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Role of dietary fish oil and plant oil supplements in ruminal lipid metabolism and fish oil induced milk fat depression in lactating cows

Doctoral Dissertation

Piia Kairenius

Luke

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Abstract

The objective of the research described in this thesis was to provide new information on the ruminal biohydrogenation of long-chain n-3 polyunsaturated fatty acids (PUFA), such as 20:5n-3, 22:5n-3 and 22:6n-3, for altering bovine milk fatty acid (FA) composition, with the potential to improve human health. Emphasis was not only placed on the potential to increase milk fat n-3, but also to modulate ruminal lipid metabolism and to explore the mechanisms driving milk fat synthesis and its regulation in lactating cows in order to understand the mechanisms and metabolic pathways underlying the diet-induced changes in milk fat depression (MFD), milk FA composition and specific FA intermediates and end products associated with MFD. Experiments documented in I–IV encompassed detailed investigations of ruminal (I-III) and mammary (IV) lipid metabolism.

Experiment reported in I was conducted to build up methods for the analysis of long-chain 20- to 22-carbon FA intermediates formed during ruminal biohydrogenation of n-3 PUFA. The detailed analysis of fish oil (FO) and omasal digesta of lactating cows fed FO enabled the structure identification of 27 previously unidentified 20- to 22-carbon FA intermediates, containing at least one *trans* double bond. No conjugated 20-carbon FA were detected in omasal digesta. Results demonstrated that the hydrogenation of 20:5n-3, 22:5n-3 and 22:6n-3 in the rumen proceeds via two principal mechanisms that involve sequential reduction or isomerisation of *cis* double bonds closest to carboxyl group and provided clear evidence of extensive biohydrogenation of 20:5n-3, 22:5n-3 and 22:6n-3 in cows fed FO.

Experiments documented in II-IV involved two physiological studies in which, the effects of dietary FO supplements alone (II; IV) or in combination (III) with sunflower (rich in 18:2-6; SFO) or linseed (rich in 18:3n-3; LFO) oil on animal performance (II-IV), ruminal lipid metabolism (II; III), microbial ecology in the rumen (II; III) and milk fat composition (IV) were investigated in lactating cows. Dietary FO supplements increased the intakes of 20:5n-3, 22:5n-3, 22:6n-3 and total FA (II; III), whereas decreased dry matter intake (II; III). Dietary oil supplements decreased (II) or had no effect (III) on ruminal volatile FA concentrations, but FO at high amounts (II) or when supplemented with plant oils (III) promoted an increase in molar proportions of propionate (II; III) and butyrate (II) at the expense of acetate (II; III).

Supplements of FO modified ruminal metabolism of 16- and 18-carbon PUFA, causing increases in *trans* 16:1, *trans* 18:1 and *trans* 18:2 flow and a decrease in 18:0 at the omasum, and at high amounts promoted *trans*-10 18:1 accumulation at the expense of *trans*-11 18:1. Dietary FO had no substantial influence on ruminal outflow of conjugated linoleic acid (CLA). Extensive ruminal biohydrogenation of 20:5n-3, 22:5n-3 and 22:6n-3 resulted in increases in numerous 20- and 22-carbon PUFA containing at least one *trans* double bond at the omasum.

Relative to FO, ruminal metabolism of 22:6n-3 was more extensive on diets containing plant oils, whereas the biohydrogenation of 22:5n-3 and 20:5n-3 showed no difference between FO and diets containing plant oils (III). The inhibitory effects of FO on the reduction of 18-carbon PUFA to 18:0 were influenced by the source of 18-carbon PUFA in SFO and LFO. The ruminal outflow of 18:0 was lower and accumulation of *trans* 18:2 and 20- to 22-carbon FA intermediates greater for LFO than SFO. Supplements of SFO and LFO caused *trans*-10 and *trans*-11 18:1 to accumulate, *trans*-10 18:1 being the most abundant FA intermediate in SFO.

Alterations in the ruminal metabolism of FA were not associated with substantial changes in rumen protozoal counts or analysed bacterial populations known to be capable of biohydrogenation (II; III), but lowered *Butyrivibrio spp.* numbers in response to increasing levels of FO (II).

Supplements of FO decreased milk fat yield and content and increased 20:5n-3, 22:5n-3 and 22:6n-3 concentrations in milk fat (IV). Enrichment of milk long-chain n-3 PUFA was associated with decreases in 4- to 18-carbon saturated FA and several-fold increases in CLA, *trans* FA and PUFA concentrations. Dietary FO resulted in the appearance of 37 unique 20- and 22-carbon FA in milk.

FO-induced MFD (up to -40.6 % reduction in milk fat synthesis) was associated with changes in the concentrations of multiple FA in milk, in particular increases in milk fat *trans*-10 18:1 and *cis*-11 18:1 concentrations, but not with changes in the amount of *trans*-10,*cis*-12 CLA in milk and omasum or estimated milk fat melting point (IV). The negative relationship between ruminal outflow of *trans*-10 18:1 and milk fat secretion confirmed that a shift in ruminal biohydrogenation of 18-carbon FA toward *trans*-10 pathway has a role in the regulation of milk fat synthesis during FO-induced MFD. A decrease in 18:0 supply in combination with increased mammary uptake of *cis*-11 18:1, *trans*-10 18:1, and *trans* 20- and 22-carbon FA intermediates originating from the rumen may contribute to the reduction of milk fat observed during FO-induced MFD.

The dietary supplements of FO alone or in combination with plant oils increased the ruminal outflow of FA intermediates containing at least one *trans* double bond and enriched long-chain n-3 PUFA in bovine milk with associated changes in the abundance and distribution of FA. These changes may have implications for the host metabolism and the nutritional quality or ruminant-derived foods.

Keywords: biohydrogenation, rumen, fish oil, plant oil, sunflower oil, linseed oil, lactating cow, polyunsaturated fatty acid, n-3 fatty acid, conjugated linoleic acid, milk fat, *trans* fatty acid, *Butyrivibrio*, microbial ecology, gas chromatography, mass spectrometry, silver-ion thin-layer chromatography

Tiivistelmä

Kala- ja kasviöljylisäysten vaikutus lypsylehmien rasva-aineenvaihduntaan pötsissä sekä kalaöljylisäyksen aiheuttama maitorasvasynteesin heikkeneminen

Tämän väitöskirjatyön tavoitteena oli tuottaa uutta tutkimustietoa monityydyttymättömien n-3-rasvahappojen, kuten 20:5n-3, 22:5n-3 ja 22:6n-3, biohydrogenaatiosta pötsissä sekä mahdollisuuksista muokata maitorasvan koostumusta ihmisravitsemuksen kannalta suotuisammaksi. Lypsylehmien nurmisäilörehuvaltaiseen ruokintaan lisättiin kala- ja kasviöljyjä, minkä avulla selvitettiin pötsin biohydrogenaation reaktioreitteihin vaikuttavia ravitsemuksellisia säätelymekanismeja ja pyrittiin lisäämään rasvahappojen 20:5n-3, 22:5n-3 ja 22:6n-3 pitoisuuksia maidossa. Samalla tarkasteltiin pötsissä tapahtuvan biohydrogenaation väli- ja lopputuotteena muodostuvien rasvahappojen sekä maitorasvasynteesin heikkenemisen eli maitorasvan depression välisiä yhteyksiä. Väitöskirja perustuu kolmeen ravitsemusfysiologiseen tutkimukseen, joiden tulokset on julkaistu väitöskirjan osajulkaisuissa I-IV.

Ensimmäisessä tutkimuksessa, jonka tulokset on raportoitu osajulkaisussa I, tutkittiin kalaöljyä saaneiden lypsylehmien pötsistä satakertaan virtaavan ruokasulan sisältämiä pitkäketjuisia 20- ja 22-hiilisiä tyydyttymättömiä rasvahappoja sekä kehitettiin analyysimenetelmiä, joiden avulla määritettiin näiden rasvahappojen rakennetta, kuten kaksoissidosten paikkaa hiiliketjussa ja *cis-trans* rakenneisomeriaa. Erilaisia rasvahappoanalytiikan menetelmiä yhdistämällä löydettiin jopa 27 aikaisemmin tunnistamatonta pötsin biohydrogenaation väli- ja/tai lopputuotteena muodostunutta, 20- ja 22-hiiliketjun pituista rasvahappoa, jotka sisälsivät vähintään yhden *trans*-kaksoissidoksen. Satakertaan virtaavasta ruokasulasta ei havaittu lainkaan yli 20 hiiltä sisältäviä rasvahappoa, joilla olisi ollut hiiliketjussaan ns. konjugoitunut rakenne. Tulokset osoittivat, että rasvahappojen 20:5n-3, 22:5n-3 ja 22:6n-3 biohydrogenaatio pötsissä on hyvin tehokas reaktiosarja, joka alkaa pääsääntöisesti lähimpänä rasvahapon karboksyyliryhmää sijaitsevien *cis*-muotoisten kaksoissidosten pelkistyessä tyydyttyneeseen muotoon tai niiden muuttuessa *trans*-muotoon isomerisaation seurauksena.

Toisen ja kolmannen tutkimuksen tulokset on julkaistu väitöskirjan osajulkaisuissa II-IV. Tutkimusten tarkoituksena oli selvittää rehuun lisätyn kalaöljyn (II; IV), kala- ja auringonkukkaöljyn (III; runsaasti linolihappoa; 18:2n-6) sekä kala- ja pellavaöljyn (III; runsaasti α-linoleenihappoa; 18:3n-3) vaikutuksia lypsylehmien ravintoaineiden saantiin, pötsissä tapahtuvaan rasva-aineenvaihduntaan ja pötsimikrobien ekologiaan (II; III) sekä maitorasvan koostumukseen ja ravintoaineiden hyväksikäyttöön maidontuotannossa (IV). Kalaöljyn lisäys nosti odotetusti kokonaisrasvahappojen sekä rasvahappojen 20:5n-3, 22:5n-3 ja 22:6n-3 saantia, mutta vähensi lehmien kokonaiskuiva-aineen syöntiä (II; III). Kalaöljyn lisäys yhdessä kasviöljyjen kanssa ei vaikuttanut pötsissä muodostuvien haihtuvien rasvahappojen kokonaispitoisuuteen (III), mutta sen pitoisuus väheni lisättäessä lehmien rehuun pelkkää kalaöljyä (II). Kalaöljyn lisäys yksin (II) tai yhdessä kasviöljyjen kanssa (III) lisäsi pötsissä muodostuneiden propionihapon (II; III) ja voihapon mooliosuuksia (II) vähentäen etikkahapon suhteellista määrää (II; III).

Kalaöljyn lisäys muutti pötsin 16- ja 18-hiilisten monityydyttymättömien rasvahappojen biohydrogenaatiota merkitsevästi, aiheuttaen pötsistä satakertaan virtaavien *trans*-16:1, *trans*-18:1 ja *trans*-18:2 rasvahappojen kokonaismäärien lisääntymisen sekä rasvahapon 18:0 virtauksen vähenemisen. Suurimmalla kalaöljyannoksella rasvahapon *trans*-11 18:1 virtaus kääntyi laskuun ja rasvahapon *trans*-10 18:1 virtaus lisääntyi voimakkaasti. Kalaöljyn lisäys ei vaikuttanut juurikaan pötsistä ulos virtaavan konjugoidun linolihapon (CLA) kokonaismäärään, mutta lisäsi lukuisten 20:5n-3, 22:5n-3 ja 22:6n-3 rasvahappojen biohydrogenaation väli- ja lopputuotteina muodostuvien, *trans*-kaksoissidoksen sisältävien, 20- ja 22-hiilisten rasvahappojen virtausta satakertaan.

Kalaöljyn lisääminen rehuun yksin tai yhdessä kasviöljyjen kanssa ei vaikuttanut rasvahappojen 20:5n-3 ja 22:5n-3 biohydrogenaatioon pötsissä, mutta rasvahapon 22:6n-3 pelkistyminen oli perusteellisempaa silloin kun kalaöljyä annettiin yhdessä kasviöljyjen kanssa (III). Kasviöljyille ominaiset erot 18-hiilisten tyydyttymättömien rasvahappojen (18:2n-6 ja 18:3n-3 auringonkukka- ja pellavaöljyille vastaavasti) pitoisuuksissa vaikuttivat siihen, miten voimakkaasti kalaöljylisäys rajoitti 18-hiilisten tyydyttymättömien rasvahappojen pelkistymistä pötsissä rasvahapoksi 18:0 sekä siihen, minkälaisia reaktiovälituotteita epätäydellisen biohydrogenaation seurauksena pötsissä muodostui (III). Lisättäessä kala- ja pellavaöljyjen seosta rehuun pötsistä ulosvirtaavien *trans*-18:2 rasvahappojen ja *trans*-kaksoissidoksen sisältävien 20- ja 22-hiilisten rasvahappojen määrä oli suurempi ja rasvahapon 18:0 virtaus pienempi kuin lisättäessä kala- ja auringonkukkaöljyjen seosta. Molemmat kala- ja kasviöljyjen seokset aiheuttivat rasvahappojen *trans*-10 18:1 ja *trans*-11 18:1 kertymisen pötsinesteeseen, mutta lisättäessä kalaöljyä yhdessä auringonkukkaöljyn kanssa rasvahapon *trans*-10 18:1 muodostus oli runsaampaa.

Muutokset pötsin monityydyttymättömien rasvahappojen biohydrogenaatiossa eivät vaikuttaneet pötsi- ja satakertanesteestä määritettyjen alkueläinten ja pötsin biohydrogenaatioon osallistuvien bakteeripopulaatioiden kokonaismääriin (II; III), vaikka *Butyrivibrio*-ryhmään kuuluvien bakteerien määrät laskivatkin kalaöljyannosta lisättäessä (II).

Maidon rasvatuotos ja rasvapitoisuus pienenivät kalaöljyä lisättäessä (IV). Samalla maidon rasvahappokoostumus muuttui ihmisravitsemuksen kannalta suotuisammaksi. Kala- ja kasviöljyjen lisäys vähensi maitorauhasen *de novo*-synteesistä peräisin olevien tyydyttyneiden rasvahappojen, 4:0–12:0 ja 16:0, sekä rasvahapon 18:0 pitoisuuksia ja nosti *trans*-rasvahappojen ja terveyttä edistävien monityydyttymättömien rasvahappojen, kuten CLA, 20:5n-3, 22:5n-3 ja 22:6n-3, pitoisuutta maitorasvassa. Kalaöljyä saaneiden lehmien maidosta tunnistettiin yhteensä 37 erityistä 20- ja 22-hiilistä rasvahappoa, joiden erittyminen maitoon lisääntyi kalaöljyannosta nostettaessa (IV).

Kalaöljylisäyksen aiheuttama maitorasvan depressio (jopa -40.6 %:n vähennys maitorauhasen rasvasynteesissä) oli yhteydessä lukuisten maidon rasvahappojen pitoisuuksien muutoksiin (IV). Maidon *trans*-10 18:1 ja *cis*-11 18:1 rasvahappojen pitoisuudet kasvoivat maitorasvan synteesin vähentyessä, mutta ne eivät yksin selitä havaittua kalaöljyn aiheuttamaa maitorasvasynteesin heikkenemistä. *Trans*-10,*cis*-12 CLA:n osuus maidon ja satakerran ruokasulan rasvahapoista ei ollut yhteydessä maitorasvan depressioon. Lisäksi tässä tutkimuksessa kalaöljyn lisäys ei vaikuttanut laskennalliseen maito-

rasvan sulamispisteeseen eikä maitorasvan synteesin heikkeneminen näin ollen ollut seuraus maitorasvan korkeammasta sulamispisteestä.

Pötsistä ulos virtaavan *trans*-10 18:1 rasvahapon ja maitorasvan tuoton välillä oli selvä negatiivinen yhteys. Tämä vahvistaa näkemystä siitä, että maitorasvasynteesin lasku johtuu ainakin osittain kalaöljylisäyksen aiheuttamasta 18-hiilisten monityydyttymättömien rasvahappojen biohydrogenaation etenemisestä vaihtoehtoista reaktioreittiä pitkin, jolloin pötsissä muodostuu runsaasti *trans*-10 18:1 rasvahappoa ja rasvahapon 18:0 satakertavirtaus sekä eritys maitoon vähenee. Kalaöljyä saaneet lehmät käyttivät rasvahapon 18:0 saannin vähentyessä enemmän rasvahappoja *cis*-11 18:1 ja *trans*-10 18:1 sekä *trans*-kaksoissidoksen sisältäviä 20- ja 22-hiilisiä rasvahappoja maitorasvan muodostamiseen, jolloin nämä pötsin biohydrogenaation välituotteena muodostuneet *trans*-rasvahapot voivat olla osatekijänä maitorasvan depressiossa (IV).

Tulokset osoittivat, että kalaöljyn lisäys lypsylehmien rehuun, yksin tai yhdessä kasviöljyjen kanssa, aiheuttivat pötsistä ulosvirtaavien pitkäketjuisten, *trans*-kaksoissidoksen sisältävien, rasvahappojen määrän lisääntymisen. Lisäksi kalaöljylle ominaisten terveysvaikutteisten monityydyttymättömien n-3 rasvahappojen osuus maitorasvassa kasvoi. Kala- ja kasviöljyjen lisääminen lypsylehmien rehuun vaikuttaa siis paitsi eläimen aineenvaihduntaan myös märehtijäperäisten elintarvikkeiden ravitsemukselliseen koostumukseen.

Avainsanat: biohydrogenaatio, pötsi, kalaöljy, kasviöljy, auringonkukkaöljy, pellavaöljy, lypsylehmä, monityydyttymätön rasvahappo, n-3-rasvahappo, konjugoitu linolihappo, maitorasva, *trans*-rasvahappo, *Butyrivibrio*, mikrobiekologia, kaasukromatografia, massaspektrometria, hopea-ioni-ohutlevykromatografia

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List of original publications

This thesis is based on the following publications:

I Kairenius, P., Toivonen, V., and Shingfield, K. J. 2011. Identification and ruminal outflow of long-chain fatty acid biohydrogenation intermediates in cows fed diets containing fish oil. Lipids 46:587–606. https://doi.org/10.1007/s11745-011-3561-1

II Shingfield, K. J., Kairenius, P., Ärölä, A., Paillard, D., Muetzel, S., Ahvenjärvi, S., Vanhatalo, A., Huhtanen, P., Toivonen, V., Griinari, J. M., and Wallace, R. J. 2012. Dietary fish oil supplements modify ruminal biohydrogenation, alter the flow of fatty acids at the omasum, and induce changes in the ruminal *Butyrivibrio* population in lactating cows. J. Nutr. 142:1437–1448. https://doi.org/10.3945/jn.112.158576

III Kairenius, P., Leskinen, H., Toivonen, V., Muetzel, S., Ahvenjärvi, S., Vanhatalo, A., Huhtanen, P., Wallace, R. J., and Shingfield, K. J. 2018. Effect of dietary fish oil supplements alone or in combination with sunflower and linseed oil on ruminal lipid metabolism and bacterial populations in lactating cows. J. Dairy Sci. 101:1–15. https://doi.org/10.3168/jds.2017-13776

IV Kairenius, P., Ärölä, A., Leskinen, H., Toivonen, V., Ahvenjärvi, S., Vanhatalo, A., Huhtanen, P., Hurme, T., Griinari, J. M., and Shingfield, K. J. 2015. Dietary fish oil supplements depress milk fat yield and alter milk fatty acid composition in lactating cows fed grass silage-based diets. J. Dairy Sci. 98:5653–5671. http://dx.doi.org/10.3168/jds.2015-9548

The publications are referred to in the text by their Roman numerals.

All experiments were conducted at the Animal Metabolism Unit of Natural Resources Institute Finland (Luke, Jokioinen; formerly MTT Agrifood Research Finland).

Contributions

The contribution of all authors to the original articles of this thesis is described in the following table (initials of authors are listed in alphabetical order).

	I	II	III	IV
Planning the experiment	KJS PH PK SA	AV JMG KJS PK PH SA	AV KJS PH SA	AV JMG KJS PK PH SA
Conducting the experiment	KJS PK	AV AÄ KJS PK SA	AV KJS SA	AV AÄ KJS PK SA
Lipid analysis	KJS PK VT	AÄ KJS PK VT	HL KJS PK VT	AÄ HL KJS PK VT
Microbial ecology analysis		DP KJS RJW SM	KJS RJW SM	
Experimental data analysis	KJS PK	AV AÄ DP KJS PK PH RJW	AV KJS PK PH RJW SM	AV AÄ KJS PK PH TH
Manuscript preparation	KJS PK TV	AV AÄ KJS PH PK RJW TV	AV HL KJS PH PK RJW	AV KJS PH PK RJW

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AÄ = Anu Ärölä
DP = Delphine Paillard
HL = Heidi Leskinen
JMG = J. Mikko Griinari
KJS = Kevin J. Shingfield
PH = Pekka Huhtanen
PK = Piia Kairenius
RJW = R. John Wallace
SA = Seppo Ahvenjärvi
SM= Stefan Muetzel
TH = Timo Hurme
VT = Vesa Toivonen

Abbreviations

ACACA	acetyl-CoA carboxylase
Ag^+	silver ion
BHBA	ß-hydroxybutyrate
CLA	conjugated linoleic acid
CVD	cardiovascular disease
DM	dry matter
DMI	dry matter intake
DMOX	dimethyloxazoline
DNA	deoxyribonucleic acid
FAME	fatty acid methyl ester
FA	fatty acid
FID	flame ionization detector
FO	fish oil
FASN	fatty acid synthase
GC	gas chromatography
GL	glycolipid
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
iNDF	indigestible neutral detergent fibre
LDL	low-density lipoprotein
LFO	diet based on grass silage supplemented with 500 g/d of linseed oil and 200 g/d of fish oil
LO	linseed oil
MFD	milk fat depression
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MUFA	monounsaturated fatty acid
NEFA	non-esterified fatty acid
NDF	neutral detergent fibre
OBCFA	odd- and branched chain fatty acid
PCR	polymerase chain reaction
PL	phospholipid
PLS	partial least squares regression
PUFA	polyunsaturated fatty acid
SCD	stearoyl-CoA desaturase, Δ-9 desaturase
SFA	saturated fatty acid
SFO	diet based on grass silage supplemented with 500 g/d of sunflower and 200 g/d of fish oil
SO	sunflower oil
SREBP1	sterol regulatory element-binding protein
TAG	triacylgycerol
TLC	thin-layer chromatography
VFA	volatile fatty acid

The following table describes fatty acid families (e.g. omega-numbering), systematic and trivial names, shorthand abbreviations and notations of selected (and more common) short- and long-chain fatty acids used in the text.

Family[1]	Systematic name	Trivial name	Shorthand abbreviation	Shorthand notation
Saturated fatty acids[2]				
	ethanoic acid	acetic acid	2:0	
	propanoic acid	propionic acid	3:0	
	butanoic acid	butyric acid	4:0	
	hexanoic acid	caproic acid	6:0	
	octanoic acid	caprylic acid	8:0	
	decanoic acid	capric acid	10:0	
	dodecanoic acid	lauric acid	12:0	
	tridecanoic acid		13:0	
	tetradecanoic acid	myristic acid	14:0	
	pentadecanoic acid		15:0	
	hexadecanoic acid	palmitic acid	16:0	
	heptadecanoic acid	margaric acid	17:0	
	octadecanoic acid	stearic acid	18:0	
	nonadecanoic acid		19:0	
	eicosanoic acid	arachidic acid	20:0	
	heneicosanoic acid		21:0	
	docosanoic acid	behenic acid	22:0	
	tricosanoic acid		23:0	
	tetracosanoic acid	lignoceric acid	24:0	
	pentacosanoic acid		25:0	
	hexacosanoic acid	cerotic acid	26:0	
	heptacosanoic acid	heptacosylic acid	27:0	
	octacosanoic acid	montanic acid	28:0	

Family[1]	Systematic name	Trivial name	Shorthand abbreviation	Shorthand notation
Monounsaturated fatty acids[2]				
n-7	*cis*-9-hexadecenoic acid	palmitoleic acid	*cis*-9 16:1	16:1n-7
n-4	*all-cis*-9,12-hexadecadienoic acid		*cis*-9,*cis*-12 16:2	16:2n-4
n-4	*all-cis*-6,9,12-hexadecatrienoic acid		*cis*-6,*cis*-9,*cis*-12 16:3	16:3n-4
n-3	*all-cis*-7,10,13-hexadecatrienoic acid	HTA		16:3n-3
n-3	*all-cis*-4,7,10,13-hexadecatetraenoic acid			16:4n-3
n-1	*all-cis*-6,9,12,15-hexadecatetraenoic acid			16:4n-1
n-9	*cis*-9-octadecenoid acid	oleic acid	*cis*-9 18:1	18:1n-9
	trans-9-octadecenoic acid	elaidic acid	*trans*-9 18:1	
	trans-10-octadecenoic acid		*trans*-10 18:1	
n-7	*cis*-11-octadecenoic acid	*cis*-vaccenic acid	*cis*-11 18:1	
	trans-11-octadecenoic acid	vaccenic acid	*trans*-11 18:1	
Polyunsaturated 18-carbon fatty acids[2]				
n-6	*all-cis*-9,12-octadecadienoic acid	linoleic acid, LA	*cis*-9,*cis*-12 18:2	18:2n-6
	trans-9,*trans*-12-octadecadienoic acid	linoelaidic	*trans*-9,*trans*-12 18:2	
n-6	*all-cis*-6,9,12-octadecatrienoic acid	γ-linolenic acid, GLA	*cis*-6,*cis*-9,*cis*-12 18:3	18:3n-6
n-3	*all-cis*-9,12,15-octadecatrienoic acid	α-linolenic acid, ALA	*cis*-9,*cis*-12,*cis*-15 18:3	18:3n-3
	cis-9,*trans*-11,*cis*-15-octadecatrienoic acid		*cis*-9,*trans*-11,*cis*-15 18:3	
n-6	*all-cis*-3,6,9,12-octadecatetraenoic acid	γ-stearidonic acid		18:4n-6
n-3	*all-cis*-6,9,12,15-octadecatetraenoic acid	stearidonic acid, SDA		18:4n-3
Conjugated linoleic acids[2]				
	cis-9,*trans*-11-octadecadienoic acid	rumenic acid	*cis*-9,*trans*-11 CLA	
	trans-10,*cis*-12- octadecadienoic acid		*trans*-10,*cis*-12 CLA	
Polyunsaturated long-chain 20-, 21-, 22- and 24-carbon fatty acids[2]				
n-9	*cis*-11-eicosenoic acid	gondoic acid	*cis*-11 20:1	20:1n-9
n-7	*cis*-13-eicosenoic acid	paullinic acid	*cis*-13 20:1	20:1n-7
n-6	*all-cis*-11,14-eicosadienoic acid		*cis*-11,*cis*-14 20:2	20:2n-6
n-3	*all-cis*-14,17-eicosadienoic acid		*cis*-14,*cis*-17 20:2	20:2n-3

Family[1]	Systematic name	Trivial name	Shorthand abbreviation	Shorthand notation
n-9	*all-cis*-5,8,11-eicosatrienoic acid	mead acid		20:3n-9
n-6	*all-cis*-8,11,14-eicosatrienoic acid	dihomo-γ-linolenic acid, DGLA		20:3n-6
n-3	*all-cis*-11,14,17-eicosatrienoic acid	ETE		20:3n-3
n-6	*all-cis*-5,8,11,14-eicosatetraenoic acid	arachidonic acid, AA		20:4n-6
n-3	*all-cis*-8,11,14,17-eicosatetraenoic acid	ETA		20:4n-3
n-3	*all-cis*-5,8,11,14,17-eicosapentaenoic acid	EPA		20:5n-3
n-3	*all-cis*-6,9,12,15,18-heneicosapentaenoic acid	HPA		21:5n-3
n-9	*cis*-13-docosenoic acid	erucic acid	*cis*-13 22:1	22:1n-9
n-7	*cis*-15-docosenoic acid		*cis*-15 22:1	22:1n-7
n-6	*all-cis*-13,16-docosadienoic acid			22:2n-6
n-3	*all-cis*-13,16,19-docosatrienoic acid			22:3n-3
n-4	*all-cis*-10,13,16-docosatrienoic acid			22:3n-4
n-9	*all-cis*-4,7,10,13-docosatetraenoic acid			22:4n-9
n-6	*all-cis*-7,10,13,16-docosatetraenoic acid	adrenic acid		22:4n-6
n-3	*all-cis*-10,13,16,19-docosatetraenoic acid			22:4n-3
n-6	*all-cis*-4,7,10,13,16-docosapentaenoic acid	osbond acid, n-6 DPA		22:5n-6
n-3	*all-cis*-7,10,13,16,19-docosapentaenoic acid	clupanodonic acid, n-3 DPA		22:5n-3
n-3	*all-cis*-4,7,10,13,16,19-docosahexaenoic acid	DHA		22:6n-3
n-9	*cis*-15-tetracosenoic acid	nervonic acid	*cis*-15 24:1	24:1n-9
n-7	*cis*-17-tetracosenoic acid		*cis*-17 24:1	24:1n-7
n-6	*all-cis*-9,12,15,18-tetracosatetraenoic acid			24:4n-6
n-6	*all-cis*-6,9,12,15,18-tetracosapentaenoic acid			24:5n-6
n-3	*all-cis*-9,12,15,18,21-tetracosapentaenoic acid			24:5n-3
n-3	*all-cis*-6,9,12,15,18,21-tetracosahexaenoic acid	nisinic acid		24:6n-3

[1]In the omega nomenclature the double bond positions are counted from the methyl end of the carbon chain. For example, the n-3 FA have a "signature" double bond in the third carbon from the methyl end of the molecule (omega-3 or n-3 FA). [2]Fatty acids consist of carbon atom chains that have a methyl group (CH3) at one end and a carboxylic acid group (COOH) at the other. Two main classes of FA are saturated and unsaturated fatty acids, which are further divided to monounsaturated and polyunsaturated fatty acids.

Contents

1. Introduction

Lipids in human nutrition. Lipids and fatty acids (FA) play an important role in human health and nutrition. Ruminant derived foods are an important and versatile source of nutrients, such as amino acids, calcium and bioactive lipids in the human diet (Givens and Shingfield, 2006; Shingfield et al., 2013). As ruminant derived foods are also rich in saturated fatty acids (SFA) there has been an increased interest in developing nutritional strategies for altering the composition of milk and meat fat to improve long-term human health (Givens and Shingfield, 2006; Shingfield et al., 2008b).

Clinical studies implicate an excessive consumption of medium-chain SFA and *trans* FA and low intake of polyunsaturated FA (PUFA) and n-3 PUFA as risk factors for cardiovascular disease (CVD) and other metabolic diseases (Meier et al., 2019; Mensik et al., 2003; Willet et al., 1993). It is well established that SFA, in particular myristic acid (14:0) and palmitic acid (16:0), and possibly also lauric acid (12:0), decrease the relative proportions of high-density lipoprotein (HDL) and increase low-density lipoprotein (LDL) cholesterol and increase the risk of CVD in humans (Hu et al., 2001; Givens, 2008).

In contrast, dietary consumption of long-chain n-3 PUFA, such as α-linolenic acid (18:3n-3), eicosapentaenoic acid (20:5n-3), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3) is beneficial for human health and has been shown to induce protective effects against CVD (Wang et al., 2006; Harris et al., 2008; 2009; Palmquist, 2009). There is also evidence to indicate that moderate increases in the consumption of n-3 PUFA improves immune function, prevent certain inflammatory diseases and neurological disorders and may prevent certain cancers (Wang et al., 2006; Harris et al., 2009; Palmquist, 2009; Yashodhara et al., 2009; Deckelbaum and Torrejon 2012). Fatty acid 18:3n-3 must be obtained from the diet as it cannot be synthesised by humans. Synthesis of 20:5n-3 and 22:6n-3 from 18:3n-3 in humans is limited (Burdge and Calder, 2005) and therefore the diet is the principal source of long-chain n-3 PUFA.

Milk and dairy products as a source of lipids. The increased awareness of the association between diet and health has led to nutritional quality becoming an important determinant of consumer food choices. Milk and dairy products are the main source of 12:0, 14:0, 16:0 and total SFA in the human diet and also make a significant contribution to *trans* FA intake. Lowering the concentration of these nutritionally undesirable FA and increasing the content of specific bioactive lipids recognized as having potential or putative beneficial effects on human health (such as *cis*-9 18:1, *cis*-9,*trans*-11 conjugated linoleic acid (CLA), 18:3n-3, and n-3 PUFA) in ruminant milk, meat and dairy products offer an opportunity to improve long-term human health without requiring changes in consumer eating habits. Developing food products and dietary regimens that promote human health is also central for preventing and reducing the economic and social impact of chronic diseases.

Nutritional strategies to modulate lipid metabolism and increase the total unsaturated FA and n-3 PUFA content of milk in lactating cows have often included supplementing feeding rations with plant oils, oilseeds, marine lipids (e.g. fish oil (FO) and ma-

rine algae) and rumen protected lipids (Kliem and Shingfield, 2016), and selecting different forage sources (e.g. red clover, pasture, grass hay and grass silage; Halmemies-Beauchet-Filleau et al., 2013b) and concentrate components (e.g. feeds high in rapidly fermentable carbohydrates such as cereal grains) with plant oils (Loor et al., 2004; Ventto et al., 2017) and using different forage:concentrate ratios (e.g. high or low levels of starch/forage; Kliem and Shingfield, 2016). These dietary approaches are not only used for improving the nutritional quality of milk, but also to alter ruminal or tissue lipid metabolism and achieve a desired response, such as a decrease in milk fat synthesis to explore the mechanisms driving milk fat synthesis and its regulation in ruminants. Addition of lipids to the ruminant diet offers opportunities to increase the partitioning of energy towards body tissues during early lactation with the intention of reducing the extent and duration of negative energy balance in high producing dairy cows (Qin et al., 2018), as well as a model for studying the nutritional regulation of mammary lipogenesis, particularly lipogenic gene expression (Bernard et al., 2018).

Bovine milk fat. Bovine milk contains approximately 3-5 % fat depending on e.g. breed, state of lactation, management, and feeding strategies of dairy cows. Milk fat is secreted from mammary epithelial cells as milk fat droplets (milk fat globules), that contain mainly triacylglycerols (TAG; 96-98 %; Jensen, 2002), and smaller amounts of di- and monoacylglycerols (about 2 % of the lipid fraction), cholesterol esters (CE; less than 0.5 %), phospholipids (PL; including sphingolipids; about 1 %) and non-esterifield FA (NEFA; about 0.1 %) constitute the globule membrane (a protein rich polar lipid coat) surrounding the milk fat droplet (Jensen, 2002; Shingfield et al., 2010a; Bernard et al., 2018).

Milk fat TAG are synthesised from more than 500 individual FA (Jensen, 2002; Lindmark Månsson, 2008). Fatty acids are a wide group of compounds with different chain lengths, branching, degree of unsaturation (number of double bonds; Figure 1A), configuration of double bonds (*cis*-*trans*; Figure 1A) and other functional and structural groups (Rodriguez-Estrada et al., 2014). In most cases, double bonds next to each other in FA are separated by a single methylene group (methylene interrupted), but some naturally occurring FA have conjugated double bond system, that is, two double bonds separated by one single bond (Figure 1B). Most natural FA have double bonds in the *cis* configuration. *Trans* double bonds arise from ruminal biohydrogenation or industrial processes (hydrogenation and refining processes; Shingfield et al., 2008b). A summary of FA nomenclature and shorthand notations of the most common FA used in scientific publications of this thesis is presented as a separate table in the beginning of this work.

The predominant SFA in milk are 14:0, 16:0 and stearic acid (18:0). These SFA account for 75 % of the total FA, with a further 21 % occurring as MUFA of which the most prevalent is oleic acid (*cis*-9 18:1). Only 4-5 % of total FA in milk fat are PUFA, occurring mainly as linoleic acid (18:2n-6; ca. 1-3 %) and 18:3n-3 (0.5-2 %; Jensen, 2002; Lindmark Månsson, 2008). Approximately 3 % of the FA in milk are *trans* FA, especially Δ4-16 *trans* 18:1 isomers (Jensen, 2002), of which *trans*-11 18:1 is the most abundant (Precht and Molkentin, 1999; Shingfield et al., 2008b). Although it is still uncertain if dairy *trans* FA

are as harmful as *trans* FA from plant-derived industrial fats and oils (Kleber et al., 2016), there is some evidence that the distribution of *trans* 18:1 and *trans* 18:2 isomers differs between ruminal and industrial *trans* FA (e.g. Shingfield et al., 2008b; Lock et al., 2005).

Milk fat contains also a number of bioactive FA, including butyric acid (4:0), odd- and branched-chain FA (OBCFA), and *cis*-9,*trans*-11 CLA, which have been suggested to possess anti-inflammatory, anti-obesity, anti-carcinogenic, anti-mutagenic, anti-adipogenic and anti-diabetogenic properties, and have been reported to improve different biomarkers of cardiovascular health in animal models and *in vitro* studies with human cell lines (Huth et al., 2006; Shingfield et al., 2008b; Gebauer et al., 2011). Typically, milk fat contains 2-5 % of 4:0, 2-3 % of OBCFA, and 0.3-0.6 % of *cis*-9,*trans*-11 CLA, but concentrations are subject to considerable variation.

"Conjugated linoleic acid'' is a collective term to describe a mixture of geometric and positional isomers of 18:2 containing a conjugated double bond (Figure 1B). Dairy products are the main source of CLA in the human diet, with the *cis*-9,*trans*-11 isomer accounting for 70–80 % of total CLA intake (Lawson et al., 2001), as *cis*-9,*trans*-11 is the major isomer of CLA in bovine milk (ca. 65.6–88.9 % of total CLA; Shingfield et al., 2008b). The most abundant OBCFA in ruminant milk fat are usually isomers of tridecanoic acid (*iso* 13:0), tetradecanoic acid (*iso* 14:0), pentadecanoic acid (15:0, *iso* 15:0 and *anteiso* 15:0), hexadecanoic acid (*iso* 16:0) and heptadecanoic acid (17:0, *iso* 17:0 and *anteiso* 17:0) (Bauman et al., 2016; Vlaeminck et al., 2006). These originate mainly from microbial OBCFA synthesis in the rumen but also from *de novo* synthesis (15:0 and 17:0) in ruminant tissues, including the mammary gland (Vlaeminck et al., 2006).

Milk fat is poor source of long-chain n-3 PUFA, and naturally almost devoid of n-3 very long-chain PUFA, specifically 20:5n-3 and 22:6n-3 (on average, 0.06 and 0.03 % of total FA, respectively; IV). Marine lipids, FO or marine algae in particular, have been commonly used as sources of n-3 long-chain PUFA in dairy cow rations to increase the milk fat content of these FA. The concentration of ≥ 18-carbon FA, including 18:2n-6, 18:3n-3, 20:5n-3, and 22:6n-3, in milk is dependent on the absorption of these FA from the small intestine and uptake by the mammary gland.

Ruminal lipid metabolism. After ingestion, dietary plant lipids, such TAG, PL and glycolipids (GL), are released from their structural components through mastication and microbial digestive processes within the rumen, and FA are released from these lipids by the action of microbial lipases (called as lipolysis) (Harfoot and Hazlewood, 1988; Jenkins et al., 2008).

Bacterial breakdown of dietary lipids is quite rapid and rather complete for most unprotected lipids (85-95 %) as observed earlier in the rumen of lactating cows fed grass hay (Bauchart et al., 1990), fresh grass (Halmemies-Beauchet-Filleau et al., 2013a) or grass silage-based diets (Halmemies-Beauchet-Filleau et al., 2013a). A number of factors related to diet and rumen environment, such as high level of PUFA intake or low rumen pH, that are known to inhibit the activity and growth of certain bacteria in the rumen, have been determined to influence the rate and extent of lipolysis in the rumen (Palmquist et al., 2005; Lourenço et al., 2010).

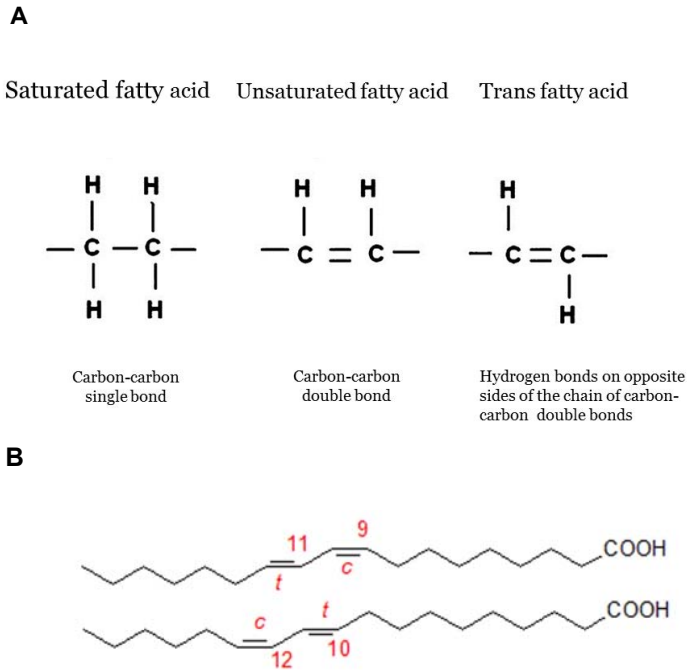

Figure 1. Structural differences between *cis* and *trans* fatty acids in A) Dhaka, V., Gulia, N., Ahlawat, K.S. and Khatkar, B.S., 2011 J. Food Sci. Technol. 48:5:534-541, Copyright (2011) Springer Nature, and a conjugated double bond system in B) AOAC Lipid Library, 2019 cited on 27 December 2019.

After lipolysis, the next step in the transformation of unsaturated FA in the rumen is biohydrogenation. During biohydrogenation, rumen microbes convert unsaturated FA to SFA via a stepwise reduction process during which *cis*-double bonds of unsaturated FA are first isomerised to *trans* FA intermediates (called as a *cis-trans* isomerisation) followed by hydrogenation of the double bonds in the carbon chain to yield saturated end products. Numerous, previously reviewed (Palmquist et al., 2005; Jenkins et al., 2008; Shingfield et al., 2010a; 2013), *in vivo* and *in vitro* studies have elucidated in detail the major biohydrogenation pathways of plant-derived 18:2n-6 and 18:3n-3 (Figure 2; Shingfield and Griinari, 2007). Under normal rumen conditions biohydrogenation of 18:2n-6 and 18:3n-3 is considered to proceed via isomerisation of the *cis*-12 double bond resulting in the formation of conjugated 18:2 or 18:3, respectively. Conjugated FA intermediates, such as *cis*-9,*trans*-11 CLA and *cis*-9,*trans*-11,*cis*-15 18:3, respectively, are temporary metabolites and are further reduced to 18:0 as the final end product with *trans*-11 18:1 (vaccenic acid) as a common intermediate. The final reduction step is considered to

be rate limiting and therefore *trans* 18:1 intermediates can accumulate (Harfoot and Hazlewood, 1988; Griinari and Bauman, 1999; Bauman and Griinari, 2001).

Besides general biohydrogenation pathways of 18:2n-6 and 18:3n-3 described in Figure 2, numerous minor biohydrogenation pathways exist in the rumen that are dependent on the ruminal microbial ecosystem, resulting in the formation of a wide range of biohydrogenation FA intermediates, such as several 18:3, 18:2, CLA and *trans* 18:1 isomers (Shingfield et al., 2010a). Although most of these ruminal FA intermediates are further hydrogenated to 18:0, some of them escape from the rumen depending on feeding management, and are absorbed in the small intestine, and yet further incorporated into ruminant milk and tissue lipids.

Dietary lipid supplementation in the diet of ruminants. Dietary supplements of plant oils rich in 18:2n-6 (e.g. sunflower oil; SO) (Shingfield et al., 2008a) and 18:3n-3 (e.g. linseed oil; LO) (Loor et al., 2004) or FO (Shingfield et al., 2003) are known to increase *trans*-11 18:1 formation in the rumen of lactating cows. Feeding plant-derived 18:2n-6 or 18:3n-3 together with FO further increases *cis*-9,*trans*-11 CLA concentrations in milk from lactating cows (Whitlock et al., 2002; Palmquist and Griinari, 2006; Shingfield et al., 2006a), but the enrichment varies depending on the composition of the basal diet and the amount and source of lipid supplements. The increases in *cis*-9,*trans*-11 CLA in milk in FO diets are accompanied by elevated proportions of *trans* 18:1 and *trans* 18:2 isomers (Chilliard et al., 2001; Shingfield et al., 2003; Loor et al., 2005a), indicating that ruminal biohydrogenation of 18-carbon PUFA to saturated end product, 18:0, is incomplete (Shingfield et al., 2003; Lee et al., 2008; Shingfield et al., 2010b). Furthermore, dietary supplements of oils containing 18:2n-6 or 18:3n-3, but not FO, lower the proportions of 12:0, 14:0, and 16:0 in milk fat (Shingfield et al., 2013).

Oil supplements can also be used to increase the ruminal outflow of long-chain n-3 FA intermediates available for absorption and incorporation into milk and meat. Several studies have shown that FO can be used to increase the 20:5n-3 and 22:6n-3 concentrations in ruminant milk and tissue lipids (Scollan et al., 2006; Chilliard et al., 2007; Palmquist, 2009). However, the level of enrichment of n-3 PUFA on milk and meat is limited. A number of detailed physiological studies in ruminants have indicated that 20:5n-3 and 22:6n-3 are hydrogenated extensively in the rumen (Shingfield et al., 2003; 2010; Lee et al., 2008) and disappear during incubations with rumen fluid *in vitro* with the extent of hydrogenation being dependent on the amount of FO supplementation (Dohme et al., 2003; AbuGhazaleh and Jenkins, 2004; Wasowska et al., 2006), but the metabolic pathways involved and the intermediates formed are not well known. Only few studies, including the experiments reported in this thesis (II; III), have indicated that the biohydrogenation of long-chain PUFA in FO or marine algae result in the formation and accumulation of numerous 20- and 22-carbon biohydrogenation intermediates containing at least one *trans* double bond in cattle (Shingfield et al., 2003; 2010; Lee et al., 2008) and in nonlactating sheep (Toral et al., 2010; 2012), but the effects of 18-carbon PUFA supply on ruminal long-chain FA metabolism and microbial communities in ruminants fed FO are not well established.

Role of microbial population in ruminal biohydrogenation. The main members of the microbial population in the rumen are comprised of bacteria, bacteriophages, protozoa, fungi and archaea that live in a symbiotic relationship and are collectively responsible for microbial fermentation in ruminants. The microbial ecology related to ruminal biohydrogenation has attracted considerable interest as PUFA are considered detrimental to rumen microbes (Maia et al., 2010; Huws et al, 2015; Enjalbert et al., 2017). Bacteria, rather than protozoa, are thought to be responsible for ruminal biohydrogenation, but relatively few strains capable of biohydrogenation have been identified (Harfoot and Hazlewood, 1988; Lourenço et al., 2010). The identification of specific microbial species able to catalyse one or more reactions remains challenging, as biohydrogenation intermediates and products may also be exchanged between populations.

Among cultivated bacteria, all members of the *Butyrivibrio* group form *cis*-9,*trans*-11 CLA from 18:2n-6 much more rapidly than do other species, but only *B. proteoclasticus* is shown to be capable of reducing *trans*-11 18:1 to 18:0 (Jeyanathan et al., 2016). Previous reports highlight that the effects of FO on ruminal biohydrogenation in growing cattle are associated with changes in the ruminal bacterial community known to be capable of biohydrogenation (Kim et al., 2008; Huws et al., 2010; 2011). In addition, populations of specific bacteria in the rumen were found to be altered by marine algae supplements in lactating sheep fed diets containing SO (Toral et al., 2012), but there are no reports on the effects of FO with plant oils on the relative abundance of key biohydrogenating bacteria in cattle.

Ruminal biohydrogenation of PUFA is a complex process comprising of isomerisation, desaturation and hydrolysation reactions and different biohydrogenation pathways. A different set of FA intermediates may be produced under specific feeding strategies, such as diets causing milk fat depression (MFD), which may shift the ruminal biohydrogenation towards a higher production of *trans*-10 18:1 at the expense of *trans*-11 18:1 (Figure 2; Shingfield and Griinari, 2007). A shift toward *trans*-10 biohydrogenation pathway in the rumen is more pronounced in cows fed diets containing relatively high concentrations of plant-derived PUFA (Griinari et al., 1998; Piperova et al., 2000; Loor et al., 2005b), high-starch, low-fibre diets (Piperova et al., 2002; Zened et al., 2013), high-concentrate diets containing plant oils (Loor et al., 2004; Ventto et al., 2017), or diets containing high marine lipids (e.g. II; Toral et al., 2016a).

Milk fat synthesis in the mammary gland. Under normal dietary and physiological conditions, ca. 40 % of total FA in milk fat originates from *de novo* synthesis in the mammary gland, while the rest originate from the direct mammary uptake of circulating plasma FA (Chilliard et al., 2000). Substrates for mammary *de novo* synthesis are mainly acetate (2:0) and ß-hydroxybutyrate (BHBA) derived from fermentation of feed components in the rumen (Lock and Bauman, 2004). Substrates are used by the mammary epithelial cells in the presence of two key enzymes, acetyl-CoA carboxylase (ACACA) and FA synthase (FASN) (Shingfield et al., 2010a) to synthesize short- and medium-chain FA, providing all 4:0 to 12:0, most of the 14:0 (ca. 95 %), and about half (ca. 50 %) of 16:0 secreted in milk (Chilliard et al., 2000). The direct uptake of preformed FA provides re-

maining of the 16- and all of the ≥ 18-carbon long-chain FA in milk fat (ca. 60 %; Chilliard et al., 2000).

Fatty acids of 10- to 20-carbon atoms may be desaturated in the mammary epithelial cells by Δ9-desaturase complex (stearoyl-CoA desaturase; SCD) that is responsible for introducing a *cis* double bond at position Δ9 of FA. Special attention has been directed to SCD, which is the main enzyme responsible for converting SFA into MUFA (18:0 to *cis*-9 18:1). The SCD activity plays an important role in ruminants because it reverses ruminal biohydrogenation (i.e. saturation) of dietary MUFA and PUFA in the rumen. In ruminants, 60 % of *cis*-9 18:1 (derived from 18:0), 50-56 % of *cis*-9 16:1 (derived from 16:0) and 90 % of *cis*-9 14:1 (derived from 14:0), and > 60 % of *cis*-9,*trans*-11 CLA (derived from *trans*-11 18:1) originate from mammary SCD activity (Bernard et al., 2018).

Most of the CLA isomers appearing in milk fat are derived directly from the biohydrogenation of diet 18:2n-6 and 18:3n-3 in the rumen (Baumgard et al., 2000; Bauman and Griinari, 2003). However, 64-97 % of *cis*-9,*trans*-11 CLA and also a majority of *trans*-7,*cis*-9 CLA secreted in milk originates from Δ9-desaturation of *trans*-11 18:1 (Griinari et al., 2000; Corl et al., 2001; Mosley et al., 2006) and *trans*-7 18:1 (Corl et al, 2002; Piperova et al., 2002), respectively, in the mammary gland (Figure 2; Griinari and Bauman, 1999).

Milk fat depression in lactating cows is characterised by decreases in milk fat within a few days without changes in milk yield or other milk components (e.g. Bauman and Griinari, 2003; Shingfield and Griinari, 2007). Several theories have been proposed to explain diet-induced MFD, including 1) increased formation of biohydrogenation intermediates that inhibit, directly or indirectly, milk fat synthesis, 2) lowered availability of 18:0 for endogenous *cis*-9 18:1 synthesis via SCD in the mammary gland in marine lipid-induced MFD, and 3) an increase in the supply of *trans* FA formed in the rumen contributing to lower milk fat synthesis by increasing the milk fat melting point above body temperature, exceeding the capacity to maintain milk fat fluidity and thereby lower the rate of fat removal in mammary epithelial cells (Loor et al., 2005a; Shingfield and Griinari, 2007; Gama et al., 2008; Harvatine et al., 2009). Of these, the most widely accepted hypothesis is the biohydrogenation theory of MFD (Bauman and Griinari, 2001) which suggests that the changes in ruminal lipid metabolism increase the formation of specific FA biohydrogenation intermediates from dietary PUFA that directly inhibit milk fat synthesis (Bauman and Griinari, 2001; 2003).

These specific FA intermediates associated with MFD often derive from the alternative *trans*-10 biohydrogenation pathway and contain a double bond in the *trans* configuration at the 10th carbon atom from the carboxyl end. Because these biohydrogenation intermediates, e.g. *trans*-10 18:1, *trans*-10,*cis*-12 CLA and *trans*-10,*cis*-15 18:2 are available for absorption and incorporation into milk fat, they are suggested to cause reductions in milk fat content resulting in MFD (Bauman and Griinari, 2003; Shingfield and Griinari, 2007).

Trans-10,*cis*-12 CLA formed during the isomerisation of 18:2n-6 in the rumen (Wallace et al., 2007) is the only intermediate shown unequivocally to lower milk fat synthe-

sis in lactating cows (Baumgard et al., 2000; 2002; Glasser et al., 2010; Harvatine and Bauman, 2011), but it does not, in isolation, explain MFD in cows fed diets containing FO (Loor et al., 2005a; Gama et al., 2008; Toral et al., 2015). Earlier findings involving postruminal infusions of FA, such as a mixture of CLA isomers, suggest that other intermediates, including *cis*-10,*trans*-12 CLA (Saebø et al., 2005), *trans*-9,*cis*-11 CLA (Perfield et al., 2007), and possibly *trans*-10 18:1 (Shingfield et al., 2010a; Harvatine et al., 2009; Conte et al., 2018) may also inhibit milk fat synthesis in the lactating cow. However, it is probable that other, yet unidentified, biohydrogenation intermediates or metabolites or other additional mechanisms contribute to the regulation of mammary lipogenesis and MFD in cows fed diets containing marine lipids (Loor et al., 2005a; Gama et al., 2008; Shingfield and Griinari, 2007).

Even though dietary FO supplements cause MFD, direct measurements of biohydrogenation intermediates formed in the rumen of lactating cows under these circumstances are limited. Characterizing the changes in ruminal biohydrogenation and microbial ecology is central in understanding the mechanisms underpinning physiological responses to lipid supplements containing long-chain PUFA in lactating ruminants and the impact of different nutritional strategies on composition of milk fat, which is a major source of SFA in the human diet (Eilander et al., 2015). Dairy products are a significant source of fat in the diet in most developed countries highlighting the need to understand how to produce milk with nutritionally more beneficial FA composition.

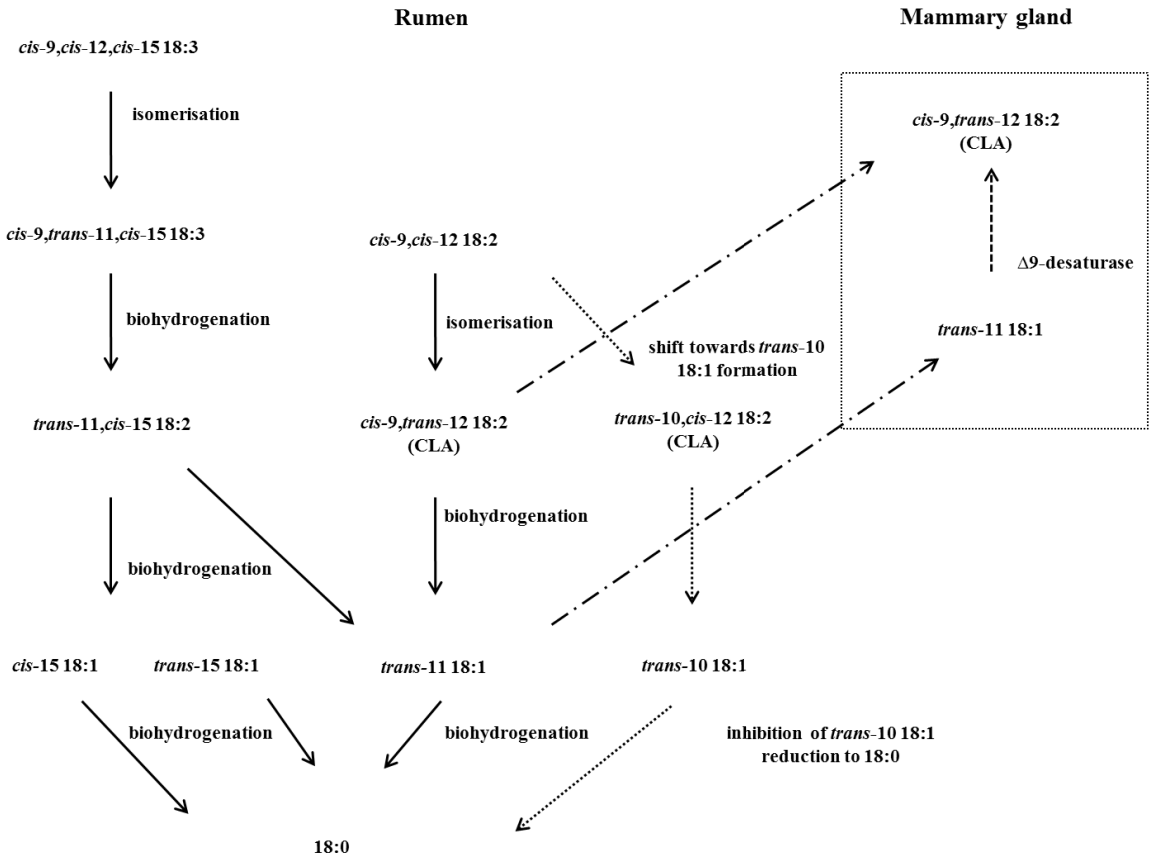

Figure 2. Major pathways of 18:2n-6 and 18:3n-3 metabolism in the rumen and changes (dashed arrows) in biohydrogenation pathway of 18:2n-6; so called *trans*-10 shift that occur on diets causing milk fat depression (modified from Shingfield and Griinari, 2007 Eur. J. Lipid Sci. Technol. 109:799-816). Arrows pointing towards mammary gland indicate the important role of Δ9-desaturase (SCD) in the appearance of *cis*-9,*trans*-11 CLA in ruminant milk fat (modified from Griinari and Bauman, 1999 in Advances in Conjugated Linoleic Acid Research Vol. 1, pp. 180-200; Bauman and Griinari, 2001 Livest. Prod. Sci. 70:15-29).

2. Aims of the thesis

The general aim of this thesis was to provide new information on the metabolism of 18-carbon MUFA and PUFA and long-chain 20- to 22-carbon n-3 PUFA, such as 20:5n-3 and 22:6n-3, in the rumen of lactating cows by studying the transformations occurring through supplementation of dairy cows' diet with FO alone or with plant oils rich in 18:2n-6 or 18:3n-6. The research findings reported in this work also provided further insight into the possible causes for FO-induced MFD and varying effects of FO on milk FA composition in lactating cows by describing the mechanisms involved in the ruminal biohydrogenation, microbial ecology and mammary lipid metabolism. In addition, this research helped to explain the appearance of FA biohydrogenation products in milk (and meat) with putative biological activity relevant to the prevention of chronic diseases in humans and novel long-chain FA intermediates whose bioactivity is still unknown.

For individual experiments, the objective was to build up methods for the analysis of long-chain 20- to 22-carbon FA intermediates formed during ruminal biohydrogenation of n-3 PUFA by combining different analytical techniques. These procedures were applied in the detailed analysis of different feed and oil supplements, omasal digesta and milk fat. The experiments were designed to investigate the effects of dietary FO supplements alone (I; II) or in combination with plant oils rich in 18:2n-6 or 18:3n-3 (III) on the ruminal and mammary lipid metabolism in order to understand the mechanisms and metabolic pathways underlying the diet-induced changes in milk fat depression (MFD), milk FA composition and specific FA intermediates and end products associated with MFD. To achieve this objective, experiments documented in I-IV encompassed detailed investigations of animal performance (II; III), ruminal lipid metabolism (I-III) and bacterial populations (II; III), the relationships between the flow of FA at the omasum and secretion of milk FA (IV) and changes in milk fat synthesis (IV) to provide further insight into the possible causes of FO-induced MFD, and evaluated the potential of dietary FO supplements to enrich long-chain n-3 FA in milk (IV). All the experimental treatments were formulated to meet the objectives of providing new information on changes in ruminal biohydrogenation of long-chain polyenoic n-3 FA and milk fat synthesis, metabolic pathways involved and specific FA intermediates and end products associated with MFD.

The main hypotheses tested in this research were:

i) Dietary FO supplements alone or in combination with plant oils inhibit the biohydrogenation of unsaturated FA in the rumen and alter the flow of specific 16- to 22-carbon FA intermediates, 20:5n-3, 22:5n-3 and 22:6n-3 at the omasum.

ii) Biohydrogenation of long-chain n-3 PUFA in FO may proceed via different reduction mechanism than the well-known biohydrogenation pathways of 18:2n-6 and 18:3n-3.

iii) Dietary plant-derived 18-carbon PUFA lower the ruminal biohydrogenation of PUFA, including 20:5n-3, 22:5n-3 and 22:6n-3, and increase the ruminal outflow of *trans*-11 18:1.

iv) The effect of FO supplements on microbial biohydrogenation may differ depending on whether 18:2n-6 or 18:3n-3 is the main source of 18-carbon PUFA.

v) Dietary FO supplements alone or in combination with plant oils modify rumen microbial communities and induce changes in the abundance of key rumen bacterial populations known to be capable of biohydrogenation.

vi) Dietary FO supplements increase the ruminal outflow of biohydrogenation intermediates that have not been previously identified, and these intermediates are transferred into milk fat. FO supplements also enrich CLA, 20:5n-3, 22:5n-3 and 22:6n-3 concentrations in milk fat.

vii) The effect of dietary FO on the flow of *trans* FA intermediates at the omasum, observed changes in milk FA composition and calculated milk fat melting point are related to FO-induced MFD.

3. Materials and methods

3.1. Experimental animals and designs

The experiments documented in I-IV were conducted as three separate experiments (Table 1). All the experimental procedures used are described in detail in I-IV, and only a brief outline is presented herein. Experiments were performed with multiparous Finnish Ayrshire dairy cows in late or mid-lactation fitted with rumen cannulae, except for exp. 2, in which 2 Finnish Ayshire and 2 Holstein-Friesian were used. The cows averaged 197 days in milk (SD 9.40), 589 kg live weight (SD 16), and 27 kg milk yield (SD 1.14) at the beginning of the experiments. Experiments were conducted as a balanced 4 × 4 Latin Square; except for exp. 1 that was carried out according to a design where all cows were fed grass silage-based diet with no additional lipid during the first experimental period followed by the same basal diet supplemented with 250 g/d FO during the second period. The experimental design with 14-d periods used in exp. 1 may be criticized due to the confounding effects of treatment with time. However, time-related effects associated with short experimental periods were considered rather minor defect compared with the importance of the detailed FA composition analyses of FO and omasal digesta combined with measurements of nutrient flow at the omasum that provided the first quantitative estimates on the ruminal outflow of long-chain n-3 PUFA biohydrogenation intermediates in lactating cows. For exp. 2 and 3, experimental periods lasted for 28 and 21 days, respectively.

3.2. Experimental treatments

All experimental treatments comprised restrictively fermented grass silage and a cereal-based concentrate (forage:concentrate ratio 60:40, on a dry matter (DM) basis) offered at 95 % of *ad libitum* intake measured during 14 d before the start of the experiment. Forages were prepared from primary growths of mixed timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) in experiments reported in I and III, and from the primary growth of tall fescue (*Festuca arundinacea*) in II and IV. The ultra-refined herring and mackerel oil (FO; EPAX 3000 TG, Pronova Biocare AS, Norway) was used in all experiments (I-IV) as a dietary supply of very long-chain n-3 PUFA. In the experiment documented in III, SO (Raisioagro Ltd., Finland) and LO (Elixi Oil Ltd., Finland) were used as sources of 18:2n-6 and 18:3n-3, respectively. All oil supplements were fed in equal amounts twice a day by mixing thoroughly with concentrate ingredients just before feeding.

Experiment reported in I was conducted to characterize the intermediates formed during ruminal biohydrogenation of long-chain PUFA in FO. The structure and composition of 20- to 24-carbon FA in FO and the omasal digesta was determined and ruminal outflow quantified in cows fed a basal diet containing no additional oil (control) or supplemented with 250 g/day of FO (treatment FO). Experiment documented in II and IV

involved a physiological study in which the effects of incremental dietary FO supplementation (0, 75, 150, or 300 g FO/d) on the flow of FA at the omasum (II), changes in the key bacterial species known to be capable of biohydrogenation (II), milk production (IV), and milk FA composition (IV) were investigated. Experiment reported in III investigated the effects of diet containing no additional oil (control), FO alone (200 g FO/day; treatment FO) or FO in combination with SO (rich in 18:2-6; 200 g FO and 500 g of SO/day; treatment SFO) or LO (rich in 18:3n-3; 200 g of FO and 500 g of LO/day; treatment LFO) on the flow of FA at the omasum and bacterial populations in lactating cows (III).

3.3. Experimental measurements

Feed intake was determined daily as the difference between the amount of feeds offered and the amount of refused feeds. For rumen fermentation measurements rumen fluid was collected at regular intervals through rumen cannula using a vacuum pump and a flexible tube (II; III). In addition, subsamples of filtered ruminal fluid were collected for visual assessment of protozoal numbers (II; III). Rumen bacterial populations known to be capable of biohydrogenation were determined from omasal digesta by quantitative polymerase chain reaction (qPCR) analysis (II; III). In I-III, the omasal sampling tehnique in combination with a triple marker system were used to assess nutrient flow entering the omasal canal as previously described by Ahvenjärvi et al. (2000). Diet digestibility was measured by total faecal collection (II; III). Daily milk yields of all experimental cows were recorded throughout each experiment. Samples for the analysis of milk composition in IV were collected and composited according to yield over two consecutive milkings on d 17, 20, 24, and 27 of each experimental period. Ruminal administration of LiCo-EDTA as indigestible marker to estimate flow of FA at the omasum in the experiment reported in II were found to alter milk fat composition (Shingfield et al., 2006b); therefore, only samples of milk collected on d 17, immediately before marker administration, were submitted for detailed FA analysis (IV). A triple marker system based on Cr-EDTA, Yb-acetate, and indigestible neutral detergent fibre (iNDF) as markers for liquid, small, and large particles of omasal digesta, respectively, were used in experiment reported in III. For all experiments, cows were housed in individual tie-stalls within a dedicated metabolism unit with continuous access to water and milked twice daily.

Fatty acid content of grass silage, concentrates, and omasal digesta was determined using internal and external standards (I-IV). Tritridecanoin (T-135; Nu-Chek-Prep Inc., Elysian, MN) was used as an internal standard for feeds (I-IV) and milk (IV) and tridecanoic acid (13:0, N-13A, Nu-Check-Prep Inc.) for omasal digesta (I-III). Trihexadecanoin (T-5888, Sigma-Aldrich, St. Louis, MO) was used to set up an external calibration curve for feed ingredients (I-III) and milk (IV) and hexadecanoic acid (S-4751, Sigma-Aldrich) for omasal digesta (I-III). Fatty acid methyl esters (FAME) of lipids in oil supplements and freeze-dried samples of silage and concentare were prepared in a one-step extraction-methylation procedure (Shingfield et al., 2003). The freeze-dried samples of reconstituted omasal digesta were adjusted to 2.0 by hydrochloric acid and extracted in duplicate

with a mixture (3:2; vol:vol) of hexane and isopropanol. Fatty acid methyl esters were prepared using a two-step base-acid catalysed procedure as described in I. Lipids in milk samples were extracted in duplicate using a mixture of ammonia, ethanol, diethyl ether, and hexane (0.2:2.5:2.5, vol/vol) and converted to FAME using methanolic sodium methoxide as a catalyst (Shingfield et al., 2003). Fatty acid methyl esters were quantified using a gas chromatography (GC) and a high-performance liquid-chromatography (HPLC) (I-IV). Fatty acid methyl esters were quantified using a GC (6890; Agilent Technologies, Wilmington, DE) fitted with a 100-m fused silica capillary column (CP-Sil 88; Chrompack 7489, Chrompack International BV, Middelburg, the Netherlands) using a temperature gradient program and hydrogen as the carrier gas (Shingfield et al., 2003). Individual isomers of 18:1 and 18:2 were further resolved in a separate analysis under isothermal conditions at 170°C (Shingfield et al., 2003). Under these conditions, *trans*-10,*cis*-15 18:2 and *trans*-11,*cis*-15 18:2 eluted as a single peak (I; II; IV). To resolve these isomers in experiment reported in III, analysis of FAME was repeated using a ionic liquid column (SLB-IL111; 100 m × 0.25 mm i.d., 0.2 µm film thickness, Sigma-Aldrich) and helium as a carrier gas (Alves and Bessa, 2014; Ventto et al., 2017).

Fatty acids were initially identified based on retention time comparisons with authentic FAME standards: Nu-Chek-Prep GLC #463; 21:0, #N-21-M; 23:0, #N-23-M; 24:0, #N-24-M; *trans*-11 20:1, #U-64-M; *cis*-12-21:1, #U-85-M; *cis*-14 23:1, #U-87-M, Larodan Fine Chemicals 18:4n-3, #10-1840; *cis*-9 20:1, #20-2001-1-4; 20:4n-3, #20-2024-1; 21:3n-3, #20- 2103-1-4; 21:5n-3, #20-2105-1-4; *trans*-13 22:1, #20-2210-9; 22:5n-6, #20-2265-7; 23:5n-3, #20-2305-1-4; 24:5n-3, #20-2405-4; 25:0, #20-2500-7; 29:0, #20-2900-7; 9-O-18:0, #14-1800-5-4; 12-O-18:0, #14-1800-6-4; 13-O-18:0, #14-1800-7-4; 16-O-18:0, #14-1800-8-39 (Malmö, Sweden), and Sigma-Aldrich 26:0, #H-6389; 27:0, #H-6639; 28:0, #O-4129; 30:0 #T-1902; mixture of 18:2n-6 isomers, #L-8404; mixture of conjugated 18:2 isomers, #O-5632; mixture of 18:3n-3 isomers, #L-6031. Fatty acid methyl esters not available as commercial standards were identified based on the fractionation of FAME by complementary silver-ion (Ag^+) thin-layer chromatography (TLC) and GC-mass spectrometry (MS) analysis of FAME and corresponding 4,4-dimethyloxazoline (DMOX) derivatives (I-IV). The distribution of CLA isomers was determined by Ag^+-HPLC (I-IV). Analysis was repeated using 2.0 % (vol:vol) acetic acid in heptane to resolve *cis*-10,*trans*-12 CLA and *trans*-10,*cis*-12 CLA (Ventto et al., 2017).

3.4. Statistical analyses

The statistical methods for each experiment have been described in detail in I-IV, only a brief outline is presented herein. Differences in FA flow at the omasum and ruminal FA balance between the control and FO treatments in I were evaluated statistically by a paired *t* test (SAS Institute Inc., Cary, NC, USA). A 95 % confidence interval was used as the default for the hypothesis test and assumptions of data normality were validated using the univariate procedure of SAS.

For II-IV, data were analysed by ANOVA with a statistical model that included the fixed effects of period and treatment and the random effect of cow using the mixed procedure of SAS. Measurements of rumen pH and fermentation characteristics were analysed by ANOVA for repeated measures with a model that included the fixed effect of period, treatment, time and treatment × time interactions and the random effect of cow assuming an Auto Regressive Order One Covariance Structure. Denominator degrees of freedom were calculated using the Satterthwaite option and the Kenward-Rogers method. For II and IV, sums of squares were further separated into polynomial contrasts to test for the significance of linear and quadratic responses to dietary FO, and for III into single degree of freedom orthogonal contrasts to test the effects due to oil supplementation (control vs. FO, SFO, and LFO), addition of 18-carbon PUFA-rich plant oils to the FO (FO vs. SFO and LFO), and different sources of 18-carbon PUFA (SFO vs. LFO).

Associations of FA flow at the omasum with milk fat yield and the output of FA in milk for individual cows were evaluated by linear regression analysis using REG procedure of SAS in IV. In addition, relationships of milk fat content and milk fat yield with concentrations of all identified FA in milk were further analysed by partial least squares regression (PLS) using the PLS procedure of SAS, with milk fat yield and milk fat content as response variables and the proportion of individual FA in milk as an explanatory variable to produce correlation loading plots (IV). The number of factors was set to 2 (sum of FA in milk based on carbon chain length and degree of unsaturation, 18-carbon FA, and isomers of 20- to 22-carbon FA) or all 3 factors (for all identified FA) using the leave-one-out cross validation procedure, based on minimizing the predicted residual sum of squares.

3.5. Summary of experimental designs

Table 1 Description of experiments reported in this thesis

Publ	Exp.	Animals	Design	Dietary ingredients	Treatments	Measurements and objectives
I	1	5 rumen fistulated dairy cows	2 subsequent periods, 2 diets	Grass silage (GS) Standard concentrate (C)	GS:C ratio = 60:40	Ruminal metabolism of long-chain fatty acid (FA) in FO
				No additional lipid (Control) 250 g/d additional fish oil (FO)	Control FO	Identification of novel long-chain FA intermediates on the digesta of cows fed FO
II, IV	2	4 rumen fistulated dairy cows	4 × 4 Latin Square	Grass silage (GS) Standard concentrate (C)	GS:C ratio = 60:40	Effect of incremental amounts of FO in the GS based diet on o DM intake o Ruminal lipid metabolism o Rumen fermentation characteristics o Rumen microbial communities o Milk production o Milk FA composition o Milk fat depression o Transfer efficiency of FA from feed to milk
				No additional lipid (Control) 75 g/d additional FO (FO75) 150 g/d additional FO (FO150) 300 g/d additional FO (FO300)	Control FO75 FO150 FO300	
III	3	4 rumen fistulated dairy cows	4 × 4 Latin Square	Grass silage (GS) Standard concentrate (C)	GS:C ratio = 60:40	Effect of FO supplementation alone or in combination with plant oils rich in 18:2n-6 and 18:3n-3 in the GS based diet on o DM intake o Ruminal lipid metabolism o Rumen fermentation characteristics o Rumen microbial communities
				No additional lipid (Control) 200 g/d additional FO 200 g/d FO plus 500 g/d sunflower oil (SFO) 200 g/d FO plus linseed oil (LFO)	Control FO SFO LFO	

4. Results and discussion

4.1. Analysis of long-chain fatty acids by chromatography

As a consequence of the complex nature of ruminant derived lipids and FA, the analytical methods used are important when studying lipid metabolism of dairy cows. This chapter describes the analytical methods and challenges of this thesis work.

Chromatography is one of the most effective analytical procedures for separating and analysing the properties of lipids. In chromatographic analysis molecules are separated based on different affinities while passing through a matrix. After being separated, the concentration of each of the molecules is determined as they pass by a suitable detector (e.g., UV-visible, fluorescence, or flame ionization) or the molecules are collected in different fractions for further analysis usually by other chromatographic methods. Various forms of chromatography are available to analyse the lipids in feeds and biological samples, such as tissues and body fluids, e.g. TLC (I), GC (I-IV), and HPLC (I-IV).

Thin layer chromatography is used mainly to separate and determine the concentration of different types of lipid groups, e.g. free FA, tri-, di-, and monoacylglycerols, cholesteryl esters, and PL. A TLC plate is coated with a suitable absorbing material. A small amount of the lipid sample to be analysed is applied onto the TLC plate. The different lipid fractions are separated on the basis of their affinity for the absorbing material. It is possible to identify the lipid fractions in the sample by using reference compounds. Fractions are scraped off from the plate and analysed further using e.g. GC-flame ionization detection (FID) or GC-MS. For details see more information from the LipidWeb, 2019.

It is difficult to analyse intact lipid molecules and free medium- and long-chain FA using GC because they are not very volatile. For this reason, the free FA and esterified FA for example in TAG, PL and GL are usually derivatized prior to analysis to increase their volatility. The most commonly used FA derivatives in GC analysis are FAME. As the FAME pass through the GC capillary column, they are separated based on differences in their molecular weights and polarities, and the FAME are quantified using FID or MS as detectors.

The identification of biohydrogenation intermediates of FA by GC is challenging due to the lack of reference compounds and small concentration of some intermediates. The chromatographic resolution may also be insufficient due to the extensive number of FA compounds present in the samples. Especially FAME that differ in double bond position and double bond configuration may co-elute in GC analysis. Long capillary columns with highly polar cyanopropyl siloxane stationary phase, e.g. the 100m long CP-Sil 88 column (I-IV), can be used in the successful separation of most of the *cis* and *trans* 18:1, 18:2 and 18:3 isomers that are found in ruminant-derived samples. However, even with this column type different GC oven temperature programs are sometimes needed to achieve a sufficient separation. However, in recent years the SLB-IL111 column has been report-

ed to provide improvements on the resolution of FAME that are co-eluting with highly polar cyanopropyl siloxane stationary phases (Alves and Bessa, 2014).

Gas chromatography in combination with MS (I-IV) has been used most often for structure determination of FA as different derivatives, such as the use of nitrogen containing derivatives, e.g. picolinyl (Harvey, 1984; 1992; Christie et al., 1986; Christie, 1998) and DMOX (Zhang et al., 1988; Spitzer, 1997) derivatives. In this research project FA were quantified as FAME using GC-FID and reference FAME compounds were used in identification. Methyl ester and DMOX derivatives of FA were also analysed by GC-MS using electron ionization in order to characterize the FA structures (I-IV). The various ionic species produced from a given FA derivative by electron ionization are separated according to mass/charge (m/z) ratio in which z = 1 in a magnetic field, and a spectrum is obtained that shows the masses of the fragment ions and their abundances relative to the most abundant ion (base ion).

Initial identification of FA carbon chain length and number of double bonds in experimental samples were based on GC–MS analysis of FAME. For example molecular ions at m/z 324, 322, 320, 318, 316, 338, 334, 332, 330, 352, 350, 348, 346, 344, 342, 358, 372 and 370 confirmed the appearance of 20:1, 20:2, 20:3, 20:4, 20:5, 21:1, 21:3, 21:4, 21:5, 22:1, 22:2, 22:3, 22:4, 22:5, 22:6, 23:5, 24:5 and 24:6 methyl esters, respectively, but the mass spectrum did not contain sufficient characteristic ion fragments to locate the position of double bonds or other specific structural characteristics. Therefore, the structure of long-chain FA contained in FO, omasal digesta and milk fat from cows fed FO were formally identified based on GC–MS analysis of DMOX derivatives (I-IV). As an example, the mass spectrum of the DMOX derivative of *trans*-9,*cis*-14,*cis*-17 20:3 is shown in Figure 3. The McLafferty ion at m/z = 113 is the expected base peak, accompanied by a prominent ion at m/z = 126. Fragmentation of the carbon chain of the FA gives rise to a series of abundant ions that are 14 atomic mass units (amu) apart. The molecular ion (m/z = 359) was used to define the chain length and number of double bonds, and three ions separated by gaps of 12 amu at m/z 196 and 208; 264 and 276; 304 and 316 (Figure 3) were used to locate double bonds at positions Δ9, Δ14, and Δ17, respectively. From the obtained spectra it was possible to confirm that the peaks were FA with a certain molecular weight using the McLafferty ions and molecular ions, but in all cases it was not possible to deduce the detailed structure of the FA, e.g. locate the double bond positions, in which case the peak was considered as an unidentified FA. Characteristic ion fragments in the mass spectrum of DMOX derivatives used to locate the position of double bonds of 20-, 21- and 22-carbon unsaturated FA in omasal digesta are listed in detail in I. The signals from diagnostic ions usually become weaker and the number of interfering ions from other fragments increases with the increasing number of double bonds. Thus, interpretation of spectra from highly unsaturated FA is challenging. Some of the biohydrogenation intermediates of highly unsaturated FA were present in minor amounts in the samples obtained in this research and thus it was often difficult to acquire spectra of sufficient quality.

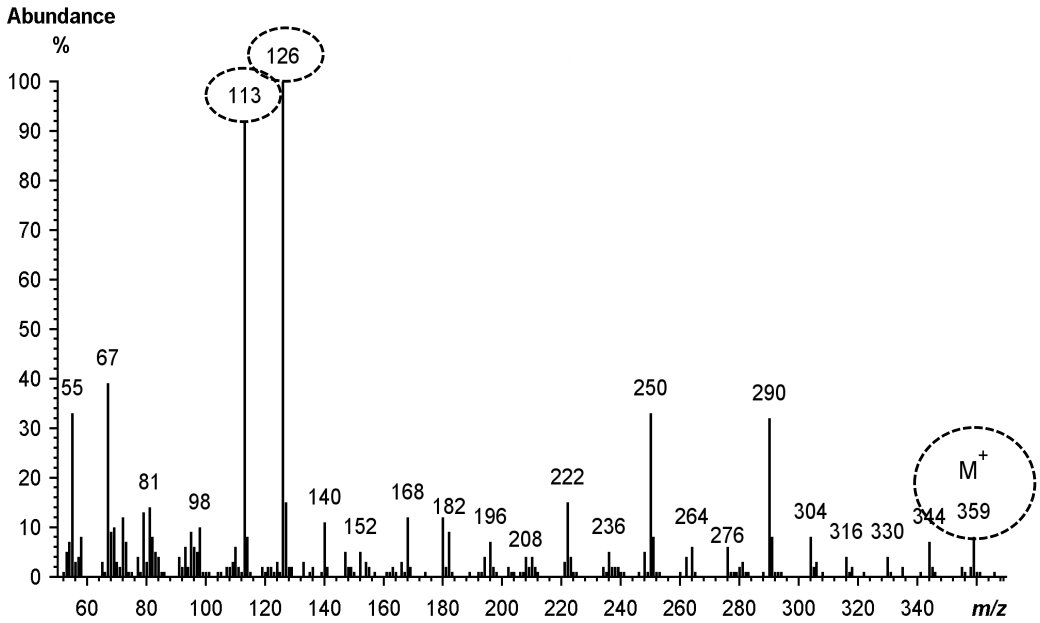

Figure 3. Gas chromatography-electron ionization mass spectrum of the 4,4-dimethyloxaline (DMOX) of *trans*-9,*cis*-14,*cis*-17-20:3 detected in omasal digesta of cows fed grass silage based diet containing fish oil (modified from I).

By cross-referencing peaks in the total ion chromatogram of DMOX and FAME derivatives with the relative retention time and elution order of FAME in the GC-FID chromatogram, minor FA in omasal digesta and milk could be identified. While it is generally accepted that DMOX derivatives exhibit chromatographic properties comparable to those of FAME (Spitzer, 1997; Christie, 1998) under the chromatographic conditions used in this thesis work we observed slight variations in the relative retention and elution order of FAME and DMOX derivatives which introduced major challenges in the attempts to identify the structure of minor components in samples containing a highly complex mixture of FA. An extensive overview of selected regions of the total ion chromatogram for DMOX derivatives prepared from total lipid in omasal digesta of cows fed the FO treatment is given in I. For example, during the analysis of DMOX derivatives 18:3n-3 eluted after *cis*-13 20:1 and 22:0 eluted before *cis*-9,*trans*-11,*cis*-15 18:3, *cis*-14,*cis*-17 20:2 and *trans*-9,*trans*-14,*trans*-17 20:3, whereas the reverse was true during the analysis of methyl esters. Relative retention times of *trans*-11,*cis*-14,*cis*-17 20:3, 20:3n-3, 20:5n-3, *cis* (Δ11-15) 22:1, *trans*-12,*trans*-17 22:2, Δ10,13,17-22:3, *trans*-10,*trans*-13,*cis*-16,*cis*-19 22:4, 22:4n-3, *trans*-5,*cis*-10,*cis*-13,*cis*-16,*cis*-19 22:5, 23:0, 24:0, *cis*-16 25:1 and 26:0 were also found to differ when analysed as a methyl ester or DMOX derivative.

The number and positions of double bonds can also be determined by pre-fractionations of the samples by different HPLC or TLC techniques. Development of Ag^+-TLC plates with hexane and diethyl ether allowed 18-, 20, and 22-carbon *cis*-monoenoic

isomers to be resolved from *trans* monoenoic methyl esters, but did not result in the complete separation of methyl esters of long-chain dienoic and trienoic FA (I). In addition, a fraction containing FA with four to six double bonds remained at or near the origin. However, fractionation of FAME according to the degree of unsaturation and double bond geometry by Ag^+-TLC allowed *cis* and *trans* monoenoic FA to be completely resolved, confirming the configuration of double bonds for 20:1 and 22:1 isomers.

Despite the fractionation of FAME prior to the conversion of DMOX derivatives, it was not possible to elucidate the structure of all 20- and 22- carbon PUFA containing two of more double bonds due to a very low abundance and/or co-elution with other components. A mixture of hexane and diethyl ether was used to develop Ag^+-TLC plates based on reports that have shown this solvent system to resolve *trans* 18:1 and *cis* 18:1 isomers in bovine milk fat (Precht and Molkentin, 1996; Cruz-Hernandez et al., 2004; 2006), and therefore applied in the analysis of omasal digesta due to the occurrence of *cis* and *trans* 20:1 and *cis* 22:1 isomers. Previous investigations have reported the separation of methyl esters of *cis* 20:1, *cis* 22:1, *trans* 20:1 and *trans* 22:1, and geometric isomers of 20:5n-3 and 22:6n-3 by Ag^+-TLC using plates developed with a mixture (50:50, v/v) of toluene and hexane (Wilson et al., 2000) or a mixture (85:15, v/v) of toluene and methanol (Fournier et al., 2006a; 2006b) but the use of alternative solvents was not explored further in our studies. Methyl esters of 22-carbon FA containing four or more double bonds were not resolved by Ag^+-TLC, which, combined with the inability of GC–MS analysis of DMOX derivatives to distinguish between geometric isomers, meant that inferences on double bond geometry of specific biohydrogenation intermediates in omasal digesta of cows fed FO had to be drawn on the basis of retention times and order of elution relative to authentic standards during GC analysis (I). The main drawbacks of the above-mentioned methods (Ag^+-TLC, GC-FID, GC-MS) are that pre-fractionation of samples, preparation of derivatives and visual interpretation of mass spectra are laborious processes.

Techniques applied to the analysis of FA composition allowed carbon chain length and double bond position for most of the 20- and 22-carbon biohydrogenation intermediates in omasal digesta to be identified, but the structure of several minor FA remained unknown (I). Furthermore, double bond geometry of most polyenoic biohydrogenation intermediates had to be inferred rather than unequivocally determined. Additional investigations based on GC–MS analysis of other FA derivatives including picolinyl esters (Christie et al., 1986; Christie, 1998), or analysis of FAME for example by covalent adduct chemical ionization tandem MS (Michaud et al., 2003; Gómez-Córtes et al., 2009), two-dimensional GC analysis (Vlaeminck et al., 2007), GC-Fourier infrared spectroscopy-MS (Wahl et al., 1994), reversed-phase HPLC (Banni et al., 1996) and Ag^+-HPLC (Fournier et al., 2006b) could further help in verifying and characterizing the double bond geometry of polyenoic biohydrogenation intermediates formed during the hydrogenation of long-chain unsaturated FA in the rumen.

4.2. Impact of dietary fatty acids on nutrient intake, digestibility and rumen fermentation characteristics

4.2.1. Fatty acids in feeds

Dairy diets are mixtures of fresh or conserved forages and concentrates, all of which contain lipids. These lipids can be characterised as structural or polar membrane lipids, such as GL and PL, free FA, TAG, and sterol esters. In forages, GL and PL predominate, whereas the main components in cereals, oil seeds, animal fats, and by-product feeds are TAG (Harfoot and Hazlewood, 1988). Diets consumed by lactating cows are low in fat content, generally containing only about 40 to 50 g/kg DM of total fat. The predominant PUFA in ruminant diets are 18:2n-6 and 18:3n-3, with 18:2n-6 being a major component of maize silage, plant oilseeds and cereals, whereas 18:3n-3 is a major component of grass forages and linseed (Elgersma, 2015). Moreover, some plant oilseeds provide MUFA (mainly *cis*-9 18:1), whereas marine products (FO, marine algae) provide long-chain 20- and 22-carbon n-3 PUFA (mainly 20:5n-3 and 22:6n-3; Chilliard et al., 2007). Summary of mean FA composition (g/100 g FA) and total FA content (g/kg of DM) of some common feed ingredients and oil supplements is presented in Table 2.

4.2.1.1. Fatty acids in grass silage and concentrates

Consistent with previous reports (Halmemies-Beauchet-Filleau et al., 2013a; Elgersma, 2015), for all forages, 18:3n-3 represented on average 46–62 % of total FA, followed by 16:0 (14–17 %) and 18:2n-6 (12-19 %) (I–IV). Typically, concentrates, in particular cereals and plant oilseeds, contain relatively high concentrations of 18:2n-6, *cis*-9 18:1 and 16:0 (Elgersma, 2015). In the experiments reported in I and III, concentrate supplements fed were comprised of rolled barley, solvent extracted rapeseed meal and molassed sugar beet pulp and therefore the lipid in the diets was relatively rich in 18:2n-6 (on average, 37 % of total FA), *cis*-9 18:1 (29 %) and 16:0 (16 %) with few differences in FA composition and content between these experiments. However, in experiment documented in II and IV the content of *cis*-9 18:1 was 36 % because rapeseed expeller was used instead of solvent extracted rapeseed meal and concentrate supplement contained rolled oats.

4.2.1.2. Fatty acids in oil supplements

In experiment reported in III, SO and LO were fed in combination with FO as sources of plant-derived 18:2n-6 and 18:3n-3, respectively. As expected, ultra-refined herring and mackerel oil supplements fed in I-III were relatively rich in 20:5n-3, 22:5n-3, 22:6n-3, and appropriate sources of several other long-chain PUFA not contained in other feed ingredients. The FA composition of refined FO is in a good agreement with other published values in the literature showing that marine lipids from fish, mammals, plankton or algae are rich sources of long-chain (20- to 22-carbon) PUFA, of which 20:5n-3 and 22:6n-3 are the most important (Ackman, 1992). However, the FA composition of FO may vary great-

ly according to fish species, season and geographical location, with values of 4–32 % of total FA for 20:5n-3 and 2–27 % for 22:6n-3, and there may be also a great variability for the other major FA, i.e. 14:0, 16:0, 16-, 18-, 20- and 22-carbon MUFA (Belling et al., 1997; Moffat and McGill, 1993; Vlieng and Body, 1988).

4.2.2. Effect on nutrient intake and digestibility

Increasing levels of FO (II) and when supplementing the FO diet with plant oils (III) decreased intakes of grass silage DM, total DM, organic matter, neutral detergent fibre (NDF), potentially digestible NDF and crude protein, which were lowered yet further when FO was fed with plant oils (III), even though cows were fed at a restricted level of intake in both cases (II; III). It is well established that FO and FO plus plant oils cause dose-dependent decreases in intake in lactating cows (Keady et al., 2000; Palmquist and Griinari, 2006; II; III), but the mechanisms involved are not well defined. The adverse effects of higher PUFA intake in cows have been attributed to several mechanisms including changes in ruminal fermentation pattern and microbial communities involved in fibre digestion as well as a tendency to shift the site of nutrient digestion from the rumen to the intestines, and elevated plasma gut peptide concentrations (Allen, 2000).

The decrease in DM intake to oil supplements were not accompanied by adverse effects on ruminal or total tract nutrient digestion, but seemed to increase whole tract DM digestion (II; III), which may be explained by longer ruminal retention time caused by lower intake (Huhtanen and Kukkonen, 1995). Overall, our data suggest that inclusion of FO alone and in combination with plant oils in the diet has no negative impact on rumen function, consistent with earlier studies indicating that moderate amounts of FO (Lee et al., 2008; Shingfield et al., 2010b), plant oils (Bayat et al., 2018; Shingfield et al., 2008a) or their blend (Toral et al., 2010) do not depress nutrient digestion in ruminants.

As expected, dietary oil supplements increased the intakes of several MUFA and PUFA (II; III). Supplementing the diet with FO increased the intake of most long-chain PUFA other than 18:2n-6, including 16:2n-4, 16:3n-4, 16:4n-1, 18:1n-9, 18:4n-3, 20:3n-3, 20:4n-6, 20:4n-3, 20:5n-3, 21:5n-3, 22:3n-3, 22:5n-3, and 22:6n-3 (II; III), whereas the intakes of *cis*-9 18:1 and 18:2n-6 were substantially higher for SFO than LFO by design (III), and accordingly 18:3n-3 intake was greater for LFO compared with SFO (III).

4.2.3. Effect on rumen fermentation characteristics

Dietary FO supplements alone or in mixture with plant oils had no effect on mean rumen pH (6.58–6.68) (II; III). This is consistent with previous findings in cattle fed different lipid sources, including FO (Keady and Mayne, 1999) and SO (Shingfield et al., 2008a), whereas in some cases FO supplementation of 250 g/day (Shingfield et al., 2003) or 420 g/day (Toral et al., 2016a) has been reported to increase ruminal pH in lactating cows.

Ammonia N concentrations were not affected by oil supplements (II; III) consistent with earlier findings in lactating cows fed FO (Shingfield et al., 2003; Toral et al., 2016a) or in nonlactating sheep fed a high-concentrate diet supplemented with a combination

of FO (10 g/kg) and SO (20 g/kg; Toral et al., 2009). However, previous investigatios have demonstrated that increasing level of dietary SO tends to decrease ammonia N concentrations in the rumen of lactating cows (Shingfield et al., 20008b), whereas supplementing the diet with FO increases it over sampling time in cattle (Keady and Mayne, 1999).

In the experiments of the present thesis, oil supplements decreased (II) or had no effect (III) on ruminal volatile FA (VFA) concentrations, but FO at high amounts (II) or when supplemented with plant oils (III) promoted an increase in molar proportions of propionate (3:0) (II; III) and butyrate (4:0) (II) at the expense of acetate (2:0) (II; III). From these findings, reduced acetate and total VFA concentrations and increases in propionate confirmed earlier observations of rumen fermentation characteristics in lactating cows (Doreau and Chilliard, 1997; Shingfield et al., 2003; Toral et al., 2016a). On the contrary with observed increases in the ruminal proportions of butyrate in our research (II), butyrate concentrations have been also reduced in lactating sheep (Frutos et al., 2018) and goats (Toral et al., 2016a) fed FO. Observed decreases in ruminal butyrate to FO supplements have often been attributed to the adverse effects of unsaturated FA on the growth and microbial activity of specific populations of rumen cellulolytic bacteria, including *Butyrivibrio*-like bacteria, which produce butyrate and are known to be sensitive to the bacteriostatic effects of PUFA (Jenkins et al., 2008), and the selective changes in the ruminal bacterial community capable of biohydrogenation may partly explain the variable effects of FO supplements on rumen VFA profiles (Belenguer et al., 2010). Among long-chain FA, unsaturated FA are more detrimental to rumen microbes than saturated ones (Harfoot and Hazlewood, 1988).

The main effects on rumen fermentation characteristics were relatively minor in response to 75 or 150 g FO/d, the main changes occurring in diets supplemented with 300 g FO/d (II). At relatively low amounts FO has usually rather small effects on rumen fermentation parameters in lactating cows, but higher levels of FO or FO fed with SO, have been shown to induce larger changes in the relative proportions of gluconeogenic (3:0 as a major hepatic gluconeogenic substrate) and lipogenic (also called as ketogenic compounds, including 2:0 and 4:0) fermentation end products (Palmquist and Griinari, 2006; Shingfield et al., 2003; 2008b). Furthermore, observed changes in molar VFA proportions were more pronounced for SFO and LFO compared with FO, with no evidence of differences due to the source of plant oil (III).

Table 2 Mean fatty acid composition (g/100 g fatty acids) and total fatty acid content (g/kg of dry matter) of some common feed ingredients and oil supplements

	14:0	16:0	16:1 *cis*-9	18:0	18:1 *cis*-9	18:2n-6	18:3n-3	20:1 *cis*-11	20:5n-3	22:5n-6	22:5n-3	22:6n-3	∑Fatty acids	Reference
Grass silage[1]	0.4	16	0.3	1.3	2.9	16	55						22	I, IV
Maize silage	0.5	17	0.3	2.3	21	49	5.6	0.2					24	Shingfield et al., 2011
Red clover silage[2]	0.6	21	0.1	5.6	3.9	19	37						31.8	Vanhatalo et al., 2007
Grass hay[3]	0.5	21	0.3	1.9	3.3	19	40						8.6	Halmemies-Beauchet-Filleau et al., 2013a
Alfalfa	2.4	25		5.6	2.3	14	41							Toral et al., 2016b
Barley		20		1	12	58	9						26	Jakobsen, 1999
Maize grain		13		2	33	50	2						45	Jakobsen, 1999
Oats		19		1	33	44	3						44	Jakobsen, 1999
Wheat		21		2	14	58	5						22	Jakobsen, 1999
Camelina oil	0.1	5.6	0.1	2.4	12	16	37	15					954	Bayat et al., 2015
Linseed oil		4.2		2.7	17	16	58	0.2					953	IV
Maize oil	0.1	13	0.1	1.9	32	49	0.2	0.3						Ai et al., 2014
Olive oil		10	0.7	2.7	77	5.4	0.5	0.2						Ai et al., 2014
Palm kernel oil[4]	15	17		2	15	1								Jakobsen, 1999
Palm olein[5]	1.0	40	0.3	4.2	43	11	0.3							Dorni et al., 2018
Rapeseed oil		4.5		1.4	61	23	9.9							Rego et al., 2009
Safflower oil		6.7		2.3	15	76							953	Bell et al., 2006
Soybean oil		12		3.9	25	54	5.2							Dorni et al., 2018
Sunflower oil	0.1	6.1	0.1	3.6	27	60	0.1	0.2					902	IV
Fish oil[6]	7.3	15	7.8	2.8	9.9	1.3	1.0	1.5	16	0.3	1.8	10	942	I, II, IV
Marine algae[7]	9.9	25	1.6	5.8					1.4	15	0.3	37	955	Toral et al., 2012

[1]Mixture of meadow fescue x timothy (*Festuca pratensis* x *Phleum pretense*); [2]The mean of fatty acid content of early and late cut of red clover (*Trifolium pratense*); [3]The mean of fatty acid content of grass hays from two different experiments; [4]Palm kernel oil is derived from the kernel of the palm fruit of E. Guineesis, and contains also a substantial amount of medium-chain fatty acids (8:0, 10:0, and 12:0); [5]Palm oil is is extracted from the flesh of the fruit of E. Guineesis. When the semi-solid palm oil is refined, it separates into liquid palm olein and palm stearine. Although semi-solid palm oil and liquid palm olein are produced from the same plant and share many similar properties, the main difference between them is their chemical state at room temperature. [6]Ultra-refined herring and mackerel oil used in the experiments 1-3; [7]Fatty acid composition reported as % of free fatty acids.

4.3. Lipid metabolism in the rumen

4.3.1. General changes in the omasal flow of fatty acid biohydrogenation products and saturated fatty acids

Changes in omasal flow of NEFA in response to FO supplements were characterized by increases in 14:0, 16:0, total 16:1, 16:2, 18:1, 18:2, MUFA, PUFA, and a wide range of *trans* FA (II; Figure 4). Accordingly, inclusion of FO alone or plant oils with FO in the diet increased the flow of 16:0, total 16:1, 18:1, 18:2, *trans* FA, MUFA, and PUFA at the omasum (III; Figure 4). In addition, increasing levels of FO decreased omasal flow of 18:0 and total SFA (II), although when compared with the control, oil supplements had no effect on the amount of 18:0 and SFA leaving the rumen (III). The same was true when FO was compared with SFO and LFO, but the flows of 18:0 and SFA differed between SFO and LFO (III). Moreover, plant oils increased the amount of *trans* 18:1, total 18:2, *trans* FA, MUFA, and PUFA at the omasum compared with FO alone, with the amounts of total 18:2, *trans* FA, and PUFA being higher for LFO than SFO (III). These findings are consistent with previous investigations in lactating (Shingfield et al., 2003) and growing cattle (Kim et al., 2008; Lee et al., 2008, Shingfield et al., 2010b; 2011) indicating that dietary PUFA are generally having a common influence on ruminal metabolism by inhibiting the last step of biohydrogenation and resulting in the accumulation of several monoenoic, dienoic and polyenoic FA in the rumen.

Incremental levels of FO in the diet increased the amount of certain OBCFA at the omasum, including *iso* 13:0, *anteiso* 13:0, *iso* 15:0, *anteiso* 15:0, 3,7,11,15-tetra-methyl-16:0, *cis* (Δ6, 7, 10 and 11) 17:1, *trans*-10 17:1, and *cis* (Δ10–12) 19:1 (II), that originate from membrane lipid of rumen bacteria, and have been suggested to represent a proxy of the microbial community in the rumen (Vlaeminck et al., 2006; Fievez et al., 2012). However, dietary oil supplements had relatively minor effects on ruminal escape of OBCFA (III). In cattle, marine lipids in the diet has been reported to decrease (Boeckaert et al., 2007), have no effect (Or-Rashid et al., 2008), or increase (Boeckaert et al., 2008) concentrations of ruminal OBCFA, indicating that dietary lipid supplements in lactating cows may have variable effects on ruminal outflow of specific OBCFA.

Consistent with previous reports in sheep (Kitessa et al., 2001; Toral et al., 2010), FO in the diet increased the ruminal outflow of 10-OH-18:0+9-O-18:0, varying from 0.12-0.85 (control) to 0.43-1.41 g/d (75-300 g/d FO) (I-II), and 10-O-18:0, varying from 1.48-2.00 (control) to 5.97-19.4 g/d (75-300 g FO/d; I-II), but had no effect on 13-O-18:0 leaving the rumen (I-III). Inclusion of plant oils with FO enhanced yet further the ruminal outflow of 10-O-18:0, being 0.77, 7.29, 32.3 and 17.3 g/d for the control, FO, SFO and LFO respectively (III). Incubations of FA substrates with rumen fluid or pure cultures of rumen bacteria have shown that *cis*-9 18:1 (Hudson et al., 1995; Jenkins et al., 2006; McKain et al., 2010) and *trans*-10 18:1 (McKain et al., 2010) can be hydrated to yield 10-OH-18:0, which is further oxidized to 10-O-18:0. Rumen bacteria are also known to be

capable of converting 18:2n-6 to *cis*-9,13-OH 18:1 (Hudson et al., 1998). Identification of 9-0-18:0, 10-OH-18:0, 10-O-18:0 and 13-O-18:0 in omasal digesta of cows fed FO (I-III) provided clear evidence that dietary FO causes an increase in ruminal outflow of oxygenated 18-carbon FA in cattle, but it remains uncertain if the accumulation of oxygenated 18-carbon FA is due to higher *cis*-9 18:1 intakes and/or ruminal *trans*-10 18:1 concentrations (Jenkins et al., 2006; McKain et al., 2010), or whether one or more FA in FO promote the hydration of unsaturated 18-carbon PUFA, or alternatively inhibit further tranformations of 18-carbon oxygenated FA in the rumen.

4.3.2. Biohydrogenation intermediate products of 16-carbon fatty acids

Supplementing the diet with FO increased the flow of 16-carbon FA (Figure 4) and resulted in the appearance of several *trans*-16:1 (Δ6–14) and *trans*-16:2 (Δ9,14; 10,14; 11,15) isomers at the omasum (II; III) confirming earlier findings in cattle (Shingfield et al., 2010b; 2011) that one or more FA in FO inhibit also the complete reduction of 16-carbon unsaturated FA to 16:0 in the rumen. The metabolic fate of 16-carbon unsaturated FA in the rumen is not well defined, but comparisons of the FA composition of FO and omasal digesta suggest that the formation of *trans*-16:1 and -16:2 isomers originate from incomplete biohydrogenation of 16:2n-4, 16:2n-7, 16:3n-4, 16:4n-1 and 16:4n-3 present in FO (II; Shingfield et al., 2010b; 2011). Inclusion of plant oils with FO altered the biohydrogenation of 16-carbon unsaturates, promoting the formation of *trans*-10-containing products, such as *trans*-10 16:1 (-, 0.54, 0.70 and 1.30 g/d for control, FO, SFO and LFO, respectively) and *trans*-10,*trans*-14 16:2 (-, 0.10, 0.15 and 0.26 g/d, respectively) in the rumen (III), suggesting that the mechanisms involved are common to both 16- and 18-carbon unsaturated FA (refer to next chapter 4.3.3).

4.3.3. Biohydrogenation intermediate products of 18-carbon fatty acids

Incubations with rumen fluid have established that very long-chain PUFA originating from FO, such as 20:5n-3 and 22:6n-3, inhibit the complete hydrogenation of 18-carbon unsaturated FA causing *trans* 18:1 isomers to accumulate (AbuGhazaleh and Jenkins, 2004; Klein and Jenkins, 2011). The data presented in this research confirms the contribution of dietary supply of unprotected FO to the inhibition of ruminal 18-carbon FA metabolism *in vivo* in lactating cows (I-II; Toral et al., 2016a). Supplementing the diet with additional FO alone resulted in higher flows (g/d) of 18:1 and 18:2 isomers at the omasum (Figure 4 and 5), changes characterized by an increase in *cis* (Δ11, 13, 15, 16) 18:1, *trans* (Δ6–8, 10–13, 15) 18:1, *cis*-9,*trans*-12 18:2, *trans*,*cis* (Δ9,12; 10,15; 11,15; 12,15) 18:2, and *trans*,*trans* (Δ9,14; 10,15; 11,14) 18:2 at the omasum (II; III; Table 3 for most *trans* 18-carbon MUFA and PUFA). The ruminal outflow of non-conjugated 18:2 biohydrogenation intermediates, such as 18:2n-6 (6.48, 4.59, 3.87 and 6.84 g/d for 0, 75, 150 and 300 gFO/d, respectively), unresolved *trans*-11,*cis*-15 18:2 and *trans*-10,*cis*-15 18:2 (Table 3), and *trans*,*trans* (Δ11,14; 11,15) 18:2 (Table 3), increased especially when supplementing the diet with incremental levels of FO (II). At the same time the flow of

18:3n-3 (1.85, 1.34, 1.07 and 1.41 g/d, respectively) at the omasum decreased, but had no effect on *cis*-9,*trans*-11,*cis*-15 18:3 leaving the rumen (II). These findings are consistent with previous investigations in lactating (Shingfield et al., 2003) and growing cattle (Kim et al., 2008; Lee et al., 2008; Shingfield et al., 2010b; 2011), indicating that one or more FA in dietary FO inhibit the complete hydrogenation of 18-carbon unsaturated FA to 18:0 in the rumen, resulting in the accumulation of numerous *trans* 18:1 and *trans* 18:2 biohydrogenation intermediates (Table 3). On the other hand, increased ruminal outflow of *cis* 18:1 isomers, *cis*-13 18:1 particularly, may also indicate that supplementation of marine lipid sources rich in 22:6n-3 not only limited *trans* 18:1 (Figure 4; Table 3) but also *cis* 18:1 saturation (e.g., II; Toral et al., 2012; 2017).

Inclusion of plant oils with FO increased the amount of *cis* (Δ15, 16) 18:1, *trans* (Δ6–8, 10) 18:1 (Figure 5; Table 3), and 18:2 intermediates other than *cis*-11,*cis*-14 18:2 compared with FO (III). Furthermore, the source of plant oil had a major influence on the amounts of specific 18:1, such as *cis* (Δ11, 15) 18:1, *trans*-11 18:1 (Figure 5; Table 3) and 18:2 intermediates leaving the rumen (III). Despite of a similar intake of 18-carbon PUFA and similar flow of *trans* 18:1, the flow of 18:0 (Figure 4) at the omasum was lower and accumulation of *trans* 18:2 intermediates (Table 3) greater for LFO than SFO (III). Such findings indicate that the inhibitory effects of FO on the reduction of 18-carbon FA to 18:0 are influenced by the relative amounts of 18:2n-6 and 18:3n-3 in the diet, i.e. the number of double bonds in the 18-carbon FA supplements. Oil supplements decreased flow of 18:2n-6 (17.8, 11.9, 9.94 and 6.76 g/d for control, FO, SFO and LFO, respectively) and 18:3n-3 (7.42, 5.86, 2.67 and 4.37 g/d, respectively) at the omasum, with the amount of 18:3n-3 being lower when plant oils were fed compared with FO alone (III). Comparison of these findings with other published research results is challenging because direct measurements of biohydrogenation intermediates formed in the rumen of lactating cows under *in vivo* conditions are limited, especially when FO has been used as a lipid source in a combination with plant oil supplements rich in 18:2n-6 and 18:3n-3.

Fish oil supplemented treatments (II; III) had no substantial influence on the flow of *cis*-9,*trans*-11 CLA (Table 3) or total CLA (Figure 5; Table 3) at the omasum, but altered the relative abundance of positional and geometric isomers of Δ9,11 and Δ10,12 CLA in omasal digesta (Table 3), confirming that *trans*-10 18:1 (Figure 5; Table 3) would not arise from the common biohydrogenation pathway of 18:2n-6 (Figure 2). These findings are consistent with previous investigations in ruminants fed diets containing FO alone (Shingfield et al., 2003) or in a combination with plant oils rich in 18:2n-6 or 18:3n-3 (Toral et al., 2010; Shingfield et al., 2011). Irrespective of diet, *cis*-9,*trans*-11 was the major isomer of CLA in omasal digesta (58-72 and 26-51 % of total CLA for II and III, respectively), confirming previous reports in lactating cows (Shingfield et al., 2003; 2008a; Loor et al., 2005c).

Incremental levels of FO in the diet increased the omasal flow of *trans*-8,*cis*-10 CLA, but lowered the amount of *trans*,*trans* (Δ11,13; 12,14; 11,13) at the omasum (II; Table 3). Compared with control, the omasal flow of *trans*-7,*cis*-9 CLA and *trans*,*trans* (Δ8,10; 9,11; 10,12) CLA increased, and *trans*-11,*cis*-13 CLA and *trans*-11,*trans*-13 CLA

decreased in response to oil supplementation (III; Table 3). Supplementing the diet with incremental levels of FO had no effect on *trans*-10,*cis*-12 CLA flow, but quadratically increased *trans*-9,*cis*-11 CLA at the omasum, reaching a maximum for additional FO of 150 g/d (II; Table 3). However, relative to FO, plant oils plus FO resulted in higher *trans*-10,*cis*-12 CLA and *trans*,*trans* (Δ8,10; 10,12) CLA and lower *trans*-7,*cis*-9 CLA at the omasum (III; Table 3). Furthermore, the ruminal outflow of *trans*-10,*cis*-12 CLA was greater for SFO than LFO (III), whereas the reverse was true for the flows of *trans*-11,*cis*-13 CLA and *trans*,*trans* (Δ11,13; 12,14; 13,15) CLA (III; Table 3). Typically, FO has minimal influence on ruminal *trans*-10,*cis*-12 CLA formation (Shingfield et al., 2003; 2010), whilst on the contrary increases in dietary 18:2n-6 content often promote *trans*-10,*cis*-12 CLA synthesis (Sackmann et al., 2003; Shingfield et al., 2008a). Similarly, *trans*-11,*cis*-13 CLA and *trans*-11,*trans*-13 CLA at the omasum were higher on LFO than SFO (III; Table 3), confirming previous studies *in vitro* (Jouany et al., 2007; Honkanen et al., 2016). All these findings together indicate that geometric Δ11,13 CLA isomers are formed during incomplete biohydrogenation of 18:3n-3.

The major pathway of ruminal 18:3n-3 metabolism involves an initial isomerisation to *cis*-9,*trans*-11,*cis*-15 18:3 and sequential reduction of double bonds to yield *trans*-11,*cis*-15 18:2 and *trans*-11 18:1 as intermediates (Figure 2). Relative to the control, oil supplements had no effect on *cis*-9,*trans*-11,*cis*-15 18:3 (1.15, 1.20, 0.79 and 3.60 g/d for control, FO, SFO and LFO, respectively) at the omasum, whereas the flow was higher on LFO than SFO (III) due to the higher intake of 18:3n-3 in LFO. Relative to SFO, the amounts of alternative novel *trans* 18:2 isomers, including *trans*-10,*cis*-15 18:2 and *trans*,*trans* (Δ9,14; 11,15; 10,15; 9,12) 18:2 were increased on LFO (III; Table 3), providing further evidence that multiple products are formed during the biohydrogenation of 18:3n-3 (Jouany et al., 2007; Alves and Bessa, 2014). Inclusion of LO with FO altered the major pathways described for 18:3n-3, resulting in *trans*-10,*cis*-15 18:2 replacing *trans*-11,*cis*-15 18:2 (III; Table 3) as the major 18:2 intermediate escaping the rumen, which provides more support to the existence of alternative *trans*-10 pathway of 18:3n-3 metabolism in the rumen (Alves and Bessa, 2014; Ventto et al., 2017). In II, revisiting the analysis of 18:2 isomers in omasal digesta of experimental cows revealed that *trans*-10,*cis*-15 18:2 was erroneously reported to coelute with *trans*-9,*cis*-12 18:2, *cis*-12,*trans*-16 18:2 and *cis*-8,*cis*-12 18:2, but the revised identification is taken into account in Table 3.

Supplementing the diet with incremental levels of FO resulted in increases in *trans* 18:1 at the omasum (II; Figure 4; Table 3), with the flows of most isomers, other than *trans*-10 18:1, reaching a maximum to additional FO of 150 g/d, whereas 300 g FO/d promoted the largest increase in the flow of *trans*-10 18:1 (II; Figure 5; Table 3). In growing cattle, dietary FO supplements are known to cause a progressive increase in *trans* 18:1 at the duodenum, with no clear indication that ruminal accumulation of *trans*-10 18:1 would be as pronounced as in the research presented in this thesis (Kim et al., 2008; Lee et al., 2008; Shingfield et al., 2010b). However, alterations in ruminal biohydrogenation pathways leading to an increase in *trans*-10 18:1 formation are known to

occur typically in cows fed high-starch, low-fibre diets with or without supplements of plant oils or oilseeds (Shingfield and Griinari, 2007). Under these circumstances, both *trans*-10 18:1 and *trans*-10,*cis*-12 CLA accumulate in the rumen (Shingfield and Griinari, 2007) which is thought to be related to low rumen pH (Fuentes et al., 2009). In contrast, diet supplemented with 300 g FO/d caused a shift in ruminal 18-carbon FA biohydrogenation, resulting in extensive accumulation of *trans*-10 18:1 at the omasum (II; Figure 5; Table 3), accounting for 33.6 % of total *trans* 18:1, in the absence of changes in *trans*-10,*cis*-12 CLA at the omasum (II; Table 3) or decreases in rumen pH (II).

Previous investigations *in vitro* have demonstrated that *trans*-10 18:1 is formed from 18:2n-6 via reduction of Δ10,12 CLA isomers (Kepler et al., 1966; McKain et al., 2010) and may also be formed by isomerisation of *cis*-9 18:1 (Mosley et al., 2002). However, detailed analysis of lipid in omasal digesta of cows fed FO (I-III) highlighted the possibility that *trans*-10 18:1 may also be formed from the reduction of other precursors *in vivo*, including *trans*-8,*trans*-10 CLA, *trans*,*cis* (Δ10,14; 10;15) 18:2, and *trans*-10,*trans*-14 18:2 (II; III; Table 3). Despite of the apparent accumulation of *trans*-10 18:1 at the omasum with dietary FO, *trans*-11 was the major 18:1 isomer in omasal digesta, accounting for 49.1 and 40.3 %, 41.0, 40.0, 54.2 and 39.6 % and 46.7, 51.3, 26.2 and 39.4 % of total *trans* 18:1 for each experimental treatments in I, II and III, respectively (Figure 5; Table 3).

Interestingly, relative to SFO, the amount of *trans*-11 18:1 increased on LFO, whereas the reverse was true for the flow of *trans*-10 18:1 (4.95, 5.45, 44.3 and 35.6 % of total *trans* 18:1 for control, FO, SFO and LFO, respectively) when compared SFO with the LFO (III; Figure 5; Table 3). In previous investigations, supplements of sunflower seeds or linseeds in combination with FO have been shown to elevate the proportion of *trans*-11 18:1 in ruminal (AbuGhazaleh et al., 2003) and duodenal digesta (Shingfield et al., 2011) of cattle. Some of these apparent discrepancies between our results and earlier investigations can be explained by differences in the effects of lipid supplements on the major biohydrogenation pathways in the rumen due to variation in 18-carbon FA intake and physical form of supplemental lipids (oil vs. oil seeds). However, ruminal accumulation of *trans*-10 18:1 when supplementing FO with sources of 18:2n-6 or 18:3n-3 is consistent with earlier reports in lactating cows fed FO (II; Shingfield et al., 2003; Loor et al., 2005c) or high-concentrate diets with plant oils (Loor et al., 2004; Ventto et al., 2017). These findings indicate that one or more FA in FO inhibit the reduction of *trans* 18:1 (as well as *trans* 18:2) intermediates by ruminal micro-organisms, promoting the formation of *trans*-10 18:1 and other *trans*-10 containing intermediates such as *trans*-10,*cis*-12 CLA and *trans*-10,*cis*-15 18:2 (Table 3). However, the underlying causes remain unknown.

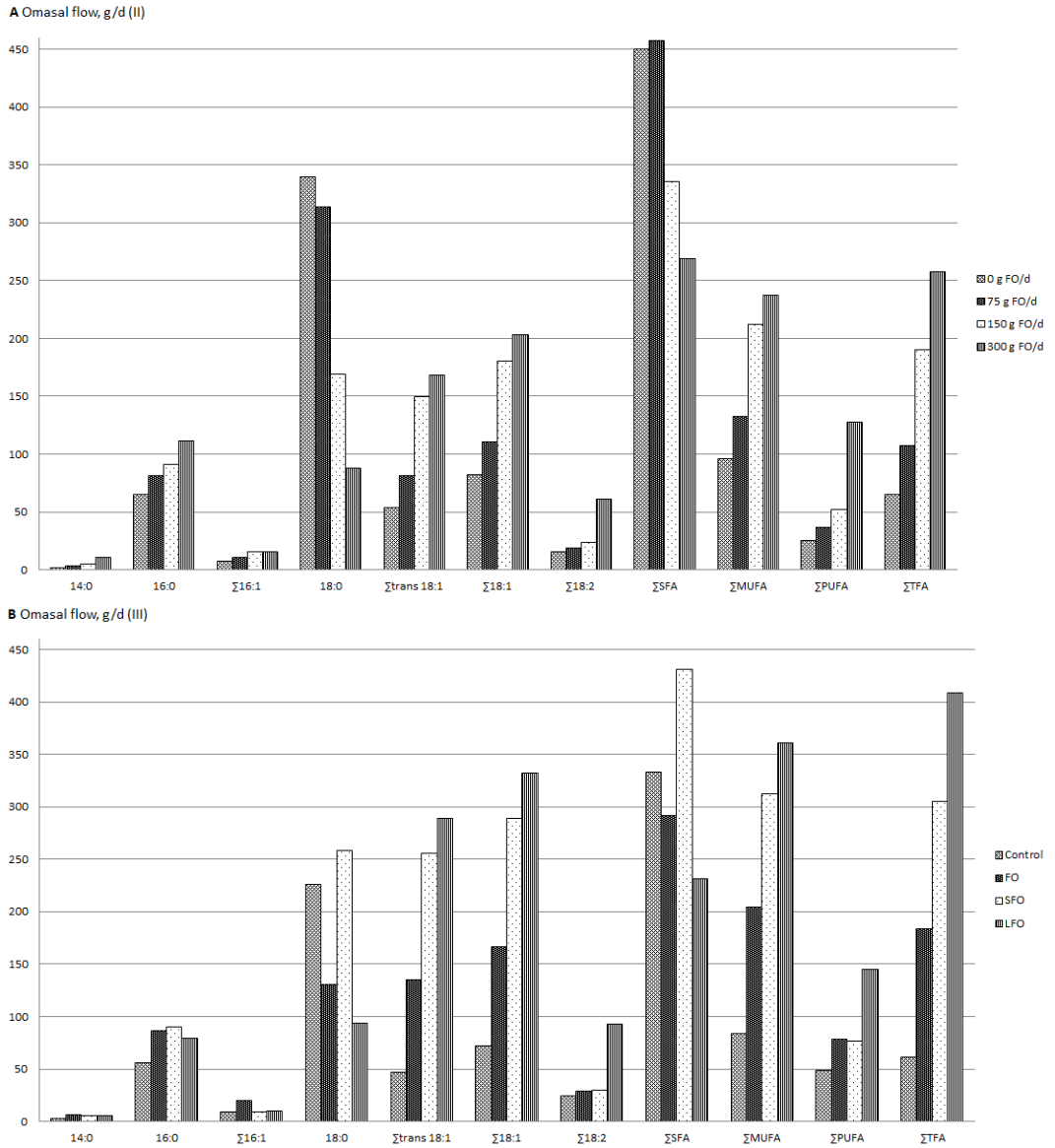

Figure 4. Effect of experimental treatments on the omasal flow of selected fatty acids and fatty acid groups at the omasum in lactating cows fed grass silage-based diets in A) II and B) III.

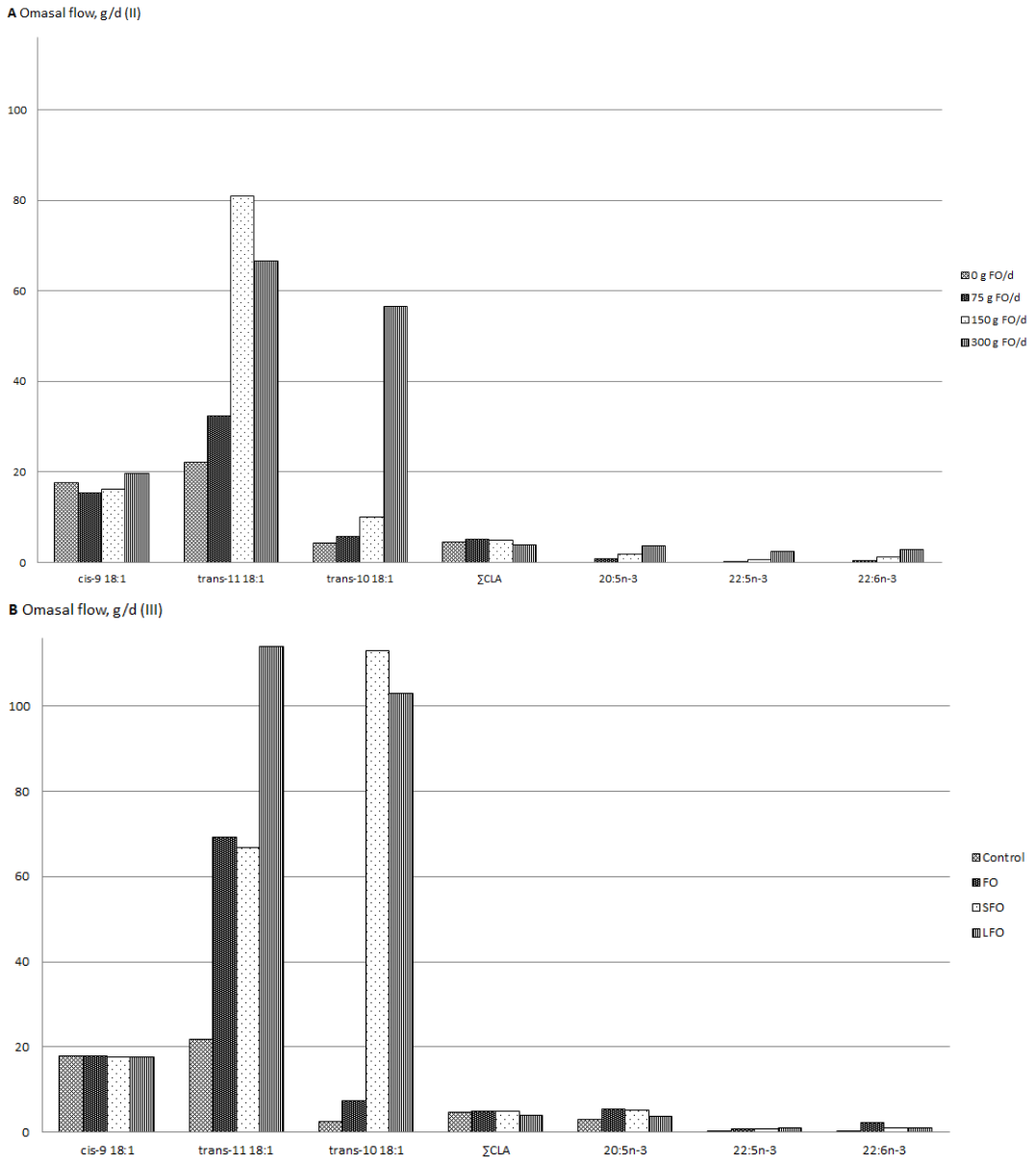

Figure 5. Effect of experimental treatments on the omasal flow of selected unsaturated fatty acids at the omasum in lactating cows fed grass silage-based diets in A) II and B) III.

Table 3 Effect of dietary fish oil supplements on the flow of selected 18-carbon fatty acids containing at least one *trans* double bond at the omasum or duodenum in lactating or growing cattle fed grass-silage based diets

	Shingfield et al., 2003		II				Shingfield et al., 2010b				Shingfield et al., 2011				III				Loor et al., 2005c		
	C	FO	C	FO 75	FO 150	FO 300	C	FO 82	FO 163	FO 245	C	FO	L	LFO	C	FO	SFO	LFO	FO	L	S
Sampling site	Omasum		Omasum				Duodenum				Duodenum				Omasum				Omasum		
Animal	Dairy cow		Dairy cow				Steer				Steer				Dairy cow				Dairy cow		
Oil inclusion rate, g/d	0	250	0	75	150	300	0	82	163	245	0	266	265	FO132 L132	0	200	FO200 S 500	FO200 L500	428	860	965
Forage inclusion rate, g/kg DM	600	600	580	580	580	580	600	600	600	600	600	600	600	600	600	600	600	600	350	350	350
Duration, d	14	14	28	28	28	28	21	21	21	21	21	21	21	21	21	21	21	21	28	28	28
Animals per group	5	5	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	3	3
Flow, g/d																					
∑*trans* 18:1	38.2	182	53.6	80.7	149	168	40.3	68.8	107	116	24.5	84.4	108	125	46.3	135	255	289	213	293	282
*t*4	0.45	0.65	0.62	0.63	0.74	0.42	0.41	0.47	0.52	0.42	0.23	0.69	0.15	0.56	0.66	0.71	0.86	0.81	0.80	1.92	2.03
*t*5	0.37	0.56	0.44	0.50	0.62	0.42	0.30	0.39	0.39	0.34	0.16	0.42	0.11	0.42	0.48	0.60	0.72	0.69	0.67	1.39	1.74
*t*6,-7,-8	1.67	6.65	3.46	5.14	7.94	6.83	2.6	4.2	5.6	5.7	1.58	4.67	5.08	5.60	2.14	6.57	11.5	10.3	6.80	12.1	14.1
*t*9	1.07	6.21	2.02	3.54	6.37	5.72	1.8	3.3	4.4	4.7	1.09	2.84	4.45	4.88	1.36	5.38	11.9	6.67	4.60	6.39	7.83
*t*10	1.71	14.3	4.25	5.59	9.95	56.4	3.3	4.3	5.0	6.0	2.05	3.91	19.2	6.44	2.29	7.36	113	103	84.1	32.8	114
*t*11	17.0	121	22.0	32.3	80.8	66.5	21.6	38.7	69.5	79.1	13.8	51.2	66.7	82.9	21.6	69.2	66.8	114	84.7	115	87.3
*t*12	2.21	9.41	4.31	7.59	11.8	9.41	2.8	5.9	7.8	7.9	1.47	4.79	5.85	8.70	2.73	9.80	11.7	11.5	9.01	15.8	15.3
*t*13,(-14)	6.46	14.0	11.3	17.2	21.0	15.9	2.1	3.3	4.3	4.3	1.01	4.47	2.68	4.77	7.02	19.6	23.2	26.1	13.7	60.3	23.3
*t*15	3.15	5.70	5.24	8.21	9.20	6.68	2.5	4.7	5.5	5.1	1.40	5.76	3.25	6.25	3.65	8.98	9.68	11.5	6.20	29.0	9.57
*t*16[1]	3.99	3.05	-	-	-	-	2.8	3.7	3.5	2.7	1.63	5.63	0.98	4.29	4.37	6.39	5.40	5.60	2.53	18.2	6.57
∑CLA	4.36	3.50	4.44	5.13	4.92	3.82	0.39	0.52	0.47	0.37	0.38	3.18	0.27	0.55	4.67	4.76	4.85	3.89	3.94	6.50	8.31
*c*9,*t*11	2.86	2.08	2.99	3.23	2.83	2.75	0.23	0.28	0.28	0.21	0.19	0.63	0.11	0.26	3.24	3.76	3.74	2.56	0.40	1.39	2.13
*c*10,*t*12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.13	0.21	0.29
*c*11,*t*13	0.01	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.33	0.11	0.19
*c*12,*t*14	0.05	0.0	0.05	0.03	0.0	0.01	0.0	0.0	0.0	0.0	0.0	0.04	0.01	0.0	-	-	-	-	-	-	-
*t*7,*c*9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	0.04	0.01	0.01	-	-	-
*t*8,*c*10	-	-	0.01	0.0	0.08	0.11	0.01	0.01	0.01	0.02	0.0	0.01	0.01	0.01	-	-	-	-	0.27	0.21	0.29

	Shingfield et al., 2003		II				Shingfield et al., 2010b				Shingfield et al., 2011				III				Loor et al., 2005c		
	C	FO	C	FO 75	FO 150	FO 300	C	FO 82	FO 163	FO 245	C	FO	L	LFO	C	FO	SFO	LFO	FO	L	S
*t*9,*c*11	-	-	0.14	0.79	0.89	0.28	-	-	-	-	-	-	-	-	-	-	-	-	0.27	0.21	0.29
*t*10,*c*12	0.10	0.02	0.08	0.02	0.03	0.04	0.01	0.02	0.01	0.01	0.02	0.02	0.03	0.01	0.08	0.01	0.21	0.08	0.27	0.32	1.84
*t*11,*c*13[1]	0.46	0.20	0.22	0.25	0.19	0.05	0.03	0.03	0.03	0.01	0.03	1.19	0.01	0.10	0.64	0.46	0.13	0.39	0.27	0.85	0.29
*t*13,*c*15	-	-	-	-	-	-	-	-	-	-	0.0	0.06	0.0	0.00	-	-	-	-	-	-	-
*t*7,*t*9	0.0	0.05	0.07	0.11	0.27	0.17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*t*8,*t*10	0.01	0.10	0.05	0.07	0.13	0.07	0.01	0.01	0.01	0.02	0.01	0.01	0.02	0.01	0.02	0.04	0.07	0.07	-	-	-
*t*9,*t*11	0.22	0.55	0.21	0.23	0.33	0.23	0.05	0.08	0.07	0.05	0.05	0.16	0.06	0.08	0.19	0.25	0.41	0.29	-	-	-
*t*10,*t*12	0.05	0.06	0.05	0.03	0.03	0.05	0.03	0.03	0.02	0.02	0.03	0.06	0.01	0.01	0.04	0.03	0.14	0.13	-	-	-
*t*11,*t*13	0.40	0.09	0.41	0.21	0.09	0.05	0.02	0.03	0.02	0.01	0.03	0.69	0.01	0.03	0.32	0.09	0.06	0.17	-	-	-
*t*12,*t*14	0.19	0.08	0.18	0.14	0.07	0.04	0.02	0.02	0.02	0.01	0.01	0.29	0.01	0.03	0.13	0.07	0.06	0.15	-	-	-
*t*13,*t*15	-	-	-	-	-	-	-	-	-	-	0.0	0.02	0.0	0.0	0.02	0.01	0.01	0.03	-	-	-
∑*trans* 18:2	-	-	-	-	-	-					-	-	-	-	1.46	4.34	4.26	21.3	-	-	-
*c*9,*t*12[2]	-	-	0.16	0.16	0.25	0.65	-	-	-	-	-	-	-	-	0.14	0.37	0.51	1.11	0.53	3.09	1.16
*c*11,*t*15	-	-	0.11	0.20	0.36	2.80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*t*9,*c*12[3]	0.18	1.60	0.27	0.53	0.87	1.83	-	-	-	-	-	-	-	-	0.24	0.82	1.60	4.35	1.33	3.30	1.26
*t*10,*c*15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	0.81	5.20	35.8	-	-	-
*t*11,*c*15[4]	2.81	18.8	3.73	5.38	8.96	31.5	1.34	2.34	3.29	3.62	0.77	6.73	4.45	7.87	3.72	8.56	6.74	21.5	17.9	50.1	8.31
*t*12,*c*15	-	-	0.24	0.38	0.39	1.14	-	-	-	-	-	-	-	-	0.24	0.60	0.68	1.32	-	-	-
*t*9,*t*12	0.03	0.93	0.09	0.16	0.26	0.68	0.32	0.57	0.99	1.25	0.20	2.88	1.50	2.91	-	-	-	-	1.47	2.98	0.68
*t*10,*t*15[5]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.15	0.49	1.02	8.54	-	-	-
*t*11,*t*15[6]	-	-	0.87	1.43	2.73	4.87	-	-	-	-	-	-	-	-	0.96	3.16	2.66	10.2	-	-	-
*t*11,*t*14[7]	-	-	0.27	0.39	0.72	1.69	-	-	-	-	-	-	-	-	0.36	0.87	0.84	3.70	-	-	-

[1]Co-elutes with *cis*-9,*cis*-11 18:2 in Loor et al., 2005c; [2]Co-elutes with *cis*-9,*trans*-14 18:2 in II; [3]Co-elutes with *cis*-12,*cis*-16 18:2 and *trans*-8,*cis*-12 18:2 in II, and co-elutes with *cis*-12,*cis*-16 18:2 and *trans*-11,*cis*-16 18:2 in III; [4]*Trans*-11,*cis*-15 18:2 co-elutes with *trans*-10,*cis*-15 18:2 in II; [5]Co-elutes with *trans*-9,*trans*-12 18:2; [6]Co-elutes with *trans*-9,*trans*-14 18:2; [7]Co-elutes with *trans*-10,*trans*-13 18:2 in II, co-elutes with *trans*-10,*trans*-13 18:2, *cis*-9,*trans*-13 18:2 and *cis*-10,*trans*-14 18:2 in III; C, control diet; CLA, conjugated linoleic acid; FO, fish oil; L, linseed oil; S, sunflower oil; For clarity purposes, abbreviated names of fatty acids are reported in the table (*c*, *cis*; *t*, *trans*).

4.3.4. Biohydrogenation intermediate products of 20- to 22-carbon fatty acids

Incubations with mixed rumen bacteria have demonstrated that 20:5n-3 and 22:6n-3 disappear over time (Dohme et al., 2003; AbuGhazaleh and Jenkins, 2004; Wasowska et al., 2006; Aldai et al., 2012; 2018; Escobar et al., 2016; Toral et al., 2018c), but to date, only few investigations have characterized 20- and 22-carbon intermediates formed during ruminal biohydrogenation of marine lipid sources high in 22:5n-3 and/or 22:6n-3, and evaluated the putative pathways under *in vivo* conditions (e.g. I-III; Toral et al., 2010; 2012).

Detailed analysis of lipid in omasal digesta of cows fed grass silage-based diets supplemented with different levels of refined FO (I-III) along with measurements of ruminal FA balance (I) and nutrient flow at the omasum (I-III; Shingfield et al., 2003) provided the first quantitative estimates of the ruminal outflow of long-chain 20- to 22-carbon PUFA intermediates in lactating cows. In total, 27 previously unidentified 20-, 21- and 22-carbon FA containing at least one *trans* double bond (Table 4) plus several unique all-*cis* long-chain PUFA not contained in refined FO were detected in variable concentrations in omasal digesta of cows fed FO for the first time (I; II), with the implication that the hydrogenation of long-chain unsaturated FA in the rumen involves a complicated series of metabolic reactions and the formation of numerous biohydrogenation intermediates.

Overall, the data demonstrated extensive ruminal biohydrogenation of 20:5n-3, 22:5n-3 and 22:6n-3 *in vivo* resulting in the accumulation of numerous 20-, 21- and 22-carbon unsaturated FA at the omasum (I-III). The total omasal flows of 20-, 21- and 22-carbon PUFA varied between 5.66-46.5, 0.00-1.00 and 3.78-25.8 g/d, respectively, for all experimental treatments in this research (I-III; Figure 6). The differences in the ruminal biohydrogenation of 20:5n-3, 22:5n-3 and 22:6n-3 and the different flows of total 20-, 21- and 22-carbon intermediates at the omasum on LFO versus SFO (III; Figure 6), provided a clear evidence that the source of 18-carbon PUFA (18:2n-6 vs. 18:3n-3) influences the kinetics of ruminal 20:5n-3, 22:5n-3, and 22:6n-3 metabolism. It is well established that during incubations with rumen contents, 18:2n-6 inhibited 20:5n-3 and 22:6n-3 biohydrogenation (Wasowska et al., 2006). In line with this, studies in growing cattle (Shingfield et al., 2011) and nonlactating sheep (Toral et al., 2010) fed diets containing FO and plant oils have also demonstrated that the supply of 18-carbon PUFA may influence the extent of ruminal 20- and 22-carbon PUFA biohydrogenation.

Table 4 Effect of dietary fish oil supplements on the proportion of selected 20- and 22-carbon fatty acids containing at least one *trans* double bond in the omasal or ruminal fluid in ruminants

Sampling site	I		Toral et al., 2010[1]		II				III				Toral et al., 2012				
	C	FO	C	SFO	C	FO 75	FO 200	FO 300	C	FO	SFO	LFO	C	S	SMA1	SMA2	SMA3
Sampling site	Omasum		Rumen		Omasum				Omasum				Rumen				
Species	Bovine		Ovine		Bovine				Bovine				Ovine				
Oil inclusion rate, g/d	none	250	none[2]	FO(10) S(20)	none	75	150	300	none	200	FO200 S500	FO200 L500	none	S (25)	S(25)[2] MA(8)	S(25)[2] MA(16)	S(25)[2] MA(24)
Forage inclusion rate, g/kg DM	600	600	350	350	580	580	580	580	600	600	600	600	485	485	485	485	485
Duration, d	14	14	11	11	28	28	28	28	21	21	21	21	28	28	28	28	28
Animals per group	5	5	5	5	4	4	4	4	4	4	4	4	5	5	5	5	5
Fatty acid profile (g/100 g fatty acids)																	
***trans* 20:1**																	
*t*6,-7,-8	-	-	0.02	0.02	-	-	-	-	0.02	0.01	0.01	0.01	0.03	0.01	0.01	0.02	0.02
*t*9,-10	0.01	0.04	-	0.04	0.01	0.04	0.06	0.07	0.01	0.06	0.03	0.03	-	-	-	-	-
*t*11	0.02	0.07	<0.01	0.07	0.03	0.06	0.12	0.08	0.03	0.15	0.07	0.08	-	-	-	-	-
*t*12	0.01	0.08	<0.01	0.07	0.02	0.06	0.12	0.11	0.01	0.15	0.09	0.11	0.01	0.01	0.01	0.01	0.01
*t*13	0.02	0.11	<0.01	0.08	0.02	0.09	0.19	0.10	0.01	0.22	0.09	0.10	0.01	<0.01	0.01	0.01	0.01
*t*14	0.02	0.09	0.03	0.11	0.02	0.11	0.20	0.10	0.02	0.25	0.10	0.09	0.02	0.01	0.01	0.02	0.02
*t*15	0.03	0.25	<0.01	0.14	0.04	0.30	0.55	0.23	0.02	0.66	0.32	0.17	-	-	-	-	-
***trans* 20:2**																	
*c*10,*t*15[3]	-	0.04	-	0.03	-	0.02	0.05	0.06	<0.01	0.13	0.07	0.07	-	-	-	-	-
*t*11,*c*15	-	0.16	-	-	-	0.01	0.05	0.18	-	0.07	0.05	0.18	-	-	-	-	-
*t*11,*c*17	-	0.08	<0.01	0.03	-	0.01	0.04	0.08	-	-	-	-	-	-	-	-	-
*t*13,*c*17	-	0.36	<0.01	0.03	0.02	0.09	0.21	0.69	0.20	0.45	0.42	0.42	-	-	-	-	-
*t*14,*c*17	-	0.18	<0.01	0.02	-	0.05	0.11	0.15	<0.01	0.19	0.10	0.05	0.01	<0.01	<0.01	0.01	<0.01
*t*9,*t*15	-	0.24	-	-	0.03	0.10	0.17	0.27	0.06	0.30	0.20	0.28	-	-	-	-	-
*t*10,*t*16[4]	-	0.21	-	-	-	-	0.06	0.09	-	0.07	0.04	0.09	-	-	-	-	-
*t*11,*t*15	-	0.15	-	-	0.05	0.08	0.11	0.15	-	0.07	0.05	0.12	-	-	-	-	-
*t*13,*t*17	-	0.07	-	-	-	0.01	0.04	0.09	<0.01	0.04	0.04	0.08	-	-	-	-	-
***trans* 20:3**																	
*c*11,*c*14,*t*17	-	0.07	-	-	-	0.02	0.04	0.12	-	-	-	-	-	-	-	-	-
*t*11,*c*14,*c*17	-	0.14	-	-	0.05	0.21	0.18	0.41	-	-	-	-	-	-	-	-	-

Sampling site	I		Toral et al., 2010[1]		II				III				Toral et al., 2012				
	C	FO	C	SFO	C	FO 75	FO 200	FO 300	C	FO	SFO	LFO	C	S	SMA1	SMA2	SMA3
*t*11,*t*14,*c*17	-	-	-	-	-	-	-	-	-	0.04	0.03	0.04	-	-	-	-	-
Δ10,14,17[5]	-	0.05	-	-	-	-	0.02	0.09	-	-	-	-	-	-	<0.01	<0.01	0.01
Δ11,14,17[5]	-	0.03	-	-	-	-	0.01	0.05	-	0.01	0.01	0.02	-	-	<0.01	<0.01	0.01
Δ11,14,18[5]	-	0.01	-	-	0.03	0.06	0.05	0.02	-	0.03	0.01	0.01	0.02	0.01	0.01	0.01	0.02
*t*10,*t*14,*c*7	-	0.13	-	-	-	0.05	0.06	0.33	-	-	-	-	-	-	0.02	<0.01	0.01
*c*9,*t*14,*t*17	-	-	-	-	-	-	-	-	0.64	0.66	0.83	0.36	-	-	-	-	-
*t*9,*c*14,*c*17	-	-	-	-	-	-	-	-	0.01	0.17	0.11	0.13	-	-	-	-	-
*t*9,*c*14,*t*17	-	-	-	-	-	-	-	-	-	0.03	0.22	0.04	-	-	-	-	-
*t*9,*t*14,*t*17	-	0.03	-	-	-	-	-	-	0.01	0.01	0.02	0.04	-	-	<0.01	0.01	0.01
*t*10,*t*14,*t*17	-	0.05	-	-	-	0.01	0.03	0.07	-	-	-	-	-	-	-	-	-
***trans* 20:4**																	
*t*7,*c*11,*c*14,*c*17	-	0.02	-	-	-	0.04	0.05	0.15	-	0.06	0.04	0.03	-	-	-	-	-
***trans* 22:2**																	
*t*12,*t*17	-	0.03	-	-	<0.01	0.03	0.06	0.05	0.09	0.12	0.08	0.09	-	-	-	-	-
*t*13,*t*18	-	-	-	-	<0.01	0.03	0.05	0.05	-	-	-	-	-	-	-	-	-
***trans* 22:3**																	
*c*10,*t*14,*c*19	-	0.08	-	-	0.02	0.02	0.04	0.11	0.06	0.07	0.04	0.09	0.04	0.03	0.01	0.03	0.02
*t*12,*c*16,*c*19	-	0.14	-	-	-	0.04	0.12	0.23	<0.01	0.19	0.14	0.15	-	-	0.12	0.05	0.05
***trans* 22:4**																	
*c*7,*t*13,*c*16,*c*19	-	0.05	-	-	-	0.02	0.02	0.04	<0.01	0.03	0.01	0.03	-	-	0.01	0.09	0.07
*t*8,*c*13,*c*16,*c*19	-	0.13	-	-	-	0.01	0.07	0.30	0.18	0.24	0.15	0.22	-	-	-	-	-
*t*10,*t*13,*c*16,*c*19	-	0.03	-	-	-	0.01	0.04	0.12	-	-	-	-	-	-	-	-	-
***trans* 22:5**																	
(*t*)5,*c*10,*c*13,*c*16,*c*19[6]	-	0.05	-	-	-	-	-	-	-	0.26	0.06	0.11	-	-	0.12	0.11	0.13

[1]Values are mean of experimental days 3 and 10; [2]Intake of dry matter or lipid supplements not reported. Concentrations of oil in the diet (g/kg diet dry matter) indicated in parentheses; [3]Co-elutes with *trans*-11,*cis*-17 20:2 in III; [4]Co-elutes with 21:0 in II; [5]Retention time comparisons inferred a *cis*,*trans*,*trans* or *trans*,*cis*,*trans* double bond configuration; [6]*cis* or *trans* double bond configuration undetermined in III, refer to Δ5,10,13,16,19 22:5; C, control diet; CLA, conjugated linoleic acid; FO, fish oil; L, linseed oil; MA1-3, increasing levels of marine algae in the diet, concentrations of marine algae in the diet (g/kg diet dry matter) indicated in parentheses; S, sunflower oil; For clarity purposes, abbreviated names of fatty acids are reported in the table (c, *cis*; t, *trans*).

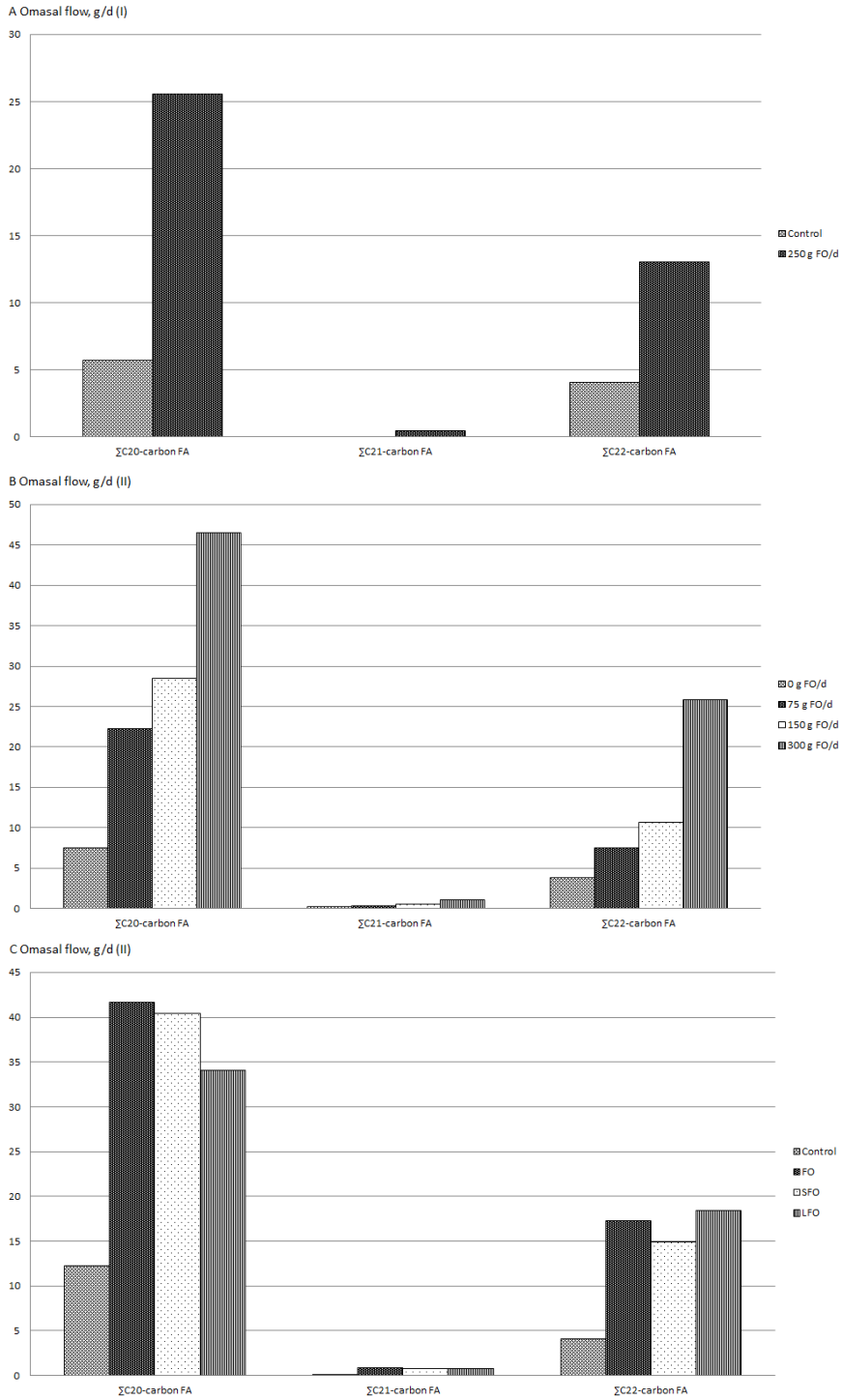

Figure 6. Effect of experimental treatments on the total omasal flow of 20-, 21- and 22-carbon fatty acids at the omasum in lactating cows fed grass silage-based diets in A) I, B) II and C) III.

Ruminal outflows of all-*cis* 20-, 21-, and 22-carbon PUFA, most of them not supplied from the diet, such as 20:2n-3, 20:2n-6, 20:3n-3, 20:3n-6, 20:4n-3, 21:3n-3, 21:4n-3, 22:2n-6, 22:3n-3, 22:3n-6, 22:4n-3, and 22:4n-6, exceeded intake, which would indicate these long-chain PUFA as intermediates of 20:5n-3, 22:5n-3 or 22:6n-3 metabolism. Inclusion of plant oils plus FO decreased 20:4n-6, 21:5n-3, 22:4n-6 and 22:6n-3 at the omasum and increased ruminal outflow of 22:4n-3 compared with FO (III). Furthermore, LFO resulted in higher flows of 22:4n-6, 22:4n-3 and 22:5n-3, but decreased the amount of 22:3n-3 compared with SFO (III).

The formation of 18-carbon FA intermediates containing a conjugated double bond system (Figure 1B) is proposed to represent as an initial step of the main biohydrogenation pathways of 18-carbon PUFA (Lee and Jenkins, 2011; Alves and Bessa, 2014; Honkanen et al., 2016) and it is assumed that the ruminal metabolism of 20:5n-3, 22:5n-3 and 22:6n-3 would follow the same pattern involving isomerisation of *cis* double bond(s) to form intermediates with 5 or 6 double bonds, containing at least one *trans* double bond (Jenkins et al., 2008) and/or formation of conjugated 20- and 22-carbon intermediates that are sequentially reduced to their saturated end products in this biochemical process. The reduction of the *cis* double bond closest to the carboxyl group (*cis*-5 in 20:5n-3, *cis*-7 in 22:5n-3) is proposed to represent an initial step of the main biohydrogenation pathway of 20:5n-3, 21:5n-3 and 22:5n-3 PUFA in the rumen (I; II; Toral et al., 2010; 2018c; Jeyanathan et al., 2016; Escobar et al., 2016).

Extensive investigations have not yielded any substantive evidence that the carbon chain of PUFA is elongated or shortened during biohydrogenation in the rumen (Harfoot and Hazlewood, 1988; Jenkins et al., 2008). Therefore, e.g. the appereance of 21:3n-3 and 21:4n-3 in omasal digesta of cows fed dietary FO supplements must arise from the hydrogenation of 21:5n-3 in FO via specific mechanism that involves the sequential reduction of the *cis*-6 and *cis*-9 double bonds in 21:5n-3 and 21:4n-3, respectively (I-III). However, definitive information on the biohydrogenation of 22:6n-3 is still lacking. Some reports show much lower importance of above-mentioned reduction mechanism for ruminal 22:6n-3 metabolism compared to that of 20:5n-3 and 22:5n-3 (Aldai et al., 2018; Toral et al., 2018c) than the others (Jeyanathan et al., 2016). This discrepancy between investigations, together with the high accumulation of numerous unique 20- to 22-carbon PUFA intermediates in the rumen (I-III), could indicate that the ruminal metabolism of very long-chain n-3 PUFA may differ within ruminant species as recently outlined after comprehensive incubations of 20:5n-3, 22:5n-3 and 22:6n-3 with rumen contents of cows and ewes (Toral et al., 2018c).

Omasal digesta of cows fed FO was devoid of conjugated 20:5, 22:5 or 22:6 biohydrogenation intermediates (I-III), in agreement with earlier studies reporting no detection of conjugated 20- and 22-carbon FA isomers *in vivo* with cows, sheep, and goats fed FO or marine lipids (Toral et al., 2010; 2012; 2016a) or *in vitro* incubations of 20:5n-3 and 22:6n-3 (Aldai et al., 2012; Escobar et al., 2016; Jeyanathan et al., 2016). These findings, suggest a different pathway for the biohydrogenation of long-chain 20- and 22-carbon n-3 PUFA compared to that of 18-carbon PUFA. However, these observations are

challenged by the quite recent identification and characterization of minor conjugated 22:6 isomers (Aldai et al., 2018) as well as the appearance of conjugated Δ11,13,17,19-22:4 intermediate in ovine and bovine rumen inoculum (Toral et al., 2018c) after *in vitro* incubations of 20:5n-3/22:6n-3 and 22:5n-3, respectively. The reason for the discrepancy of the results between our research and the *in vitro* investigations of Aldai (2018) and Toral (2018a) may be the complexity of *in vivo* experiments conducted with FO compared with the more straightforward *in vitro* approach with pure 20:5n-3, 22:5n-3 and 22:6n-3. Therefore, the transient formation of conjugated double bond structures as a result of ruminal long-chain 20- and 22-carbon PUFA metabolism cannot be unequivocally excluded (Figure 7 and Figure 8).

Dietary FO supplements alone (I-III) or in combination with plant oils (III) resulted in corresponding increases in numerous polyenoic 20- and 22-carbon intermediates containing at least one *trans* double bond escaping the rumen (Table 4). The most abundant *trans* 20- and *trans* 22-carbon FA intermediates found in FO digesta were *trans*-13,*cis*-17 20:2 (II; III), *trans*-10,*trans*-14,*cis*-17 20:3 (II), *trans*-7,*cis*-11,*cis*-14,*cis*-17 20:4 (II; III), *trans*-12,*cis*-16,*cis*-19 22:3 (III), *cis*-7,*trans*-13,*cis*-16,*cis*-19 22:4 (III) and *trans*-8,*cis*-13,*cis*-16,*cis*-19 22:4 (III). In addition, inclusion of plant oils plus FO increased multiple *trans*,*cis* and *trans*,*trans* 20- and 22-carbon FA intermediates at the omasum, including (Δ11,15; 13,17) 20:2 and *trans*-11,*cis*-14,*trans*-17 20:3, compared with FO (III). Furthermore, LFO resulted in higher flows of *trans*-11,*cis*-15 20:2, *trans*,*trans* (Δ10,16; 11,15; 13,17) 20:2, *cis*-10,*trans*-14,*cis*-19 22:3 and *trans*-8,*cis*-13,*cis*-16,*cis*-19 22:4, but decreased the amounts of *trans*-14,*cis*-17 20:2 and *cis*-9,*trans*-14,*trans*-17 20:3, compared with SFO (III). The total flow at the omasum varied between 8.62-23.5 and 1.02-5.58 g/d for *trans* 20- and *trans*-22-carbon PUFA, respectively, in different levels of FO (I–III; Table 4), demonstrating that ruminal biohydrogenation of ≥ 20-carbon PUFA to saturated end products is incomplete and highly variable, involving the formation of several *trans* PUFA intermediates via different biochemical pathways.

Even though oil supplements increased 20-, 21-, and 22-carbon PUFA intake, the amounts of 20:0, 21:0, and 22:0 reaching the omasum were not substantially higher than the control (I–III), indicating that ruminal biohydrogenation of 20- and 22-carbon unsaturated FA to saturated end products is incomplete. These findings are in agreement with earlier reports in cattle (Shingfield et al., 2003; 2010; Lee et al., 2008) and sheep (Toral et al., 2010) fed FO or marine lipids. In accordance to this we observed also increases in ruminal outflow of several *trans* 20:1 and *trans* 22:1 isomers irrespective of oil supplemented diets (I-III; Table 4). Changes in the flow of *trans* 20:1 biohydrogenation intermediates were also accompanied by an increase in the amount of *cis* (Δ11,13-15) 20:1 at the omasum (I-III) that may reflect ruminal escape of these FA contained in FO, or formation of these isomers during the hydrogenation of 20-carbon PUFA in the rumen. Ruminal escape of FA in FO may also account for the increase in *cis* (Δ11,13,15) 22:1 in FO omasal digesta (I-III), but the possibility that one or more of these isomers is formed during the penultimate step of 22-carbon PUFA hydrogenation in the rumen cannot be excluded.

The biological significance of the formation and accumulation of numerous 20- and 22-carbon FA containing at least one *trans* double bond in the rumen of cows fed FO remains uncertain. It is not clear whether long-chain n-3 PUFA by themselves or the *trans* double bond containing FA intermediates formed during the hydrogenation of very long-chain n-3 PUFA in the rumen, might affect the ruminal metabolism of other, mainly 18-carbon PUFA. Total ruminal outflow of specific 20- and 22-carbon biohydrogenation intermediates was of the same magnitude as the total intake of 20:5n-3, 22:5n-3 and 22:6n-3 (I–III) and it is probable that most of these intermediates are also incorporated into milk fat and tissue lipids, albeit at low concentrations.

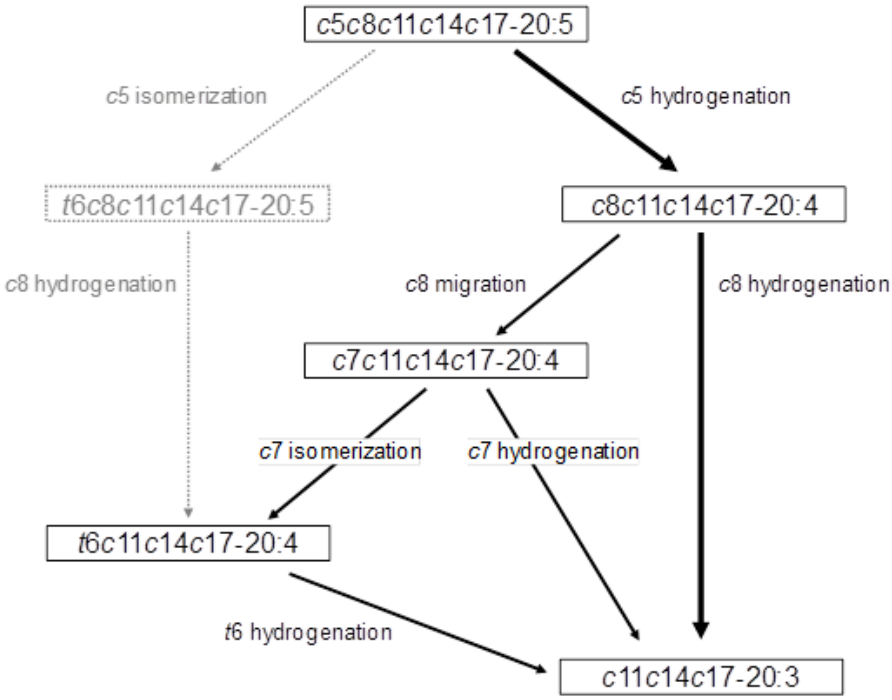

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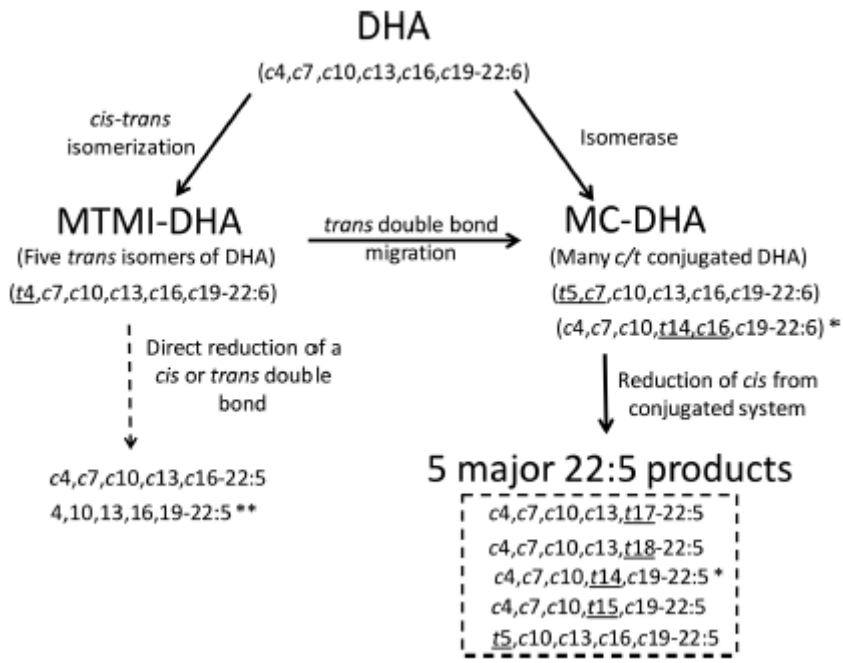
Figure 7. Putative pathways describing initial 20:5n-3 biohydrogenation *in vitro*. Thick arrows highlight the potentially major pathway; grey arrows and text represent a hypothetical pathway involving the formation of a conjugated 20:5 intermediate (not identified in digesta yet). For clarity purposes, abbreviated names of fatty acids are reported in the figure (c, *cis*; t, *trans*).



Adapted with permission from Aldai, N., Delmonte, P., Alves, S.P., Bessa R.J.B. and Kramer, J.K.G. 2018. J. Agric. Food Chem. 66:842-855. Copyright (2018) American Chemical Society.

Figure 8. Proposed pathways of DHA (22:6n-3) metabolism using sheep rumen fluid. One of the five possible MTMI-DHA and two of the MC-DHA metabolites are shown as representative products, as well as the five 22:5 products identified, all of which contain an isolated *trans* double bond. (*) Members of the pathway of DHA metabolism that resulted in the formation of the major 22:5 product in the incubation mixture. (**) Product reported by Jeyanathan et al.2016 (BMC Microbiol. 16:104) that could not be confirmed. MTMI-DHA, mono *trans* methylene interrupted-DHA; MC-DHA, monoconjugated DHA.

4.3.5. The extent of apparent ruminal biohydrogenation of dietary unsaturated fatty acids

Mean estimates of 18:2n-6 and 18:3n-3 biohydrogenation reported in the literature vary between 70-95 and 85-100 %, respectively (Doreau and Ferlay, 1994; Chilliard et al., 2007). In this thesis the apparent biohydrogenation of PUFA was calculated as [(intake, g/d – flow at the omasum, g/d)/intake, g/d] (I-III). The amounts of most unsaturated 18-carbon FA at the omasum were lower than their respective intake, indicating extensive ruminal biohydrogenation of 18-carbon FA for all experimental treatments in this research (I–III). Mean estimates of *cis*-9 18:1, 18:2n-6 and 18:3n-3 biohydrogenation were 87, 96 and 98 %, respectively, in cows fed FO (I; II) and 90, 97 and 98 %, respectively, in cows fed FO plus plant oils (III), consistent with previously reported estimates of 88–89 % in lactating cows fed grass-silage based diets containing moderate levels of FO (250 g FO/d; Shingfield et al., 2003). These estimates were marginally higher than corresponding values measured in the basal diet (on average 82, 94 and 97 %, respectively; I–III)

and slightly greater than earlier estimates of 58–97 % reported in cattle (Lee et al., 2008; Shingfield et al., 2010b; 2011).

Oil supplements increased *cis*-9 18:1, 18:2n-6 and 18:3n-3 biohydrogenation in the rumen, which was further enhanced when FO was compared with SFO and LFO (III) consistent with the phenomenon previously reported in growing cattle fed diets containing plant oils and FO (Shingfield et al., 2011). However, there was no difference in the extent of ruminal *cis*-9 18:1 and 18:2n-6 biohydrogenation between the SFO and LFO treatments, but the extent of ruminal 18:3n-3 biohydrogenation was substantially greater for LFO than SFO (III), confirming earlier reports in growing cattle (Shingfield et al., 2011) and nonlactating sheep (Toral et al., 2010) that dietary supply of 18-carbon PUFA may influence the extent of n-3 PUFA biohydrogenation in the rumen.

The long-chain 20- and 22-carbon n-3 PUFA were extensively biohydrogenated in the rumen, confirming earlier reports in lactating cows (e.g. Doreau and Chilliard, 1997; Shingfield et al., 2003). However, the extent of 20:5n-3, 22:5n-3 and 22:6n-3 biohydrogenation (98, 88, and 98 %, respectively) at present work (I–III) was higher than previously reported mean estimates of 92 and 89 % for 20:5n-3 and 22:6n-3, respectively, in lactating cows fed a high-concentrate diet containing FO (Loor et al., 2005c), and mean values of 95, 71 and 96 % for 20:5n-3, 22:5n-3 and 22:6n-3, respectively, reported in cattle fed grass-silage based diets supplemented with moderate levels of FO (ca. 50-270 g FO/d; Lee et al., 2008; Shingfield et al., 2010b; 2011). Variations in the extent of long-chain PUFA metabolism have been suggested to reflect e.g. differences in dietary fibre content and concentration of FO in the rumen (Gulati et al., 1999).

Limited data exists reporting mean values of ruminal biohydrogenation for unique long-chain n-3 FA originating from FO, such as 20:4n-3 and 21:5n-3, varying between 67-100 % (Shingfield et al., 2003; I; II) and 73-81 % (I–III), respectively. Consistent with these findings, the ruminal metabolism of very long-chain all-*cis* polyenoic FA, such as 22:4n-6, 23:5n-3, 24:5n-3, and 24:6n-3, appeared to be highly extensive as well, ca. 87-100 % at present work (I).

Increasing levels of FO decreased the extent of ruminal biohydrogenation of 20:5n-3 (-, 93.8, 92.5, and 91.7 % for 0, 75, 150 and 300 g FO/d, respectively; II), 22:5n-3 (-, 86.8, 79.8, and 51.0 %, respectively; II), and 22:6n-3 (-, 93.8, 92.0, and 89.6 %, respectively; II), whereas the dietary supply of plant oils with FO resulted in more extensive metabolism of 22:6n-3 compared with FO (-, 89.9, 95.6, and 94.9 %, for control, FO, SFO and LFO, respectively; III). The effect of diets containing plant oils and FO on the ruminal biohydrogenation of 20:5n-3 (-, 82.6, 84.0, and 88.2 %, respectively; III) seemed to be similar, but the effect was not significantly different between diets. However, the biohydrogenation of 22:5n-3 (-, 78.5, 87.3, and 83.5 %, respectively; III) was less extensive in LFO than SFO, but showed no difference between FO and diets containing plant oils, offering no support to the lowered biohydrogenation of 22:5n-3 in response to the dietary inclusion of plant-derived 18-carbon PUFA as hypothesized in the beginning of this work (refer to the 3rd hypothesis in chapter 2).

4.3.6. Rumen microbial ecology

Dietary FO treatments (II; III) had no effect on protozoal numbers in the rumen. Of the species known to be capable of biohydrogenation, the group *B. proteoclasticus* was the most abundant, comprising up to 0.74 and 1.27 % of total bacteria for II and III, respectively. The group encompassing *B. fibrisolvens* and known *Pseudobutyrivibrio spp.* were the next most numerous (0.55–0.96 and 0.93 to 1.47 % of total bacterial DNA, respectively), whereas *B. hungatei* represented only a small proportion (0.002-0.04 and 0.04–0.05, respectively) of total *Butyrivibrio* + *Pseudobutyrivibrio* group. Reported numbers of the group *Butyrivibrio* + *Pseudobutyrivibrio* in the experiments presented in this thesis were higher than earlier estimates across a range of FO or marine diets in ruminants (Boeckaert et al., 2008; Huws et al., 2010; 2011; Toral et al., 2012). This may reflect the differences between the analysis of reconstituted omasal digesta in our studies, containing large-particle, small-particle, and liquid fractions in amounts representative of that truly entering the omasal canal, and analysis of isolated samples of ruminal digesta in the previous studies. However, the effect of dietary FO on the numbers of *B. fibrisolvens* + *Pseudobutyrvibrio* (II) is consistent with several observations reporting a decline in *B. fibrisolvens* in the presence of marine lipids or 22:6n-3 (Liu et al., 2011; Potu et al., 2011; Gudla et al., 2012; Toral et al., 2012). Even though diets containing FO alone or in combination with plant oils altered the amounts of 18-carbon biohydrogenation intermediates and 18:0 at the omasum, no other changes in analysed ruminal bacterial populations were observed by treatment except a decrease in the abundance of *Oribacterium spp*. on LFO compared with SFO (III).

The population sizes of *Streptococcus bovis* and *Propionibacterium acnes*, ruminal species known to hydrate rather than hydrogenate *cis*-9 18:1 and 18:2n-6 (Hudson et al., 2000; Kim et al., 2008), were extremely small in omasal digesta and not affected by treatment (II; III). *S. bovis* was present in 0.01 and 0.005 % (average) of the bacterial community for II and III, respectively, but the numbers of *P. acnes* were several orders of magnitude lower (approximately ≤ 0.001 % of total bacteria). *Megasphaera elsdenii* was below the limit of detection (10−7 of total bacteria) in all samples (II; III). The atypical type strain of *B. fibrisolvens*, (which historically is the type strain, ATCC 19171), was present in 0.01 % of the population when incremental levels of FO was fed (II), but below the limit of detection in all samples with diets containing no oil, FO alone or FO plus plant oils (III). Because *B. proteoclasticus* is the only known bacterium capable of reducing 18:1 isomers to 18:0, it might have been anticipated that the decrease in 18:0 at the omasum to FO (II) or other oil treatments (III) would have been accompanied by a fall in *B. proteoclasticus* numbers. However, there was no evidence of such an association in this (II; III) or other published reports in the literature (Kim et al., 2008; Huws et al., 2010; 2011; Gudla et al., 2012). Nevertheless, FO has been reported to decrease the abundance of different bacteria belonging to the *Butyrivibrio* group, including *B. proteoclasticus*, in continuous cultures (AbuGhazaleh and Ishlak, 2014).

Changes in microbial ecology associated with the *trans*-10 shift in cows fed 300 g FO/d are difficult to explain. *Butyrivibrio spp.* catalyze the reduction of *trans*-

10,*cis*-12 CLA to *trans*-10 18:1 (McKain et al., 2010), but did not result in the accumulation of *trans*-10,*cis*-12 CLA or other 10,12 geometric CLA isomers. Although *M. elsdenii* has been reported to form *trans*-10,*cis*-12 CLA from 18:2n-6 (Kim et al., 2002), there is also evidence to show that no strain of *M. elsdenii* carries out that reaction (Wallace et al., 2006). Nevertheless, the numbers of *M. elsdenii* in the experiments of this thesis were below the limit of detection (II; III). However, *P. acnes* catalyses the reduction of 18:2n-6 to *trans*-10,*cis*-12 CLA (Liavonchanka et al., 2006) and FO supplements tended to cause a dose-dependent increase in this population. However, it remains unclear if *P. acnes* could be solely responsible for *trans*-10,*cis*-12 CLA and *trans*-10 18:1 formation, because the specific activities of this bacterium have not been reported. Alternatively, the growth and activity of a microorganism capable of converting *trans*-11 18:1 to *trans*-10 18:1 may be promoted by one or more FA in FO, but the identity of such species is not known.

It has often been assumed that the main FA in FO are responsible for the inhibitory effects on biohydrogenation. Based on our observations *in vivo* (II; III) and earlier incubations of FA substrates with rumen fluid (Wasowska et al., 2006) it seems that FO inhibits ruminal biohydrogenation through a mechanism that is not solely explained by effects on *B. fibrisolvens*. Other bacteria, such as *Ruminococcus albus*, *Eubacterium spp.*, and *Treponema spp.* (Yokoyama and Davis, 1971; Kemp et al., 1975), and strains of O*ribacterium* (S. Muetzel and R. J. Wallace, unpublished data, personal communication) have been described to convert 18:2n-6 to *cis*-9,*trans*-11 CLA and *trans*-11 18:1. Including FO in the diet of growing cattle has been shown to induce changes in the number of other rumen bacterial strains, such as *Anaerovirbrio lipolytica*, *Fibrobacter succinogenes*, and *Ruminococcus flavefaciens* (Huws et al., 2010), suggesting a broader influence on ruminal lipid metabolism (Kim et al., 2008; Huws et al., 2010). Furthermore, a negative relationship between DNA concentrations from *B. proteoclasticus* strains and 18:0 at the duodenum was reported in earlier studies (Kim et al., 2008; Huws et al., 2010). No such relationship was observed in our research (II; III). It is possible, therefore, that unknown microbial species, not quantified here, are involved in FA biohydrogenation. Alternatively, direct inhibition of the biohydrogenation pathway, which does not lead to changes in the biohydrogenating community but alters the biohydrogenation products, could be the principal mode of action of the plant PUFA (Maia et al., 2007; Lourenço et al., 2010).

4.4. Lipid metabolism in the mammary gland

4.4.1. Effect of dietary fatty acids on milk production and composition

Supplements of FO decreased yields of energy-corrected milk, milk fat and protein, and milk fat content (IV) confirming earlier observations of milk production characteristics in lactating cows (Donovan et al., 2000; Keady et al., 2000; Rego et al., 2005). It is well established that dietary FO supplements often lower milk yield, with the decrease being dependent on the amount (Donovan et al., 2000; Rego et al., 2005) and source of FO

(Keady et al., 2000). Observed decrease in milk yield, together with depressed DMI (refer to chapter 4.2.2), in response to lipid supplements have often been attributed to the adverse effects of unsaturated FA on the growth of specific populations of rumen cellulolytic bacteria (Jenkins, 1993).

Diets supplemented with 300 g FO/d lowered milk protein yield, whereas 150 g FO/d decreased milk protein content consistent with earlier findings in lactating cows (Offer et al., 1999; Donovan et al., 2000; Keady et al., 2000). In high amounts, FO tended to lower milk lactose secretion, whereas earlier studies have reported variable effects on milk lactose output (Offer et al., 1999; Loor et al., 2005a; Donovan et al., 2000; Keady et al., 2000).

Increases in FO supplementation progressively decreased milk fat content and yield, which represents a typical response in lactating cows (Offer et al., 1999; Donovan et al., 2000; Keady et al., 2000; Rego et al., 2005) and is associated with the effects of long-chain PUFA in FO on ruminal lipid metabolism (Shingfield et al., 2003; Loor et al., 2005c). Compared with 0 g FO/d, milk fat yield and content were decreased by 40.6 and 30.1 %, respectively, on diets supplemented with 300 g FO/d. Specific responses on milk fat content during FO-induced MFD are discussed more detail in chapter 4.4.3.

4.4.2. Fatty acids in milk

Analysis of fractionated FAME and corresponding DMOX derivatives by GC-FID and GC-MS allowed 196 baseline-separated or coeluting FA to be detected in the milk fat GC-FID chromatogram as presented in IV. Of these, 174 were able to be identified, including 37 unique 20- and 22-carbon intermediates not previously reported in milk from cows fed FO. Overall, dietary FO supplements elevated milk PUFA, mono- and polyenoic *trans* FA concentrations (IV), and at high amounts altered the distribution of individual *trans* 16- to 22-carbon FA isomers (IV), which can be attributed to the incomplete biohydrogenation of unsaturated 16- to 22- carbon PUFA in the rumen (II).

Earlier reports had relied on the same elution order in GC analysis and isomer composition of partially hydrogenated plant oils to identify changes specifically in milk *trans* 18:2 isomers to FO (Shingfield et al., 2003; Loor et al., 2005a), but in the experiments presented in this thesis a combination of preparative Ag^+-TLC of FAME and GC-MS analysis of FAME and corresponding DMOX derivatives was used to analyze milk fat composition to avoid these uncertainties (IV).

4.4.2.1. Saturated fatty acids and *cis*-9 18:1 in milk

Indirect comparison of dietary FO supplements on milk fat synthesis and milk FA composition of lactating cows reported in the literature is presented in Table 5. In this thesis work, supplements of FO progressively decreased the relative proportions of short- and medium-chain SFA (4- to 16-carbon FA; IV) and 18:0 in milk (IV; Table 5) that were accompanied by an increase in 14:0, 16:0 and 18:0 at the omasum (II; Figure 4). Previous investigations in lactating cows fed FO have reported similar decreases in the propor-

tions of milk FA synthesized *de novo* (Donovan et al., 2000; Keady et al., 2000; Rego et al., 2005), which can be explained by the inhibitory effects of long-chain FA on ACACA activity and the *de novo* synthesis of short- and medium-chain SFA in mammary secretory cells with the effects being more potent when the number of carbon atoms and/or the degree of unsaturation, especially the number of *trans* double bonds in the carbon chain, increases (Chilliard et al., 2000). It is also well established that FO lowers mammary mRNA abundance for *ACACA* and *FASN* in lactating cows (Ahnadi et al., 2002). Furthermore, in our study dietary FO decreased *cis* 18:1 concentration in milk that were, for the most part, due to decreases in *cis*-9 18:1 (IV; Table 5). Observed decreases in milk 18:0 and *cis* 18:1 concentrations are consistent with previous findings in cows fed FO (Offer et al., 1999; Donovan et al., 2000; Rego et al., 2005), due to lowered availability of 18:0 for direct incorporation in milk fat or for endogenous synthesis of *cis*-9 18:1 in the mammary gland.

Basal activity of SCD, evaluated using the milk 14:1/14:0 desaturation index as a proxy (Bernard et al., 2008), was not affected by FO inclusion (IV; Table 5). However, observed increases in *cis*-9 18:1/18:0 concentration ratio (IV; Table 5), without substantial changes in *cis*-9 18:1 at the omasum (II; Figure 5) could be considered as evidence that the alterations in the *cis*-9 18:1:18:0 ratio may potentially arise from changes in SCD substrate specificity. Reports of FO having a direct effect on *SCD* transcription are equivocal. Inclusions of FO (15 g/kg DM) and soybean oil (30 g/kg DM) have been reported to decrease (Harvatine and Bauman, 2006) or increase (Invernizzi et al., 2010) mRNA abundance for *SCD1* in mammary gland tissue. When added to the diet or fed as rumen protected form, FO tended to decrease mammary *SCD* mRNA abundance (Ahnadi et al., 2002), whereas *SCD1* transcription was more recently demonstrated to be unaffected by abomasal infusion of FO (Dallaire et al., 2014).

Dietary FO inclusion had rather small or no effect on the milk fat OBCFA concentrations (IV) consistent with observed OBCFA flows at omasum (II) and confirmed recent observations in lactating sheep fed marine lipids (Toral et al., 2018b). However, these findings are questioned by some other investigations that have reported also increases (Toral et al., 2015) or decreases (Toral et al., 2018c) in milk OBCFA in lactating cows or sheep, respectively. The inconsistency between these studies may reflect changes in duodenal flow of rumen bacteria, because FA synthesis by rumen bacteria is considered to be the main source of OBCFA in milk fat (Vlaeminck et al., 2006).

4.4.2.2. 16-carbon fatty acids in milk

Dietary FO supplements increased milk fat *cis* 16:1 and *trans* 16:1 concentrations, with the majority of the increase due to enrichment of *cis*-9 16:1 and *trans* (Δ9-12) 16:1. In addition, increases in 10-O-16:0 and all identified milk 16:2 isomers were observed. These findings are in line with previous investigations in lactating ruminants fed diets supplemented with FO (Toral et al., 2015; 2018b). Increases in 16:1 (II; Figure 4) and 16:2 isomers at the omasum (II) were attributed to incomplete biohydrogenation of

16:2n-4, 16:3n-4, 16:4n-1, and 16:4n-3 reflecting the distribution of 16-carbon intermediates in milk (Destaillats et al., 2000).

4.4.2.3. 18-carbon fatty acids in milk

Incremental amounts of FO in the diet had no effect on milk 18:2n-6 or 18:3n-3 (Table 5), but altered the distribution of non-conjugated 18:2 and 18:1 FA intermediates in milk fat, changes characterized by substantial increases in the total concentrations of 18:2 isomers containing one or two *trans* double bonds (0.85, 1.23, 1.91 and 3.73 g/100g FA for 0, 75, 150 and 300 g FO/d, respectively) and *trans* 18:1 isomers (Table 5) confirming earlier findings in lactating cows (Chilliard et al., 2001; Shingfield et al., 2003; Loor et al., 2005a; Toral et al., 2015). Most of the increase in total milk *trans* FA content to FO (52.3, 77.4, 122 and 115 g/d, respectively; IV) was associated with specific enrichment of *trans* 18:2 (Δ9,11; Δ9,12; Δ11,15) and *trans* 18:1 (Δ8–12) isomers (IV) which can be attributed to the incomplete biohydrogenation of unsaturated 18-carbon FA in the rumen (II; Table 3). Previous studies *in vitro* have demonstrated that both 20:5n-3 and 22:6n-3 inhibit the reduction of 18-carbon unsaturated FA to 18:0 causing *trans* 18:1 and *trans* 18:2 intermediates to accumulate (AbuGhazaleh and Jenkins, 2004; Klein and Jenkins, 2011).

Complementary Ag^{+}-TLC fractionation of FAME and GC-MS analysis of DMOX derivatives indicated that FO results in the appearance of 21 minor *trans* 18:2 isomers in milk not reported previously (IV). Even though separation using the CP-Sil 88 capillary column was not possible, further work in our laboratory based on GC-MS and GC-FID analysis with the 100-m SLB-IL111 capillary column (Alves and Bessa, 2014) has allowed *trans*-10,*cis*-15 18:2 to be isolated in milk fat of cows fed 200 g of FO/d alone or in combination with 500 g of SO or LO/d (Kairenius et al., unpublished). Observed increases in milk *trans*-10,*cis*-15 18:2 secretion (0.00, 0.18, 1.28 and 5.99 g/d for control, FO, SFO and LFO, respectively) in experiment 3 (Kairenius et al., unpublished), have provided further evidence that FO alters the biohydrogenation of 18-carbon PUFA, and plant-derived oil supplements can be used to increase the ruminal outflow of spesific 18-carbon PUFA intermediates (III) available for absorption and incorporation into milk and meat fat.

Incremental supplementation of FO elevated milk fat *cis*-9,*trans*-11 CLA content in a quadratic manner, reaching a maximum on the level of 150 g FO/d (from 0.61 to 2.15 g/100 g of FA for 0 and 150 g/d of FO, respectively) but not on 300 g FO/d supplementation level. This was a consequence of the apparent alterations in ruminal biohydrogenation on the diet supplemented with 300 g FO/d, which caused *trans*-10 18:1 to accumulate with no change in *trans*-11 18:1 leaving the rumen (II; Figure 5; Table 3). The supplementation level of 300 g FO/d further increased *trans*-10 18:1 (from 1.06 to 4.20 g/100 g of FA for 150 and 300 g/d FO, respectively) with no change in *trans*-11 18:1 concentration (from 5.31 to 5.46 g/100 g of FA, respectively) (Table 5). Increases in milk *cis*-9,*trans*-11 CLA content were consistent with previous findings in lactating cows (Offer et al., 1999; Donovan et al., 2000; Shingfield et al., 2003; Rego et al., 2005) and can

be explained by an increase in the supply of *trans*-11 18:1 (II; Figure 5; Table 3) for endogenous *cis*-9,*trans*-11 CLA synthesis in the mammary gland in 150 g/d of FO supplementation level (Griinari et al., 2000).

Trans-7,*cis*-9 CLA was not detected in omasal digesta (II; Table 3) but it increased in milk reaching a maximum on the level of 150 g FO/d (from 0.80 to 1.05 g/100 g of FA for 0 and 150 g/d FO, respectively; IV), which can be explained by greater amounts of *trans*-7 18:1 at the omasum (II; Table 3) being used for endogenous synthesis of *trans*-7,*cis*-9 CLA in the mammary gland (Corl et al., 2002). Secretion of *cis*-9,*trans*-12 18:2, *cis*-9,*trans*-13 18:2, and *cis*-9,*trans*-14 18:2 in milk (IV) also exceeded the flow at the omasum (II; Table 3), providing further evidence that these isomers are formed via the action of SCD on *trans* (Δ12-14) 18:1 in the bovine mammary gland (Griinari et al., 2000; Shingfield et al., 2008b).

For all treatments, *trans*-11 18:1 was quantitatively the most important 18:1 isomer in milk fat, accounting for proportionately 0.35, 0.40, 0.48, and 0.40 of total *trans* 18:1 for 0, 75, 150 and 300 g/d of dietary FO, respectively, although at high amounts, FO increased milk *trans*-10 18:1 concentration several-fold as noted above (up to 0.31 of total *trans* 18:1 for 300 g FO/d; IV). Both of these observations are in close agreement with other reports in lactating cows fed FO (e.g. Loor et al., 2005a; Palmquist and Griinari, 2006; Toral et al., 2015). Specific effects of FO on milk *trans*-10 18:1 content, and the level of other *trans*-10 containing FA isomers in milk of cows experiencing FO-induced MFD are discussed more detail in chapter 4.4.3.

In the experiment presented in IV, cows fed FO produced milk containing higher concentrations of FA containing a hydroxyl or oxo group located on carbons 9 and 10 relative to the carboxyl group, including 10-OH-18:0 and 10-O-18:0, but no other significant changes in the amounts of 13- and 15-O-18:0 in milk were observed. The enrichment of 10-O-18:0 in FO-milk is consistent with previous findings in lactating ruminants fed rations supplemented with marine lipids (Bichi et al., 2013; Toral et al., 2014; 2015; 2018b), but the number of *in vivo* experiments reporting concentrations of oxygenated 18-carbon FA in bovine milk with diet-induced MFD is limited (IV; Toral et al., 2015; Leskinen et al., 2019).

4.4.2.4. 20- to 22-carbon fatty acids in milk

Dietary FO supplements enriched 20:5n-3, 22:5n-3 and 22:6n-3 in milk (IV; Table 5) and resulted in the appearance of multiple 20- to 22-carbon FA in milk (IV). Increases in milk long-chain n-3 PUFA concentrations are consistent with several previous reports in lactating cows fed FO under different feeding strategies (Table 5). However, the main findings in most of these reports are related to the FO-induced changes in the distribution of 18-carbon FA in milk of cows fed FO (e.g. Shingfield et al., 2003; Loor et al., 2005a), and the number of investigations reporting the abundance of *cis* and *trans* polyenoic FA in milk is limited (IV; Toral et al., 2015).

Milk from cows fed incremental levels of FO contained *cis*-14 20:1, *trans* (Δ9-15) 20:1, positional and geometric isomers of 20:2 (n = 9) and 20:3 (n = 7), and an unusual 20:4 intermediate not contained in FO (IV). Dietary FO supplements increased also milk 20:2n-6, 20:3n-3, 20:4n-6, 20:4n-3, *cis*-11 22:1 and *cis*-13 22:1 concentrations and the abundance of 22:2 (n = 2), 22:3 (n = 2), and 22:4 (n = 3) isomers containing at least one *trans* double bond. In total, the appearance of 56 novel 20-, 21-, or 22-carbon intermediates containing at least a single *trans* double bond and several all-*cis* long-chain unsaturated FA were found to be increased in milk from cows fed FO (IV), consistent with other findings in lactating cows (Toral et al., 2015). No conjugated 20-, 21-, and 22-carbon FA were detected in milk (IV; Toral et al., 2015), consistent with an absence of these intermediates in omasal digesta of experimental cows offered FO-supplemented diets (I-III).

Incremental amounts of FO resulted in dose-dependent increases in 20- and 22-carbon *trans* FA concentrations (range 0.16–1.61 and 0.01–0.28 g/100 g of FA, respectively) in milk, with *trans* 20:1, *trans* 20:2 and *trans* 20:3 isomers being the most abundant (0.18, 0.27, and 0.17 g/100 g of FA, respectively), confirming other observations in cows fed FO (Toral et al., 2015). A close relationship between flow at the omasum (II) and output in milk (IV) suggests that most, if not all, of the unusual minor 20- to 22-carbon FA in milk from cows fed FO originated from the rumen rather than elongation and desaturation of FA in nonmammary tissues or possible endogenous synthesis in the mammary gland. However, possible benefits of milk 20- to 22-carbon FA to human health need to be considered in the context of the overall changes in milk FA to FO.

4.4.2.5. Transfer efficiency of long-chain n-3 fatty acids from feed to milk

The transfer efficiency of long-chain PUFA from the diet into milk was determined by evaluating the slope of regression of the amount of 20:5n-3, 22:5n-3 and 22:6n3 in milk versus intake. Supplements of FO elevated milk 20:5n-3, 22:5n-3 and 22:6n-3 concentrations in a dose-dependent manner, increases that were associated with a mean apparent transfer efficiency of 1.3, 8.1, and 1.8 %, respectively, being consistent with values in the literature, ranging between 1.4-3.3 and 1.9-4.8 % for 20:5n-3 and 22:6n-3, respectively (Lock and Bauman, 2004; Rego et al., 2005; Palmquist and Griinari, 2006; Toral et al., 2015), but much lower than the apparent transfer efficiency of 25.5 % for 22:5n-3 reported for lactating cows fed FO (Toral et al., 2015). Typically transfer efficiencies reported in the literature are calculated from concentrations of the individual FA in diet and milk, but also estimates evaluated by regression are reported, being 5.7 and 0.98 % for 20:5n-3 and 22:6n-3, respectively (Palmquist and Griinari, 2006). Even though it is generally accepted that apparent transfer of 20:5n-3, 22:5n-3 and 22:6n-3 is rather low, reported literature estimates are subject to considerable variation (Palmquist and Griinari, 2006).

Dietary FO supplements increase 20:5n-3 and 22:6n-3 concentrations in ruminant milk fat and tissue lipids (Palmquist, 2009), but enrichment is limited. The rather low

apparent transfer of 20:5n-3 and 22:6n-3 from the diet into milk in this, and other reports in lactating cows fed dietary FO supplement, can in the most part be attributed to the extensive biohydrogenation of these highly unsaturated FA in the rumen (Shingfield et al., 2013). However, the difference in the enrichment of these long-chain n-3 PUFA in milk also reflects the preferential incorporation of PUFA into plasma CE and PL instead of TAG, which is the primary source of FA for milk fat synthesis (Shingfield et al., 2013).

It is also well established that the use of different feeding strategies to increase milk fat n-3 PUFA content via conversion of dietary 18:3n-3 to very long-chain n-3 PUFA, such as 20:5n-3 and 22:6n-3, has little or no effect on milk fat levels of 20:5n-3 and 22:6n-3 (Palmquist, 2009) because of limited activity of Δ5- and Δ6-desaturase, enzymes responsible for this conversion, and elongase in the mammary gland of lactating cows (Hagemeister et al., 1991). A higher efficiency of transfer for 22:5n-3 compared with 20:5n-3 or 22:6n-3, is postulated to be in part a result of the potential elongation of 20:5n-3 to 22:5n-3 and retroconversion of 22:6n-3 to 22:5n-3 in body tissues (Palmquist, 2009).

4.4.3. Milk fat depression

Dietary FO decreased milk fat synthesis and milk fat content up to -40.6 and -30.1 %, respectively (IV; Table 5) consistent with earlier findings in lactating cows fed FO (Table 5) or marine products (Ahnadi et al., 2002) alone or in combination with plant lipids (Invernizzi et al., 2010; Angulo et al., 2012).

Incremental levels of FO decreased progressively the proportions of short- and medium chain SFA and their secretion in milk (IV; Table 5). However, oil supplements decareased (II) or had no effect (III) on rumen VFA concentrations, but at high amounts FO promoted an increase in molar propionate and butyrate proportions at the expense of acetate (II). These alterations in rumen function may have contributed to the observed differences in the proportions of short- and medium-chain SFA in milk fat (IV). Plasma acetate and BHBA were not analysed in our experiments, but earlier investigations have reported changes in the mammary uptake of these metabolites in cows fed FO (Loor et al., 2005a) and high-concentrate diets (Loor et al., 2005b). However, possible changes in rumen fermentation, e.g. shift in the rumen VFA profile toward less acetate and more propionate, plasma concentrations and mammary uptake of acetate and BHBA, and reductions in *de novo* FA synthesis cannot fully explain reductions in milk fat synthesis (Bauman and Griinari, 2003; Loor et al., 2005b).

To explore in more detail the possible mechanisms underlying the FO-induced MFD in lactating cows, relationship between FA flow at the omasum and milk FA output was investigated, but ruminal outflows of *trans*-9,*cis*-11 CLA (II; Table 3) and *trans*-10,*cis*-12 CLA (II; Table 3) did not explain the lowered milk fat synthesis (IV). Decreases in milk fat output in response to FO were not either associated with increases in milk *trans*-10,*cis*-12 CLA (IV; Table 5), in agreement with earlier findings reporting extremely low concentrations of milk *trans*-10,*cis*-12 CLA in response to FO (Loor et al., 2005a). However, these observations are challenged by a recent meta-analysis that investigated correla-

tion between MFD and milk fat proportions of multiple biohydrogenation intermediates, and reported the highest correlation of MFD with *trans*-10 18:1 and *trans*-10,*cis*-12 CLA (Conte et al., 2018). No *cis*-10,*trans*-12 CLA was detected in omasal digesta (I; II; Table 3) or in milk (IV), consistent with other findings in lactating cows experiencing diet-induced MFD (Shingfield et al., 2003; Ventto et al., 2017; Leskinen et al., 2019).

Previous investigations *in vivo* have also demonstrated the potential role of *trans*-10,*cis*-15 18:2 in MFD (Alves and Bessa, 2014; Ventto et al., 2017; Leskinen et al., 2019), although no direct measurements of the potential antilipogenic effects of *trans*-10,*cis*-15 18:2 on milk fat synthesis have been reported. However, further studies using mice adipocyte cell cultures have shown that *trans*-10,*cis*-15 18:2 isolated from beef fat does not exert same anti-adipogenic properties as *trans*-10,*cis*-12 CLA (Vahmani et al., 2016). *Trans*-10,*cis*-15 18:2 co-eluted with *trans*-11,*cis*-15 18:2 on the CP-Sil 88 column used for GC-analysis of FA in omasal digesta (I; II; Table 3) and milk fat (IV; Table 5), and no definitive conclusions of the incremental levels of dietary FO on *trans*-10,*cis*-15 18:2 formation can be drawn based on the results of this thesis. However, the appearance of *trans*-10,*cis*-15 18:2 in the omasal digesta, as well as increased omasal flow of *trans*-10,*cis*-15 18:2 in response to inclusion of additional level of 200 g FO/d alone or in combination with plant oils (0.00, 0.81, 5.20 and 35.8 g/d for control, FO, SFO and LFO, respectively; III) demonstrates the potential involment of ruminal *trans*-10,*cis*-15 18:2 formation in lactating cows fed diets often associated with MFD (III). In support of this, the milk fat synthesis (-16.7, -41.4, and -48.7 %) and milk fat content (-11.8, -19.3, and -27.7 %) decreased in experiment 3 for FO, SFO and LFO compared with diet containing no oil, respectively (Kairenius et al., unpublished).

The inhibitory effects of dietary FO supplementation on milk fat synthesis were associated with a remarkable increase in *trans*-10 18:1 at the omasum from control diet to increasing levels of FO (II; Table 3) and elevated *trans*-10 18:1 concentrations in milk fat, respectively, with the greatest increase to the additional level of 300 g FO/d (IV; Table 5). These findings are in agreement with previous reports supporting the potential role of *trans*-10 18:1 in FO-induced MFD (Bauman and Griinari, 2003; Shingfield and Griinari, 2007; Conte et al., 2018). Across all treatments, a close negative association existed between *trans*-10 18:1 at the omasum and milk fat secretion (IV), which may be considered evidence that increased ruminal formation of *trans*-10 18:1 contributes to FO-induced MFD in lactating cows.

Even though increases in milk *trans*-10 18:1 content is a consistent feature of diet-induced MFD in lactating cows (Bauman and Griinari, 2003; Shingfield and Griinari, 2007; Conte et al., 2018), previous reports on the physiological effects of *trans*-10 18:1 in the lactating cow are inconsistent. Abomasal infusion of 42.6 g/d of *trans*-10 18:1 was shown to have no influence on milk fat secretion (Lock et al., 2007), findings that have been challenged on the basis that the enrichment of *trans*-10 18:1 in milk during postruminal infusions was too low (1.11 g/100 g FA; Lock et al., 2007) for possible effects on mammary lipogenesis to be detected (Kadegowda et al., 2008). Further investigations have demonstrated that postruminal infusion of a mixture of 18:1 methyl esters

supplying 92.1 g/d of *trans*-10 18:1 over a 5-d period induced an approximate 20 % decrease in milk fat yield, associated with a higher proportion of *trans*-10 18:1 in milk (on average 4.37 g/100 g FA; Shingfield et al., 2009). Even though a direct cause and effect could not be established, comparisons with reports in the literature and the relative abundance of constituents in the methyl ester preparation infused implicated *trans*-10 18:1 as the isomer responsible.

In most cases a dramatic deacrease in milk fat yield (even up to -50%; Invernizzi et al., 2010) is associated with a lower expression of most of the lipogenic genes involved in bovine mammary *de novo* synthesis, uptake of preformed long-chain FA, and TAG synthesis and lower expression of transcription factor SREBP1 that is a regulatory element of genes involved in major mammary lipogenic pathways (Bernard et al., 2018). Incubations of mammary epithelial cells with *trans*-10 18:1 have been shown to decrease lipogenic gene expression of FASN, SCD, and SREBF1 (Kadegowda et al., 2009), suggesting that *trans*-10 18:1 may be active in the mammary gland. In support of this, a close negative association between milk fat secretion and concentration of *trans*-10 18:1 in milk was observed in the loading plots for correlations between milk FA composition and milk fat content and yield in our study (IV).

Decreases in milk fat secretion with increasing FO supplementation were also associated with an increase in milk fat *cis*-11 18:1 concentration (from 0.46 to 1.27 g/100 g of FA) consistent with a negative relationship between these parameters reported previously for cows with FO-induced MFD (Gama et al., 2008; Toral et al., 2015). *Cis*-11 18:1 is a component of bacterial and dietary lipids and can be synthesized from the elongation of *cis*-9 16:1, and the enrichment of *cis*-11 18:1 in milk on FO treatments can be explained by an increase in *cis*-11 18:1 at the omasum (from 2.53 to 10.5 g/d; II). Earlier experiments established that FO promotes ruminal escape of *cis*-11 18:1 (I; Shingfield et al., 2003), but the biological activity of *cis*-11 18:1 as a potent milk fat inhibitor in ruminants is equivocal. Incubations with *cis*-11 18:1 were shown to decrease lipogenesis in bovine adipocytes (Burns et al., 2012), whereas postruminal infusion of a mixture of 18:1 isomers supplying up 12.4 g/d of *cis*-11 18:1 in cows increased concentrations of this isomer in milk from 0.59 to 1.50 g/100 g of FA but had no effect on milk fat synthesis (Shingfield et al., 2007).

During FO-induced MFD, decreases in milk fat synthesis are accompanied by lowered proportions of 18:0 and *cis*-9 18:1 in milk fat and higher *trans* 18:1 concentrations (Offer et al., 1999; Donovan et al., 2000; Table 5). Several reports have suggested that decreases in the availability of 18:0 combined with an increase in *trans* 18:1 isomers, as well as the regulation of TAG synthesis to maintain milk fat fluidity, may explain, or at least contribute to, the decrease in milk fat synthesis (Loor et al., 2005a; Gama et al., 2008; Toral et al., 2018a,b). It is well established that *trans* FA have higher melting points than their *cis* counterparts, being thus more similar to SFA from the physical and functional point of view. In addition, the melting point of FA depends on the chain length and the degree of unsaturation (Ratnayake and Galli, 2009). The larger the num-

ber of carbon atoms, the higher the melting point, and the larger the number of double bonds, the lower the melting point.

However, in high amounts FO had no effect on calculated mean milk fat melting point (IV; Table 5), despite of the substantial changes in milk FA composition (IV). This is in a good agreement with previous findings in lactating ruminants fed FO (Toral et al., 2013; 2018a), but in contrast with some other reports in lactating cows demonstrating that dietary FO supplements may also increase (Gama et al., 2008) or decrease (Toral et al., 2015) calculated mean milk fat melting point. However, the inconsistency between our results and previous findings in the literature is difficult to explain. As previous meta-analysis concluded, the variations in milk fat melting point in response to a wide range of dietary lipid supplements are much smaller than the variation in milk FA composition, indicating that the maintenance of milk fat melting point within a normal physiological range has a role in the regulation of milk fat synthesis during diet-induced MFD (Toral et al., 2013).

Activity of SCD in the mammary gland of ruminants is thought to serve as a mechanism to maintain and regulate the fluidity properties of milk fat for efficient secretion from the mammary gland by reducing the melting point of FA, and SCD activity is often estimated by calculating desaturation indexes that are based on the product/substrate FA ratios for the relevant desaturase FA pairs, *cis*-9 14:1/14:0, *cis*-9 16:1/16:0 and *cis*-9 18:1/18:0, in particular. In this research, dietary FO supplements progressively increased milk fat *cis*-9 18:1/18:0 concentration ratios (by 5.17, 16.7, and 24.7 % compared to control; IV; Table 5), without substantial changes in *cis*-9 18:1 at the omasum (II; Figure 5), but not *cis*-9 14:1/14:0 ratios (IV; Table 5), a proxy used for mammary SCD activity (Bernard et al., 2008). This, together with a shortage of available 18:0 (II; Figure 4), caused by the inhibition of the last step of ruminal biohydrogenation, indicates that substrate specifity of SCD may alter FO-induced MFD.

In addition, more recently, studies conducted with lactating sheep, have demonstrated that dietary supplementation of 18:0 with FO, regardless of the 18:0 doses, were not able to reverse the negative effect of FO on MFD (Toral et al., 2018a,b). Although the reasons underlying the lack of response to dietary 18:0 are uncertain, these findings suggest that changes in SCD-activity, supply of 18:0 for Δ9-desaturation or milk fat melting point may not, at least alone, explain FO-induced MFD.

Cows experiencing FO-induced MFD produced milk containing higher concentrations of 10-OH-18:0, 10-O-16:0 and 10-O-18:0 (refer to chapter 4.4.2.3). Partial least square regression analysis indicated that a positive association existed between FO-induced MFD and the milk fat proportions of oxygenated 18-carbon FA, particularly of 10-oxo-18:0 (IV; Table 5), consistent with other findings in lactating ruminants (Bichi et al., 2013; Toral et al., 2015; Frutos et al., 2018). Although marine lipids seem to enrich the concentration of oxygenated FA in milk of ruminants there is no conclusive evidence to establish a direct cause and effect of these FA metabolites on mammary lipogenesis. However, all these findings together suggest the potential involvement of oxygenated 18-carbon FA, with 10-oxo-18:0 in particular, in FO-induced MFD, but the association

between FO-induced MFD and 13- and 15-oxo-18:0 seems to be less relevant (IV; Toral et al., 2015; Frutos et al., 2018).

Further exploration of the data, mainly associations between the flow of selected 16- to 22-carbon FA at the omasum to FO treatments (II) and milk fat yield (IV) provided further insight into the possible causes for FO-induced MFD and varying effects of FO on milk FA composition in lactating cows. In the loading plots for correlations between milk FA composition and milk fat yield and content (IV), several close negative associations were identified between milk fat secretion and concentrations of multiple long-chain 16-, 18-, 20-, and 22-carbon PUFA containing one or more *trans* double bonds. This indicates that *trans*-10,*cis*-12 CLA alone cannot explain FO-induced MFD.

In addition, cows fed FO and experiencing MFD produced milk containing higher concentrations of other FA containing a *trans* double bond or a hydroxy or oxo group located on carbons 9 and 10 relative to the carboxyl group that included *trans*-10 16:1, *trans*-10,*trans*-14 16:2, unresolved *trans*-10,*cis*-15 and *trans*-11,*cis*-15 18:2, *trans*-10,*trans*-12 CLA, *trans*-10,*cis*-12 CLA, *trans*-10,*trans*-14,*trans*-17 20:3 and 10-OH-18:0 (IV). Furthermore, *trans*-8,*trans*-10 CLA, *trans* (Δ9,10) 20:1, and *trans*-10,*trans*-16 20:2 were located close to *trans*-10 18:1, but vertically opposite in the correlation loading plot (IV). Overall, the PLS analysis suggested that FO-induced MFD may arise from changes in the concentrations of multiple FA in milk, including a decrease in 18:0 supply in combination with increased mammary uptake of *cis*-11 18:1, *trans*-10 18:1 and mono and polyenoic *trans* 20- and *trans* 22-carbon FA (IV).

Table 5 Indirect comparison of dietary fish oil supplements on milk fat synthesis and milk fatty acid composition of lactating cows

Reference	Shingfield et al., 2003[1]		Rego et al., 2005[2]			Loor et al., 2005a[3]		IV[4]				Gama et al., 2008[5]			Toral et al., 2015[6]	
	C	FO250	C	FO160	FO320	C	FO270	C	FO75	FO150	FO300	C	FO200-HF	FO200-LF	C	FO420
Fish oil source	none	herring + mackerel	none	sardine	sardine	none	menhaden	none	herring + mackerel	herring + mackerel	herring + mackerel	none	salmon	salmon	none	anchovy
Fish oil inclusion rate, g/d	0	250	0	160	320	0	270	0	75	150	300	0	200	200	0	420
Forage inclusion rate, g/kg DM	600	600	*ad lib*	*ad lib*	*ad lib*	660	660	580	580	580	580	LF	HF	LF	400	400
Duration, d	14	14	28	28	28	28	28	28	28	28	28	21	21	21	26	26
Animals per group	5	5	4	4	4	6	6	4	4	4	4	4	4	4	4	4
Milk fat content, g/kg	46.0	42.8	34.8	29.9	23.4	35.4	25.1	41.2	38.9	33.0	28.8	38.6	28.5	26.2	33.4	23.4
Response[7]		-6.96		-16.4	-32.8		-29.1		-5.58	-19.9	-30.1		-26.2	-32.1		-29.9
Milk fat yield, g/d	788	602	920	780	580	783	567	997	945	846	592	730	450	440	992	706
Response[7]		-23.6		-15.2	-37.0		-27.6		-5.22	-15.1	-40.6		-38.4	-39.7		-28.8
Milk fat melting point, °C	-	-	-	-	-	-	-	38.7	38.4	38.7	38.3	36.1	38.4	37.2	37.3	34.2
Fatty acid profile (g/100 g fatty acids)																
4:0	4.58	2.42	3.90	3.13	3.14	1.91	1.83	3.30	3.47	3.17	2.86	-	-	-	3.39	3.86
6:0	2.23	1.66	1.06	0.95	0.79	1.92	1.75	2.05	2.02	1.77	1.59	-	-	-	2.38	1.95
8:0	1.11	1.08	0.86	0.70	0.59	1.28	1.23	1.25	1.20	1.05	0.94	-	-	-	1.30	1.00
10:0	2.22	2.81	1.92	1.54	1.36	3.43	3.54	2.47	2.27	2.06	1.88	-	-	-	2.80	2.15
∑4:0,6:0,8:0, 10:0	10.1	7.97	7.74	6.32	5.88	8.54	8.35	9.07	8.96	8.05	7.27	9.60	9.15	8.60	9.87	8.96
12:0	2.40	3.39	2.48	2.02	1.77	4.27	4.49	3.39	3.07	2.88	2.72	-	-	-	3.03	2.42
14:0	10.2	13.3	9.47	9.36	8.84	13.9	14.8	12.0	11.6	11.4	10.9	-	-	-	11.2	10.1
16:0	24.7	33.3	23.2	23.1	22.6	34.2	31.8	30.1	29.7	28.2	27.1	-	-	-	30.1	25.4
∑12:0,14:0,16:0	37.3	50.0	35.2	34.5	33.2	52.4	51.1	45.5	44.4	42.5	40.7	48.8	56.0	48.8	44.3	37.9
18:0	19.5	4.43	12.0	10.4	7.68	8.69	2.71	10.1	8.90	6.06	4.25	7.88	2.03	2.49	10.2	3.17
10-O-18:0	-	-	-	-	-	-	-	0.11	0.15	0.67	0.73	-	-	-	0.03	0.75
13-O-18:0	-	-	-	-	-	-	-	0.02	0.02	0.04	0.03	-	-	-	0.02	0.03
*c*9-18:1	18.1	4.84	23.6	20.1	15.7	15.8	6.05	17.5	16.2	12.2	8.76	22.3	8.87	10.8	16.8	7.56

Reference	Shingfield et al., 2003[1]		Rego et al., 2005[2]			Loor et al., 2005a[3]		IV[4]				Gama et al., 2008[5]			Toral et al., 2015[6]	
	C	FO250	C	FO160	FO320	C	FO270	C	FO75	FO150	FO300	C	FO200-HF	FO200-LF	C	FO420
∑*trans* 18:1	4.5	14.4	5.92	8.53	12.0	3.01	13.8	3.91	6.13	11.1	13.5	-	-	-	3.34	14.5
*t*10	0.21	1.01	-	-	-	0.27	1.76	0.34	0.46	1.06	4.20	0.65	2.57	3.76	0.42	4.10
*t*11	1.80	9.39	-	-	-	1.08	9.17	1.37	2.43	5.31	5.46	1.92	8.60	11.4	1.25	7.17
∑CLA	0.56	1.85	2.25	3.23	3.63	0.63	3.36	0.76	1.20	2.38	2.26	-	-	-	0.72	2.83
*c*9,*t*11	0.39	1.66	-	-	-	0.56	3.20	0.61	1.03	2.15	2.07	0.67	2.74	3.21	0.59	2.56
*t*9,*c*11	-	-	-	-	-	0.01	0.04	-	-	-	-	-	-	-	0.02	0.10
*t*10,*c*12	<0.01	<0.01	-	-	-	-	-	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.02	<0.01	0.01
*t*9,*t*11	0.010	0.014	-	-	-	0.03[a]	0.08[a]	0.01	0.02	0.02	0.02	-	-	-	0.02	0.01
*t*10,*t*12	<0.01	0.01	-	-	-	-	-	<0.01	<0.01	<0.01	0.01	-	-	-	<0.01	0.010
*t*11,*c*15 + *t*10,c15 18:2[8]	0.19	1.56	-	-	-	0.07	1.01	0.24	0.34	0.64	2.04	-	-	-	0.11	1.32
18:2n-6	0.90	1.25	2.51	1.99	0.65	1.88	1.36	1.22	1.18	1.21	1.17	3.08	1.62	2.24	2.28	1.96
18:3n-3	0.42	0.45	0.99	1.06	1.03	0.28	0.31	-	-	-	-	0.19	0.17	0.20	0.65	0.56
20:5n-3	0.05	0.11	0.07	0.18	0.33	0.08	0.36	0.06	0.06	0.07	0.17	0.05	0.30	0.27	0.10	0.47
22:5n-3	0.0	0.22	0.07[b]	0.17[b]	0.36[b]	0.14	0.40	0.09	0.08	0.10	0.18	-	-	-	0.16	0.44
22:6n-3	0.0	0.10	0.06	0.17	0.43	0.04	0.17	0.03	0.03	0.05	0.10	0.11	0.56	0.54	0.05	0.27
Fatty acid ratios																
*c*9-14:1/ 14:0	0.06	0.04	-	-	-	0.08	0.07	0.10	0.10	0.10	0.11	0.06	0.06	0.08	0.08	0.08
*c*9-18:1/ 18:0	0.94	1.18	-	-	-	1.82	2.23	1.74	1.83	2.03	2.17	2.88	4.54	4.47	1.73	2.61
*c*9,*t*11 CLA/*t*11-18:1	0.23	0.18	-	-	-	0.52	0.23	0.45	0.44	0.41	0.39	0.39	0.31	0.31	0.41	0.37
*t*10-18:1/*t*11-18:1	0.12	0.11	-	-	-	0.25	0.19	0.25	0.19	0.20	0.77	0.34	0.30	0.33	0.34	0.57

[1]Dry matter intake (DMI) 11.0 and 9.33 kg/d for C and FO250 treatments, respectively; [2]DMI not reported; [3]DMI 19.8 and 16.2 kg/d for C and FO270 treatments, respectively;[4]DMI 19.2, 18.9, 18.3 and 16.0 kg/d for treatments FO0, FO75, FO150 and FO300, respectively; [5]DMI 19.8, 13.3 and 11.4 kg/d, for treatments C, FO200-HF and FO200-LF, respectively, [6]DMI 10.2 and 8.52 kg/d for C and FO420 treatments, respectively; [7]Response calculated as [(treatment – control)/control]*100; [8]*Trans*-11,*cis*-15 18:2 co-elutes with *trans*-10,*cis*-15 18:2 in IV and Toral et al., 2015, but the possible co-elution not reported in Shingfield et al., 2003 or Loor et al., 2005a; *ad lib*, cows at pasture; C, control diet; CLA, conjugated linoleic acid; FO, fish oil; HF, high fibre diet containing 40 % NDF; LF, low fibre diet containing 26 % NDF; [a]Includes also *trans*-8,*trans*-10 CLA and *trans*-10,*trans*-12 CLA isomers; [b]Only 22:5 reported; For clarity purposes, abbreviated names of fatty acids are reported in the table (*c*, *cis*; *t*, *trans*).

5. Conclusions

Based on the results of this research the conclusions and appropriate implications are following:

i) Dietary FO with or without plant oil supplements modified ruminal biohydrogenation of 16- and 18-carbon unsaturated FA, causing dose-dependent increases in *trans* 16:1, *trans* 18:1 and *trans* 18:2 flow and a concomitant decrease in 18:0 at the omasum, and at high amounts promoted *trans*-10 18:1 accumulation at the expense of *trans*-11 18:1. Supplements of FO increased also the flow of 20:5n-3, 22:5-3 and 22:6n-3 at the omasum and resulted in corresponding increases in numerous 20- and 22-carbon unsaturated FA containing one or more *trans* double bonds at the omasum, providing clear evidence of extensive metabolism of 20:5n-3 and 22:6n-3 in the rumen of lactating cows.

ii) No conjugated 20-carbon FA were detected in experimental digesta samples, which suggests that biohydrogenation of long-chain PUFA does not involve formation of intermediates containing a conjugated double bond system. Nevertheless, the hydrogenation of 20:5n-3, 21:5n-3 and 22:6n-3 in the rumen proceeds via two principal mechanisms that involve sequential reduction or isomerisation of *cis* double bonds closest to carboxyl group.

iii) The plant derived 18-carbon PUFA influenced the ruminal metabolism of long-chain n-3 PUFA in the rumen of lactating cows. The biohydrogenation of *cis*-9 18:1, 18:2n-6 and 18:3n-3 in cows fed FO diets with or without of plant oils was higher than biohydrogenation of these FA originating from ingredients of a control diet, being even greater when plant oils were fed with FO. The ruminal metabolism of 22:6n-3 was more extensive on diets containing higher amounts of 18-carbon PUFA, whereas the biohydrogenation of 22:5n-3 showed no difference between FO and diets containing plant oils. Ruminal outflow of 20:5n-3 was not altered when plant oils were added to FO. Supplements of FO plus plant oils shifted the ruminal biohydrogenation towards a higher production of *trans*-10 18:1 at the expense of *trans*-11 18:1.

iv) The inhibitory effects of FO on the reduction of 18-carbon FA to 18:0 were influenced by the relative amounts of 18:2n-6 and 18:3n-3 in the diet. Despite of a similar intake of 18-carbon PUFA and similar flow of *trans* 18:1, the flow of 18:0 at the omasum was lower and accumulation of *trans* 18:2 and 20- to 22-carbon FA intermediates greater for LFO than SFO. Supplementing FO with sources of 18:2n-6 or 18:3n-3 caused *trans*-10 and *trans*-11 18:1 to accumulate, and, on the SFO treatment, *trans*-10 18:1 was the most abundant biohydrogenation intermediate escaping the rumen.

v) Alterations in the amount of FA intermediates at the omasum or ruminal biohydrogenation pathways were not associated with substantial changes in rumen protozoal counts or analysed bacterial populations known to be capable of biohydrogenation, but lowered *Butyrivibrio spp.* numbers in response to incremental levels of FO.

vi) Detailed analysis of lipid in omasal digesta and milk fat of cows fed FO enabled the structure identification of 27 and 37, respectively, previously unidentified 20- to 22-carbon FA intermediates containing at least one *trans* double bond, and the detection of *cis*-14 20:1, 20:2n-3, 21:4n-3 and 22:3n-6 not contained in FO. Dietary FO supplements can be used to enrich 20:5n-3, 22:5n-3 and 22:6n-3 in milk, with associated decreases in 4- to 18-carbon SFA, several-fold increases in CLA, mono- and polyenoic *trans* FA, and PUFA concentrations. Changes in the abundance and distribution of 16-, 18, 20-, and 22-carbon FA containing a single or several *trans* double bonds in milk were analogous with alterations in the ruminal supply of n-3 PUFA, with enrichment of *trans* 18:1 and *trans* 18:2 being quantitatively the most important.

vii) Increasing levels of FO decreased milk fat yield (up to -40.6 %) and milk fat content (up to -30.1%). Fish oil-induced MFD was associated with changes in the concentrations of multiple FA in milk, in particular increases in milk fat *trans*-10 18:1 and *cis*-11 18:1 concentrations. Decreases in milk fat yield in response to FO were not related to changes in milk *trans*-10,*cis*-12 CLA concentration, estimated milk fat melting point or the amounts of *trans*-9,*cis*-11 CLA and *trans*-10,*cis*-12 CLA at the omasum. In addition, no *cis*-10,*trans*-12 CLA was detected in omasal digesta or milk fat. The negative relationship between ruminal outflow of *trans*-10 18:1 and milk fat secretion confirmed that a shift in ruminal 18-carbon FA biohydrogenation toward *trans*-10 biohydrogenation pathway has a role in the regulation of milk fat synthesis during FO-induced MFD. A decrease in 18:0 supply in combination with increased mammary uptake of *cis*-11 18:1, *trans*-10 18:1, and *trans* 20- and 22-carbon FA intermediates originating from the rumen may contribute directly or indirectly to the reduction of milk fat observed during FO-induced MFD.

6. Future research

This thesis work provided a comprehensive analysis of FA metabolites formed in the rumen under different oil-supplemented dietary strategies, especially during FO-induced MFD. This, together with measurements of changes in rumen bacterial population, allowed the identification of potential candidates for inhibitors of milk fat synthesis. However, the current theories of diet-induced MFD, including the biohydrogenation theory, remain incomplete. Several experiments support the potential role of *trans*-10 18:1 in MFD, but no studies have been performed to investigate the potential direct role of *trans*-10,*cis*-15 18:2 or oxo-FA, particularly 10-oxo-18:0, in milk fat synthesis. To confirm the role of these specific compounds in MFD, abomasal infusion studies using a relatively pure forms of *trans*-10,*cis*-15 18:2 and oxo-FA, as well as *trans*-10 18:1, together with lipogenic gene expression studies are needed.

Further research using functional metagenomics and/or metabolomics approaches may provide a greater insight into the diet-induced changes in microbiota and bacterial biohydrogenation within the rumen. The *trans*-11 to *trans*-10 shift in microbial biohydrogenation is not well understood. *Butyrivibrio spp.* are still the best-known *trans*-11 producing ruminal bacteria, however, the ruminal bacteria responsible for *trans*-10 formation are unclear. The results of this thesis and inconsistent results in literature suggest that other, yet uncultured, bacteria might be involved in this process or that specific rumen conditions are needed to produce *trans*-10 containing compounds.

Plasma lipids and metabolites were not analysed in the experiments of this thesis. Quantitative analysis of FA from plasma lipids could provide a deeper insight into the factors contributing in mammary lipogenesis during FO-induced MFD and reveal the possible changes in the composition and concentration of circulating lipids, mammary uptake (jugular-mammary venous differences) and *de novo* synthesis of FA, as well as alterations in desaturation of FA in the mammary gland. These analyses could provide results to understand in more detail the mechanisms underlying the diet-induced MFD in cows. Measurements of BHBA, NEFA, plasma glucose and hormones influencing energy metabolism such as insulin and leptin, could also deepen the understanding of the biological processes related to metabolic disorders.

The impact of diets and nutritional factors inducing graded levels of MFD on genes and gene networks regulating mammary and tissue lipogenic and desaturation pathways could be used to explore the mechanisms driving important processes of lactating cows (e.g. lactation, energy metabolism). This information could be used in establishing solutions for better management of energy balance in dairy cows during late pregnancy and early lactation to improve health, well-being and reproduction performance, as well as in providing new insights for potential strategies to reduce the risk of MFD in dairy cows.

The lactating cow represents a unique model to investigate both acute and long-term regulation of MFD, since temporal changes in milk fat secretion and lipogenic gene expression can be readily monitored in the same animal through the collection of sequential milk samples and a series of tissue biopsies e.g. from subcutaneous adipose,

liver and mammary tissues. Studies in cows fed diets causing MFD are of particular value for investigations on the nutritional regulation of genes that encode for enzymes involved in *de novo* FA synthesis, FA uptake, lipid transport, esterification and desaturation. Examining the impact of diet on changes in mammary gene expression and the interrelation between gene expression in adipose, liver and the mammary gland could further the development of long-term breeding strategies for more optimal production of milk constituents and enhanced milk FA composition.

Milk production from ruminants is coming under increasing criticism due to the need to simultaneously reduce ruminal methane emissions and the overall impact on the environment, improve the health and welfare of ruminant livestock, and produce increasing amounts of dairy products of high nutritional quality that promote long-term human health. Fundamental research regarding the role of ruminant-derived food in health maintenance and disease prevention and opportunities to develop health promoting ruminant-derived foods containing lower proportions of medium-chain SFA and higher concentrations of *cis* MUFA, PUFA and bioactive lipids, including *trans*-11 18:1, CLA, 20:5n-3, 22:5n-3 and 22:6n-3, without increasing levels of *trans* FA in milk, are needed. In addition, development of commercially suitable applications, efficient and safe methods to protect PUFA from ruminal biohydrogenation without impairing their bioavailability for absorption should also be investigated in more detail.

There are indications that dairy products may lower the risk of mortality and CVD (Dehghan et al., 2018) and ruminant *trans* FA may be beneficial for human health (Da Silva et al., 2015). However, little is still known about the nutritional health impact of ruminal and industrial *trans* 16- to 22-carbon FA and this deserves more research effort. A higher consumption of industrial *trans* FA in the human diet is known to be associated with increased cardiovascular risk, with some evidence from clinical trials implicating 18-carbon PUFA containing more than one double bond as being particularly harmful (Lemaitre et al., 2006; Muhlenbeck et al., 2017). It is possible that increases in milk polyenoic *trans* FA may offset some of the expected benefits to human health from the enrichment of 20:5n-3 and 22:6n-3 in ruminant derived foods, but this remains a question. Further evidence from human clinical and cohort studies would be needed to assess the impact of *trans* FA originating from different sources (ruminal vs. industrial *trans* FA) for public health.

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