE: root mean square error.
[3]FPCM: fat- and protein-corrected milk.

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Milk yield averaged 41.2 ± 7.3 kg/d with 3.81 ± 0.55 % fat, 3.14 ± 0.24 % protein, and 4.82 ± 0.29 % lactose. Shifting from 80% grass silage to 80% maize silage in the diet linearly increased (P = 0.085) milk yield by 3.4 kg/d, whereas fat content linearly decreased (P = 0.099) by 0.3%. Shifting from a high-forage to a high-concentrate diet linearly increased (P < 0.001) milk yield by 8.2 kg/d and linearly decreased (P < 0.001) fat content by 0.8%. Increasing proportion of CL in the diet did not affect milk yield and fat content.

Changing F/C ratio in the diet had a quadratic relationship (P = 0.012) with fat- and protein-corrected milk (FPCM) yield, with the highest FPCM yield achieved at a 50:50 F/C ratio. In addition, there was an interaction (P = 0.032) between forage type and F/C ratio for FPCM yield. When F/C ratio was 35:65, FPCM yield reached a plateau for diets containing 80% grass silage in the diet. However, when F/C ratio was 65:35, FPCM yield was higher when 20% grass silage was included in the diet.

There were interactions between forage type and CL proportion (P = 0.031) and between F/C ratio and CL proportion (P = 0.052) for milk protein content. At the 80% grass silage diet, milk protein content showed the highest level when 1% CL was included, whereas at the 80% maize silage diet, milk protein content showed the highest level in combination with 5% CL. A F/C ratio of 35:65 resulted in the highest milk protein content in combination with 1% CL.

None of the main factors affected milk lactose content.

Milk fatty acid profile

Individual treatment means for milk FA profile are shown in Table 6. Equations for response surfaces are derived for selected milk FA, viz. C4:0 to C14:0 saturated FA (C4-C14), C14:0, C16:0, C18:0, *cis*-9-C18:1, C18:2*n*6, and C18:3*n*3, and selected biohydrogenation intermediates, viz. *trans*-10-C18:1, *trans*-11-C18:1, *trans*-13+14-C18:1, *trans*-15-C18:1, *cis*-15-C18:1, *trans*-11-*cis*-15-C18:2, and *cis*-9,*trans*-11-C18:2 (Table 7).

When shifting from 80% grass silage to 80% maize silage in the diet, the proportions of *trans*-10-C18:1 (P = 0.035) and C18:2*n*6 (P = 0.002) in milk fat linearly increased by 0.34 and 0.21 g/100 g FA, respectively, whereas the proportions of *trans*-11,*cis*-15-C18:2 (P = 0.084) and C18:3*n3* (P < 0.001) linearly decreased by 0.08 and 0.14 g/100 g FA, respectively. Increasing the forage proportion in the diet linearly increased (P < 0.001) the proportion of C18:0 by 1.67 g/100 g FA and decreased (P = 0.004) the proportion of *trans*-13+14-C18:1 by 0.34 g/100 g FA. The F/C ratio in the diet showed a quadratic relationship with C4-C14 (P = 0.011), C14:0 (P = 0.090), C16:0 (P = 0.050), *trans*-10-C18:1 (P = 0.007), *trans*-11-C18:1 (P = 0.032), *trans*-15-C18:1 (P = 0.075), *cis*-15-C18:1 (P = 0.006), *trans*-11,*cis*-15-C18:2 (P = 0.006), C18:2*n*6 (P = 0.014), and C18:3*n*3 (P = 0.027) proportions in milk fat. At the medium level of grass silage (50% grass silage), the lowest proportions of *trans*-10-C18:1 and *trans*-15-C18:1 were achieved when the diet contained 55% to 65% forage. The lowest proportions of *cis*-15-C18:1, *trans*-11,*cis*-15-C18:2, and C18:3*n*3 were achieved when the diet contained a 50:50 F/C ratio, whereas the lowest proportion of C18:2*n*6 was achieved when the diet contained a 65:35 F/C ratio. Proportions of C4-C14, C14:0, C16:0, and *trans*-11-C18:1 showed a maximum level when the diet contained a 50:50 F/C ratio. An increasing proportion of CL in the diet linearly increased proportions of C18:0 (P < 0.001), *trans*-11-C18:1 (P < 0.001),

trans-13+14-C18:1 ($P < 0.001$), and *cis*-9,*trans*-11-C18:2 ($P = 0.046$) in milk fat by 2.03, 0.21, 0.52, and 0.04 g/100 g FA, respectively. In contrast, the proportions of C14:0 ($P = 0.084$) and C16:0 ($P < 0.001$) linearly decreased with an increasing proportion of CL in the diet. The proportion of CL showed a quadratic relationship with C4-C14 ($P = 0.094$), C18:2*n*6 ($P = 0.017$), and C18:3*n*3 ($P = 0.073$) proportions in milk fat; the proportion of C4-C14 reached a minimum at 3% CL, whereas the proportion of C18:2*n*6 reached a maximum at 3% CL and the proportion of C18:3*n*3 reached a plateau at 5% CL

Interactions between F/C ratio and CL proportion were found for *trans*-10-C18:1 ($P = 0.023$), *trans*-15-C18:1 ($P = 0.039$), *cis*-15-C18:1 ($P = 0.014$), *trans*-11,*cis*-15-C18:2 ($P = 0.066$), and C18:3*n*3 ($P = 0.034$) proportions in milk fat. From 80% to 20% of grass silage, the proportion of *trans*-10-C18:1 (1.64 to 1.98 g/100 g FA), *trans*-15-C18:1 (0.75 g/100 g FA), *cis*-15-C18:1 (0.63 g/100 g FA) *trans*-11,*cis*-15-C18:2 (0.68 to 0.59 g/100 g FA), and C18:3*n*3 (1.08 to 0.93 g/100 g FA) showed the highest levels when the diet contained 5% CL in combination with a 35:65 F/C ratio.

Interactions between forage type and F/C ratio were found for the proportion of C4-C14 ($P = 0.032$), C14:0 ($P = 0.033$), and *cis*-9-C18:1 ($P = 0.045$) in milk fat. The combination of a high forage proportion with 80% grass silage or a low forage proportion with 20% grass silage in the diet gave the highest *cis*-9-C18:1 proportions (19.81 and 21.94 g/100 g, respectively), whereas these combinations resulted in the lowest C4-C14 (20.12 and 21.51 g/100 g FA, respectively) and C14:0 proportions (9.59 and 10.17 g/100 g FA, respectively).

Discussion

The aim of the current study was to simultaneously evaluate different levels of CL supplementation in combination with variation in the characteristics of the basal diet (forage type and F/C ratio) on intake, production performance and milk FA profile. Multiple mechanisms regulate DMI of ruminants, but DMI generally declines with increasing NDF, especially forage NDF, content of the diet (Allen, 2000). Increasing the concentrate proportion linearly increased DMI in the current study, which is in agreement with a lower NDF content for the high concentrate diets. In addition, DMI was strongly correlated with NE_L intake ($r = 0.98$, $P < 0.001$). Abrahamse et al. (2008b) observed a significantly higher DMI when maize silage proportion in the diet increased at the expense of grass silage and Kliem et al. (2008) also found a linear increase in DMI when replacing grass silage with maize silage. These results were all in agreement with the results of the current study. The absence of an effect of CL supplementation on DMI in the current experiment is in agreement with a recent review (Petit, 2010), reporting no effect of feeding up to 15% whole linseed on DMI of dairy cows in early lactation. Chilliard et al. (2009) indeed showed no effect on DMI when 12.4% of whole linseed was included in the diet, whereas an equal amount of linseed FA fed as extruded linseed or linseed oil did result in a decreased DMI, with a greater decrease for cows fed linseed oil. It was therefore concluded that processing of oilseeds might affect DMI, which might be related to the increased availability of oil in the rumen (Petit, 2010). In the current study, the amount of crushed linseed was probably not high enough to cause rumen disturbances resulting in decreased DMI. Intake of C18:2*n*6 and C18:3*n*3

Table 6. Treatment means for milk fatty acid profile (g/100 g fatty acids) for cows fed diets with varying forage type (20, 50, and 80% grass silage; DM basis), forage to concentrate ratio (35, 50, and 65% forage; DM basis), and proportion of crushed linseed (1, 3, and 5% CL; DM basis)

Milk fatty acid profile, g/100 g FA	Forage type (grass silage %)												
	20				50					80			
	F/C ratio (forage %)												
	35	50		65	35		50	65		35	50		65
	Crushed linseed (%)												
	3	1	5	3	1	5	3	1	5	3	1	5	3
C4:0	2.70	3.21	3.07	2.94	2.83	2.88	3.18	3.03	3.66	3.13	3.28	3.37	3.43
C6:0	1.73	2.18	2.17	1.99	1.88	1.87	2.08	2.00	2.19	2.08	2.13	2.11	2.06
C8:0	1.10	1.39	1.47	1.28	1.22	1.18	1.30	1.24	1.27	1.31	1.34	1.23	1.19
C10:0	2.52	3.19	3.44	2.90	2.81	2.66	2.86	2.74	2.58	2.97	3.01	2.53	2.42
C11:0	0.06	0.07	0.07	0.05	0.07	0.06	0.05	0.04	0.03	0.06	0.06	0.05	0.03
C12:0	3.12	3.65	4.05	3.34	3.64	3.21	3.28	3.12	2.83	3.61	3.51	2.77	2.64
C13:0	0.07	0.08	0.10	0.08	0.09	0.08	0.07	0.07	0.06	0.09	0.07	0.06	0.06
Iso-C13:0	0.02	0.02	0.03	0.02	0.02	0.03	0.02	0.02	0.03	0.02	0.02	0.02	0.02
Anteiso-C13:0	0.07	0.07	0.09	0.07	0.10	0.06	0.07	0.07	0.06	.08	0.08	0.06	0.05
C14:0	9.82	11.23	11.60	10.98	11.10	10.00	10.56	10.62	10.08	10.74	10.91	9.78	9.99
Iso-C14:0	0.05	0.06	0.06	0.07	0.06	0.05	0.07	0.10	0.09	0.05	0.10	0.09	0.10
Cis-9-C14:1	0.93	0.92	1.12	0.97	1.28	0.82	0.89	0.89	0.80	0.97	0.93	0.80	0.81
C15:0	0.73	0.82	0.83	0.80	0.90	0.81	0.78	0.86	0.79	0.84	0.87	0.73	0.88
Iso-C15:0	0.14	0.17	0.16	0.18	0.15	0.14	0.18	0.21	0.19	0.15	0.19	0.18	0.19
Anteiso-C15:0	0.37	0.39	0.36	0.37	0.34	0.36	0.37	0.40	0.39	0.36	0.40	0.36	0.37
C16:0	28.39	31.41	27.75	28.68	31.95	27.53	29.31	29.58	26.30	29.32	30.95	27.75	29.07
Cis-9-C16:1	1.90	1.72	1.44	1.62	1.84	1.56	1.56	1.91	1.34	1.58	1.67	1.57	1.68
C17:0	0.46	0.49	0.47	0.54	0.49	0.44	0.47	0.58	0.47	0.45	0.50	0.44	0.53
Cis-9-C17:1	0.16	0.14	0.11	0.15	0.12	0.12	0.13	0.20	0.12	0.12	0.13	0.14	0.19
C18:0	10.50	9.98	10.91	10.96	8.64	10.99	11.04	11.04	13.01	9.68	10.33	12.35	11.40
Total *trans*-C18:1[1]	6.46	4.46	5.84	4.91	4.59	8.06	5.36	3.93	6.20	7.10	4.40	5.29	4.71
Trans-4-C18:1	0.03	0.03	0.02	0.03	0.03	0.02	0.03	0.02	0.04	0.03	0.03	0.03	0.03
Trans-5-C18:1	0.02	0.02	0.01	0.02	0.02	0.01	0.02	0.02	0.03	0.02	0.02	0.02	0.02
Trans-6+7+8-C18:1	0.53	0.37	0.41	0.39	0.39	0.57	0.41	0.31	0.45	0.53	0.37	0.37	0.33
Trans-9-C18:1	0.39	0.29	0.31	0.30	0.31	0.42	0.32	0.25	0.34	0.40	0.28	0.29	0.26

Table 6. Continued.

Milk fatty acid profile, g/100 g FA	Forage type (grass silage %)												
	20				50					80			
	F/C ratio (forage %)												
	35	50		65	35		50	65		35	50		65
	Crushed linseed (%)												
	3	1	5	3	1	5	3	1	5	3	1	5	3
Trans-10-C18:1	1.67	0.62	0.57	0.46	0.63	1.88	0.58	0.35	0.45	1.35	0.42	0.37	0.34
Trans-11-C18:1	1.01	1.10	1.43	1.19	0.95	1.20	1.30	1.12	1.58	1.50	1.18	1.30	1.37
Trans-12-C18:1	0.56	0.42	0.55	0.50	0.45	0.66	0.52	0.37	0.61	0.60	0.40	0.53	0.45
Trans-13+14-C18:1	1.19	0.86	1.35	1.02	1.03	1.76	1.16	0.74	1.42	1.48	0.93	1.16	0.91
Trans-15-C18:1	0.51	0.34	0.55	0.47	0.35	0.79	0.48	0.34	0.60	0.58	0.34	0.57	0.46
Trans-16+*cis*-14-C18:1	0.58	0.41	0.63	0.54	0.44	0.74	0.55	0.43	0.68	0.60	0.43	0.66	0.55
Total *cis*-C18:1[2]	22.58	19.55	19.32	21.61	20.50	20.61	20.81	22.23	21.76	18.98	20.08	22.63	22.74
Cis-9-C18:1	21.11	18.43	18.12	20.34	19.28	18.90	18.55	21.08	20.37	17.50	19.00	21.38	21.53
Cis-11-C18:1	0.64	0.53	0.46	0.50	0.59	0.58	0.51	0.56	0.44	0.54	0.50	0.43	0.50
Cis-12-C18:1	0.32	0.29	0.36	0.38	0.30	0.33	0.37	0.26	0.45	0.40	0.27	0.36	0.30
Cis-13-C18:1	0.10	0.07	0.07	0.09	0.08	0.10	0.08	0.09	0.09	0.10	0.08	0.08	0.10
Cis-15-C18:1	0.41	0.21	0.31	0.30	0.24	0.70	0.31	0.23	0.41	0.44	0.23	0.38	0.32
Total non-conjugated C18:2[3]	3.67	2.70	2.86	2.91	3.01	3.79	2.94	2.63	2.93	3.50	2.64	2.86	2.72
Trans-9,*trans*-12-C18:2	0.04	0.01	0.01	0.01	0.03	0.05	0.02	0.01	0.02	0.03	0.02	0.01	0.01
Trans-11,*cis*-15-C18:2	0.41	0.18	0.30	0.28	0.22	0.70	0.32	0.26	0.48	0.49	0.24	0.39	0.42
Cis-9,*cis*-12-C18:2	2.28	1.87	1.69	1.78	1.93	1.95	1.76	1.71	1.44	1.99	1.69	1.60	1.55
Total conjugated C18:2[4]	0.57	0.53	0.69	0.60	0.60	0.60	0.62	0.54	0.69	0.74	0.57	0.59	0.63
Cis-9,*trans*-11-C18:2	0.56	0.53	0.68	0.59	0.59	0.59	0.60	0.52	0.67	0.73	0.55	0.57	0.59
Trans-10,*cis*-12-C18:2	0.00	0.00	0.01	0.01	0.01	0.02	0.02	0.02	0.03	0.01	0.02	0.02	0.03

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Table 6. Continued.

Milk fatty acid profile, g/100 g FA	Forage type (grass silage %)												
	20				50					80			
	F/C ratio (forage %)												
	35	50		65	35		50	65		35	50		65
	Crushed linseed (%)												
	3	1	5	3	1	5	3	1	5	3	1	5	3
Cis-9,*cis*-12,*cis*-15-C18:3	0.82	0.51	0.80	0.74	0.61	1.00	0.79	0.72	0.86	0.89	0.68	0.99	0.89
FA ≥ C:20[5]	0.47	0.45	0.47	0.51	0.41	0.46	0.47	0.52	0.43	0.45	0.43	0.50	0.46
C20:0	0.11	0.10	0.10	0.11	0.10	0.11	0.12	0.12	0.14	0.10	0.12	0.12	0.12
C20:1	0.06	0.05	0.04	0.05	0.05	0.06	0.05	0.05	0.05	0.06	0.04	0.05	0.05
C20:2	0.02	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.04	0.01
C21:0	0.03	0.02	0.03	0.02	0.03	0.04	0.02	0.02	0.02	0.03	0.02	0.02	0.02
C22:0	0.04	0.05	0.09	0.08	0.00	0.04	0.05	0.06	0.01	0.04	0.00	0.06	0.06
Total unknown	0.59	0.59	0.72	0.68	0.69	0.64	0.68	0.64	0.79	0.68	0.68	0.72	0.66
SFA[6]	60.03	66.39	64.64	63.26	64.18	60.48	63.80	63.56	62.09	62.99	65.58	62.07	62.38
C4-C14[7]	20.99	24.84	25.79	23.43	23.49	21.79	23.26	22.75	22.62	23.84	24.18	21.78	21.72
OBCFA[8]	2.11	2.31	2.27	2.32	0.35	2.15	2.21	2.55	2.23	2.24	2.42	2.11	2.43
MUFA[9]	31.92	26.70	27.77	29.17	28.25	31.11	28.67	29.02	30.14	28.70	27.12	30.36	30.00
PUFA[10]	5.30	3.97	4.57	4.51	4.48	5.59	4.59	4.17	4.70	5.35	4.15	4.68	4.46
UFA[11]	37.22	30.67	32.34	33.68	32.73	36.70	33.26	33.19	34.84	34.05	31.27	35.04	34.46

[1]Total *trans*-C18:1: ∑ (*trans*-4-C18:1, *trans*-5-C18:1, *trans*-6+7+8-C18:1, *trans*-9-C18:1, *trans*-10-C18:1, *trans*-11-C18:1, *trans*-12-C18:1, *trans*-13+14-C18:1, *trans*-15-C18:1, *trans*-16+*cis*-14-C18:1).
[2]Total *cis*-C18:1: ∑ (*cis*-9-C18:1, *cis*-11-C18:1, *cis*-12-C18:1, *cis*-13-C18:1, *cis*-15-C18:1).
[3]Total non-conjugated C18:2: ∑ (*trans*-9,*trans*-12-C18:2, *cis*-9,*trans*-13-C18:2, *trans*-8,*cis*-13-C18:2, *cis*-9,*trans*-12-C18:2, *trans*-9,*cis*-12-C18:2, *trans*-11,*cis*-15-C18:2, *cis*-9,*cis*-12-C18:2, *cis*-9,*cis*-15-C18:2).
[4]Total conjugated C18:2: ∑ (*cis*-9,*trans*-11-C18:2, *trans*-10,*cis*-12-C18:2).
[5]Total ≥ C20:0: ∑ (C20:0, C20:1, C20:2, C20:3*n*3, C20:4*n*6, C21:0, C22:0, *cis*-13-C22:1, C22:5, C22:6, C24:0).
[6]Saturated fatty acids: ∑ (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C24:0).
[7]C4-C14 saturated fatty acids: ∑ (C4:0, C6:0, C8:0, C10:0, C12:0, C14:0).
[8]Odd and branched chain fatty acids: ∑ (*iso*-C13:0, *anteiso*-C13:0, *iso*-C14:0, *anteiso*-C14:0, *iso*-C15:0, *anteiso*-C15:0, C15:0, *iso*-C16:0, *anteiso*-C16:0, *iso*-C17:0, *anteiso*-C17:0, C17:0, *cis*-9-C17:1).
[9]Mono unsaturated fatty acids: ∑ (*cis*-9-C14:1, *cis*-9-C16:1, Total *cis*-C18:1, Total *trans*-C18:1, C20:1, *cis*-13-C22:1).
[10]Poly unsaturated fatty acids: ∑ (Total non-conjugated C18:2, Total conjugated C18:2, C18:3*n*6, C18:3*n*3, C20:2, C20:3*n*3, C20:4*n*6, C22:5, C22:6).
[11]Unsaturated fatty acids: ∑ (MUFA, PUFA).

Table 7. Effects of varying forage type (20, 50, and 80% grass silage; DM basis), forage to concentrate ratio (35, 50, and 65% forage; DM basis), and proportion of crushed linseed (1, 3, and 5% CL; DM basis) on selected milk fatty acids (g/100 g fatty acids)[1]

Dependent variable	Intercept	Grass silage %	Forage %	CL %	Forage % x Forage %	CL % x CL %	Grass silage % x CL %	Forage % x CL %	Grass silage % x Forage %	RMSE[2]
C4-C14[3]	4.71	0.1100	0.7673	-1.4526	-0.0065	0.2067	ns	ns	-0.0026	0.6197
	(5.354)	(0.0500)	(0.2020)	(0.6767)	(0.0020)	(0.1095)			(0.0010)	
C14:0	4.79	0.0460	0.2200	-0.1365	-0.0017	ns	ns	ns	-0.0011	0.0819
	(2.249)	(0.0225)	(0.0914)	(0.0712)	(0.0009)				(0.0004)	
C16:0	28.61	ns	0.1768	-1.0133	-0.0021	ns	ns	ns	ns	1.1373
	(2.216)		(0.0910)	(0.0750)	(0.0009)					
C18:0	6.56	ns	0.0555	0.5051	ns	ns	ns	ns	ns	0.4440
	(0.562)		(0.0092)	(0.0731)						
Trans-10-C18:1	4.51	-0.0057	-0.1494	0.5236	0.0014	ns	ns	-0.0088	ns	0.1023
	(1.110)	(0.0023)	(0.0418)	(0.1600)	(0.0004)			(0.0032)		
Trans-11-C18:1	0.02	ns	0.0418	0.0523	-0.0004	ns	ns	ns	ns	0.0046
	(0.422)		(0.0171)	(0.0141)	(0.0002)					
Trans-13+14-C18:1	1.32	ns	-0.0111	0.1290	ns	ns	ns	ns	ns	0.0238
	(0.179)		(0.0032)	(0.0240)						
Trans-15-C18:1	0.66	ns	-0.0180	0.1555	0.0002	ns	ns	-0.0017	ns	0.0059
	(0.278)		(0.0106)	(0.0390)	(0.0001)			(0.0008)		
Cis-9-C18:1	22.89	-0.1194	-0.0705	ns	ns	ns	ns	ns	0.0025	0.3433
	(3.171)	(0.0586)	(0.0619)						(0.0012)	
Cis-15-C18:1	0.97	ns	-0.0361	0.1730	0.0004	ns	ns	-0.0024	ns	0.0057
	(0.303)		(0.0115)	(0.0429)	(0.0001)			(0.0008)		
Trans-11,*cis*-15-C18:2	1.08	0.0014	-0.0442	0.1631	0.0005	ns	ns	-0.0021	ns	0.0065
	(0.361)	(0.0007)	(0.0137)	(0.0511)	(0.0001)			(0.0010)		
Cis-9,*cis*-12-C18:2	3.68	-0.0036	-0.0608	0.1209	0.0005	-0.0247	ns	ns	ns	0.0166
	(0.373)	(0.0009)	(0.0155)	(0.0536)	(0.0002)	(0.0087)				
Cis-9,*trans*-11-C18:2	0.57	ns	ns	0.0122	ns	ns	ns	ns	ns	0.0002
	(0.033)			(0.0060)						

6

Table 7. Continued.

Dependent variable	Intercept	Grass silage %	Forage %	CL %	Forage % x Forage %	CL % x CL %	Grass silage % x CL %	Forage % x CL %	Grass silage % x Forage %	RMSE[2]
Cis-9,*cis*-12,cis-15-C18:3	0.71 (0.245)	0.0025 (0.0050)	-0.0191 (0.0094)	0.2196 (0.0427)	0.0002 (0.0001)	-0.0102 (0.0050)	ns	-0.0017 (0.0007)	ns	0.0076

[1]The full model included linear and quadratic effects of forage type (20, 50, and 80% grass silage; DM basis), forage to concentrate ratio (35, 50, and 65% forage; DM basis), crushed linseed (1, 3, and 5% CL; DM basis) and all 2-way interactions. The final models include significant effects ($P < 0.10$). The SE of the coefficient is given within parenthesis.

[2]RMSE: root mean square error.

[3]C4-C14 saturated fatty acids ∑ (C4:0, C6:0, C8:0, C10:0, C12:0, C14:0).

were affected by forage type and reflected the higher proportion of C18:3*n*3 in grass silage versus the higher proportion of C18:2*n*6 in maize silage. In addition, intake of C18:2*n*6 greatly increased when the diet shifted towards a higher concentrate proportion, whereas intake of C18:3*n*3 strongly increased when the diet contained a higher CL proportion.

Milk yield was influenced by both forage type and F/C ratio, but not by CL supplementation, which was consistent with the effect on DMI. For milk fat content, however, the opposite relationship was found for forage type and F/C ratio. Fat content decreased when the diet contained 80% maize silage compared with 80% grass silage and a higher concentrate proportion. Chilliard et al. (2007) reported a larger decrease in milk fat content when vegetable oils were added to a maize silage based diet compared with a grass silage based diet, which was mainly related to an increased proportion of *trans*-10-C18:1 in milk fat. Indeed, an increased proportion of *trans*-10-C18:1 in milk fat related to increased dietary starch and decreased NDF contents are associated with a reduction in milk fat content (Nielsen et al., 2006), which was confirmed in the current study. It should be noted that *trans*-10-C18:1 has often been associated with milk fat depression although this FA is thought to play no regulatory role in milk FA synthesis (Lock et al., 2007). Proportion of *trans*-10-C18:1 has rather been empirically related to milk fat depression probably in relation to its association with CLA, notably *trans*-10, *cis*-12-C18:2 that does play a regulatory role (Shingfield and Griinari, 2007). However, in addition to *trans*-10, *cis*-12-C18:2 also other biohydrogenation intermediates might play a regulatory role in milk FA synthesis (Loor et al., 2005). Milk protein content showed interactions between F/C ratio and proportion of CL and between forage type and proportion of CL. This was probably related to the relationship between milk protein content and the supply of glucogenic nutrients (relation between starch content and milk protein content was R^2 = 0.40; Jenkins and McGuire, 2006).

Responses in milk FA profile from lipid supplementation largely depend on characteristics of the lipid (source, physical form, and inclusion rate) and on characteristics of the basal diet (forage type and F/C ratio; Chilliard et al., 2007). To our knowledge, the current experiment was the first experiment to simultaneously vary crushed linseed supplementation, forage type, and F/C ratio to be able to identify and quantify interactions between these factors on milk FA profile in high producing dairy cows. Various biohydrogenation intermediates (*trans*-C18:1, *cis*-C18:1 and non-conjugated and conjugated C18:2 and C18:3 isomers) are formed from dietary C18:2*n*6 and C18:3*n*3 (Chilliard et al., 2007). In the current study supplementation of different levels of CL to a basal diet varying in forage type and F/C ratio affected the proportions of biohydrogenation intermediates in milk fat. Interactions were found between CL supplementation and F/C ratio for proportions of C18:3*n*3, *trans*-11,*cis*-15-C18:2, *trans*-10-C18:1, *trans*-15-C18:1, and *cis*-15-C18:1 in milk fat, with the highest levels achieved when the diet contained 5% CL and a 35:65 F/C ratio. These increased levels of C18:3*n*3 and biohydrogenation intermediates are in agreement with results of Loor et al. (2005b), who found increased proportions of *trans*-10-C18:1, *trans*-11-C18:1, *trans*-11,*cis*-15-C18:2, and total C18:3 isomers for the high concentrate diet with supplemental linseed oil (3% of DM). Previously, Sterk et al. (2010) showed that rumen biohydrogenation kinetics of crushed linseed did not differ from biohydrogenation kinetics of linseed oil. Loor et al. (2004) suggested that the increased dietary

starch content in high concentrate diets affects ruminal FA metabolism resulting in increased biohydrogenation intermediates produced in the rumen and consequently secreted in milk fat.

Diets with high starch and low fiber contents that are supplemented with poly-unsaturated FA can inhibit mammary gland short-chain FA synthesis (Kliem et al., 2008). In the current study interactions between forage type and F/C ratio were found for the proportions of C4:0 to C14:0 in milk fat, with lower levels achieved when the diet contained a high forage proportion in combination with 80% grass silage or a low forage proportion in combination with 80% maize silage. In addition, the proportion of C4:0 to C14:0 the current study reached a minimum when 3% CL was included in the diet, whereas the proportions of C14:0 and C16:0 in milk fat linearly decreased with increasing CL proportion. During diet-induced milk fat depression, the secretion of all FA in milk is decreased, but the decrease is disproportionally higher for the FA synthesized de novo (Shingfield and Griinari, 2007). Shingfield and Griinari (2007) summarized the major theories explaining diet-induced milk fat depression and the researchers concluded that the direct inhibition of milk fat synthesis in the mammary gland by elevated biohydrogenation intermediates was able to explain most cases. In the current study the decreased proportions of C4:0 to C14:0 saturated FA, C14:0, and C16:0 in milk fat were also in accordance with the increased proportions of the biohydrogenation intermediates.

No interactions were found between CL supplementation and forage type for the selected milk FA. Chilliard et al. (2007) suggested rumen biohydrogenation to be less complete when adding vegetable oils to a maize silage based diet compared with addition to a grass silage based diet. In the current study, this was not confirmed, which might be related to the relatively low starch content of the maize silage resulting in a relatively low maximum starch content of 209 g/kg DM in the treatment diets. However, several linear effects of forage type on milk FA were found. Shifting from 80% grass silage to 80% maize silage linearly increased *trans*-10-C18:1 and C18:2*n*6, whereas *trans*-11,*cis*-15-C18:2 and C18:3*n*3 proportions linearly decreased. Kliem et al. (2008) found increased proportions of *trans*-C18:1 isomers, total conjugated C18:2, and C18:2*n*6, and a decreased proportion of C18:3*n*3 in milk fat when replacing grass silage with maize silage in a diet without supplemental oil.

Glasser et al. (2008) suggested that changes in ruminal biohydrogenation are caused by changes in starch content of the diet affecting ruminal pH and microbial populations. However, Loor et al. (2004) suggested that changes in ruminal biohydrogenation can follow changes in dietary starch content without an effect on ruminal pH. This might be related to the content of dietary NDF (physically effective NDF) in addition to the content of dietary starch playing an important role in the estimation of ruminal pH (Zebeli et al., 2008). Also, changes in dietary starch content might induce small alterations in the microbial population that are able to affect ruminal biohydrogenation (Loor et al., 2004). Starch and NDF availability and their effects on buffering capacity and alterations in the microbial population in the rumen are linked with a shift in the production of isomers with a *trans*-11- to a *trans*-10- double bond (Loor et al., 2004). An increase in milk *trans*-10-C18:1 was commonly found with either high concentrate diets or maize silage based diets that were supplemented with poly-unsaturated FA rich oils (Chilliard et al., 2007). In the current study, increasing starch content the diet indeed increased *trans*-10-C18:1 proportion in milk fat (R^2 = 0.50) and the increased *trans*-10-C18:1 proportion was strongly related to the decreased milk fat content (R^2 = 0.81). The increased *trans*-10-C18:1 proportion

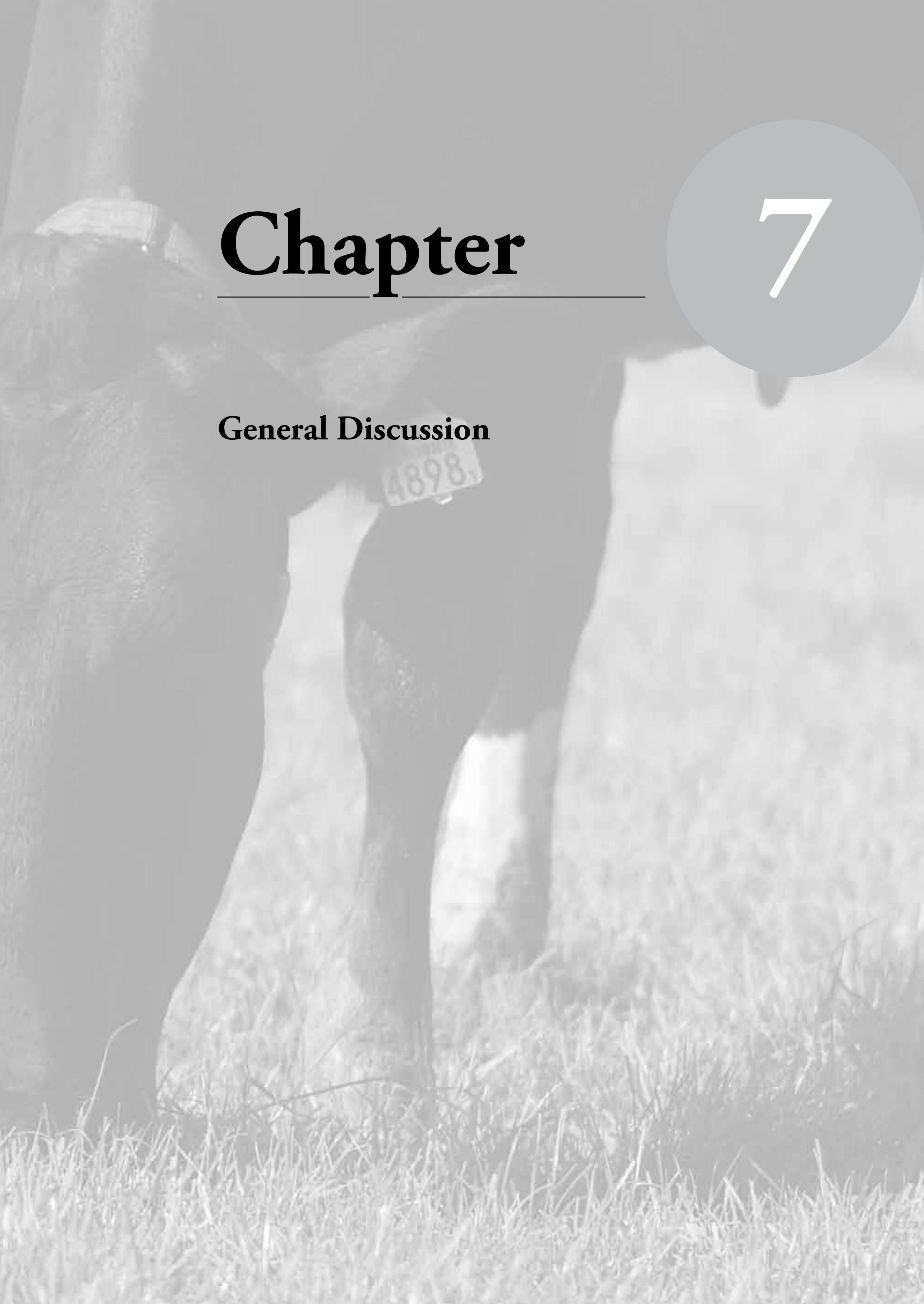
in relation to high starch diets might be related to changes in the bacterial population. Nielsen et al. (2006) reported that high grain diets promote the growth of the bacterial strain *Megasphera elsdenii* YJ-4 (Kim et al., 2002) in combination with a decrease in the main cellulose digesting bacterial strain *Butyrivibrio fibrisolvens* (Klieve et al., 2003). These different bacterial strains convert C18:2*n*6 and C18:3*n*3 through different biohydrogenation routes. *Megasphera elsdenii* YJ-4 can convert C18:2*n*6 to *trans*-10,*cis*-12-C18:2 and *trans*-10-C18:1 (Bauman and Griinari, 2001), whereas the bacterial strain *Butyrivibrio fibrisolvens* converts C18:2*n*6 to *cis*-9,*trans*-11-C18:2 and *trans*-11-C18:1 (Harfoot and Hazlewood, 1997). Shingfield et al. (2005) suggested that starch content and the ratio of starch to NDF in the diet are important determinants of the *trans*-C18:1 isomer profile in milk due to the effects on the relative abundance and activity of specific populations of bacteria in the rumen.

Conclusions

Increasing the proportion of CL in combination with varying forage type and F/C ratio in the diet of high producing dairy cows affects intake, production performance, and milk FA profile. Interactions were found between CL supplementation and F/C ratio for proportions of C18:3*n*3 and several biohydrogenation intermediates in milk fat, with the highest levels achieved when the diet contained 5% CL and a 35:65 F/C ratio. There were no interactions between CL supplementation and forage type for the selected milk FA. However, several linear effects of shifting from 80% grass silage to 80% maize silage on milk FA were found. Transfer efficiencies of C18:2*n*6 and C18:3*n*3 were highest at 1% CL supplementation and decreased quadratically (C18:2*n*6) and linearly (C18:3*n*3) with increasing CL supplementation. Transfer efficiency of C18:2*n*6 was additionally decreased with increasing maize silage and concentrate proportion in the diet. This study showed that the effect of adding crushed linseed on the proportions of several FA in milk fat, including C18:2*n*6 and C18:3*n*3, depends significantly on the F/C ratio and forage type (grass silage versus maize silage) in the basal diet. In addition, this study showed that in FA research other feed characteristics like forage type and F/C ratio could influence the final impact of a supplemental fat source on milk FA profile.

Acknowledgments

The authors would like to thank the staff of trial farm Nötcenter Viken, Lantmännen, Sweden for their assistance in animal care, feeding and sampling. The laboratory staff of the Animal Nutrition Group, Wageningen University is greatly acknowledged for their help in chemical analysis. The financial support of the Food and Nutrition Delta program of Senter Novem (Den Haag, the Netherlands), Royal FrieslandCampina (Amersfoort, The Netherlands), Agrifirm Group (Apeldoorn, The Netherlands), Agrifirm Innovation Center (Apeldoorn, The Netherlands), and Barenbrug Holland B.V. (Oosterhout, The Netherlands) is gratefully acknowledged.



Chapter 7

General Discussion

Introduction

The research presented in this thesis aimed to improve the milk fatty acid (FA) profile of dairy cows by altering the rumen biohydrogenation processes to increase unsaturated fatty acids (UFA), such as *cis*-9,*cis*-12,*cis*-13-C18:3 (C18:3*n*3) and *cis*-9,*trans*-11-C18:2 (conjugated linoleic acid isomer; CLA). In Chapter 2 it was shown that the inclusion of different fat sources affect milk FA profile and that the technological form (such as oil, seed, or protected sources), additional inclusion of fish oil, and characteristics of the basal diet (such as main forage type and amount of NDF) could influence the effect of these fat sources on milk FA profile. In Chapter 3 several technological and chemical treatments of linseed including addition of docosahexaenoic acid (C22:6*n*3; DHA) were evaluated in vitro. The results showed that the extent of biohydrogenation of C18:3*n*3 from linseed could be reduced when linseed was crushed followed by formaldehyde treatment and when whole linseed was extruded. The addition of DHA to linseed oil showed that the extent of biohydrogenation of C18:3*n*3 was high, but the complete biohydrogenation towards C18:0 was inhibited, resulting in increased proportions of biohydrogenation intermediates. From the results of this in vitro experiment, the most promising treatments were selected to be studied in the in vivo experiment described in Chapters 4 and 5. In this experiment cows were fed crushed linseed, extruded whole linseed, formaldehyde treated linseed oil, or linseed oil in combination with marine algae rich in DHA. Omasal C18:3*n*3 flow was higher in cows fed extruded whole linseed, whereas plasma and milk C18:3*n*3 proportions were higher in cows fed formaldehyde treated linseed oil. In line with the in vitro results in Chapter 3, complete biohydrogenation towards C18:0 was strongly inhibited when marine algae rich in DHA were fed. In Chapter 6, crushed linseed supplementation level was varied simultaneously with F/C ratio and forage type (grass silage versus maize silage) and it was shown that the effect of adding crushed linseed on the proportions of several milk FA, including C18:3*n*3, depended significantly on the F/C ratio and forage type in the basal diet. In this general discussion, the importance of milk fat and the opportunities to alter milk FA profile through intake, ruminal FA metabolism, and mammary gland metabolism will be discussed. The second part of this chapter discusses effects of diets containing more UFA to improve milk FA profile on animal metabolism and methane production.

Importance of milk fat and opportunities to alter milk FA profile

Changes in milk fatty acid profile

Fat is an important constituent of whole milk and contributes to its energy density. Fat also has an essential function in many of the physical properties, manufacturing qualities, and organoleptic characteristics of dairy products (Harvatine et al., 2009). Milk fat is secreted from mammary epithelial cells as lipid droplets surrounded by a protein rich polar lipid coat, called milk fat globule membrane (Mather and Keenan, 1998; Keenan, 2001; Oliverier-Bousquet, 2002; Harvatine et al., 2009). The globules contain non-polar or core lipids, such as triacylglycerides (TAG; the most important fraction; ~ 97.5%), cholesterol esters, and retinol esters (Jensen, 2002). The milk fat globule membrane consists of phospholipids, proteins, cholesterol, enzymes, etc. and forms a loose layer around the lipid droplets.

The milk fat globule membrane prevents the globules from coalescing and acts as an emulsion stabiliser (Jensen, 2002). The estimated proportion of FA in total milk lipids was calculated to be 93.3% FA (Glasser et al., 2007a). This FA proportion in milk fat is used to calculate the secretion of FA in milk fat and thereby the transfer efficiency from feed FA to milk FA.

Bovine milk includes over 400 individual FA differing mainly in chain length, chain orientation, and presence and orientation of double bonds (Jensen, 2002). Only a small part of these individual FA is present in substantial amounts. Dutch milk fat contains 70.6 g saturated FA/100 g FA (SFA), 3.9 g odd and branched chain FA/100 g FA (OBCFA), 23.5 g mono-unsaturated FA/100 g FA (MUFA), and 2.3 g poly-unsaturated FA/100 g FA (PUFA; Heck et al., 2009). This generally high proportion of SFA in bovine milk fat is traditionally associated with concern related to human health (Astrup et al., 2011). Increasing the proportion of UFA is considered an improvement of the nutritional quality of milk fat (Bauman and Lock, 2010). In addition, increasing specific FA, such as C18:3*n*3 and CLA, in bovine milk shows health promoting potential and the possibilities to increase these FA are intensively studied in this thesis.

Bovine milk FA profile is linked to intrinsic (animal species, breed, genotype, pregnancy, and lactation stage) and extrinsic (environmental) factors (Chilliard et al., 2007). The possibilities of changing milk FA profile by genetic selection have been described by Stoop (2009). There are several opportunities to implement genetic selection for milk FA profile, but results are limited and will only be achieved over generations. For example, when the 25% best performing cows in terms of UFA proportion in milk fat were to be mated with the bulls with the highest estimated breeding value for UFA proportion in milk fat, while improving the entire population (selection of cows and bulls with best estimated breeding value for UFA proportion in milk fat), an increase in UFA proportion in milk fat of approximately 5.2 g/100 g FA can be achieved in 10 years (Stoop, 2009). The effect of lactation stage is related to body fat mobilisation during the period of negative energy balance (NEB; Van Knegsel et al., 2005). Body fat mobilisation leads to increased proportions of C16:0, C18:0, and *cis*-9-C18:1 in milk fat, related to these FA being the main FA in adipose tissue (Scollan et al., 2001; Van Knegsel et al., 2007a; Zachut et al., 2010). Seasonal variation in Dutch milk FA profile is large, with decreasing SFA proportions and increasing *cis*-9-C18:1, *trans*-11-C18:1, *cis*-9,*trans*-11-C18:2, and C18:3*n*3 proportions in spring and summer compared with autumn and winter, as shown by Heck et al. (2009). These seasonal effects are strongly related to the start of the grazing period, generally in April, when cows are fed diets based on fresh grass (in summer on average 250 g/kg DM; Heck et al., 2009). During autumn, generally in October, cows are housed indoors again and fed diets generally composed of more concentrates and silages.

Changes in milk FA profile that can be achieved depend on biological limits to guarantee fluidity of the milk (Heck, 2009). Daily infusion of 500 g linseed oil to the duodenum increased the proportion of C18:3*n*3 in milk fat up to 13.9 g/100 g FA, whereas milk fat content was not affected (Petit et al., 2002a). Duodenal infusion of 160 g/d of free C18:3*n*3-rich FA even increased the proportion of C18:3*n*3 in milk fat up to 25.4 g/100 g FA (Khas-Erdene et al., 2010). This difference might be caused by the difference in supplementation of C18:3*n*3 as intact linseed oil (TAG) or as free FA. However, Litherland et al. (2005) did not find differences in milk fat secretion of C18:2*n*6 after abomasal infusion

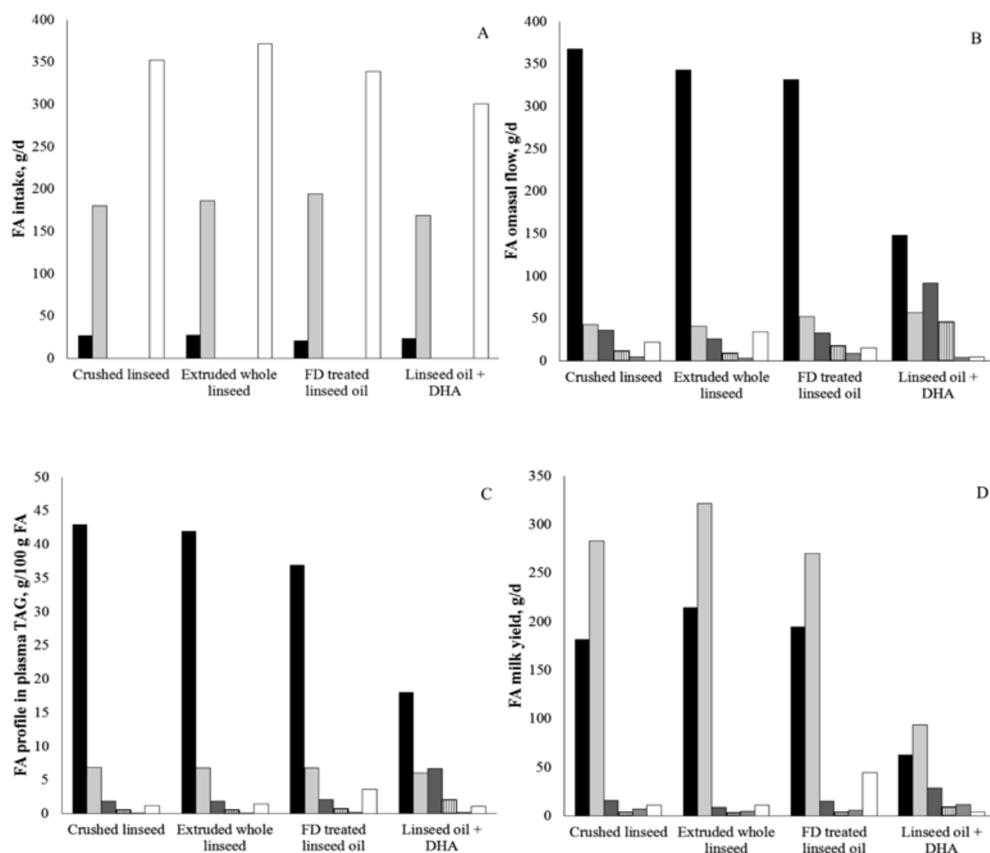


Figure 1. Intake (A), omasal flow (B), plasma triacylglycerides (TAG) (C), and milk secretion (D) of C18 FA (Chapter 4 and 5). From left to right the vertical bars represent the following C18 FA: C18:0, *cis*-9-C18:1, *trans*-11-C18:1, *trans*-11,*cis*-15-C18:1, *cis*-9,*trans*-11-C18:2, and C18:3*n*3.

of soybean oil as TAG or as free FA. The difference between the secretion of C18:3*n*3 from infusion of linseed oil (109 g/d from 245 g infused C18:3*n*3/d; Petit et al., 2002a) and from infusion of free C18:3*n*3 rich FA (170 g/d from 132 g infused C18:3*n*3/d; Khas-Erdene et al. 2010) suggests an overestimation of the milk fat C18:3*n*3 proportion for the latter study. However, the possibility to reach high C18:3*n*3 proportions in milk fat shows that by-passing ruminal FA metabolism can result in marked increases in these desirable milk FA. Figure 1 shows the intake, omasal flow, milk TAG composition, and milk secretion of C18 FA for cows fed supplemental crushed linseed, extruded whole linseed, formaldehyde treated linseed oil, and linseed oil in combination with DHA (Chapter 4 and 5). In this figure it is shown that the cows eat approximately 300 – 370 g C18:3*n*3/d, whereas omasal flow of C18:3*n*3 is only 5 to 35 g C18:3*n*3/d. The profile of C18 FA in omasal flow is strongly related to the profile in plasma TAG, whereas the profile again changes significantly when secreted into milk fat. Changes from feed intake to omasal flow are explained by the extensive biohydrogenation of C18:3*n*3, whereas changes from plasma TAG to secreted milk are explained by desaturation of C18:0 and *trans*-11-C18:1.

Changes in dietary fatty acid intake

Diets for dairy cows are generally composed of forages, either fresh or conserved, and concentrates. A general characteristic of dairy cow diets is that they are high in fibre (generally > 300 g/kg DM cell wall constituents) and low in lipids (generally < 70 g/kg DM total fat; Palmquist et al., 2005). The supply of FA to dairy cows can be influenced by changing the composition of the diet. In addition, ruminal FA metabolism is significantly influenced by ruminal pH, which is related to the ratio between structural fibre and rapidly fermentable carbohydrates in the diet (Boeckaert et al., 2008a) and reflects the balance between acid production (i.e. VFA and lactate) and acid removal through neutralisation (buffer capacity) and absorption within the rumen (Allen et al., 1997; Zebeli et al., 2008). The lipid fraction in leaves of herbs and grasses ranges from 30 to 100 g/kg DM and lipids are mainly located in the photosynthetic tissues (Elgersma et al., 2006, Khan et al., 2009). Fresh grass contains high proportions of C18:3*n*3. However, during field wilting of grass prior to ensiling or hay making, oxidative losses of the PUFA occur via the lipoxygenase system, a defence mechanism of the plants initiated in damaged tissue (Dewhurst et al., 2006). These oxidative losses lead to substantially lower C18:3*n*3 contents in conserved grasses compared with fresh pasture. Fatty acids in maize silage originate from membrane lipids in the leaves and stems (C18:3*n*3) and storage lipids in the kernels (*cis*-9-C18:1 and C18:2*n*6), resulting in high levels of *cis*-9-C18:1 and C18:2*n*6 in mature maize silage harvested for high DM and starch yield (Khan et al., 2011). The FA composition in concentrates will differ depending on the raw material composition, e.g. grains are generally rich in *cis*-9-C18:1 and C18:2*n*6. Supplemental fat sources can be used in dairy diets to increase energy intake and to change milk FA profile. These supplemental fat sources can be rich in either C16:0 (palm oil sources), C18:0 (animal fat sources), *cis*-9-C18:1 (canola sources), C18:2*n*6 (soybean and sunflower sources), C18:3*n*3 (linseed sources), C20:5*n*3 (fish oil sources), or C22:6*n*3 (marine algae, fish oil sources). In addition, many by-products of the food industry, highly variable in quality and FA composition, may be included in the ration of dairy cows (Palmquist et al., 2005).

Changes in ruminal fatty acid metabolism

As shown in Figure 1, dietary FA are extensively altered in the rumen, resulting in marked differences between FA intake (mostly UFA) and FA outflow (mostly SFA), as a result of the rumen microbial population performing two major processes: lipolysis and biohydrogenation (Jenkins et al., 2008). Dietary lipids, characterised as structural or polar lipids (glycolipids, phospolipids), free FA, TAG, and sterol esters (Yang and Fujita, 1997), are first subject to lipolysis of their ester linkages by microbial lipolytic enzymes (except for the free FA). Then, the free UFA are subject to biohydrogenation, which requires free UFA to proceed (Harfoot and Hazlewood, 1997). Lipolysis and biohydrogenation are affected by the lipid source (amount and composition of FA, technological form) and the characteristics of the basal diet (forage type, forage to concentrate ratio (F/C ratio), fibre content, and starch content). Especially simultaneous changes in these dietary characteristics have a major impact on ruminal FA metabolism (Palmquist et al., 2005; Chapter 6).

In Figure 2 the relationship between the intake of C18:3*n*3 and the milk yield of C18:3*n*3 is presented based on the data of individual cows from Chapters 4, 5, and 6. It is clearly shown that

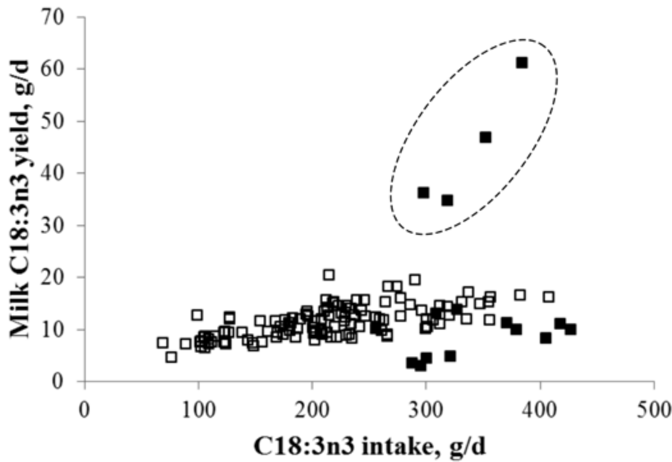


Figure 2. Relationship between C18:3*n*3 intake and C18:3*n*3 secretion in milk. Individual cow data are from Chapter 4 and 5 (■) and Chapter 6 (○). Data within the dashed circle are from cows fed formaldehyde treated linseed oil.

biohydrogenation of C18:3*n*3 is extensive and milk secretion of C18:3*n*3 reaches a maximum of approximately 20 g/d, whereas only protected sources (formaldehyde treated linseed oil; data within dashed circle) achieve higher milk C18:3*n*3 secretion.

In Chapter 2 prediction equations (Equation 1 and 2) for the proportion of C18:3*n*3 in milk fat were derived, in which effects of technological form of linseed (including added fish oil), main forage type, and contents of C18:3*n*3 and NDF in the total diet were used as class and continuous variables.

(1) Milk C18:3*n*3 (g/100 g FA) = 3.12 – 0.132 x C18:3*n*3 (g/kg DM) – 0.0056 x NDF (g/kg DM) + 0.00034 x C18:3*n*3 (g/kg DM) x NDF (g/kg DM; equation for linseed fed as seed; intercept and slope for C18:3*n*3 need to be adjusted for different forms of linseed supply)

(2) Milk C18:3*n*3 (g/100 g FA) = 3.88 – 0.187 x C18:3*n*3 (g/kg DM) – 0.0076 x NDF (g/kg DM) + 0.00048 x C18:3*n*3 (g/kg DM) x NDF (g/kg DM; equation for diets fed a supplemental source of linseed and maize silage as the main forage type in the diet; intercept and slope for C18:3*n*3 need to be adjusted for different main forage types in the diet)

From these equations it is clear that the dietary content of both C18:3*n*3 and NDF are important determinants for the extent of biohydrogenation and eventually secretion of C18:3*n*3 in milk. Proportion of C18:3*n*3 is influenced by technological form of supplemental linseed, addition of fish oil FA, and main forage type in the basal diet (Figure 3; Chapter 2). Equations 1 (Figure 4A) and 2 (Figure 4B) were evaluated with treatment means from Chapters 4, 5, and 6 and it is shown that Equation 1 can predict the C18:3*n*3 proportion in milk fat for treatment means from Chapters 4 and 5 perfectly (R^2 = 1.00). However, for the low C18:3*n*3 proportions there was an over-prediction

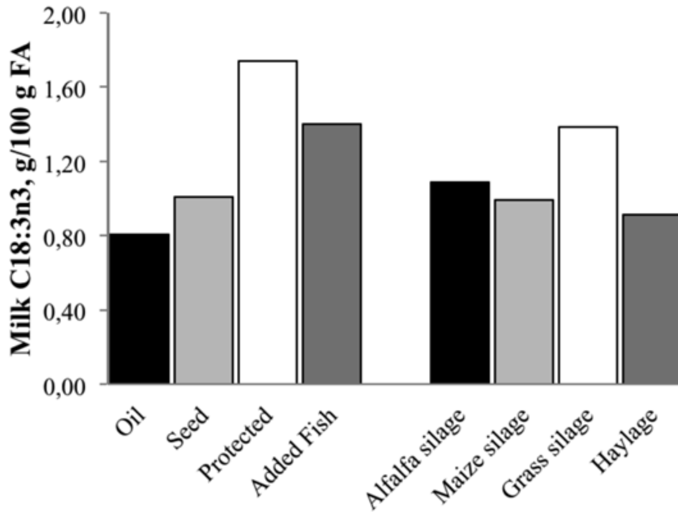


Figure 3. Least squares means for diets supplemened with linseed fed as different technological forms or fed to diets with different main forage types. Data are adjusted for the random effect of experiment, unequal variances among experiments and the means of the continuous variables C18:3*n*3 and NDF content (Chapter 2).

of the observed C18:3*n*3 proportion, whereas for the high C18:3*n*3 proportion there was an underprediction of the observed C18:3*n*3 proportion. The proportion of C18:3*n*3 in milk fat for treatment means from Chapter 6 could only be predicted poorly (R^2 = 0.22). The evaluation of Equation 2 showed that the C18:3*n*3 proportion in milk fat from treatment means from both Chapters 4 and 5 (R^2 = 0.09) and Chapter 6 (R^2 = 0.00) could not be predicted correctly (Figure 4B). The variation in forage type in Chapter 6 (80% grass silage versus 50/50% grass/maize silage versus 80% maize silage;

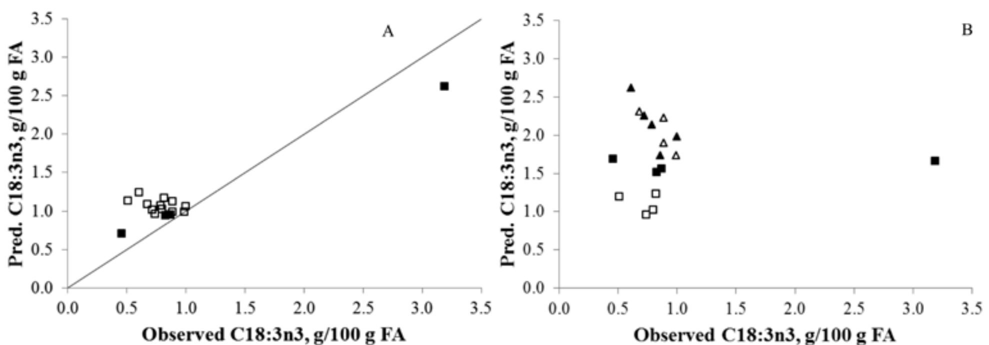


Figure 4. Observed versus predicted proportion of C18:3*n*3 in milk fat. Figure 4A: Equation 1 evaluated with treatment means from Chapter 4 and 5 (■) and Chapter 6 (□). The solid line is the Y = X regression. Figure 4B: Equation 2 evaluated with treatment means from Chapter 4 and 5 (■) and Chapter 6 with 80% maize silage (% of forage DM; □), 50% maize silage/50% grass silage (% of forage DM; ▲) and 80% grass silage (% of forage DM; △).

proportions of forage DM) could not be modelled with Equation 2. However, the regression slope for increasing grass proportion in the forage proportion of the diet was positive (coefficient = 0.0025) as shown in the response surface equation derived in Chapter 6. In addition, data from Chapter 2 derived with Equation 2 resulted in a higher ($P = 0.06$) proportion of C18:3*n*3 in milk fat for linseed supplemented diets with grass silage as the main forage type (1.39 g/100 g FA) compared with maize silage (0.99 g/100 g FA; Figure 3).

In Chapter 6 a response surface equation (Equation 3) for the proportion of C18:3*n*3 in milk fat was derived based on forage type in the diet (grass silage versus maize silage; Grass %; expressed as proportion of total forage DM), F/C ratio (Forage %), and proportion of crushed linseed (CL %).

(3) Milk C18:3*n*3 (g/100 g FA) = 0.71 + 0.0025 x Grass % - 0.0191 x Forage % + 0.2196 x CL % + 0.0002 x Forage %^{2} – 0.0102 x CL %^{2} – 0.0017 x Forage % x CL %

In Equation 3, main forage type (grass versus maize silage, Grass %), F/C ratio (Forage %) and proportion of crushed linseed in the diet (CL %) are the determinants for secretion of C18:3*n*3 in milk fat. Figure 5 shows the relationships between F/C ratio and proportion of crushed linseed in the diet for diets containing maize silage (Figure 5A) or grass silage (Figure 5B) as the main forage type (Chapter 6). Evaluation with the treatment means from Chapters 4 and 5 showed that Equation 3 could poorly account for the variation in proportion of C18:3*n*3 in milk fat achieved in this experiment ($R^2 = 0.08$). This variation in proportion of C18:3*n*3 in milk fat (Chapter 4 and 5) was related to the technological form of the linseed and this was not modelled in Equation 3, but in Equation 1 as shown in Figure 4A as discussed above.

Although significant effects of changes in the basal diet are found, the extent of biohydrogenation of C18:3*n*3 is high for linseed treatments in Chapter 4 (90.9 to 98.5%) which is in agreement with different linseed treatments in an experiment by Gonthier et al. (92.9% to 96.6%; 2004). The extent

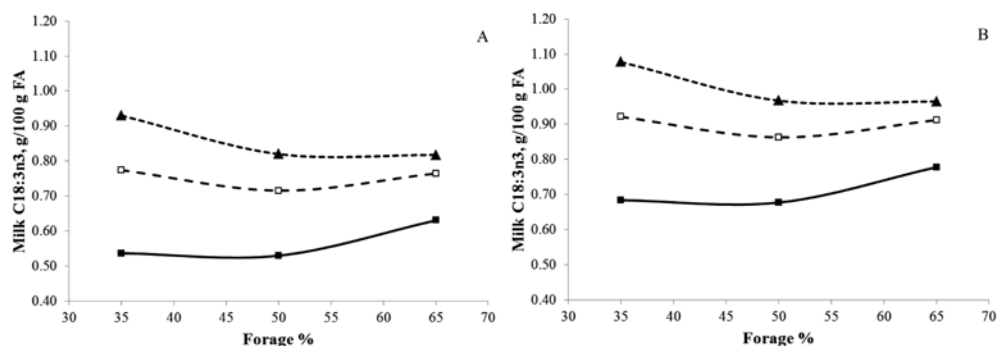


Figure 5. Relationship between forage % in the diet and C18:3*n*3 secretion in milk (Chapter 6) for diets containing 80% maize silage (A), or 80% grass silage in the forage proportion of the diet (DM basis; B). Data are for diets containing 1% crushed linseed (─■─), 3% crushed linseed (- - □- -), and 5% crushed linseed (--▲--).

of biohydrogenation can be described as a function of the FA pool size, the ruminal retention time, and the hydrogenation capacity of the microbial population (Harvatine and Allen, 2006). The hydrogenation capacity depends on the species and concentration of the microbial population in combination with the rumen environment. Due to the changes in NDF and starch contents in the diets, changes in the microbial population in the rumen will likely have appeared. These changes in microbial fermentation can be characterised by a decreased rumen pH and shifts in the rumen pattern of VFA produced (lower acetate and higher propionate proportions; Fuentes et al., 2009). Although not studied, it was speculated that an increased starch content in the diet decreases the activity / number of *Butyrivibrio fibrisolvens* in the rumen which may alter biohydrogenation routes (Klieve et al., 2003, Nielsen et al., 2006). Biohydrogenation of C18:3*n*3 results in a number of biohydrogenation intermediates, which vary largely under influence of diet composition (Chilliard et al., 2007). In the different studies described in this thesis, mainly C18:1 and C18:2 isomers were identified and these isomers showed marked differences related to the fat source and basal diet composition. When a F/C ratio of 35:65 was fed, increasing crushed linseed proportion in the diet increased proportions of *trans*-11,*cis*-15-C18:2, *cis*-9,*trans*-11-C18:2, *trans*-10,*cis*-12-C18:2, *trans*-10-C18:1, *trans*-11-C18:1, *trans*-13+14-C18:1, *trans*-15-C18:1, and *cis*-15-C18:1 in milk fat (Chapter 6). Proportions of several of the biohydrogenation intermediates and C18:3*n*3 in milk fat showed interactions between F/C ratio and proportion of crushed linseed in the diet, suggesting a lower complete biohydrogenation of C18:3*n*3 when a high concentrate diet (65% concentrates) was combined with the highest supplementation of crushed linseed (5%). In vitro, these effects were confirmed by Fuentes et al. (2009) who showed a lower extent of biohydrogenation of *cis*-9-C18:1, C18:2*n*6, and C18:3*n*3 in a high concentrate (30:70 F/C ratio) compared with a low concentrate (70:30 F/C ratio) diet. When a minimum level of fibre (approximately 30%) in a diet containing a high concentrate proportion is guaranteed, rumen pH is less affected and thus bacteria responsible for biohydrogenation respond less to the high concentrate proportion (Fuentes et al., 2009). In addition to fibre (physically effective NDF), rumen degradable starch from grain sources and DMI have to be considered to estimate the effect on rumen pH (Zebeli et al., 2008) and consequently on ruminal FA metabolism.

In Figure 6 the relationship between the intake of C18:3*n*3 and the milk yield of *cis*-9,*trans*-11-C18:2 (Figure 6A) based on the data of individual cows from Chapters 4, 5, and 6 is presented. From this Figure it can be seen that there is no clear relationship between the intake of C18:3*n*3 and the secretion of *cis*-9,*trans*-11-C18:1 in milk. However, the response surface equation derived in Chapter 6 (Equation 4) shows that the proportion of *cis*-9,*trans*-11-C18:2 increased with increasing proportion of crushed linseed in the diet.

(4) Milk *cis*-9,*trans*-11-C18:2 (g/100 g FA) = 0.57 + 0.0122 x CL %

Cis-9,*trans*-11-C18:2 in milk fat partly originates from rumen outflow (biohydrogenation intermediate), whereas the largest part originates from desaturation of *trans*-11-C18:1 in the mammary gland. This is confirmed by the strong relationship between the *trans*-11-C18:1 and *cis*-9,*trans*-11-C18:1 proportions in milk fat (Figure 6B). Cows receiving linseed oil in combination with

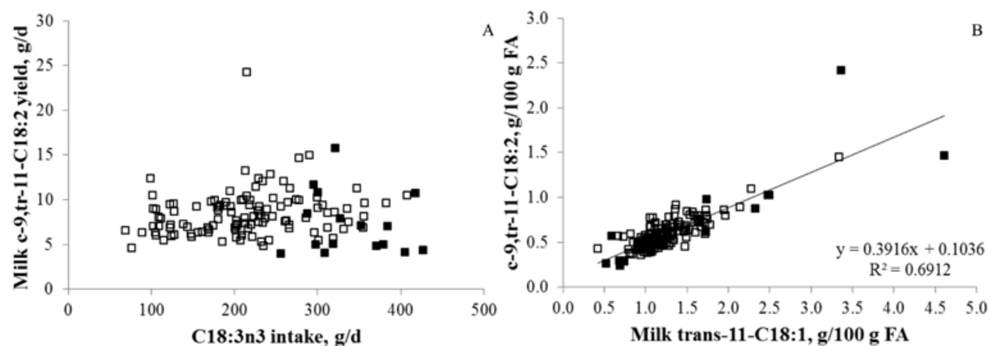


Figure 6. Relationship between C18:3*n*3 intake and *cis*-9,*trans*-11-C18:2 secretion in milk (A) and relationship between proportion of *trans*-11-C18:1 and *cis*-9,*trans*-11-C18:1 in milk fat (B). Individual cow data are from Chapter 4 and 5 (■) and Chapter 6 (□).

marine algae enriched in DHA had higher *trans*-11-C18:1 and *cis*-9,*trans*-11-C18:2 proportions in milk fat (Chapter 4 and 5).

The addition of fish oil (Chapter 2) or marine algae enriched in DHA (Chapter 3, 4, and 5) to diets containing a source of soybean, sunflower, or linseed was also an important factor in the research described in this thesis. The addition of fish oil and marine algae affect the hydrogenation capacity of the microbial population. Proportions of C18:0 decreased and proportions of *trans*-FA increased both in vitro (Vlaeminck et al., 2008; Chapter 3) and in vivo (Boeckaert et al., 2008a, b; Chapter 4 and 5) after supplementation of marine algae rich in DHA to diets with (Chapter 3, 4, and 5) or without (Vlaeminck et al., 2008; Boeckaert et al., 2008a, b) supplemental linseed oil. Boeckaert et al. (2008b) related the inhibition of biohydrogenation of *trans*-FA to C18:0 to alterations in the bacterial community, specifically bacteria from the *Butyrivibrio* group. The authors concluded that dietary marine algae affected non-cultivated species, clustering between the genus *Butyrivibrio* and the genus *Pseudobutyrivibrio* and that other, still uncultivated bacteria are involved in C18:0 production. In Chapter 4 a shift in rumen pattern of produced VFA was found (lower acetate and higher propionate) when marine algae rich in DHA were added to a diet containing linseed oil, which could be related to these changes in the bacterial community. However, no effects on degradation of NDF in the rumen were found and therefore the effects on the microbial population could not be confirmed (and were not measured).

Changes in mammary metabolism of fatty acids

The profile of absorbed FA is determined by the dietary FA profile and ruminal FA metabolism as described before. However, there is selectivity in the distribution of absorbed UFA in the major plasma lipid fractions, which is considered important for the distribution of the different lipid fractions to the mammary gland for milk fat synthesis (Loor et al., 2002c). Both C18:2*n*6 and C18:3*n*3 are selectively incorporated in plasma cholesterol esters and phospholipids, whereas the mammary gland primarily uses the plasma TAG and free FA fractions for milk fat synthesis (Loor et al., 2002b), resulting,

additionally to biohydrogenation, in the low transfer efficiency for C18:2*n*6 and C18:3*n*3 (Jacobs et al., 2011). When the different plasma lipid classes are analysed separately, plasma TAG and free FA fractions show a composition very much comparable to duodenal FA (Glasser et al., 2007b), which was also shown by the comparable omasal FA flows and plasma TAG FA composition in the research described in this thesis (Figure 1; Chapters 4 and 5).

Fatty acids can be desaturated through the action of the enzyme stearoyl-CoA desaturase (SCD) during intestinal absorption (in the enterocyte) and within tissues (e.g. mammary gland and adipose tissue; Glasser et al., 2007b). The activity of the SCD enzyme in desaturation of C18:0 and *trans*-11-C18:1 is supported by the close associations observed among increases of substrates and products in plasma TAG and milk fat (Figure 7), as was previously described in Loor et al. (2005b). In addition, basal activity of SCD as evaluated using the milk C14 desaturation index as a proxy was not affected by C18:3*n*3 intake (Figure 8). Jacobs et al. (2011) did not find an effect of linseed oil addition on mammary SCD1 mRNA expression determined using quantitative real-time PCR and on milk desaturation indices. Expression of SCD1 mRNA in milk somatic cells was determined for the cows in the experiment described in Chapters 4 and 5, but was not different between the different linseed treatments (Jacobs, unpublished results).

Interrelationships between rumen fermentation and mammary metabolism are important and diet-induced milk fat depression is a naturally occurring situation that involves these interrelationships (Lock et al., 2007). Under certain dietary conditions, unique *trans*-FA can be produced as a result of altered biohydrogenation pathways (Bauman and Griinari, 2003) and one of the most extensively studied *trans*-FA from these pathways, *trans*-10,*cis*-12-C18:2, is known to be a potent inhibitor of milk fat synthesis (Lock et al., 2007; Shingfield and Griinari, 2007). However, both omasal flow and milk fat proportion of *trans*-10,*cis*-12-C18:2 were very low in the experiments performed in this thesis, and thus probably not suited to estimate the effect of this isomer. In addition, over a wide range of diets causing milk fat depression, increased proportions of *trans*-10-C18:1 in milk fat have also been observed (Shingfield and Griinari, 2007). However, this negative relationship between milk fat

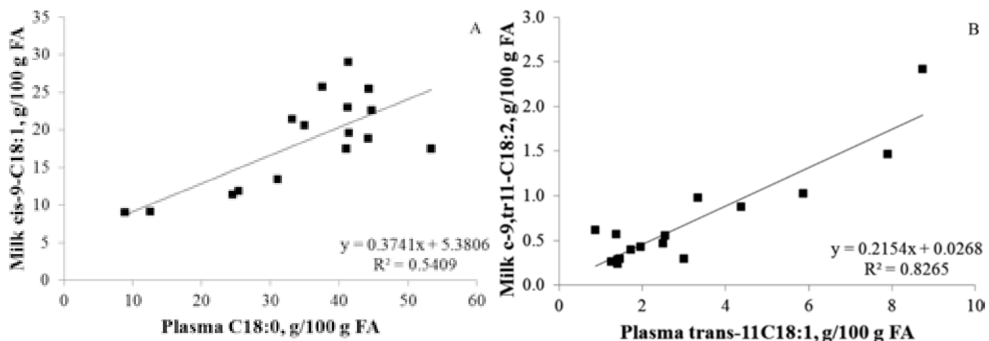


Figure 7. Relationship between proportion of C18:0 in plasma TAG and *cis*-9-C18:1 in milk fat (A) and relationship between proportion of *trans*-11-C18:1 in plasma TAG and *cis*-9,*trans*-11-C18:2 in milk fat (B; Chapter 5).

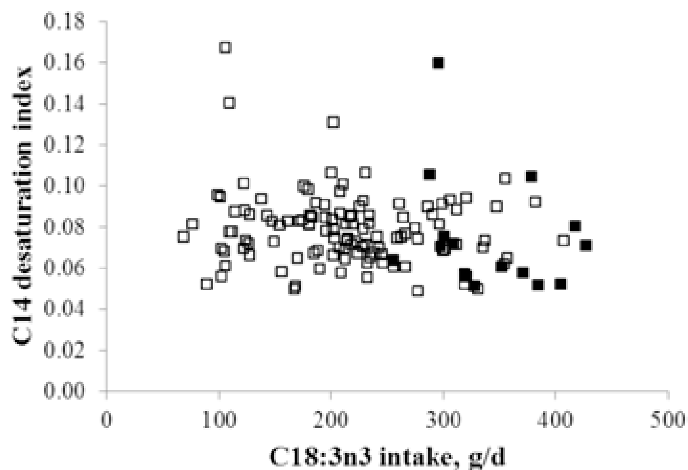


Figure 8. Relationship between C18:3*n*3 intake and C14 desaturation index [*cis*-9-C14:1/ (C14:0 + *cis*-9-C14:1)]. Individual cow data are from Chapter 5 (■) and Chapter 6 (□).

content and *trans*-10-C18:1 proportion does not imply a direct cause and effect. A close relationship between omasal flow of *trans*-10-C18:1 and milk secretion of *trans*-10-C18:1 was observed (R^2 = 0.85; Chapter 4 and 5) with a mean transfer efficiency from omasum to milk of 30.9%, which is very close to the mean transfer efficiency from duodenum to milk of 32.1% reported by Shingfield and Griinari (2007). Lock et al. (2007) concluded that administration of a pure preparation of *trans*-10-C18:1 did not affect milk fat secretion. However, there was a relatively low transfer efficiency of this pure preparation into milk fat (15%; Lock et al., 2007), which was less than expected from the transfer efficiency from omasal/duodenal flow to milk in Chapter 4 and 5, and Shingfield and Griinari (2007). Curvilinear relationships were demonstrated for rumen outflow of *trans*-10-C18:1 and milk fat content (Lock et al., 2007), and milk fat proportion of *trans*-10-C18:1 and milk fat content (Loor et al., 2005b), which were confirmed in the current thesis (Figure 9). Therefore, the role of *trans*-10-C18:1 in milk fat secretion is still not fully elucidated.

Mammary de novo synthesis (mainly from acetate and ß-hydroxybutyrate from rumen organic matter fermentation) generates the short- and medium-chain FA (C4 to C14 FA) and part of the 16-carbon FA. Because of the close relationship between the secretion of C4-C14 FA and C16 FA, Glasser et al. (2007b) concluded that mammary de novo synthesis can be estimated by the milk secretion of the sum of even-chain C4-C16 FA. The low relationship between duodenal C18 FA flow and C18 FA secretion in milk suggest that milk C18 FA secretion is not only limited by the supply of C18 FA to the mammary gland (Glasser et al., 2007b). This low relationship was confirmed with data from Chapter 4 and 5 showing a poor relation between omasal C18 FA flow and secreted C18 FA in milk (R^2 = 0.16; Chapter 4 and 5). Figure 10 shows the relationship between the milk fat secretion of C4-C16 FA and total C18 FA (Figure 10A: Glasser et al., 2007b; Figure 10B: Chapter 5 and 6). Glasser et al. (2007b) showed that cows fed diets supplemented with plant oils were on

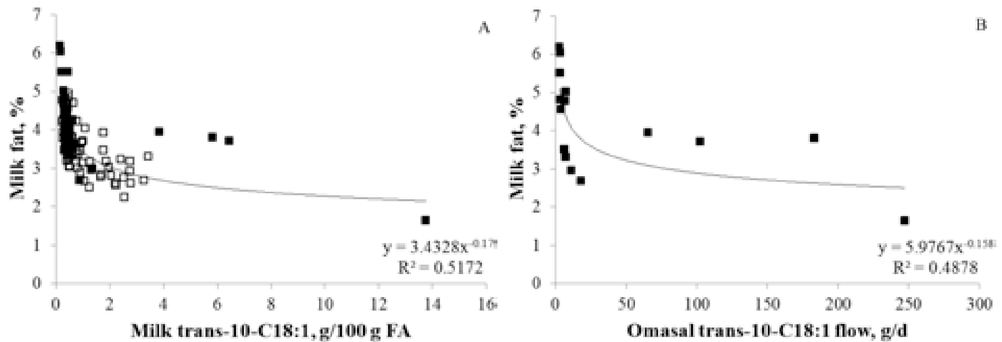


Figure 9. Relationship between milk *trans*-10-C18:1 proportion and milk fat content (A), and omasal flow of *trans*-10-C18:1 and milk fat content (B). Individual cow data are from Chapter 4 and 5 (■) and Chapter 6 (□).

the same regression line (dashed line on figure 10A; slope = 1.04, R^2 = 0.86), whereas cows fed unsupplemented diets or diets supplemented with fish oil were on a second regression line (solid line on figure 10A; slope = 0.33, R^2 = 0.60). Data from the experiments in this thesis show a comparable separation between cows supplemented with high or low levels of linseed (data from Chapter 5 with high C18:3*n*3 intake, slope = 0.90, R^2 = 0.78; data from Chapter 6 fed 1% crushed linseed, slope = 0.36, R^2 = 0.30).

Glasser et al. (2007b) hypothesised that milk fat secretion in low-lipid diets could be limited by the availability of total C18 FA, whereas in high lipid diets milk fat secretion could be limited by a low C4-C16 secretion, which is supposed to be a combined effect of substrate shortage and inhibition of de novo synthesis by long-chain FA. The relatively low availability of C4-C16 FA in high lipid diets could limit milk fat TAG synthesis (Glasser et al., 2007b), because during synthesis of TAG in the mammary gland these FA are the main FA at the *sn*-2 and *sn*-3 positions (Jensen, 2002). In contrast, Glasser et al. (2007b) hypothesised that the relatively low availability of total C18 FA in low lipid diets could result in a high milk fat melting point being a constraint for the incorporation of saturated de novo synthesised FA in milk TAG. However, a lower availability of C18:0 in combination with a higher availability of C4-C16 FA would result in a lower melting point, because of the average chain length being shorter. The data from the current experiments also do not fully support the hypothesis from Glasser et al. (2007b). The mean fraction of total C18 FA in total milk FA was 45 g/100 g for data from Chapter 5 and 39 g/100 g for data from the 1% crushed linseed supplemented diets in Chapter 6. These fractions were somewhat lower (54 g/100 g FA for plant oil supplemented diets), respectively higher (34 g/100 g FA for unsupplemented diets) compared with the results of Glasser et al. (2007b). The addition of fish oil or marine algae could limit milk fat secretion due to the decreased availability of C18:0 and *cis*-9-C18:1 (Loor et al., 2005d) and increased availability of *trans*-C18:1 increasing the melting point of the pool of long-chain FA and thereby reaching the physiological limit for milk TAG formation (Shingfield and Griinari, 2007).

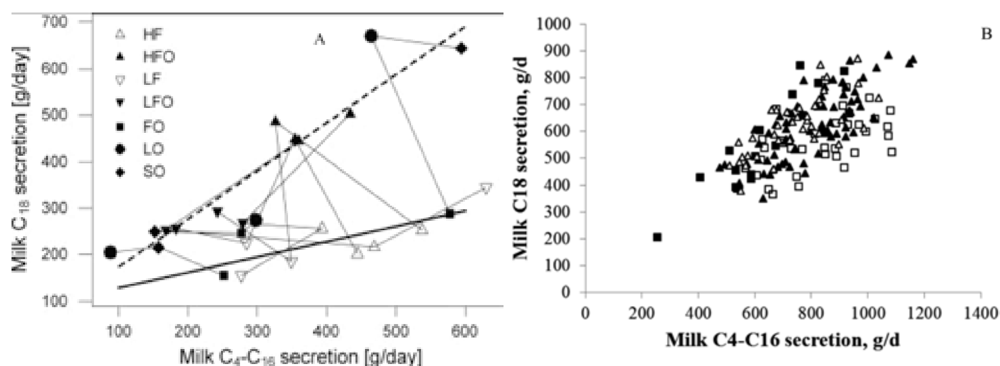


Figure 10. Relationship between milk secretion of the sum of C4-C16 FA and total C18 FA. Figure 10A: each point is the individual value for one cow (same cow is linked via a thin line) fed a diet containing high forage (HF), high forage + 3% linseed oil (HFO), low forage (LF), low forage + 3% linseed oil (LFO), diet with 2.5% fish oil (FO), diet with 5% linseed oil (LO), diet with 5% sunflower oil (SO). The dashed line is the regression line across plant oil supplemented diets and the solid line is the regression across the unsupplemented and fish oil supplemented diets (Glasser et al., 2007b). Figure 10B: Individual cow data are from Chapter 5 (■) and Chapter 6 with supplementation of 1% crushed linseed (▲), 3% crushed linseed (△) and 5% crushed linseed (Δ).

Effects of diets containing more UFA on animal metabolism and methane

Animal metabolism

Due to the increased energy requirements for milk production, which cannot be met by feed intake alone, dairy cows in early lactation experience a NEB (Van Knegsel et al., 2007b). Hormonal changes (e.g. high ratio of growth hormone to insulin) allow mobilisation of long-chain FA from adipose tissue to increase energy available for milk secretion (Drackley, 1999). Diets inducing milk fat depression might support the dairy cow to increase energy balance in early lactation by decreasing energy output via milk fat production (Castañeda-Gutiérrez et al., 2005; Odens et al., 2007). As already discussed, specific *trans*-FA produced during altered biohydrogenation pathways of PUFA, might induce milk fat depression, resulting in decreased milk fat yield without alteration of milk and milk protein yield. Several studies (Castañeda-Gutiérrez et al., 2005; Odens et al., 2007; Moallem et al., 2010; Pappritz et al., 2011) used preparations of *trans*-10,*cis*-12-C18:2 to reduce milk energy output during the period of NEB early lactation. These studies reported either no effects on energy balance (Castañeda-Gutiérrez et al., 2005; Pappritz et al., 2011) or an improved energy balance (Odens et al., 2007; Moallem et al., 2010), which was related to the dosage of *trans*-10,*cis*-12-C18:2 and the timing of the occurrence of milk fat depression immediately after calving. The experiments performed in this thesis were carried out with cows in early- to mid-lactation (52 ± 22 DIM for cows in Chapter 4 and 5, and 72 ± 17 DIM for cows in Chapter 6), which were in positive energy balance (Table 1). A significant milk fat depression was found for cows receiving linseed oil and marine algae rich in DHA (Chapter 5). In combination with a numerically lower milk yield, cows in this treatment

Table 1. Energy intake, energy in milk and energy balance (kJ/(kg$^{0.75}$ per day)) as calculated with the VEM system[1] for cows in Chapter 4 and 5.

Parameter	Dietary treatment				SEM[2]	P-value
	CL	EL	FL	DL		
Net energy intake	1152	1156	1231	1137	-	-
Calculated energy in milk	1124[a]	1145[a]	1196[a]	954[b]	32.4	0.008
Calculated energy balance	28[b]	11[b]	35[b]	183[a]	71.2	0.002

[a,b]Means within a row with different superscripts differ (P < 0.05).
[1]Van Es (1975).
[2]SEM: standard error of mean.

showed a higher calculated energy balance (Table 1). It is therefore suggested that marine algae rich in DHA might improve energy balance of dairy cows in early lactation. Hostens et al. (2009) showed a decreased energy output in milk fat for cows receiving 220 g DHA Gold (Martek Biosciences, Corp., Columbia, MD) from 21 d before calving to 100 DIM (calculated energy in milk 1258 kJ/(kg$^{0.75}$ per day) for the DHA group compared with 1329 kJ/(kg$^{0.75}$ per day) for the control group, assuming 600 kg BW for all cows). However, measured blood serum metabolites (higher BHBA, lower glucose concentrations in DHA group, and similar NEFA concentrations) and BHBA in follicular fluid (higher level in DHA group) suggested a decreased energy balance. From these results the authors concluded that although there was a decrease in energy output in milk there was no increase in energy status as measured in serum and follicular fluid. However, the effects on production characteristics during the first two weeks of lactation were small. This would be in agreement with the results of Castañeda-Gutiérrez et al. (2005) and Zachut et al. (2010), who suggested that the decrease in milk fat output should be reached immediately after calving to be able to improve energy balance. In contrast to the research based on preparations of *trans*-10,*cis*-12-C18:2 to induce milk fat depression, Zachut et al. (2010) used extruded linseed to improve energy balance. However, cows increased milk yield at the expense of milk fat content and energy balance was only improved by the increased energy intake from the extruded linseed, thus resulting in an improved energy balance but without an effect on blood metabolites (glucose, NEFA).

Due to the extensive body fat mobilisation of dairy cows in NEB, the dairy cow is predisposed to hepatic lipidosis and ketosis, because of the inability to dispose of mobilised FA via ß-oxidation or the limited capacity to export FA re-esterified into TAG from the liver (Grummer, 1993; Bell, 1995; Van Knegsel et al., 2007b). Research suggests that hepatic FA metabolism can be influenced by FA chain length and degree of unsaturation. From a series of in vitro experiments, it was suggested that increasing the length and degree of unsaturation of FA decreased hepatic TAG accumulation and down-regulated gene expression of specific proteins involved in synthesis and secretion of very low density lipoproteins that export TAG from the liver (Mashek et al., 2002; Mashek and Grummer, 2003). Further in vivo research suggests that modifications in adipose tissue metabolism by C18:3*n*3 might increase the uptake of circulating NEFA by peripheral tissues (Mashek et al., 2005; Pires and Grummer, 2008). The sensitivity of adipose tissue to insulin might be influenced by the addition of C18:3*n*3, which might decrease plasma NEFA concentrations and FA uptake by the liver (Mashek et al., 2005). Petit et al.

(2007) found lower concentrations of liver TAG in wk 4 postpartum and higher concentrations of liver glycogen at wk 2 and 4 postpartum for multiparous cows fed linseed compared with a control diet. The authors concluded that linseed fed from 6 wks before calving can provide a useful strategy to improve hepatic metabolism after calving and therefore prevent the development of lipidosis.

Supplementation of UFA might also influence immune system responses, as shown by Mach et al. (2011). These authors showed that cows fed UFA enriched diets had a down-regulation of many key genes known to be involved in cellular and humoral immune responses, pathogen-induced signalling, and cellular stress and injury. It is therefore suggested that diets containing more UFA can affect immune functions of the mammary gland, but specific research designed to confirm these hypotheses is required (Mach et al., 2011).

Supplementation of UFA in the form of linseed might also change hormone secretion related to reproduction functions. Described effects of PUFA on reproduction are: increased ovarian steroidogenesis, manipulation of insulin to stimulate ovarian follicle development, and/or inhibition of the uterine production and release of $PGF_{2\alpha}$ (Mattos et al., 2000). Inhibition of the uterine production and release of $PGF_{2\alpha}$ by *n*3 FA may result in increased embryonic survival and pregnancy rates (Petit et al., 2008). Santos et al. (2008) summarised effects of different studies feeding C18:3*n*3 or EPA and DHA and reported reduced pregnancy losses in three of five studies. However, in relation with the decreased release of $PGF_{2\alpha}$, oestradiol levels might also be decreased after feeding *n*3 FA which has a negative effect on expression of oestrus and uterine priming before oestrus (Santos et al., 2008). Results of a study in which linseed was fed to early lactation dairy cows on three commercial dairy farms showed that reproductive performance was not influenced by feeding 0.85 kg DM linseed/d compared with a control diet (Bork et al., 2010). In conclusion, inconsistent results suggest that there may be beneficial effects of *n*3 FA on reproductive performance, but these are not fully elucidated yet. From the experiments performed in this thesis, effects on reproduction could not be determined due to the low number of animals to detect effects on reproduction and due to the design of the experiments with 21 d measurement periods in a Latin square design (Chapter 4 and 5) or a Box-Behnken design (Chapter 6).

Methane production

Ruminants are responsible for 15 to 20% of total anthropogenic emissions of CH_4 and mitigation strategies are developed to reduce these emissions and improve production efficiency of ruminants (Martin et al., 2008). Feeding supplemental UFA to improve milk FA profile can affect enteric CH_4 emissions by decreasing the amount of OM fermented in the rumen, the activity of rumen methanogens, and protozoal numbers (Johnson and Johnson, 1995; Beauchemin et al., 2009), and a small reduction through biohydrogenation of UFA as a hydrogen sink (Jenkins et al., 2008). Martin et al. (2008) showed a significant decrease in CH_4 emissions when crude linseed, extruded linseed, or linseed oil were included in the diet at 57 g FA/kg of diet DM. The authors found the greatest decrease when linseed oil was added since this treatment was associated with the most pronounced reductions in feed intake and rumen substrate fermentability. The decreasing effect of supplemental fat on CH_4 emission depends on the amount and FA profile of the fat source, the technological form

of the fat source, and the composition of the basal diet (e.g. the F/C ratio; Beauchemin et al., 2009). Supplementation of different rumen available fat sources (crushed seeds) showed that canola seed and linseed reduced CH_4 expressed per unit feed DM more compared with sunflower seed and a control diet (Beauchemin et al., 2009). Often the reduction in CH_4 emissions when feeding supplemental fat is caused by a decreased DMI, ration digestibility, or a combination of both (Martin et al., 2008). Rumen apparent digestibility of OM and NDF and total tract apparent digestibility of NDF were not different for the different linseed treatments in Chapter 4. Martin et al. (2008) suggested that there might be a direct toxic effect of linseed FA on methanogens. However, Van Zijderveld et al. (2011b) found no difference in CH_4 production after feeding extruded linseed compared with a fractionated palm oil (exchanged isolipidically) at equal DMI levels, suggesting that effects of feeding linseed on methane emissions would be mainly caused by the indirect effects (e.g. fermented OM, NDF digestibility). Because of the absence of effects on DMI, OM and NDF digestibilities (Chapter 4), it is not expected that methane production in the experiments described in this thesis was significantly affected.

Milk FA profile is considered to be a potential indicator of CH_4 production (Chilliard et al., 2009; Dijkstra et al., 2011). Multiple regression equations to predict CH_4 production were presented by Chilliard et al. (2009; production in g/d) and by Dijkstra et al. (2011; production in g/kg DM) and were able to predict CH_4 production with a relatively good R^2 (R^2 = 0.73 to 0.93). Both research groups concluded that the predictions were limited to diets containing a source of C18:3*n*3, respectively diets without variation in type, composition and proportion of forage and concentrate. Therefore, more data are required to confirm the use of milk FA profile as an indicator for CH_4 production.

Conclusions

Effects of supplementing dairy cows with different fat sources (differing in amount and technological form), supplemented to basal diets varying in forage type and forage to concentrate ratio were evaluated in the research described in this thesis. Significant changes in milk FA profile can be achieved when the ration of the dairy cow is altered. In the meta-analysis it was shown that various fat sources, their technological form, and their inclusion to diets differing in forage type, could significantly change the effect on milk FA profile. Various chemically or technologically treated linseed products were evaluated in vitro and only formaldehyde treatment of crushed linseed and extrusion of whole linseed were effective in decreasing the extent of biohydrogenation of C18:3*n*3 significantly. The addition of DHA to linseed oil showed that the extent of biohydrogenation of C18:3*n*3 was high, but the complete biohydrogenation towards C18:0 was inhibited, resulting in increased proportions of biohydrogenation intermediates. In vivo the most promising linseed treatments were evaluated on FA intake, omasal FA flows, plasma FA profile, and milk FA secretion. This experiment showed that the extent of biohydrogenation of C18:3*n*3 was high for all treatments (85.9 to 98.3%), whereas extruded whole linseed resulted in the lowest extent of biohydrogenation. However, fat digestibility for the diet containing extruded whole linseed was significantly lower, resulting in no effects on C18:3*n*3 proportion in plasma TAG and C18:3*n*3 secretion in milk fat. Formaldehyde treated

linseed oil showed a comparable extent of biohydrogenation with crushed linseed, however, C18:3*n*3 proportion in plasma TAG and C18:3*n*3 secretion in milk fat were significantly increased. The addition of DHA to linseed oil showed a higher extent of biohydrogenation compared with crushed linseed, however, in agreement with the in vitro experiment in chapter 3 complete biohydrogenation towards C18:0 was inhibited resulting in increased omasal flows of biohydrogenation intermediates and increased proportions of biohydrogenation intermediates in plasma TAG and milk fat. In addition, as a consequence of the increased availability of *trans*-11-C18:1, a significantly increased *cis*-9,*trans*-11-C18:2 secretion in milk fat was achieved. Transfer efficiencies from C18:3*n*3 intake to milk secretion showed a marked increase when C18:3*n*3 was supplied in the form of formaldehyde treated linseed oil. In the last experiment changes in the basal diet (grass versus maize silage as the main forage type and F/C ratio) were simultaneously evaluated with an increasing proportion of crushed linseed in the diet. Response surface equations were derived to be able to quantify the effects of the varying factors on milk FA profile. Shifting from 80% maize silage to 80% grass silage linearly increased proportions of *trans*-11,*cis*-15-C18:1 and C18:3*n*3 in milk fat, whereas proportions of *trans*-10-C18:1 and C18:2*n*6 in milk fat linearly decreased. Significant interactions between level of crushed linseed and F/C ratio were found for C18:3*n*3 and several biohydrogenation intermediates, with the highest proportions in milk fat achieved when the diet contained 5% crushed linseed and a 35:65 F/C ratio. Overall, this study showed that the effect of supplementation of crushed linseed on several milk FA proportions, depends significantly on forage type and F/C ratio in the basal diet.

The results described in this thesis show that FA profile in milk fat is largely influenced by FA intake, FA metabolism in the rumen, lipid mobilisation, and mammary gland metabolism. Alterations of the milk FA profile towards a nutritionally more beneficial profile for human health can be achieved by changing the diet of dairy cows, thereby influencing ruminal FA metabolism, the profile of absorbed FA and eventually, the profile of FA secreted in milk fat.

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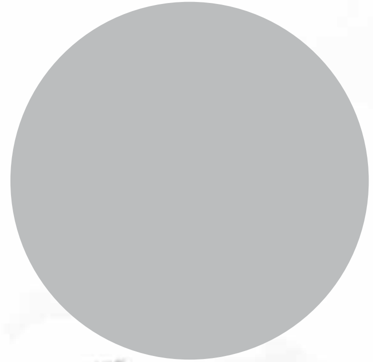
Summary
Samenvatting
Gearfetting



A substantial proportion of the daily fat intake in Western type diets of humans originates from milk and dairy products. Dutch milk fat generally consists of 70.6% saturated fatty acids, 23.5% mono-unsaturated fatty acids, and 2.3% poly-unsaturated fatty acids. Due to the large proportion of saturated fatty acids, dairy milk fat has traditionally been associated with human cardiovascular health problems. However, several specific fatty acids in milk fat, such as linolenic acid (C18:3*n*3) and conjugated linoleic acid (CLA; *cis*-9,*trans*-11-C18:2) have been associated with potential benefits for human health, but their contents in milk fat are generally low (0.5% C18:3*n*3 and 0.5% *cis*-9,*trans*-11-C18:2). Research to manipulate the milk fatty acid profile has therefore received increasing attention. Beneficial changes in the milk fatty acid profile might lead to increasing consumer acceptance of milk.

Milk fatty acids are derived from two sources: 1) mammary de novo synthesis from acetate and ß-hydroxybutyrate derived from rumen organic matter fermentation (C4:0 to C14:0 and part of C16 fatty acids); and 2) uptake of preformed fatty acids originating from the intestinal absorption of dietary, microbial, and mobilised fatty acids (part of C16 and ≥ C18 fatty acids). Changing the composition of ruminant diets provides a natural way for farmers to alter the milk fatty acid profile towards a more desirable profile. Ruminant diets are normally composed of a mix of fresh forages, conserved forages, and concentrates, and contain generally less than 70 g of lipids per kg dry matter (DM) with oleic acid (*cis*-9-C18:1), linoleic acid (C18:2*n*6), and C18:3*n*3 as the most important fatty acids. In the rumen, dietary lipids are subjected to extensive, microbial lipolysis and biohydrogenation, resulting in a high rumen outflow of free saturated fatty acids. In the mammary gland, however, these free saturated fatty acids and fatty acids that escaped rumen biohydrogenation or were partly biohydrogenated (biohydrogenation intermediates) can be transformed into unsaturated fatty acids again under influence of enzyme activity (Stearoyl Co-enzyme A Desaturase) in a process that is called desaturation.

The objective of the research described in this thesis is to improve the milk fatty acid profile of dairy cows. To achieve this objective, the main focus was on altering the diet composition and ruminal fatty acid metabolism to increase ruminal outflow of unsaturated fatty acids and consequently the secretion of unsaturated fatty acids, such as C18:3*n*3, in milk fat.

In Chapter 2, the milk fatty acid profile was evaluated in response to changes in dietary nutrient composition in relation to supplementation of different fat sources, their technological form (oil, seed, or protected), addition of fish oil, and main forage type in a meta-analysis approach. A dataset comprising 151 treatment means was built from 50 published experiments. Publications (n=47 reporting 50 experiments) reporting diet composition, nutrient composition, fatty acid composition, dry matter intake, milk yield, milk composition, and milk fatty acid profile were included in the data analyses. Mixed model regression analysis including a random experiment effect and unequal variances among experiments was used and least squares means were obtained for the different fat sources (unsupplemented, canola, soybean and sunflower, linseed, or fish oil), technological form including addition of fish oil fatty acids (oil, seed, protected, or added fish oil), and main forage type in the basal diet (alfalfa silage, barley silage, maize silage, grass silage, maize silage and haylage, or haylage). Results showed that different technological forms of supplemental canola, soybean, sunflower, or

linseed significantly affected the relationship (intercepts and coefficients) between dietary nutrient composition (fatty acid composition and NDF content) and milk fatty acid profile. This resulted in differences in several milk fatty acids for the different technological forms within fat sources supplemented to the diet. In addition, the effect of the main forage type in the diet also influenced the effect of dietary fatty acid and NDF contents on milk fatty acid profile, resulting in significant differences in several milk fatty acids for different main forage types within unsupplemented diets or diets supplemented with a source of canola, soybean, sunflower, or linseed. Thus, the effect of dietary nutrient composition on several milk FA proportions, is dependent on type and form of fat supplementation, addition of fish oil, and main forage type in the basal diet.

In Chapter 3, ruminal biohydrogenation kinetics of C18:3*n*3 from several chemically or technologically treated linseed products and docosahexaenoic acid (DHA; C22:6*n*3) addition to linseed oil were evaluated in vitro. Linseed products included in this experiment were: linseed oil, crushed linseed, formaldehyde treated crushed linseed, extruded whole linseed, extruded crushed linseed, micronized crushed linseed, lipid encapsulated linseed oil, and DHA addition to linseed oil. These products were incubated with rumen liquid using equal amounts of supplemental C18:3*n*3 and fermentable substrate (freeze-dried total mixed ration) for 0, 0.5, 1, 2, 4, 6, 12, and 24 h in a batch culture technique. Disappearance of C18:3*n*3 was measured to estimate the fractional biohydrogenation rate and lag time and calculate the effective biohydrogenation of C18:3*n*3. Technological treatment (crushing) of linseed followed by chemical treatment (formaldehyde) resulted in an effective protection of C18:3*n*3 against biohydrogenation. In addition, extrusion of whole linseed was also effective in reducing C18:3*n*3 biohydrogenation. Crushed linseed, extruded crushed linseed, micronized crushed linseed, lipid encapsulated linseed oil, and DHA addition to linseed oil did not reduce C18:3*n*3 biohydrogenation compared with linseed oil. However, the addition of DHA to linseed oil inhibited the last step of biohydrogenation from *trans*-10+11-C18:1 to C18:0, shown by a lesser proportion of C18:0 after 24 h of incubation. Regarding all evaluated linseed products, only formaldehyde treated crushed linseed and extruded whole linseed show to be of potential use in the ruminant diet to increase rumen C18:3*n*3 outflow.

In Chapter 4 and 5, the effects of the most promising linseed treatments from the in vitro experiment described in Chapter 3 were studied on omasal fatty acid flows, C18:3*n*3 biohydrogenation, plasma fatty acid composition, and milk fatty acid profile in dairy cows. The experiment was conducted as a Latin square design in which four rumen-cannulated lactating Holstein Friesian dairy cows were fed four different linseed sources: 1) crushed linseed (CL); 2) extruded whole linseed (EL); 3) formaldehyde treated linseed oil (FL); and 4) DHA in combination with linseed oil (DL), during four periods of 21 d each. Fatty acid intake, omasal fatty acid flow (estimated using Cr, Yb, and acid detergent lignin as digesta flow markers), fatty acid profile of plasma triacylglycerides, and milk production and milk fatty acid profile were determined. Average C18:3*n*3 intake was 341 ± 51 g/d. Omasal flow of C18:3*n*3 was higher for the EL treatment (33.8 g/d) compared with the CL (21.8 g/d) and FL (15.5 g/d) treatments, which were higher compared with the DL treatment (4.6 g/d). Apparent ruminal C18:3*n*3 biohydrogenation was therefore lower for the EL treatment (90.9%) compared with the CL (94.0%) and FL (95.4%) treatments, which were lower than that for the DL treatment (98.5%).

However, total tract crude fat digestibility for the EL treatment (64.8%) was lower compared with the CL treatment (71.3%) and both the EL and CL treatments were lower compared with the FL (78.5%) and DL (80.4%) treatments. In contrast to the lower C18:3*n*3 biohydrogenation for the EL treatment, the proportion of C18:3*n*3 in plasma triacylglycerides and milk fat was significantly higher for the FL treatment (3.60 and 3.19 g/100 FA, respectively) compared with the other treatments (CL: 1.22 and 0.87 g/100 g FA; EL: 1.35 and 0.83 g/100 g FA; DL: 1.12 and 0.46 g/100 g FA, respectively). From these results the transfer efficiency of C18:3*n*3 from intake to secretion in milk fat was calculated and found to be significantly higher for the FL treatment (13.1%) compared with the other linseed treatments (CL: 3.2%; EL: 3.0%; DL: 1.3%). In agreement with the inhibition of complete biohydrogenation to C18:0 in vitro, omasal flows and plasma and milk fat proportions of biohydrogenation intermediates (total *trans*-C18:1 isomers) were higher and those for C18:0 were lower for the DL treatment compared with the other treatments. In addition, the proportion of *cis*-9,*trans*-11-C18:2 in milk fat was significantly higher in the DL treatment (1.45 g/100 g FA) compared with the other treatments (CL: 0.56 g/100 g FA; EL: 0.35 g/100 g FA; FL: 0.43 g/100 g FA).

In Chapter 6 the effect of an increasing proportion of crushed linseed in the diet in combination with varying forage type (grass or maize silage) and forage to concentrate ratio on milk fatty acid profile in high-lactating dairy cows was studied. The experiment was set up as a multivariate 3-factor Box-Behnken design with proportion of crushed linseed, forage type, and forage to concentrate ratio as the main factors. Crushed linseed was supplied at 1, 3, and 5 % of diet DM, forage type was 20, 50, and 80% grass silage with the remainder being maize silage (fraction of total forage DM), and forage to concentrate ratio (DM basis) was 35:65, 50:50, and 65:35. Thirty-six Holstein and Swedish Red cows were randomly assigned to four groups which received different treatment diets during four 21-d periods. Treatment diets were formulated according to the Box-Behnken design including the centre point treatment (50% grass silage, 50:50 forage to concentrate ratio, 3% crushed linseed), which was repeated during every period. Response surface equations were derived to evaluate the effect of the main factors (linear and quadratic effects) and their interactions on several fatty acid proportions in milk fat. Proportions of C18:2*n*6 and *trans*-10-C18:1 in milk fat linearly increased when shifting from 80% grass silage to 80% maize silage, whereas proportions of C18:3*n*3 and *trans*-11, *cis*-15-C18:2 linearly decreased with this diet change. Significant interactions between the proportion of crushed linseed and the forage to concentrate ratio in the diet were found for proportions of *trans*-10-C18:1, *trans*-15-C18:1, *cis*-15-C18:1, *trans*-11,*cis*-15-C18:2, and C18:3*n*3 in milk fat, with the highest proportions of these fatty acids achieved when the diet contained 5% crushed linseed and a 35:65 forage to concentrate ratio. In contrast, no interactions were found between the proportion of crushed linseed and the main forage type for the selected milk fatty acids. From this experiment it was concluded that the effect of supplementation of crushed linseed on milk fatty acid profile, including C18:2*n*6 and C18:3*n*3 proportions in milk fat, depends on the forage to concentrate ratio and forage type in the basal diet.

Milk fat, an important constituent of whole milk, is essential in many of the physical properties, manufacturing qualities, and organoleptic characteristics of dairy products. The results obtained in this thesis show that alterations of the milk fatty acid profile towards a nutritionally more beneficial profile for human health can be achieved by changing the diet of dairy cows, thereby influencing

ruminal fatty acid metabolism, the profile of absorbed fatty acids, and ultimately the proportions of fatty acids secreted in milk fat. In the final chapter of this thesis, the relationship between linseed supplemented diets and its potential to improve animal health and reproduction and to decrease methane emission is discussed.

Melk en melkproducten vormen een belangrijk bestanddeel van Westerse diëten. Het melkvet van Nederlandse koeien bestaat gemiddeld uit 70.6% verzadigde vetzuren, 23.5% enkelvoudig onverzadigde vetzuren en 2.3% meervoudig onverzadigde vetzuren. Door dit grote aandeel verzadigde vetzuren wordt melkvet traditioneel vaak geassocieerd met hart- en vaatziekten. Specifieke meervoudig onverzadigde vetzuren, zoals linoleenzuur (C18:3*n*3) en geconjugeerd linolzuur (CLA; *cis*-9,*trans*-11-C18:2), worden echter geassocieerd met mogelijke positieve effecten op de humane gezondheid. De gehalten van deze vetzuren in melkvet zijn echter normaal gesproken laag (0.5% C18:3*n*3 en 0.5% *cis*-9,*trans*-11-C18:2). Dit heeft geleid tot een toenemende interesse in onderzoek om de vetzuursamenstelling van melk te veranderen. Positieve veranderingen van de vetzuursamenstelling van melk kunnen uiteindelijk leiden tot een betere waardering van melk en melkproducten door consumenten.

Melkvetzuren zijn afkomstig van twee bronnen: 1) de novo synthese in de uier vanuit azijnzuur en boterzuur afkomstig van de fermentatie van organische stof in de pens (C4:0 tot en met C14:0 en een gedeelte van de C16 vetzuren), en 2) opname van voorgevormde vetzuren vanuit absorptie van vetzuren uit het rantsoen, microbiële vetzuren en vetzuren afkomstig van de mobilisatie van lichaamsreserves (gedeelte van de C16 vetzuren en de vetzuren groter en gelijk aan C18). Het aanpassen van het rantsoen is voor melkveehouders een natuurlijke manier om de vetzuursamenstelling van melk te veranderen. Rantsoenen voor melkvee bestaan gewoonlijk uit een mix van ruwvoeders (vers of geconserveerd) en krachtvoeders en bevatten meestal minders dan 70 g vet per kg droge stof met oliezuur (*cis*-9-C18:1), linolzuur (C18:2*n*6) en linoleenzuur (C18:3*n*3) als de belangrijkste vetzuren. Vet is in de pens onderhevig aan intensieve microbiële lipolyse en biohydrogenatie, resulterend in een hoge pens uitstroom van vrije verzadigde vetzuren. In de uier vindt echter een tegenovergesteld proces (desaturatie) plaats onder invloed van het enzym Stearoyl Co-enzym A Desaturase, waarbij de vrije verzadigde vetzuren en de vetzuren die geheel of gedeeltelijk ontsnapt zijn aan biohydrogenatie (biohydrogenatie intermediairen) worden omgevormd tot enkelvoudig of meervoudig onverzadigde vetzuren.

De doelstelling van het onderzoek beschreven in dit proefschrift was het verbeteren van de vetzuursamenstelling van de melk van melkkoeien. De belangrijkste focus om deze doelstelling te bereiken was het aanpassen van de rantsoensamenstelling en het vetzuurmetabolisme in de pens, zodat de pens uitstroom van onverzadigde vetzuren toeneemt en hiermee de secretie van onverzadigde vetzuren, zoals C18:3*n*3, in melkvet.

In hoofdstuk 2 is het effect van aanpassingen in nutriëntensamenstelling in relatie tot verschillende vetbronnen in het rantsoen, de technologische vorm van deze vetbronnen (olie, zaad of beschermd), de additionele toevoeging van vis olie en de belangrijkste ruwvoerbron in het rantsoen op de vetzuursamenstelling van melk onderzocht met behulp van een meta-analyse. Een dataset met 151 behandelingen uit 50 gepubliceerde experimenten is gebouwd en in deze dataset zijn publicaties (47 publicaties met 50 experimenten) opgenomen die de rantsoensamenstelling, de nutriëntensamenstelling, de vetzuursamenstelling, de droge stof opname, de melkproductie, de melksamenstelling en de vetzuursamenstelling van de melk rapporteerden. Mixed model regressie analyse is gebruikt, waarbij rekening werd gehouden met het random effect van experiment en de

ongelijke variantie tussen experimenten. Vervolgens zijn de least squares means bepaald voor de verschillende vetbronnen (niet gesupplementeerd, raapzaad, sojabonen en zonnebloemzaad, lijnzaad of vis olie), technologische vorm inclusief toevoeging van vis olie (olie, zaad, beschermd of visolie) en belangrijkste ruwvoer in het basis rantsoen (luzerne silage, gerst silage, maïs silage, gras silage, maïs silage en hooi of hooi). De resultaten toonden aan dat de verschillende technologische vormen van raapzaad, sojabonen, zonnebloemzaad of lijnzaad significante invloed hadden op de relatie (intercept en coëfficiënt) tussen de nutriëntensamenstelling (vetzuursamenstelling en NDF gehalte) en de vetzuursamenstelling van de melk. Dit resulteerde in verschillen in diverse melkvetzuren voor de verschillende technologische vormen van de vetbronnen. De belangrijkste ruwvoerbron in het basisrantsoen beïnvloedde ook de relatie tussen de nutriëntensamenstelling en de vetzuursamenstelling van de melk. Dit resulteerde in verschillen in diverse melkvetzuren voor de verschillende ruwvoerbronnen in het basis rantsoen gesupplementeerd met de verschillende vetbronnen. Concluderend is het effect van de nutriëntensamenstelling op de verschillende melkvetzuren afhankelijk van het type en de vorm van de vetbron, de toevoeging van visolie en de belangrijkste ruwvoerbron in het basisrantsoen.

In hoofdstuk 3 is het effect van diverse chemische of technologische behandelingen van lijnzaad en het toevoegen van docosahexaeenzuur (DHA; C22:6*n*3) in combinatie met lijnolie op de kinetiek van C18:3*n*3 biohydrogenatie onderzocht in vitro. De geteste lijnzaad producten bestonden uit: lijnolie, geplet lijnzaad, formaldehyde behandeld geplet lijnzaad, geëxtrudeerd heel lijnzaad, geëxtrudeerd geplet lijnzaad, gemicronizeerd geplet lijnzaad, lijnolie omhuld met vet en lijnolie in combinatie met DHA. Deze producten werden gedurende 0, 0.5, 1, 2, 4, 6, 12 en 24 uur geïncubeerd met pens vloeistof in een batch cultuur techniek. De producten werden ingewogen zodat er een vergelijkbare hoeveelheid C18:3*n*3 en fermenteerbaar substraat (gevriesdroogd totaal gemixt rantsoen) werd geïncubeerd. Vervolgens werd de verdwijning van C18:3*n*3 gemeten, zodat de fractionele biohydrogenatie snelheid, de reactietijd en de effectieve biohydrogenatie van C18:3*n*3 konden worden berekend. Technologische behandeling (pletten) van lijnzaad gevolgd door chemische behandeling (formaldehyde) resulteerde in een effectieve bescherming van C18:3*n*3 tegen biohydrogenatie. Daarnaast was extrusie van heel lijnzaad ook effectief in het verminderen van de biohydrogenatie van C18:3*n*3. Geplet lijnzaad, geëxtrudeerd geplet lijnzaad, gemicronizeerd geplet lijnzaad, lijnolie omhuld met vet en lijnolie in combinatie met DHA waren niet in staat om de biohydrogenatie van C18:3*n*3 te remmen in vergelijking met lijnolie. Het toevoegen van DHA aan lijnolie remde de laatste biohydrogenatie stap van *trans*-10+11-C18:1 tot C18:0, zoals aangetoond door een lagere concentratie van C18:0 na 24 uur incubatie. Concluderend bieden alleen formaldehyde behandeld geplet lijnzaad en geëxtrudeerd heel lijnzaad een potentiële mogelijkheid in rantsoenen voor melkkoeien om de pens uitstroom van C18:3*n*3 te verhogen.

In hoofdstuk 4 en 5 zijn de vetzuurstromen door de boekmaag, de biohydrogenatie van C18:3*n*3, de plasma vetzuursamenstelling en de vetzuursamenstelling van de melk voor de meest veelbelovende lijnzaad behandelingen uit het in vitro experiment onderzocht. Het experiment was opgezet als een Latijns vierkant, waarbij vier Holstein melkkoeien voorzien van pensfistels tijdens vier perioden van 21 dagen vier lijnzaad producten gevoerd kregen: 1) geplet lijnzaad (CL), 2) geëxtrudeerd heel lijnzaad, 3) formaldehyde behandelde lijnolie (FL), en 4) DHA in combinatie met lijnolie (DL).

Tijdens het experiment zijn de vetzuuropname, vetzuurstromen door de boekmaag (met behulp van Cr, Yb en acid detergent lignin als markeerstoffen), vetzuursamenstelling van plasma triacylglyceriden en vetzuursamenstelling van melkvet bepaald. De gemiddelde opname van C18:3*n*3 was 341 ± 51 g/d, terwijl de boekmaagstroom van C18:3*n*3 hoger was voor de EL behandeling (33.8 g/d) vergeleken met de CL (21.8 g/d) en FL (15.5 g/d) behandelingen, die weer hoger waren dan de DL behandeling (4.6 g/d). De schijnbare biohydrogenatie van C18:3*n*3 in de pens was daarom lager voor de EL behandeling (90.9%) vergeleken met de CL (94.0%) en FL (95.4%) behandelingen, die weer lager waren dan de DL behandeling (98.5%). De totale schijnbare vetverteerbaarheid was echter lager voor de EL behandeling (64.8%) vergeleken met de CL behandeling (71.3%) en zowel de EL als de CL behandeling was lager vergeleken met de FL (78.5%) en DL (80.4%) behandelingen. In tegenstelling tot de lagere C18:3*n*3 biohydrogenatie voor de EL behandeling, was de concentratie van C18:3*n*3 in plasma triacylglyceriden en melkvet significant hoger voor de FL behandeling (3.60 en 3.19 g/100 g vetzuren, respectievelijk) in vergelijking met de andere behandelingen (CL: 1.22 en 0.87 g/100 g vetzuren; EL: 1.35 en 0.83 g/100 g vetzuren; DL: 1.12 en 0.46 g/100 g vetzuren, respectievelijk). De efficiëntie van C18:3*n*3 opname naar secretie in melkvet was significant hoger voor de FL behandeling (13.1%) in vergelijking met de andere lijnzaad behandelingen (CL: 3.2%; EL: 3.0%; DL: 1.3%). In overeenstemming met de remming van de complete biohydrogenatie naar C18:0 in vitro, werden hogere en lagere boekmaagstromen en concentraties in plasma en melkvet van biohydrogenatie intermediairen (totaal *trans*-C18:1 isomeren) en C18:0, respectievelijk gevonden voor de DL behandeling in vergelijking met de andere behandelingen. De concentratie van *cis*-9,*trans*-11-C18:2 in melkvet was significant hoger in de DL behandeling (1.45 g/100 g vetzuren) in vergelijking met de andere behandelingen (CL: 0.56 g/100 g vetzuren; EL: 0.35 g/100 g vetzuren; FL: 0.43 g/100 g vetzuren).

In hoofdstuk 6 is het effect van een toenemend aandeel geplet lijnzaad in combinatie met variatie in ruwvoertype (gras versus maïs silage) en ruwvoer:krachtvoer verhouding in het rantsoen op de vetzuursamenstelling van de melk van hoogproductieve koeien onderzocht. Het experiment was opgezet als een multivariate 3-factor Box-Behnken experiment met geplet lijnzaad, ruwvoertype en ruwvoer:krachtvoer verhouding als de hoofd factoren. Het aandeel geplet lijnzaad in het rantsoen was 1, 3 en 5% (droge stof basis), ruwvoertype was 20, 50 en 80% gras silage uitgewisseld met maïs silage (aandeel van totaal ruwvoer droge stof) en ruwvoer:krachtvoer verhouding was 35:65, 50:50 en 65:35 (droge stof basis). Zesendertig Holstein en Zweeds Roodbonte koeien waren random toegewezen aan vier groepen die verschillende rantsoenen kregen tijdens vier perioden van 21 dagen. De rantsoenen werden samengesteld op basis van de Box-Behnken opzet inclusief de centrale behandeling (50% gras silage, 50:50 ruwvoer:krachtvoer, 3% geplet lijnzaad) die tijdens elke periode werd herhaald. Response surface vergelijkingen werden opgesteld voor verschillende melkvetzuren om de effecten van de hoofd factoren (lineaire en kwadratische effecten) en de interacties tussen de hoofd factoren te evalueren. De verschuiving van 80% gras silage naar 80% maïs silage gaf een lineaire toename van de concentraties van C18:2*n*6 en *trans*-10-C18:1 in melkvet en een lineaire afname van de concentraties van C18:3*n*3 en *trans*-11,*cis*-15-C18:2. Er waren significante interacties tussen het aandeel geplet lijnzaad en de ruwvoer:krachtvoer verhouding in het rantsoen voor de concentraties van *trans*-10-C18:1, *trans*-15-C18:1, *cis*-15-C18:1,

trans-11,*cis*-15-C18:2 en C18:3*n*3 in melkvet, waarbij de hoogste concentraties werden bereikt als het rantsoen 5% geplet lijnzaad en een 35:65 ruwvoer:krachtvoer verhouding bevatte. Er werden geen interacties tussen het aandeel geplet lijnzaad en het ruwvoertype gevonden voor de geselecteerde melkvetzuren. Concluderend is het effect van geplet lijnzaad op diverse melkvetzuren afhankelijk van de ruwvoer:krachtvoer verhouding en het ruwvoertype in het rantsoen.

Melkvet, een belangrijk bestanddeel van rauwe melk, is essentieel voor diverse fysieke eigenschappen, bewerkingsmogelijkheden en organoleptische karakteristieken van melk en melkproducten. De resultaten van het onderzoek beschreven in dit proefschrift tonen aan dat aanpassing van de samenstelling van melkvet in de richting van een samenstelling die meer aansluit bij de nutritionele wensen voor de humane gezondheid bereikt kunnen worden door aanpassingen in het rantsoen van melkkoeien. Deze aanpassingen in het rantsoen beïnvloeden het vetzuur metabolisme in de pens, de vetzuur synthese in de uier en uiteindelijk de secretie van vetzuren in de melk. In hoofdstuk 7 van dit proefschrift wordt de relatie tussen lijnzaad supplementatie en de potentie om diergezondheid en vruchtbaarheid te verbeteren en methaan emissie te verlagen bediscussieerd.

Molke en molkprodukten binne in wichtich ûnderdiel fan it iten yn westerske lannen. It molkfet fan Nederlânske kij bestiet yn trochsnee út 70.6% fersêde fetsoeren, 23.5% inkelfâldich ûnfersêde fetsoeren en 2.3% mearfâldich ûnfersêde fetsoeren. Troch it grutte part fersêde fetsoeren wurdt molkfet faak assosjearre mei hert- en fetsykten. It lytse part oan mearfâldich ûnfersêde fetsoeren, lykas linoleensoer (C18:3*n*3; 0.5%) en konjugearre linolsoer (CLA; *cis*-9,*trans*-11-C18:2; 0.5%) wurdt lykwols assosjearre mei mooglik positive effekten op de minslike sûnens. Dat hat laat ta in tanimmende ynteresse yn ûndersyk om de gearstalling fan fetsoer yn molke te feroarjen. Positive feroaringen fan de gearstalling fan fetsoer yn molke kinne úteinlik liede ta in bettere akseptaasje fan molkprodukten troch konsuminten.

Fetsoeren yn molke komme fan twa boarnen: 1) de novo-synteze yn it jaar út jittiksoer en bûtersoer wei, dy't ûntstiet troch fermintaasje fan organyske stof yn de pânse (C4:0 oant en mei C14:0 en in part fan de C16 fetsoeren) en 2) opname fan foarfoarme fetsoeren troch absorpsje fan fetsoeren út it fretten, mikrobiële fetsoeren en fetsoeren ôfkomstich fan de mobilisaasje fan lichemsreserves (in part fan de C16 fetsoeren en fetsoeren grutter en gelyk oan C18). It oanpassen fan it fretten is foar melkfeehâlders in natuerlike wize om de gearstalling fan fetsoer yn molke te feroarjen. Fretten foar melkfee bestiet gewoanwei út in miks fan rûchfoer (farsk of konservearre) en krêftfoer en befettet meastentiids minder as 70 g fet de kilo droege stof mei oaljesoer (*cis*-9-C18:1), linolsoer (C18:2*n*6) en linoleensoer (C18:3*n*3) as de wichtichste fetsoeren. Fet hat yn de pânse te krijen mei yntinsive mikrobiële lipolyze en biohydrogenaasje, wat liedt ta in hege útstream fan frije fersêde fetsoeren. Yn it jaar fynt lykwols in proses plak dat krekt oarsom is (desaturaasje) ûnder ynfloed fan it ensym Stearoyl Co-ensym A Desaturase. Dêrby wurde de frije fersêde fetsoeren en de fetsoeren, dy't hielendal of foar in part ûntkommen binne oan biohydrogenaasje (biohydrogenaasje yntermediêren) omfoarme ta inkelfâldich of mearfâldich ûnfersêde fetsoeren.

De doelstelling fan it ûndersyk sa't dy beskreaun is yn dit proefskrift, is it ferbetterjen fan de gearstalling fan fetsoer yn 'e molke fan melkkij. De wichtichste fokus om dy doelstelling te berikken is it oanpassen fan 'e gearstalling fan it kowefretten en it metabolisme fan it fetsoer yn 'e pânse, sadat de útstream út 'e pânse fan ûnfersêde fetsoeren tanimt en dêrmei de sekreesje fan ûnfersêde fetsoeren lykas C18:3*n*3, yn molkfet.

Yn haadstik 2 is it effekt fan oanpassingen yn 'e gearstalling fan nutriïnten yn relaasje ta ferskate fetboarnen yn it fretten, de technologyske foarm fan dy fetboarnen (oalje, sied of beskerme), it addisjoneel tafoegjen fan fiskoalje en de wichtichste boarne fan rûchfoer yn it fretten op 'e gearstalling fan fetsoer yn molke ûndersocht mei help fan in meta-analyze. Der is in dataset mei 151 behannelingen út 50 publisearre eksperiminten boud en yn dy dataset binne publikaasjes (47 publikaasjes mei 50 eksperiminten) opnommen dy't de gearstalling fan it fretten, de gearstalling fan de nutriïnten en it fetsoer, de opname fan droege stof, de molkproduksje, de gearstalling fan 'e molke en it fetsoer dêryn rapportearje. Mikst-model-regresje-analyze is brûkt en dêrby is rekken hâlden mei it lokraak effekt fan it eksperimint en de ûngelikense fariaasje tusken de eksperiminten. Dêrnei binne de least squares means fêststeld foar de ferskate fetboarnen (net supplemintearre, raapsied, sojabeane en sinneblomsied, lynsied of fiskoalje), technologyske foarm ynklusyf it tafoegjen fan fiskoalje (oalje, sied, beskerme of fiskoalje) en it wichtichste rûchfoer yn it basisfretten (luzernesilaazje, koarnsilaazje, maissilaazje,

gerssilaazje, maissilaazje en hea of hea). De resultaten lieten sjen, dat de ferskate technologyske foarmen fan raapsied, sojabeane, sinneblomsied of lynsied signifikante ynfloed hiene op de relaasje (yntersept en koëffisjint) tusken de gearstalling fan de nutriïnten (gearstalling fan fetsoer en NDF gehalte) en de gearstalling fan it fetsoer yn ’e molke. Dat resultearre yn ferskillen yn ferskate fetsoeren yn molke foar de ferskate technologyske foarmen fan de fetboarnen. De wichtichste boarne fan it rûchfoer yn it basisfretten hie ek ynfloed op de relaasje tusken de gearstalling fan de nutriïnten en de gearstalling fan it fetsoer yn ’e molke. Dat resultearre yn ferskillen yn ferskate fetsoeren yn molke foar de ferskate boarnen fan it rûchfoer yn it basisfretten, supplemintearre mei de ferskate fetboarnen. De konklúzje is dat it effekt fan de gearstalling fan de nutriïnten op ferskillen yn fetsoeren yn ’e molke ôfhinklik is fan it type en de foarm fan de fetboarn, it tafoegjen fan fiskoalje en de wichtichste boarne fan it rûchfoer yn it basisfretten.

Yn haadstik 3 is it effekt fan ferskate gemyske of technologyske behannelingen fan lynsied en it tafoegjen fan docosahexaeensoer (DHA; C22:6*n*3) yn kombinaasje mei lynoalje op de kinetyk fan C18:3*n*3 biohydrogenaasje in vitro ûndersocht. De lynsiedprodukten dy’t ûndersocht binne, wiene: lynoalje, plette lynsied, mei formaldehyde behannele plette lynsied, ekstrudearre hiel lynsied, ekstrudearre plette lynsied, mikronisearre plette lynsied, lynoalje omklaaid mei fet en lynoalje yn kombinaasje mei DHA. Dy produkten waarden foar in tiid fan 0, 0.5, 1, 2, 4, 6, 12 en 24 oeren ynkubearre mei floeistof út ’e pânse yn in batch kultuertechnyk. De produkten waarden woegen, sadat der in fergelykber gewicht oan C18:3*n*3 en fermintearber substraat (fretten dat friesdroege is en folslein mingd) ynkubearre waard. Dêrnei waard it ferdwinen fan C18:3*n*3 metten, sadat de snelheid fan de fraksjonele biohydrogenaasje, de reaksjetiid en de effektive biohydrogenaasje fan C18:3*n*3 berekkene wurde koe. It technologysk behanneljen (pletten) fan lynsied, folge troch gemysk behanneljen (formaldehyde) resultearre yn in effektive beskerming fan C18:3*n*3 tsjin biohydrogenaasje. Dêrnjonken wie ekstrúzje fan hiel lynsied ek effektyf yn it ferminderjen fan de biohydrogenaasje fan C18:3*n*3. Plette lynsied, ekstrudearre plette lynsied, mikronisearre plette lynsied, lynoalje omklaaid mei fet en lynoalje yn kombinaasje mei DHA wiene net by steat om de biohydrogenaasje fan C18:3*n*3 te remjen yn fergeliking mei lynoalje. It tafoegjen fan DHA oan lynoalje remme de lêste biohydrogenaasje-stap fan *trans*-10+11-C18:1 oant C18:0, lykas oantoand troch in legere konsintraasje fan C18:0 nei 24 oeren ynkubaasje. De konklúzje is dat allinnich mei formaldehyde behannele plette lynsied en ekstrudearre hiel lynsied in potinsjele mooglikheid biede yn it fretten fan melkkij om de útstream fan C18:3*n*3 út de pânse te ferheegjen.

Yn haadstik 4 en 5 binne de streamen fan fetsoer troch de boekmage, de biohydrogenaasje fan C18:3*n*3, de plasmagearstalling fan fetsoer en de gearstalling fan fetsoer yn ’e molke foar de behannelingen fan lynsied dêr’t it meast fan te ferwachtsjen wie, út it in vitro eksperimint ûndersocht. It eksperimint wie opset as in Latynsk fjouwerkant, wêrby’t fjouwer Holstein melkkij mei pânsefistels oer in tiidsbestek fan fjouwer perioaden fan 21 dagen fjouwer lynsiedprodukten fuorre waard: 1) plette lynsied (CL), 2) ekstrudearre hiel lynsied (EL), 3) mei formaldehyde behannele lynoalje (FL) en 4) DHA yn kombinaasje mei lynoalje (DL). Ûnder it eksperimint binne de opname fan fetsoer, de streamen fan fetsoer troch de boekmage (mei help fan Cr, Yb en acid detergent lignin as markearstoffen), de gearstalling fan fetsoer fan plasma triacylglyceriden en de gearstalling fan fetsoer

fan it molkfet fêststeld. De trochsnee opname fan C18:3*n*3 wie 341 ± 51 g/d, wylst de stream fan de boekmage fan C18:3*n*3 heger wie foar de EL behanneling (33.8 g/d) yn fergeliking mei de CL (21.8 g/d) en FL (15.5 g/d) behannelingen, dy't wer heger wiene as de DL behanneling (4.6 g/d). De skynbere biohydrogenaasje fan C18:3*n*3 yn de pânse wie dêrom leger foar de EL behanneling (90.9%) yn fergeliking mei de CL (94.0%) en FL (95.4%) behannelingen, dy't wer leger wiene as de DL behanneling (98.5%). De totale skynbere fertarring fan fet wie lykwols leger foar de EL behanneling (64.8%) yn fergeliking mei de CL behanneling (71.3%) en sawol de EL as de CL behanneling wiene leger yn fergeliking mei de FL (78.5%) en DL (80.4%) behannelingen. Yn tsjinspraak mei de legere C18:3*n*3 biohydrogenaasje foar de EL behanneling, wie de konsintraasje fan C18:3*n*3 yn plasma triacylglyceriden en molkfet signifikant heger foar de FL behanneling (3.60 en 3.19 g/100 g fetsoeren, respektivelik) neffens de oare behannelingen (CL: 1.22 en 0.87 g/100 g fetsoeren; EL: 1.35 en 0.83 g/100 g fetsoeren; DL: 1.12 en 0.46 g/100 g fetsoeren, respektivelik). De doelmjittichheid fan C18:3*n*3 opname nei sekreesje yn molkfet wie signifikant heger foar de FL behanneling (13.1%) neffens de oare behannelingen fan lynsied (CL: 3.2%; EL: 3.0%; DL: 1.3%). Yn oerienstimming mei it remjen fan de hiele biohydrogenaasje nei C18:0 in vitro, waarden hegere en legere streamen yn de boekmage en konsintraasjes yn plasma en molkfet fan biohydrogenaasje yntermediêren (totaal *trans*-C18:1 isomearen) en C18:0, respektivelik fûn foar de DL behanneling neffens de oare behannelingen. De konsintraasje fan *cis*-9,*trans*-11-C18:2 yn molkfet wie signifikant heger yn 'e DL behanneling (1.45 g/100 g fetsoeren) neffens de oare behannelingen (CL: 0.56 g/100 g fetsoeren; EL: 0.35 g/100 g fetsoeren; FL: 0.43 g/100 g fetsoeren).

Yn haadstik 6 is it effekt fan in tanimmend part plette lynsied yn kombinaasje mei fariaasje yn rûchfoertype (gers tsjin maissilaazje) en rûchfoer:krêftfoer ferhâlding yn it fretten op de gearstalling fan fetsoer yn 'e molke fan heechproduktive kij ûndersocht. It eksperimint wie opset as in multyfariate 3-faktor Box-Behnken eksperimint mei plette lynsied, rûchfoertype en rûchfoer:krêftfoer ferhâlding as de haadfaktoaren. It part plette lynsied yn it fretten wie 1, 3 en 5% (droege stof basis), rûchfoertype wie 20, 50 en 80% gerssilaazje útwiksele mei maissilaazje (part fan totaal rûchfoer droege stof) en rûchfoer:krêftfoer ferhâlding wie 35:65, 50:50 en 65:35 (droege stof basis). Seisentritich Holstein en Sweedske Readbûnte kij wiene lokraak tawiisd oan fjouwer groepen dy't ferskillend fretten krigen yn fjouwer perioaden fan 21 dagen. It fretten waard gearstald op basis fan de Box-Behnken opset ynklusyf de sintrale behanneling (50% gerssilaazje, 50:50 rûchfoer:krêftfoer, 3% plette lynsied) dy't eltse perioade werhelle waard. Response surface fergelikings waarden opsteld foar ferskate fetsoeren yn molke om de effekten fan de haadfaktoaren (lineêre en kwadratyske effekten) en de ynteraksjes tusken de haadfaktoaren te evaluearjen. It ferskowen fan 80% gerssilaazje nei 80% maissilaazje joech in lineêre taname fan de konsintraasjes fan C18:2*n*6 en *trans*-10-C18:1 yn molkfet en in lineêre ôfname fan de konsintraasjes fan C18:3*n*3 en *trans*-11,*cis*-15-C18:2. Der wiene signifikante ynteraksjes tusken it part plette lynsied en de rûchfoer:krêftfoer ferhâlding yn it fretten foar de konsintraasjes fan *trans*-10-C18:1, *trans*-15-C18:1, *cis*-15-C18:1, *trans*-11,*cis*-15-C18:2 en C18:3*n*3 yn molkfet. Dêrby waarden de heechste konsintraasjes berikt as yn it fretten 5% plette lynsied siet en de ferhâlding rûchfoer:krêftfoer 35:65 wie. Der waarden gjin ynteraksjes foar de selektearre fetsoeren yn molke fûn tusken it part plette lynsied en it rûchfoertype. Konklúzje is, dat it effekt fan plette lynsied op de

ferskate fetsoeren yn molke ôfhinklik is fan de rûchfoer:krêftfoer ferhâlding en it rûchfoertype yn it fretten.

Molkfet, in wichtich part fan rauwe molke, is essinsjeel foar ferskate fysike eigenskippen, mooglikheden foar bewurking en organoleptyske karakteristiken fan molke en molkprodukten. De resultaten fan it ûndersyk yn dit proefskrift litte sjen, dat oanpassing fan 'e gearstalling fan molkfet yn 'e rjochting fan in gearstalling, dy't mear oanslút by de nutrisjonele winsken foar de minslike sûnens, berikt wurde kin troch oanpassingen yn it fretten fan melkkij. Dy oanpassingen yn it fretten hawwe ynfloed op it fetsoer metabolisme yn 'e pânse, de fetsoer synteze yn it jaar en úteinlik de sekreesje fan fetsoeren yn 'e molke.

Yn haadstik 7 fan dit proefskrift wurdt de relaasje tusken lynsied supplemintaasje en de potinsje om sûnens en fruchtberens fan bisten te ferbetterjen en de metaan-emisje te ferleegjen bediskusjearre.

Curriculum Vitae

About the author

Attje-Rieke Sterk werd geboren op 12 juli 1979 te Leeuwarden en groeide op in Mijnsheerenland. In 1997 behaalde zij haar VWO diploma aan de Christelijke Scholengemeenschap Willem van Oranje te Oud-Beijerland. In september 1997 begon ze aan de studie Landbouw en Veehouderij met de specialisatie Rundveehouderij aan de Hogere Agrarische School te Deventer. In 2001 heeft ze deze studie succesvol afgerond en in september 2001 is ze begonnen met de opleiding Zootechniek met de specialisatie Veevoeding aan Wageningen University te Wageningen. In juni 2003 is ze met lof afgestudeerd en begonnen als onderzoeker diervoeding bij CCL te Veghel. In maart 2007 is ze begonnen als parttime promovenda bij de leerstoelgroep Diervoeding aan het onderzoek dat is beschreven in dit proefschrift. Tevens bleef ze parttime werkzaam als onderzoekster rundveevoeding bij het Agrifirm Innovation Center te Apeldoorn. Na het afronden van het promotieonderzoek blijft Attje-Rieke Sterk werkzaam als onderzoekster rundveevoeding bij Agrifirm Innovation Center.

Attje-Rieke Sterk was born on July 12 1979 in Leeuwarden and grew up in Mijnsheerenland. In 1997 she graduated from the Christelijke Scholengemeenschap Willem van Oranje in Oud-Beijerland. In September 1997 she started with her BSc Animal Husbandry at the Hogere Agrarische School in Deventer with as specialisation Ruminant Husbandry. In 2001 this study was successfully completed and in September 2001 she started with the MSc Animal Sciences at Wageningen University in Wageningen with specialisation Animal Nutrition. In June 2003 she graduated with distinction and started as an animal nutrition researcher for CCL in Veghel. In March 2007 she started as a parttime PhD at the Animal Nutrition Group. The results of her study are described in the current thesis. During her PhD-project, she continued her job as a ruminant researcher for Agrifirm Innovation Center in Apeldoorn. After her graduation, Attje-Rieke Sterk will continue her activities as a ruminant researcher at Agrifirm Innovation Center.

Publications

Refereed Scientific Publications

Sterk, A., J.M.A.J. Verdonk, A.J. Mul, B. Soenen, M.L. Bezonçon, M. Frehner, and R. Losa. 2007. Effect of xylanase supplementation to a cereal-based diet on the apparent faecal digestibility in weanling piglets. Livest. Prod. Sci. 108: 269-271.

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Sterk, A., B.E.O. Johansson, H.Z.H. Taweel, M. Murphy, A.M. van Vuuren, W.H. Hendriks, and J. Dijkstra. 2011. Effects of forage type, forage to concentrate ratio, and crushed linseed supplementation on milk fatty acid profile in lactating dairy cows. J. Dairy Sci. Accepted.

Sterk, A., B. Vlaeminck, A.M. van Vuuren, W.H. Hendriks, and J. Dijkstra. 2011. Effects of feeding different linseed sources on omasal fatty acid flows and C18:3*n*3 biohydrogenation in lactating dairy cows. Submitted.

Sterk, A., J. Dijkstra, W.H. Hendriks, and A.M. van Vuuren. 2011. Effect of feeding different linseed sources on fatty acid profiles of plasma and milk fat from lactating dairy cows. Submitted.

Sterk, A., A.M. van Vuuren, W.H. Hendriks, and J. Dijkstra. 2011. Effects of different fat sources, technological forms and characteristics of the basal diet on milk fatty acid profile in lactating dairy cows – A meta-analysis. Submitted.

Contributions to Conferences and Symposia

Sterk, A., G. Kingma, B. Vlaeminck, and J. Dijkstra. 2008. Effects of supplementing crushed linseed or linseed oil on biohydrogenation of unsaturated fatty acids in vitro. In: Proceedings of the 33rd Meeting of Dutch speaking nutrition researchers (NVO), April 25, 2008, Wageningen, the Netherlands. Pages 25-26.

Sterk, A., R. Hovenier, B. Vlaeminck, A.M. van Vuuren, and J. Dijkstra. 2009. Effects of various linseed treatments on biohydrogenation of C18:3*n*3 in vitro. In: Ruminant Physiology: Digestion, Metabolism, and Effect of Nutrition on Reproduction and Welfare. Proceedings of the XIth International Symposium on Ruminant Physiology, September 6-9, 2009, Clermont-Ferrand, France. Y. Chilliard, F. Glasser, Y. Faulconnier, F. Bocquier, I. Veissier, and M. Doreau, eds., pages 364-365, Wageningen Acedemic Publishers, Wageningen, The Netherlands.

Sterk, A., J. Dijkstra, and A.M. van Vuuren. 2010. Effects of feeding different sources of linseed on production performance and plasma and milk fatty acid profiles of lactating dairy cows. In: Proceedings of the 35th ANR Forum, April 16, 2010, Lelystad, the Netherlands. Pages 69-71.

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profile in lactating dairy cows. In: Proceedings of the 36th Animal Nutrition Research Forum, April 19, 2011, Leuven, Belgium. Pages 13-14.

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Training and Supervision Plan

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Group	Animal Nutrition Group
Daily supervisors	Dr. ir. J. Dijkstra; Dr. A.M. van Vuuren
Supervisor	Prof. dr. ir. W.H. Hendriks

The Basic Package	**Year**	**Credits** *
Course on philosophy of science and/or ethics	2007	1.5
WIAS Introduction Course	2008	1.5
International conferences		
ADSA joint annual meeting, San Antonio, USA	2007	1.2
14th Discover conference: Lipids for dairy cattle, Nashville, USA	2008	1.2
6th International Symposium on Ruminant physiology, Clermont-Ferrand, France	2009	1.2
20th Discover conference: Transition cows, Urbana-Champaign, USA	2010	1.2
8th International Symposium on the Nutrition of Herbivores, Wales, UK	2011	1.2
Seminars and workshops		
ANR Forum, Ghent, Belgium	2007	0.3
WIAS science day, Wageningen, the Netherlands	2008	0.3
ANR Forum, Wageningen, the Netherlands	2008	0.3
Seminar ‘Strategies to improve health and fertility in dairy cows’, Wageningen, the Netherlands	2008	0.2
Seminar ‘Genetics of milk quality, Wageningen’, the Netherlands	2009	0.3
ANR Forum, Lelystad, the Netherlands	2010	0.3
ANR Forum, Leuven, Belgium	2011	0.3
Presentations		
Poster presentatation at ANR Forum, Wageningen, the Netherlands	2008	1.0
Oral presentation at Genetics of milk quality, Wageningen, the Netherlands	2009	1.0
Poster presentation at 6th International Symposium on Ruminant physiology, Clermont-Ferrand, France	2009	1.0
Oral presentation at ANR Forum, Lelystad, the Netherlands	2010	1.0
Oral presentation at ANR Forum, Leuven, Belgium	2011	1.0
Poster presentation at 8th International Symposium on the Nutrition of Herbivores, Wales, UK	2011	1.0

In-Depth Studies

Design of animal experiments	2007	1.0
Statistics for the life sciences	2007	1.5
Nutrition in the omics era	2008	1.0
Advances in feed evaluation science	2009	0.3
Statistics with SAS (internal course Agrifirm Innovation Center)	2010	1.0
Orientation on mathematical modelling in biology	2011	1.5

Statutory Courses

Use of Laboratory Animals	2007	3.0

Professional Skills Support Courses

Writing and presenting scientific papers	2010	1.2
Science, the press and the general public: communication and interaction	2010	1.0
Scientific writing	2011	1.8

Research Skills Training

Preparing own PhD research proposal	2006	6.0

Didactic Skills Training

Toegepaste Dierbiologie 2008-2011	2008-2011	0.5
Animal nutrition and physiology 2008-2010	2008-2010	0.6
Oral presentation Phd research, Advances in feed evaluation science, Wageningen, the Netherlands	2009	1.0
Research Master Cluster; reviewing research preproposals	2009	0.5
MSC major thesis; supervising 3 MSc students	2007-2010	6.0

Education and Training Total 45

* one ECTS credit equals a study load of approximately 28 hours

Colophon

Colophon

Design & layout
- Digit@l Xpression, Bennekom, the Netherlands

Printing
- GVO drukkers & vormgevers | Ponsen & Looijen B.V., Ede, the Netherlands

Photographs
- Sake Kooistra
- Ruud Cuypers
- Mark and Karin Jansen van der Sligte – van der Toorn (cows)

Financial support for the research described in this thesis
- Food and Nutrition Delta program of Senter Novem, Den Haag, the Netherlands
- Royal FrieslandCampina, Amersfoort, the Netherlands
- Agrifirm Group, Apeldoorn, the Netherlands
- Agrifirm Innovation Center, Apeldoorn, the Netherlands
- Barenbrug Holland B.V., Oosterhout, the Netherlands

Financial support for the publication of this thesis
- Food and Nutrition Delta program of Senter Novem, Den Haag, the Netherlands
- Royal FrieslandCampina, Amersfoort, the Netherlands
- Agrifirm Group, Apeldoorn, the Netherlands
- Agrifirm Innovation Center, Apeldoorn, the Netherlands
- Barenbrug Holland B.V., Oosterhout, the Netherlands