Universitat Autònoma de Barcelona

Departament de Ciència Animal i dels Aliments

OBTENCIÓN DE UNA LECHE ENRIQUECIDA DE FORMA NATURAL EN ÁCIDOS GRASOS OMEGA-3 Y ÁCIDO CONJUGADO LINOLEICO (CLA) SIN DISMINUCIÓN DE LA CANTIDAD DE LA GRASA LÁCTEA

Adriana Siurana Marina

Bellaterra, Septiembre 2015

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SERGIO CALMIGLIA BLANCAFORT, como Catedrático del *Departament de Ciència Animal i dels Aliments* de la *Facultat de Veterinària* de la *Universitat Autònoma de Barcelona*,

CERTIFICO:

Que la memoria titulada **"Obtención de una leche enriquecida de forma natural con ácidos grasos omega-3 y ácido conjugado linoleico (CLA) sin disminución de la cantidad de la grasa láctea"**, presentada por **Adriana Siurana Marina**, ha sido realizada bajo mi dirección y, considerándola finalizada, autorizo su presentación para que sea juzgada por la comisión correspondiente.

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Tabla de abreviaciones

Castellano

ACACA = Acetil-CoA carboxilasa
ACSL = Familia de cadena larga Acil-CoA sintetasa
ACSS = Familia de cadena corta acil-CoA sintetasa
ADPH = Adipofilin
AG = Ácidos grasos
AGPAT = Acilglicerol-3-fosfato aciltransferasa
AGPI = Ácidos grasos poliinsaturados
AGR2 = anterior gradient 2, protein disulphide isomerase family member
BTN1A1 = Butirofilin
CD36 = molécula CD36 (receptor thrombospondin)
CLA = Ácido linoleico conjugado
DGAT = Diacilglicero aciltransferasa
DGL = Depresión de la grasa láctea
FABP = Proteínas ligantes de AG
FADS = AG desaturasa
FASN = Ácido graso sintasa
GPAM = Glicerol-3-fosfato aciltransferasa
INSIG = gen inducido por insulina
LPIN = Lipin ácido fosfatídico fosfohidrolasa
LPL = Lipoproteína lipasa
LXR = Receptor X del hígado
LXRE = elementos de respuesta a LXR
MAC-T = Células epiteliales de la glandula mamaria
NEFA = Ácidos grasos no esterificados
PPAR = Receptor del factor de proliferación del peroxisoma activado
ROSI = rosiglita zona
SCAP = proteína de activación de la segmentación de SREBP
SCD = Stearoil-CoA desaturasa
SLC27A = Soluto compañia familia 27 (Proteína de transporte de AG)
SREBP = Factor de unión a elementos reguladores del esterol
T09 = T0901317
TAG = triglicéridos
THRSP = hormona tiroidea respondedora a SPOT14
TZD = tiazolidinadiona
VA = Ácido vaccénico
VLDLR = Receptor de lipoproteínas de baja densidad
XDH = Xantina dehidrogenasa

Inglés

ACACA = acetyl-CoA carboxylase
ACLY = ATP-citrate lyase
ACSL = acyl-CoA synthetase long-chain family
ACSS = acyl-CoA synthetase short-chain family
ACVR1C = activin A receptor, type IC
ADF = acid detergent fiber
ADFP = adipophilin
AGPAT = Acylglycerol-3-phosphate acyltransferase
Akt = Serine/threonine kinase
BCVFA = branched-chain volatile fatty acid
BTN = Butyrophilin
CCL2 = chemokine (C-C motif) ligand 2
CD36 = CD36 molecule (thrombospondin receptor)
CD82 = CD82 molecule
CIN = cinnamaldehyde
CLA = conjugated linoleic acid
CO = diets enriched with corn products
CP = crude protein
CTR = control
CVD = cardiovascular disease
DCN = decorin
DGAT = diacylglycerol acyltransferase
DIM = days in milk
DM = dry matter
DMI = dry matter intake
DNASE1L3 = deoxyribonuclease I-like 3
DROSHA = drosha, ribonuclease type III; PRKAG3, protein kinase, AMP-activated, gamma 3 non-catalytic subunit
EE = ether extract
EGFR = epidermal growth factor receptor
EO = essential oils
EUG = eugenol
EXT = extruded seeds
FA = fatty acids
FABP = fatty acid binding protein
FADS = fatty acid desaturase
FASN = fatty acid synthase
FC = fold change
FFQ = food-frequency questionnaire
FO = diets enriched with fish oils
FO+PO = diets enriched with fish oils plus plant oils
FOXA1 = forkhead box A1
FOXM1 = forkhead box M1
FOXO3 = forkhead box O3
GPAT = Glycerol-3-phosphate acyltransferase
HDAC9 = histone deacetylase 9
HNF1A = HNF1 homeobox A
IL17A = interleukin 17A
IL37 = interleukin 37
INHBA = inhibin, beta A
INL = lipase inhibitor
INSIG = insulin induced gene
IPA = Ingenuity Pathway Analysis
LA = linoleic acid
LCN2 = lipocalin 2
LIN = linseed diet
LIP1 = lipase 1
LIP2 = lipase 2
LNA = linolenic acid
LIPIN = lipin phosphatidic acid phosphorilase
LPL = lipoprotein lipase
LXR = liver X receptor MFD = milk fat depression
MGAT5 = mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase
MKL2 = MKL/myocardin-like 2
MTOR = mammalian target of rapamycin
NDF = neutral detergent fiber
NTRK1 = neurotrophic tyrosine kinase, receptor, type 1
OIL = oils
OM = organic matter
PAS = feeding fresh pasture
PAX5 = paired box 5
PC = principal component
PDE4D = phosphodiesterase 4D, cAMP-specific
PDPK1 = phosphoinositide-dependent kinase 1
PHVO = partially hydrogenated vegetable oils
PLA2G10 = phospholipase A2, group X
PPARG = peroxisome proliferator-activated receptor gamma
PRLR = prolactin receptor
PROC = protein C (inactivator of coagulation factors Va and VIIIa) CSF2, colony stimulating factor 2 (granulocyte-macrophage)
PS = processed seeds
PTGS2/COX-2 = cyclooxygenase-2
PTSO = Oxy-propyl-thiosulphate

PUFA = polyunsaturated fatty acids
R-MFD = resistant to milk fat depression
RA = diets enriched with rapeseed
RICTOR = rapamycin-insensitive companion of mTOR.
RNASE1 = ribonuclease RNase A family, 1 (pancreatic)
RPKM = reads per kilobase per million mapped reads
RS = raw supplements
S-MFD = sensitive to milk fat depression
SCAP = SREBP cleavage activating protein
SCD = stearoyl-CoA desaturase
SEM = standard error of the mean
SIN3A = SIN3 transcription regulator family member A
SLC27A = solute carrier family 27 (fatty acid transporter)
SMO = smoothened, frizzled class receptor
SOY = diets enriched with soybean
SREBP1 = Sterol regulatory element binding protein 1
SUN = diets enriched with sunflower
TCF12 = transcription factor 12
TEB = terminal end buds
TFF3 = trefoil factor 3 (intestinal)
THRSP = thyroid hormone responsive SPOT14
UPF2 = UPF2 regulator of nonsense transcripts homolog (yeast)
VA = vaccenic acid
VFA = volatile fatty acids
VIPR1 = vasoactive intestinal peptide receptor 1
VLDLR = very-low density lipoprotein receptor
XDH = xanthine dehydrogenase

Resumen

Se realizaron tres estudios con el fin de identificar estrategias para evitar la depresión de la grasa láctea en las vacas lecheras cuando las dietas se complementan con aceites ricos en AGPI. Una revisión bibliográfica realizada en el primer estudio mostró que las recomendaciones diarias de CLA más comúnmente reportados para humanos son 0,8 g/d (0,6 a 3,0 g/d), aunque todas las recomendaciones se han extrapolado a partir de modelos animales y los pocos estudios en humanos reportan resultados contradictorios. Se seleccionaron 69 artículos publicados, donde se alimentaron vacas lecheras con diferentes grasas y se registró el contenido de grasa de la leche y el perfil de AG. Teniendo en cuenta los cambios en CLA y el contenido de grasa láctea, la suplementación con aceites de pescado, junto con aceites vegetales sería la mejor estrategia (395 mg de *cis*-9, *trans*-11 CLA/l *vs.* 188 mg de *cis*-9, *trans*-11 CLA/l; aumento de 2,1 veces). El consumo humano medio actual estimado en Europa, Estados Unidos y Canadá es 0,21 g/d. Si suponemos un aumento de 2,1 veces en el contenido de CLA en la leche, el consumo humano promedio aumentaría de 0,21 a 0,46 g/día. Aunque hay datos suficientes en nutrición sobre las estrategias para aumentar el contenido de CLA en la leche, las necesidades humanas no han sido bien establecidas y, en base a las recomendaciones actuales, son inalcanzables incluso si toda la leche y productos lácteos se consumen como productos enriquecidos en CLA. En el segundo estudio, se llevaron a cabo dos experimentos (fermentación *in vitro* y fermentadores de doble flujo continuo) para determinar los efectos de lipasas y aceites esenciales sobre la fermentación ruminal y la aparente biohidrogenación del ácido linoleico y linolénico. Los tratamientos fueron control, lipasa 1 y 2, un inhibidor de lipasa, PTSO; Eugenol y CIN (experimento 1), y control, lipasa 1, PTSO y CIN (experimento 2) a dos niveles de pH (6.4 y 5.6). En el experimento 1, Lipasa 1 aumentó la aparente biohidrogenación de LNA y redució la eficiencia de los pasos intermedios de la biohidrogenación del LA y LNA, pero estos resultados no fueron observados en el experimento 2. El PTSO inhibió la aparente biohidrogenación de LA y LNA y disminuyó las concentraciones totales de AG volátiles en los dos experimentos. El tercer estudio se basó en el hecho de que alimentar a vacas lecheras con dietas suplementadas con lino resulta en la depresión grasa de la leche, pero hay una amplia gama de sensibilidad entre las vacas. Los objetivos de este estudio fueron comparar la expresión de mRNA en las células somáticas de la leche en vacas resistentes o sensibles a la DGL, e identificar las vías metabólicas y factores de transcripción afectados por la DGL en vacas resistentes o sensibles a la DGL. Cuatro vacas fueron seleccionados de una granja comercial después de un cambio de una dieta de control a una dieta rica en lino. Entre ellas, dos vacas (R-MFD) fueron resistentes a la DGL teniendo alto

contenido de grasa de la leche tanto en el control (4,06%) como en la dieta suplementada con lino (3,90%); y dos vacas (S-MFD) fueron sensibles a la DGL disminuyendo el contenido de grasa de la leche después del cambio a la dieta con lino (3,87-2,52%). El análisis de expresión diferencial entre S-MFD y R-MFD vacas permitió detectar un gran número de genes expresados diferencialmente en ambas dietas, CTR (n = 1316) y LIN (n = 1.888). El ánalisis de vias metabólicas y genes reguladores claves también permitió detectar un gran número de vías y genes reguladores clave expresados de forma diferente. El análisis de SNP mostró 641 SNP sólo en las vacas R-MFD y 1024 sólo en las vacas S-MFD entre los genes expresados de manera diferente en todas las comparaciones. Los resultados sugieren que las vacas R-MFD podrían estar activando un mecanismo de compensación para aumentar la síntesis de ácidos grasos en presencia de lino. En su conjunto, los resultados de esta tesis sugieren la posibilidad de evitar la depresión de la grasa láctea mediante las tecnologías transcriptomica.

Summary

Three studies were conducted in order to identify strategies to avoid milk fat depression in dairy cows when diets are supplemented with rich oils PUFA. Literature research in the first study showed that the most commonly reported intake recommendations of CLA for human are 0.8 g/d (from 0.6 to 3.0 g/d), although all recommendations have been extrapolated from animal models and the few human studies reported contradictory results. We selected published papers (n = 69) where dairy cows were fed different fats and the milk fat content and FA profile were reported. Considering the changes in CLA and milk fat content, supplementation with fish oils together with vegetable oils would be the best strategy (395 mg of *cis*-9, *trans*-11 CLA/l *vs.* 188 mg of *cis*-9, *trans*-11 CLA/l; increase of 2.1 times). The estimated current average human consumption in Europe, US and Canada is 0.21 g/d. If we assume an increase content of 2.1 times in CLA in milk, average human consumption would increase from 0.21 to 0.46 g/day. Although there is sufficient data on feeding strategies to increase CLA content in milk, human requirements have not been well established and, based on current recommendations, they are unattainable even if all milk and milk products were consumed as CLA enriched products. In the second study, two experiments (bath culture fermentation and continous culture fermenters) were conducted to determine the effects of lipases and essential oils on rumen fermentation and apparent biohydrogenation of linoleic and linolenic acids. Treatments were control, lipase 1 and 2, a lipase inhibitor, PTSO; Eugenol and CIN (experiment 1), and control, lipase 1, PTSO and CIN (experiment 2) at two pH levels (6.4 and 5.6). In experiment 1, Lipase 1 increased the apparent biohydrogenation of LNA and reduced the efficiency of intermediary steps of biohydrogenation of LA and LNA but these results were not observed in experiment 2. The PTSO inhibited the apparent biohydrogenation of LA and LNA and decreased total VFA concentrations in the two experiments. The third study was based in the fact of feeding linseed to dairy cows results in milk fat depression (MFD), but there is a wide range of sensitivity among cows. The objectives of this study were to compare the mRNA expression of transcripts expressed in milk somatic cells in cows resistant or sensitive to MFD, and to identify metabolic pathways and transcription factors affected by MFD in resistant or sensitive cows. Four cows were selected from a dairy farm after a switch from a control diet to a linseed-rich diet. Among them, two cows (R-MFD) were resistant to MFD having high milk fat content in both control (4.06%) and linseed-rich diet (3.90%); and two cows (S-MFD) were sensitive to MFD decreasing milk fat content after the change into the LIN diet (3.87 to 2.52 %). Differential expression analysis between S-MFD and R-MFD cows

allowed to detect a large number of differentially expressed genes in both diets, CTR (n = 1,316) and LIN (n = 1,888). Pathway and key gene regulator analysis also allowed detecting a large number of differently expressed pathways and key gene regulators. Analysis of SNP discovery showed 641 SNP only in R-MFD cows and 1024 only in S-MFD cows among differently expressed genes in all comparisons. Results suggest that R-MFD cows could be activating a compensatory mechanism to increase the fatty acid synthesis in linseed-rich diets. As a whole, results of this thesis suggests the possibility of avoid milk fat depression by transcriptomic technologies.

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CAPÍTULO 1

Revisión bibliográfica

1. INTRODUCCIÓN GENERAL. PROBLEMÁTICA DEL SECTOR

En los últimos años, el consumidor ha desarrollado un interés creciente en el consumo de alimentos saludables y con actividades funcionales. En este contexto, existe un gran interés en modificar el perfil de ácidos grasos (AG) de la leche, que contiene un 70% AG saturados (Jenkins y McGuire, 2006), hacia uno más insaturado para responder a las demandas de los consumidores. Los AG saturados tienden a aumentar los niveles de colesterol plasmático incrementando la incidencia de enfermedades cardiovasculares (Hu *et al.*, 2001), lo que motiva la necesidad de modificar su perfil hacia uno más insaturado. Además, diversos AG poliinsaturados (AGPI) han demostrado tener efectos beneficiosos para la salud humana. Quizás el ejemplo más claro ha sido el desarrollo del concepto y la comercialización de alimentos ricos en AG omega-3, ya que se ha demostrado que tienen efectos cardioprotectores en humanos (Kris-Etherton *et al.,* 2002). El desarrollo de estos productos se ha basado en la modificación de la relación omega-6: omega-3 de la dieta humana que en la actualidad es del 10:1, cuando las recomendaciones oficiales las establecen en 4:1. Este impulso ha permitido la comercialización de huevos, leches y otros alimentos ricos en éstos AG.

Además, otros AG también han demostrado tener efectos beneficiosos para la salud humana y se consumen de forma deficitaria, entre los que destacan algunos isómeros del ácido linoleico conjugado (CLA). Este ácido graso parece contribuir de forma positiva a los efectos saludables de la leche, como la reducción de la carcinogénesis, de la aterosclerosis, del colesterol, de la ganancia de tejido adiposo, de la diabetes mellitus tipo II y la modulación de la respuesta inmunitaria e inflamatoria, entre otros (Belury, 2002; Parodi, 2004; Pariza *et al.*, 2001). A diferencia de los AG omega-3 (que se encuentran en numerosos aceites de origen vegetal, como el lino), el CLA se produce en la naturaleza casi de forma exclusiva en los rumiantes a partir de los intermediarios de la biohidrogenación de los AGPI en el rumen, por lo que el enriquecimiento natural de alimentos con CLA sólo es posible a través del consumo de productos lácteos y cárnicos derivados de rumiantes (Ritzenthaler *et al.,* 2001).

Para obtener una leche enriquecida en omega-3 y CLA de forma natural, las estrategias nutricionales en vacas lecheras más utilizadas son la adición de aceites vegetales ricos en ácido linoleico (precursor de la síntesis de CLA) y linolénico (fuente de omega-3 y precursor de la síntesis de CLA) que resultan en la acumulación de ácido vaccénico (VA; *trans*-11 C18:1) en el rumen y la posterior desaturación del VA a *cis*-9, *trans*-11 CLA en la glándula mamaria

mediante el enzima delta-9 desaturasa. Esta síntesis endógena en la glándula mamaria es la mayor fuente de CLA en la leche.

Sin embargo, en algunas condiciones de fermentación ruminal, las vías de la biohidrogenación de los AGPI se modifican, y se producen AG intermediarios, de los cuales algunos son potentes inhibidores de la síntesis de la grasa láctea (Bauman y Griinari, 2001). Esta depresión de la grasa láctea (DGL) se ha relacionado sobre todo con el incremento del isómero *trans*-10 C18:1 en el rumen, y *trans*-10, *cis*-12 CLA en el rumen y por síntesis endógena en la glándula mamaria. Esta reducción de la grasa láctea tiene implicaciones económicas importantes en las explotaciones lecheras y en la planificación de la comercialización de leche enriquecida con omega-3 y CLA, ya que puede comprometer la normativa de garantías de composición grasa mínima de dicha leche, además de dificultar el plan de producción industrial por no disponer de suficiente grasa para elaborar otros productos lácteos.

2. QUÉ ES EL CLA

El CLA es un AGPI formado por 18 carbonos y dos dobles enlaces conjugados separados por un único enlace simple (Lawson, 2001) los cuales pueden encontrarse en las cuatro configuraciones geométricas: *cis, trans; trans, cis; cis, cis o trans, trans*, y en las posiciones: 7,9; 8,10; 9,11; 10,12; 11,13 y 12,14 (Watkins y Li, 2002). La grasa proveniente de rumiantes es la única fuente significativa de CLA en la dieta humana, ya que los productos lácteos y cárnicos aportan aproximadamente el 70 y 25 % del CLA total ingerido en la dieta, respectivamente (Ritzenthaler *et al.,* 2001). Aunque en la grasa de rumiantes pueden encontrarse todas las formas isoméricas del CLA, la principal es el *cis*-9, *trans*-11 CLA el cual representa aproximadamente entre un 75-90% del total. El segundo isómero más común es el *trans*-7, *cis*-9 CLA, representando aproximadamente un 10% del total, y cada uno del resto de isómeros representan menos del 1% del total (Lock *et al.,* 2004). Sin embargo, en casos de acidosis la concentración del isómero *trans*-10, *cis*-12 incrementa considerablemente (Enjalbert *et al.,* 2008; Choi *et al.,* 2005). A pesar de la gran diversidad de isómeros de los que está formada el CLA, los efectos fisiológicos conocidos hasta el momento son atribuidos sólo a los isómeros *cis*-9, *trans*-11 CLA y *trans*-10, *cis*-12 CLA, aunque es posible que un gran número de los otros isómeros puedan tener actividades biológicas (Pariza, 2004).

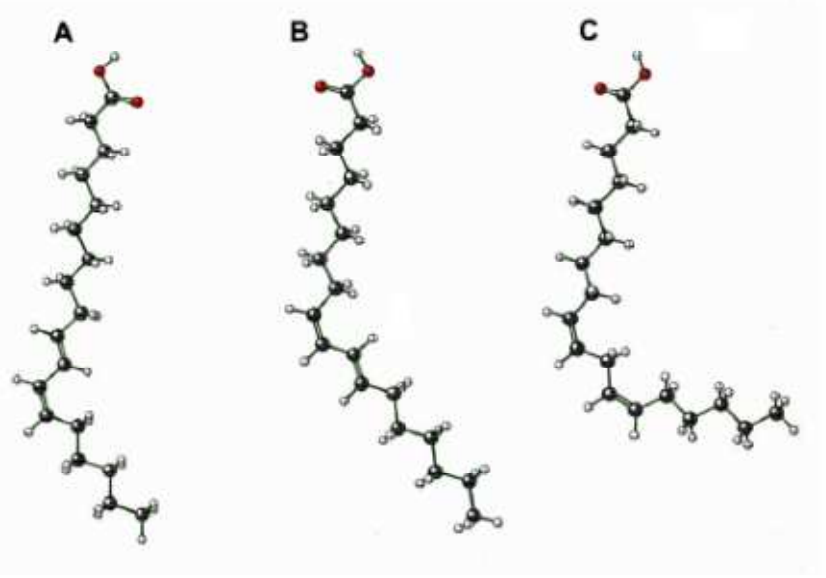

Figura 1. *Estructura química del ácido linoleico (C18:2 cis-9, cis-12) (C) y de los isómeros trans-10, cis-12 CLA (A) y cis-9, trans-11 CLA (B). Adaptado de Bauman et al. (2001).*

3. CÓMO SE FORMA EL CLA

El CLA se produce mayoritariamente en la glándula mamaria a partir de precursores que se generan en la fermentación ruminal. La producción del CLA en la leche requiere una biohidrogenación incompleta de AGPI en el rumen, proceso que es difícil de controlar. En el rumen ocurren dos procesos en el metabolismo de los lípidos. El primer paso es la hidrólisis de los enlaces éster mediante lipasas microbianas (o lipólisis) (Dawson *et al.,* 1977) y el segundo es la biohidrogenación de los AGPI.

3.1. **Lipólisis**

La hidrólisis de los enlaces éster de los triglicéridos (TAG), fosfolípidos y glicolípidos es el paso inicial del metabolismo de los lípidos provenientes de la dieta y un prerrequisito para la biohidrogenación de los AGPI. La hidrólisis de los lípidos se realiza principalmente por bacterias con una mínima participación de los protozoos, las lipasas salivares y las lipasas provenientes de las plantas. La hidrólisis es un proceso extracelular, donde el glicerol y azúcares liberados son rápidamente metabolizados por las bacterias ruminales, dejando los AG libres disponibles para su posterior transformación en la biohidrogenación ruminal. Las bacterias más importantes que participan en la lipólisis son *Anaerovibrio lipolytica* que hidroliza los TAG y *Butyrivibrio fibrisolvens* que hidroliza los fosfolípidos y glicolípidos (Harfoot y Hazlewood, 1988). Generalmente el grado de lipólisis es superior al 85%, aunque hay diversos factores que

pueden afectar al grado de lipólisis, como el aumento del contenido de grasa en la dieta, el pH ruminal bajo, y algunos antibióticos que inhiben el crecimiento y actividad de algunas bacterias reduciendo el grado de lipólisis (Van Nevel y Demeyer, 1995; 1996; Demeyer y Doreau, 1999; Beam *et al*., 2000; Fuentes *et al*., 2009, 2011).

3.2. **Biohidrogenación**

La biohidrogenación de los AGPI es la segunda transformación que sufren los lípidos dietarios en el rumen. Ésta requiere los AG libres formados en la lipólisis por lo que los factores que influyen en la lipólisis también afectaran a la biohidrogenación, y la tasa de biohidrogenación total siempre será menor que la de la lipólisis. La biohidrogenación es un proceso rápido y eficiente que sirve como mecanismo de defensa del ecosistema ruminal contra los AGPI, ya que éstos causan efectos tóxicos sobre las bacterias ruminales (Jenkins, 1993). Los sustratos mayoritarios en la biohidrogenación ruminal son los ácidos linoléico (*cis*-9, *cis*-12 18:2) y α-linolénico (*cis*-9, *cis*-12, *cis*-15 18:3), y la tasa de biohidrogenación aumenta cuando incrementa el grado de insaturación de los AG. La tasa de biohidrogenación para el ácido linoleico y linolénico en la mayoría de dietas es del 70-95% y del 85-100%, respectivamente (Doreau y Ferlay, 1994; Beam *et al.,* 2000). Las bacterias involucradas en el proceso de biohidrogenación se clasificaron en 2 grupos, A y B (Kemp y Lander, 1984). Para obtener la biohidrogenación completa de los AGPI son necesarios ambos grupos bacterianos. El grupo A hidrogena principalmente los AGPI hasta *trans* C18:1 y el grupo B hidrogena principalmente los *trans* C18:1 a ácido esteárico (Harfoot y Hazlewood, 1988). El paso inicial de la biohidrogenación de los ácidos linoleico y α-linolénico es la isomerización del doble enlace *cis*-12 a *trans*-11 que produce, en el caso del ácido linoleico, *cis*-9, *trans*-11 CLA. El segundo paso es la reducción del doble enlace *cis*-9, y el resultado de este proceso es la formación de VA. En el caso del ácido linolénico se produce primero el *cis*-9, *trans*-11*, cis*-15 18:3 seguido por una secuencia de hidrogenaciones que acaba formando *trans*-15 y *cis*-15 18:1 y VA el cual es el intermediario común para ambos AG, linoleico y α-linolénico. El último paso es la saturación y formación de ácido esteárico (C18:0) (Figura 2) (Bauman *et al.,* 2003).

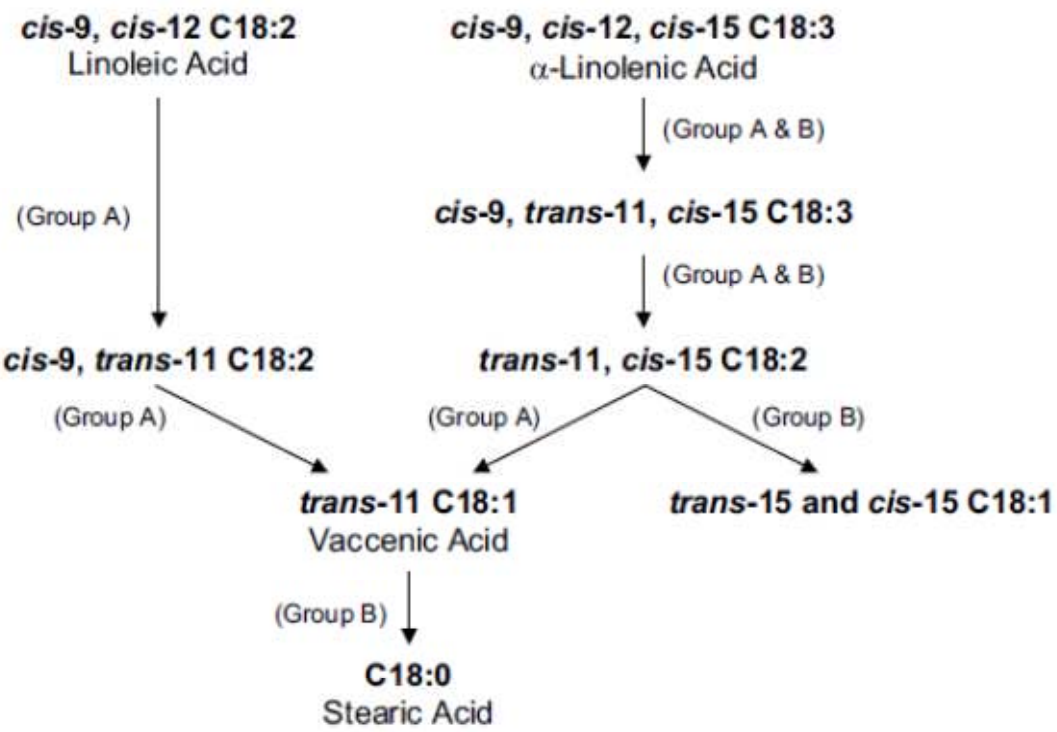

Figura 2. *Pasos de la biohidrogenación del ácido linoleico y linolénico en el rumen (adaptada de Bauman et al., 2003).*

Se ha observado que la tasa de biohidrogenación se reduce y se acumulan sus intermediaros cuando las dietas son altas en concentrado (Van Nevel y Demeyer, 1995; 1996), o contienen lípidos no protegidos (Bauman *et al.*, 2003), se reduce el pH ruminal, se añaden ionóforos o cuando se añaden aceites de pescado. Mientras que algunas de estas causas saturan la capacidad del sistema de biohidrogenar, otras modifican el medio y afectan a los grupos microbianos involucrados en algunos de los procesos de biohidrogenación. Por ejemplo, los aceites de pescado inhiben el último paso de la biohidrogenación incrementando los AG *trans* C18:1 y reduciendo el ácido esteárico C18:0 (Wachira *et al.*, 2000; Shingfield *et al.*, 2003). Así, los AGPI intermediarios acumulados en el rumen se incorporan a la grasa de los productos de rumiantes.

3.3. **Síntesis endógena de CLA**

Diversos estudios observaron que el isómero *cis*-9, *trans*-11 CLA era sólo un intermediario transitorio en el rumen y que el intermediario que realmente se acumulaba era el VA (Griinari y Bauman, 1999; Harfoot y Hazlewood, 1988). Por otro lado, otros estudios demostraron que bajo ciertas condiciones dietarías que no estaban relacionadas con el aporte de ácido linoleico, el contenido de CLA en la leche también incrementaba (Bauman *et al*., 2001;

Stanton *et al.,* 2003). Banni *et al*. (1996) demostraron que ovejas alimentadas principalmente con pasto, el cual contiene linolénico como ácido predominante y no produce *cis*-9, *trans*-11 CLA como intermediario en el rumen, resultaba en la producción de niveles elevados de CLA en la leche. A partir de estas evidencias Griinari y Bauman (1999) propusieron que la síntesis endógena en los tejidos a partir de VA mediante el enzima delta-9 desaturasa podía ser una fuente muy importante del *cis*-9, *trans*-11 CLA encontrado en la leche. Se propuso que el VA era el sustrato porque se observó una relación lineal entre el contenido en la grasa láctea de VA y *cis*-9, *trans*-11 CLA, y el coeficiente de correlación entre estos era alto, lo que podría reflejar que el VA es desaturado a CLA (Griinari y Bauman, 1999). Por lo tanto la clave para enriquecer la leche y productos lácteos en *cis*-9, *trans*-11 CLA es la acumulación de VA en el rumen que pasará a la glándula mamaria como sustrato para la formación de CLA (Figura 3) (Bauman *et al.,* 2003).

El primer estudio que mostró que el CLA se origina vía endógena fue el de Griinari *et al*. (2000) después de administrar infusiones abomasales de VA a vacas en lactación. Los resultados mostraron que el 64% del CLA en la leche se formó vía endógena. Kay *et al.* (2004) demostraron que con dietas basadas en pastos la síntesis endógena de *cis*-9, *trans*-11 CLA era del 91%. Otros estudios estimaron una síntesis endógena de *cis*-9, *trans*-11 CLA en la grasa láctea del 80% (Lock y Garnsworthy, 2002), 93% (Piperova *et al.*, 2002), 78% (Corl *et al.,* 2001) y 74% (Shingfield *et al.,* 2003). Todos los estudios llegaron a la conclusión de que la mayor fuente de *cis*-9, *trans*-11 CLA en la grasa láctea es la síntesis endógena en la glándula mamaria a partir del VA y mediante la enzima delta-9 desaturasa. El sistema desaturasa es un sistema multienzimático que incluye NADH-citocromo b5 reductasa, citocromo b5, acil-CoA sintetasa y el enzima delta-9 desaturasa. El enzima delta-9 desaturasa es el enzima clave de este sistema y su reacción introduce un doble enlace *cis* entre los carbonos 9 y 10 (Griinari y Bauman, 1999). Existen diferencias en la distribución del enzima delta-9 desaturasa entre especies y tejidos. Por ejemplo, en rumiantes en crecimiento el tejido con mayor actividad del enzima es el adiposo, mientras que en rumiantes en lactación es la glándula mamaria (Griinari y Bauman, 1999). Dentro de las diferentes especies, los rumiantes en lactación son los que tienen mayor eficacia del proceso desaturasa, mientras que en las especies no rumiantes el enzima delta-9 desaturasa funciona con muy poca eficacia, como por ejemplo en los humanos, donde la capacidad de conversión del VA en CLA es sólo del 20% (Turpeinen *et al.,* 2002). Esta baja eficacia de transformación de VA en CLA en humanos es lo que justifica la necesidad de introducir el CLA en productos habituales del consumo humano, ya que la única manera de tener un aporte significativo es mediante su incorporación en la dieta.

Hay estudios, principalmente utilizando modelos en ratas, que establecen la regulación del enzima delta-9 desaturasa. Estas investigaciones establecen que la abundancia de mRNA y la actividad enzimática son receptivas a cambios en la dieta, balances hormonales, estados fisiológicos y otros factores. Estas diferencias podrían explicar la variación individual en el contenido de CLA en la grasa de la leche observada cuando las vacas consumen la misma dieta (Griinari y Bauman, 1999).

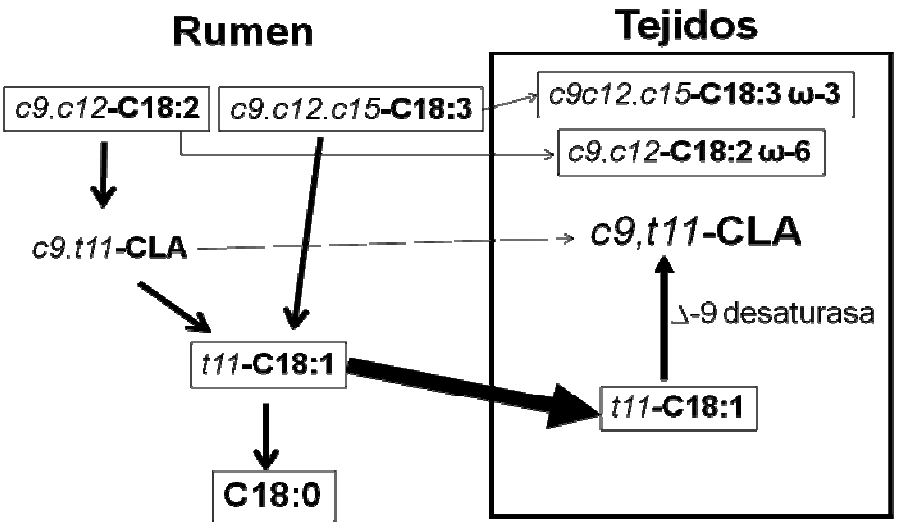

Figura 3. *Formación del isómero cis-9, trans-11 CLA en los tejidos (Adaptada de Bauman et al., 1999).*

4. ESTRATEGIAS PARA MODIFICAR EL CONTENIDO DE AGPI Y CLA EN LA LECHE

El enriquecimiento en AGPI de la leche a través de la dieta va a depender de la capacidad de estos AGPI de llegar como tales a nivel intestinal, donde van a poder ser absorbidos. Por eso, la biohidrogenación juega un papel fundamental en el aporte de AG beneficiosos para ser incorporados en la leche y es importante conocer qué factores pueden afectarla para de esta manera poder controlarlos. La dieta administrada a las vacas lecheras es el principal factor determinante del contenido de CLA de la leche y, por lo tanto, cambios en ésta pueden permitir enriquecer la leche con CLA. Griinari y Bauman (1999) propusieron que los efectos dietarios podían agruparse en 2 categorías dependiendo del mecanismo de acción:

a) Factores dietarios que aumentan los sustratos lipídicos para la producción de CLA o VA en la biohidrogenación ruminal que incluyen principalmente los aceites vegetales ricos en linoleico y linolénico, y los pastos frescos.

b) Factores dietarios que afectan a las bacterias involucradas en el proceso de biohidrogenación en el rumen como dietas con altos contenidos de concentrado y pobres en fibra que causan disminuciones del pH ruminal, responsable de cambios en la concentración de AG intermediarios, y los aceites de pescado.

Por último, puede combinarse la suplementación con sustratos lipídicos y la modificación de las bacterias ruminales.

4.1. **Suplementos lipídicos (aceites vegetales)**

Los aceites vegetales tienen diferente composición de AG dependiendo del origen y eso afecta a las concentraciones de CLA en la leche (Stanton *et al.,* 2003). Los aceites vegetales más utilizados como suplementos en dietas para vacas lecheras son los de aceite de girasol, colza, lino, soja, cacahuete, maíz, algodón, cártamo y palma (Tabla 1).

Tabla 1. *Principales ácidos grasos (g/100 g de ácidos grasos totales) presentes en diferentes aceites vegetales.*

Ingredientes	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Algodón[1]	0,8	25,3	-	2,8	17,1	53,2	0,1
Colza[1]	-	4,3	0,3	1,7	59,1	22,8	8,2
Soja[1]	0,2	10,7	0,3	3,9	22,8	50,8	6,8
Girasol[1]	0,1	5,5	-	3,6	21,7	68,5	0,1
Cacahuete[1]	-	11,5	-	3,0	53,0	26,0	-
Cártamo[1]	-	8,0	-	3,0	13,5	75,0	0,5
Oliva[1]	-	13,0	1,0	2,5	74,0	9,0	-
Lino[1]	-	6,4	-	3,1	20,1	18,2	51,4
Palma[2]	1,1	44,0	0,3	4,5	39,2	10,1	0,4
Gérmen de maiz[3]	0,5	11,4	1,2	1,7	25,3	53,3	3,3

[1]Stanton *et al.,* 2003; [2]Edem, 2002; [3]Abdelqader *et al.,* 2009

Comparando los diferentes tipos de aceites vegetales se llega a la conclusión que los aceites ricos en linoleico como el algodón, la soja, el girasol y el cártamo, producen un incremento mayor de las concentraciones de CLA en la leche, aunque otros tipos de aceites vegetales, como el lino y la colza, también se utilizan con éxito para enriquecer la leche con CLA (Stanton *et al.,* 2003). Kelly *et al.* (1998) demostraron que suplementar las dietas con aceite de girasol, rico en linoleico, era más efectivo para incrementar la concentración de CLA en la leche que suplementar las dietas con aceite de cacahuete, rico en ácido oleico (C18:1), o aceite de lino, rico en linolénico. Dhiman *et al.* (2000) demostraron que suplementando las dietas con aceite de soja, rico en linoleico, se incrementaban más las concentraciones de CLA en la leche que suplementando las dietas con aceite de lino.

Estudios in vitro han demostrado que cuando hay niveles altos de acido linoleico el patrón de biohidrogenación cambia y el ácido *trans*-11 VA se encuentra en mayor cantidad que el acido esteárico (Polan *et al.,* 1964; Harfoot *et al.,* 1973). Harfoot *et al.* (1973) encontraron que niveles altos de acido linoleico inhibían irreversiblemente la hidrogenación del ácido VA y esto resulta en un incremento en la concentración del precursor para la síntesis endógena de CLA. En cambio, cuando los niveles altos fueron de ácido linolénico, además de la acumulación de VA se acumularon el *cis*-15 y *trans*-15 C18:1 que no pueden ser desaturados a CLA. Esto podría explicar por qué las dietas ricas en linoleico, como el girasol o soja, podrían resultar en una mayor acumulación de VA y una mayor producción de CLA en la leche que las dietas ricas en linolénico, como lino (Griinari y Bauman, 1999). Independientemente del tipo de aceite vegetal, el incremento del CLA en la leche es siempre dosis-dependiente (Bauman *et al.,* 1999).

4.1.2. Método de presentación de los suplementos lípidicos

Los aceites vegetales pueden suplementarse como aceites libres, aceites protegidos o como semillas enteras o procesadas. Para lograr incrementar el contenido de CLA y VA en el rumen, los microorganismos responsables de la biohidrogenación ruminal han de tener acceso al aceite. Por esta razón se podría decir que los aceites protegidos o semillas enteras sin procesar no presentarían grandes incrementos en la producción de CLA en la leche (Stanton *et al.,* 2003). Por otro lado, los suplementos compuestos por aceites vegetales no son normalmente incluidos en las dietas para rumiantes ya que estos producen efectos inhibitorios en el crecimiento de las bacterias ruminales (Jenkins, 1993). Un método para minimizar estos efectos es ofrecer los suplementos vegetales en forma de sales de calcio o semillas sin procesar. Diversos estudios demostraron que dietas suplementadas con semillas sin procesar no aumentaron el contenido de CLA en la leche (Dhiman *et al.,* 1999; Chouinard *et al.,* 1998). Por este motivo, estudios más recientes analizaron los diferentes procesados de las semillas que aumenten el contenido de CLA en la leche causando mínimos efectos en las bacterias ruminales (Chouniard *et al.,* 2001). Para analizar los efectos de diferentes procesados de las semillas vegetales, Chouniard *et al.* (2001) ofrecieron 4 dietas suplementadas con grasa procedente de soja con diferentes procesados en las semillas. Las 4 dietas incluían semillas de soja molidas, extrusionadas, micronizadas y tostadas. La concentración de VA en la leche fue más baja con las dietas que incluían semillas molidas seguido por las dietas que incluían semillas micronizadas y tostadas, y la mayor concentración de VA en la leche se encontró con las dietas que incluían semillas extrusionadas. La leche de las vacas alimentadas con semillas extrusionadas contenía la menor concentración de linoleico y linolénico, indicando que con

este método de suplementación la biohidrogenación de estos ácidos era más completa, y concluyeron que utilizar semillas extrusionadas parece ser una manera efectiva de suplementar con AGPI causando menos efectos en las bacterias ruminales. En este experimento también se estudió el efecto del tratamiento de calor en el proceso de la extrusión. Así, ofrecieron una dieta con semillas enteras y tres dietas con semillas extrusionadas a diferentes temperaturas, 120º, 130º y 140ºC. Las tres dietas con semillas extrusionadas resultaron en un incremento similar de CLA en la leche, indicando que la temperatura a la cual se realiza el procesado no afecta a la concentración de CLA en la leche producida. Otros estudios han obtenido resultados similares con semillas procesadas de soja, algodón y colza (Dhiman *et al.,* 1999; Lawless *et al.,* 1998; Solomon *et al.,* 2000; Stanton *et al.,* 1997). Dhiman *et al.* (2000) compararon dietas enriquecidas con semillas de soja enteras, semillas de soja procesadas por calor, aceite de soja o aceite de lino, y los resultados mostraron que las dietas ricas en linoleico y linolénico podían incrementar el contenido de CLA en la leche cuando el aceite era accesible para los microorganismos del rumen. Así, las dietas suplementadas con semillas enteras no aportaron ningún incremento en el CLA de la leche comparado con las dietas control, y las dietas suplementadas con aceites, aunque causaban una disminución en la grasa láctea, resultaron en un incremento mayor de CLA en la leche que las dietas suplementadas con semillas procesadas.

4.2. **Modificación de las poblaciones microbianas**

4.2.1. Aceites de pescado

La suplementación de la dieta con harinas o aceites de pescado incrementa la concentración de CLA en la leche. Además, cantidades iguales de aceites de pescado incrementan más efectivamente la concentración de CLA en la leche que los aceites vegetales (Chouinard *et al.,* 1998). La biohidrogenación de los AG de cadena larga que contienen los aceites de pescado, el ácido ecosapentaenoico (EPA, C20:5 n3) y el docosahexaenoico (DHA, C22:6 n3), no aumenta la producción de CLA o VA en el rumen y la leche directamente, sino que el aumento de VA se produce por la inhibición del crecimiento de las bacterias que reducen el VA a esteárico, o a través de la inhibición de las enzimas reductasas de estas bacterias (Stanton *et al.,* 2003).

4.2.2. Nivel de forraje y riesgo de acidosis

Cuando las dietas contienen altos contenidos de concentrado y son pobres en fibra los resultados referentes a la concentración de CLA en la leche de los estudios son contradictorios: en algunos aumenta (Chouniard *et al.,* 1998) y en otros disminuye (Griinari *et al.,* 1998). Cuando se aumenta el concentrado en la dieta, disminuye el pH ruminal que es el causante de los cambios en las poblaciones microbianas del rumen. La disminución del pH provoca un incremento en el contenido de *trans*-18:1 en el rumen y la leche (Kalscheur *et al.,* 1997; Griinari *et al.,* 1998), pero el perfil de los AG *trans* cambia y el ácido *trans*-10 C18:1 pasa a ser el isómero predominante (Griinari *et al.,* 1998). En la leche se observa una disminución del isómero *cis*-9, *trans*-11 CLA pero un aumento del isómero *trans*-10*, cis*-12 CLA, que puede llegar a ser el mayoritario.

4.2.3. Ionóforos

Los ionóforos inhiben el crecimiento de las bacterias Gram positivas, y algunas de éstas están involucradas en el proceso de biohidrogenación. Fellner *et al.* (1997) observaron que la adición de ionóforos en fermentadores de flujo continuo inhibía la biohidrogenación del acido linoleico, lo cual resultó en una disminución de ácido esteárico y un incremento de los isómeros del ácido C18:1 en el contenido ruminal. Aunque esto se observó *in vitro*, en estudios *in vivo* en los cuales se suplementaron las dietas con ionóforos se observaron resultados variables en la concentración de CLA en la leche (Sauer *et al.,* 1998; Dhiman *et al.,* 1999; Chouinard *et al.,* 1998).

4.3. **Variación individual**

La variación individual entre animales es una de las mayores causas de diferencia en el contenido de CLA en la leche. Esta variación entre animales se relaciona con una variación individual de la expresión del gen del enzima delta-9 desaturasa y por lo tanto se observan diferencias en el contenido de CLA en la leche que se mantienen en el tiempo en cada animal aunque los animales estén alimentados con la misma dieta (Peterson *et al.,* 2002).

5. DEPRESIÓN DE LA GRASA LÁCTEA

La DGL es un síndrome de baja grasa láctea en el cuál el contenido de grasa láctea se ve reducido mientras otros componentes de la leche y la producción de leche no se alteran. Peterson *et al.* (2003) observaron una reducción del porcentaje (25%) y la cantidad (27%) de grasa en vacas alimentadas con dietas altas en concentrado y bajas de forraje, mientras que el consumo, la producción de leche, y la producción de proteína y lactosa no se vieron alterados. Además, en el mismo estudio se observó una disminución parecida de los AG provenientes de la síntesis *de novo* y de los AG provenientes de la captación desde la circulación sanguínea. Harvatine y Bauman (2006) observaron una disminución similar de la grasa láctea del 38% en vacas alimentadas con dietas que inducen la DGL (bajas en fibra y altos contenidos de aceites). Además, en este estudio los AG fueron clasificados según la longitud de su cadena en >16 carbonos, 16 carbonos, y <16 carbonos, y todas las fracciones estaban reducidas. Por lo tanto, durante la DGL se observa una disminución de ambos AG, *de novo* síntesis y captados de la circulación sanguínea, sin modificación en el resto de componentes lácteos.

Bauman y Griinari (2001) propusieron la teoría de la biohidrogenación basada en el concepto que bajo ciertas condiciones dietarías, como dietas bajas en fibra o dietas suplementadas con aceites ricos en AGPI, las vías de la biohidrogenación ruminal se alteran a causa de cambios en los procesos de fermentación microbiana, y se producen AGPI intermediarios, de los cuales algunos son potentes inhibidores de la síntesis de la grasa láctea. Esta DGL se ha relacionado sobre todo con el incremento del isómero *trans*-10 18:1 en el rumen y *trans*-10, *cis*-12 CLA por síntesis endógena en la glándula mamaria a partir de la desaturación de *trans*-10 18:1 (Figura 4) (Griinari *et al.,* 1998). Diversos estudios han confirmado esta teoría concluyendo que dietas que causan el síndrome de DGL están relacionas con el incremento del isómero especifico, *trans*-10 18:1 (Griinari *et al.,* 1998; Ärölä *et al.,* 2002; Avramis *et al.,* 2002; Offer *et al.,* 1999, 2001; Piperova *et al.,* 2000).

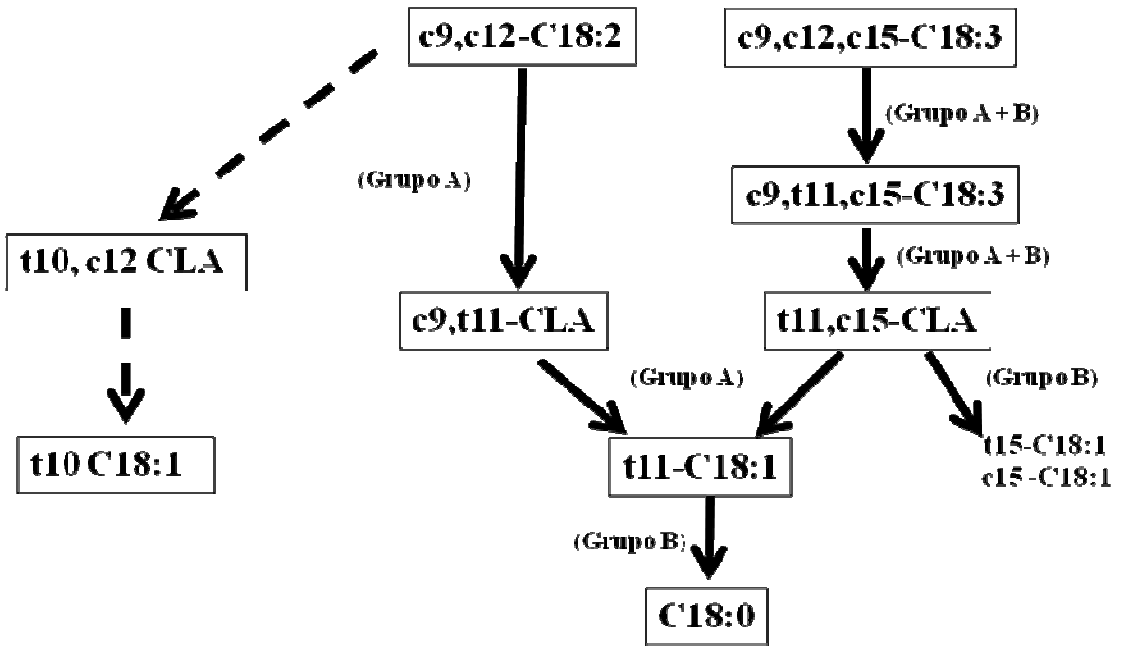

Figura 4. *Pasos de la biohidrogenación del ácido linoleico y linolénico en el rumen. (Grupo A y grupo B) Grupo de bacterias según la clasificación de Kemp y Lander (1984) involucradas en cada paso del proceso de biohidrogenación (Adaptado de Bauman et al., 2003).*

Se han desarrollado varios experimentos en los últimos años que demuestran que los AG *trans* son capaces de inhibir la síntesis de grasa láctea. Gaynor *et al.* (1994) infundieron grasa *cis* y *trans* en el abomaso de vacas lactantes y observaron que la producción de leche fue similar entre tratamientos, pero el porcentaje y la producción de grasa láctea fueron menores en el tratamiento *trans*. Diversos estudios han demostrado una reducción en el contenido de grasa láctea en vacas que habían recibido infusiones abomasales de una mezcla de isómeros del CLA demostrando un efecto inhibitorio del CLA en la producción de grasa láctea en vacas lecheras (Chouinard *et al.*, 1999a; Chouinard *et al.*, 1999b; Loor y Herbein, 1998). Para identificar isómeros específicos, Baumgard *et al.* (2000) trataron a vacas lecheras con infusiones abomasales de *cis*-9, *trans*-11 CLA o *trans*-10, *cis*-12 CLA y observaron una disminución en el porcentaje y la cantidad de grasa láctea sólo con el isómero *trans*-10, *cis*-12 CLA demostrando que éste es un isómero responsable de la DGL, mientras que el isómero *cis*-9, *trans*-11 CLA no está relacionado. Además, Baumarg *et al.* (2001) observaron una reducción de la grasa láctea progresiva a medida que se incrementaba la dosis de *trans*-10, *cis*-12 CLA mediante infusiones abomasales en vacas lecheras.

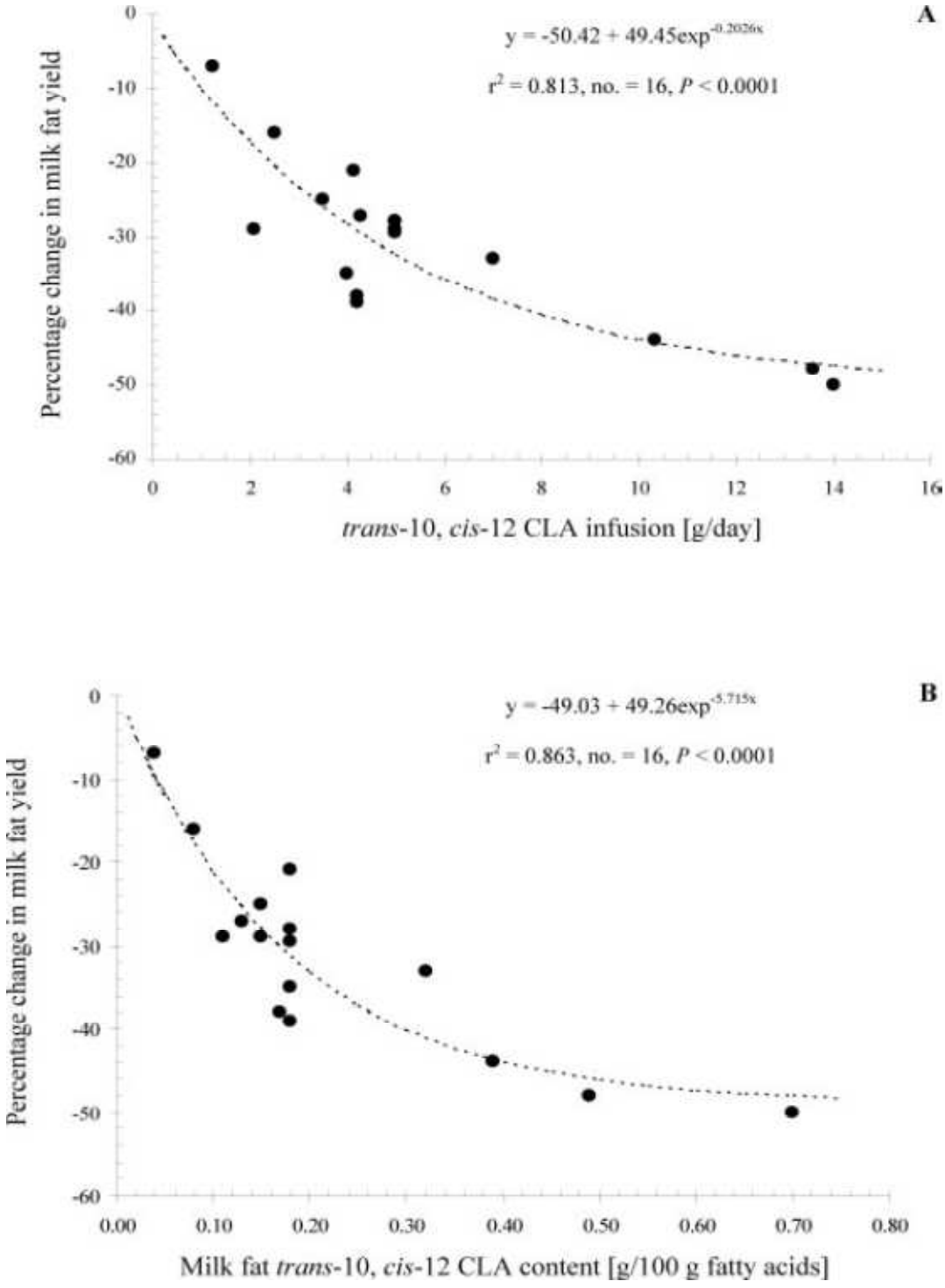

Figura 5. *Relación entre infusiones abomasales de trans-10, cis-12 CLA y el porcentaje de cambio en la cantidad de grasa láctea (A) y entre el contenido de trans-10, cis-12 CLA en la grasa láctea y el cambio relativo en la cantidad de grasa láctea durante infusiones abomasales de trans-10, cis-12 CLA (B) (Adaptado de Shingfield y Griinari, 2007).*

A pesar de la evidencia que el isómero *trans*-10, *cis*-12 CLA está implicado en la DGL, ésta puede ocurrir en ausencia o con un incremento pequeño del isómero *trans*-10, *cis*-12 CLA, indicando que otros AGPI intermediarios de la biohidrogenación o otros mecanismos están implicados en la DGL (Bauman y Griinari, 2003). En dietas suplementadas con aceites de pescado (Loor *et al.*, 2005; Shingfield *et al.*, 2003) o en dietas ricas en concentrados que contienen aceites vegetales (Loor *et al.*, 2004; 2005) se demostró que el contenido de *trans*-10, *cis*-12 CLA en omaso o duodeno era menos de 1,5 g/d, indicando que otros AGPI han de estar también relacionados con la DGL. Uno de los isómeros que se ha observado aumentado en las dietas que causan la DGL es el *trans*-9, *cis*-11 CLA (Shingfield *et al.*, 2006; Roy *et al.*, 2006). Perfield *et al.* (2007) demostró mediante infusiones abomasales que el isómero *trans*-9, *cis*-11 CLA era uno de los responsables de la DGL y en cambio el isómero *trans*-9, *trans*-11 CLA no tenía ningún efecto en la cantidad de la grasa láctea, aunque inhibió el enzima Δ-9 desaturasa en la glándula mamaria, indicado por la modificación del índice de desaturación de la grasa láctea. Otro isómero encontrado como responsable de la DGL es el *cis*-10, *trans*-12 CLA. Saebo *et al.* (2005), mediantes infusiones abomasales de una mezcla de isómeros de CLA, observaron que el isómero *cis*-10, *trans*-12 CLA era uno de los responsable de la DGL, mientras que el isómero *trans*-10, *trans*-12 no tenia efectos en la síntesis de la grasa láctea aunque alteraba el índice de desaturación de la grasa láctea. Comparados con una dosis similar de *trans*-10, *cis*-12 CLA, el isómero *cis*-10, *trans*-12 CLA fue igual o incluso ligeramente más efectivo reduciendo la producción de grasa láctea, mientras que el *trans*-9, *cis*-11 CLA fue aproximadamente la mitad de potente (Saebo *et al.*, 2005; Perfield *et al.*, 2007). Uno de los isómeros que se ha relacionado con dietas que inducen la DGL es el isómero *trans*-10 18:1, y éste se ha identificado como un potente inhibidor de la síntesis de la grasa láctea (Hinrichsen *et al.*, 2006; Loor *et al.*, 2005). Sin embargo, aunque existe una estrecha relación entre la reducción de la grasa láctea y el contenido de *trans*-10 18:1 en leche, esto solo indica un cambio en el perfil de AG derivados de la biohidrogenación debido a las dietas que causan la DGL, pero no implica una regulación directa de la síntesis de la grasa láctea (Shingfield y Griinari, 2007). Lock *et al.* (2007) demostraron que infusiones abomasales del isómero *trans*-10 18:1 no tuvieron efecto en la porcentaje o cantidad de la grasa láctea, indicando que este isómero no está involucrado directamente en la regulación de la síntesis de la grasa láctea. La implicación de otros isómeros en la DGL, como *trans*-8, *cis*-10 CLA; *cis*-9, *trans*-11 CLA; *trans*-9, *trans*-11 CLA; *trans*-10, *trans*-12 CLA, *cis*-11, *trans*-13 CLA, *trans*-9 C18:1, *trans*-11 C18:1 y *trans*-12 C18:1 no se ha podido demostrar (Perfield *et al.*, 2004; 2007; 2006; Saebo *et al.*, 2005; Baumgard *et al.*, 2000; Rindsig y Schultz, 1974; Griinari *et al.*, 2000).

Por lo tanto, hasta el momento se han identificado 3 isómeros responsables de la DGL. El isómero *trans*-10, *cis*-12 CLA como el principal responsable y más estudiado hasta el momento; y los isómeros *trans*-9, *cis*-11 CLA y *cis*-10, *trans*-12 CLA que también podrían reducir la síntesis de grasa láctea, aunque los resultados deben ser tomados con precaución ya que únicamente corresponden a un estudio, a una única dosis de infusión y se han probado como mezclas de isómeros de CLA. Por lo tanto, es necesaria más investigación con estos isómeros puros y a diferentes dosis para establecer su potencia, y clarificar su papel en la regulación de la síntesis de la grasa láctea.

6. VARIACIÓN INDIVIDUAL

Por otro lado, el contenido graso de la leche varía de forma considerable a lo largo del año y según las condiciones de manejo de la explotación. No existe bibliografía que especifique la variabilidad intrínseca y estacional del perfil de AG minoritarios (principalmente CLA y los isómeros intermediarios ruminales). El conocimiento de esta variabilidad es importante para establecer criterios de cumplimiento de especificaciones así como para establecer medidas regulares de control y seguimiento de la producción (control de puntos críticos).

7. SÍNTESIS DE LA GRASA LÁCTEA

La síntesis de grasa láctea en la glándula mamaria requiere la coordinación de los enzimas involucrados en la síntesis de AG *de novo,* en la captación de AG desde la circulación sanguínea, desaturación, esterificación, y la formación, transporte y secreción de los glóbulos grasos de la leche. La grasa de la leche contiene predominantemente TAG (más del 95%), seguido aproximadamente por un 2% de diacilglicéridos, y otros lípidos que incluyen pequeñas cantidades de fosfolípidos (1%), colesterol (0,5%) y AG libres (0,1%) (Jensen y Newberg, 1995). La grasa láctea contiene aproximadamente 400 AG diferentes. Debido a la hidrogenación de los AGPI de la dieta durante la biohidrogenación ruminal, alrededor del 70% son AG saturados, principalmente ácido palmítico (C16:0) (30%), seguido por el ácido mistírico (C14:0) y el ácido esteárico (C18:0) (MacGibbon y Taylor, 2006). Además, el 25% de los AG son monoinsaturados siendo el ácido oleico (C18:1) el predominante. Los AGPI son una pequeña porción del total de AG de la grasa láctea, siendo los principales el ácido linoleico (C18:2) y α-linolenico (C18:3) (MacGibbon and Taylor, 2006; Precht and Molkentin, 1995) (Tabla 2).

Estos AG provienen de dos fuentes: la síntesis *de novo* en la glándula mamaria y la captación de AG circulantes en la sangre originarios de la dieta o de la movilización de grasa corporal (Dils, 1986; Neville and Picciano, 1997). Los AG de cadena corta y media (C4:0 a C14:0) se forman casi exclusivamente mediante la síntesis *de novo* en la glándula mamaria, mientras que los AG de cadena larga (más de 16 carbonos) provienen de la captación de éstos desde el torrente circulatorio. Los AG de 16 carbonos provienen a partes iguales desde las 2 fuentes (Bauman y Griinari, 2003).

Tabla 2. *Perfil de ácidos grasos expresados como porcentaje usando el peso total de ácidos grasos en vacas lecheras suizas en 2001 (7 granjas y 4 muestreos durante 2001) (Adaptada de Mansson 2008).*

Ácido graso (g/100g de AG totales)	Media	DS	Valor mínimo observado	Valor máximo observado	Variación estacional
C4:0	4.4	0.1	4.0	5.1	n.s.
C6:0	2.4	0.1	2.1	2.9	n.s.
C8:0	1.4	0.1	1.2	1.9	n.s.
C10:0	2.7	0.2	2.4	3.5	*
C12:0	3.3	0.2	3.0	4.1	**
C14:0	10.9	0.5	10.0	12.1	***
C15:0	0.9	0.0	0.8	1.1	n.s.
C16:0	30.6	0.9	28.7	34.1	**
C17:0	0.4	0.0	0.4	0.5	**
C18:0	12.2	0.4	10.3	13.3	n.s.
C20:0	0.2	0.0	0.2	0.2	n.s.
Ácidos grasos saturados totales	*69.4*	*1.7*	*67.1*	*74.4*	***
C10:1	0.3	0.0	0.2	0.4	n.s.
C14:1	0.8	0.4	0.4	1.3	**
C16:1	1.0	0.0	0.9	1.8	n.s.
C17:1	0.1	0.0	<0.1	0.3	n.s.
C18:1	22.8	1.0	19.7	24.7	***
Ácidos grasos insaturados, cis, totales	*25.0*	*1.0*	*22.2*	*26.7*	**
C18:2	1.6	0.1	1.4	1.8	n.s.
C18:3	0.7	0.0	0.6	0.9	**
Ácidos grasos poliinsaturados, cis, totales	*2.3*	*0.1*	*2.0*	*2.5*	n.s.
C16:1t	0.4	0.1	0.3	0.4	***
C18:1t	2.1	0.7	2.0	3.3	***
C18:2t	0.2	0.0	0.1	0.5	n.s.
Ácidos grasos trans totales	*2.7*	*0.7*	*0.6*	*3.9*	***
CLA	0.4	0.1	0.3	0.5	***

n.s.: No significante; *p<0.05; **p<0.01; ***p<0.001.

7.1. **Síntesis *de novo***

La síntesis de AG *de novo* proviene de los sustratos acetato y β-hidroxibutirato. El acetato es convertido a acetil-CoA mediante la familia de cadena corta acil-CoA sintetasa (ACSS). La conversión de Acetil-CoA a Manolil-CoA se produce mediante el enzima Acetil-CoA carboxilasa (ACACA) considerado el paso limitante de la síntesis de AG *de novo* (Ha and Kim, 1994). El Acetil-CoA y butiril CoA (proveniente del β-hidroxibutirato) son los sustratos para el ácido graso sintasa (FASN), que producirá principalmente ácido palmítico (C16:0) y, en rumiantes, también AG de cadena corta (Palmquist, 2006).

7.2. **Captación de AG circulantes en sangre**

Los AG de cadena larga captados por la glándula mamaria proceden de los AG no esterificados (NEFA) ligados a albumina o de las lipoproteínas circulantes. Los dos genes importantes involucrados en este proceso son el Receptor de lipoproteínas de baja densidad (VLDLR) y la lipoproteína lipasa (LPL). Las VLDL se anclan al endotelio mamario donde los TAG son hidrolizados mediante la LPL para liberar AG libres (Fielding y Frayn, 1998). Los AG liberados se internalizan mediante difusión pasiva, captación de AG mediante proteínas y mediante el mecanismo flip-flop (Doege y Stahl, 2006). La difusión pasiva juega un papel pequeño comparado con las otras dos formas de internalización de AG, aunque no ha sido bien estudiado en la glándula mamaria. Las principales proteínas involucradas en la captación de AG en animales no rumiantes incluyen la molécula CD36 (receptor thrombospondin) (CD36) y la proteína soluto compañia familia de proteínas 27 (Proteína de transporte de AG) (SLC27A) (Doege y Stahl, 2006). Se cree que el gen CD36 participa en el proceso de secreción de la grasa láctea, pero también tiene un papel importante en la síntesis de grasa láctea ya que se cree que éste está involucrado además en la internalización de AG en las células de la glándula mamaria (Bionaz y Loor, 2008). La expresión de la isoforma SLC27A6 aumenta en el primer mes de lactación, sugiriendo que tiene un papel en la captación de NEFA (Bionaz y Loor, 2008b). La sobreexpresión de CD36 y SLC27A6 durante la lactación indica que estas dos proteínas cooperan durante la internalización de AG en las células de la glándula mamaria (Bionaz y Loor, 2008). Además, el gen CD36 también se encuentra localizado con la familia de Acil-CoA sintetasas y las proteínas ligantes de AG (FABP). La internalización de AG mediante la captación de AG a partir de proteínas es un proceso complejo y coordinado que requiere la participación de múltiples genes/proteínas (Bionaz y Loor, 2008).

Los AG internalizados en las células epiteliales de la glándula mamaria son activados mediante la familia de cadena larga acil-CoA sintetasa (ACSL) de los cuales la isoforma ACSL1 es predominante en la glándula mamaria, indicando su importancia en la síntesis de la grasa láctea (Bionaz y Loor, 2008b).

El transporte intracelular de los AG es debido principalmente a FABP3, que además se ha demostrado que puede prevenir la inhibición de ACACA y stearoil-CoA desaturasa (SCD) causada por los AG activados, demostrando que FABP3 está relacionado con SCD y tiene un papel importante en proveer AG para SCD (Bionaz y Loor, 2008).

7.3. **Desaturación de AG**

Los AG que llegan a la glándula mamaria son principalmente saturados debido a la biohidrogenación ruminal. Parte de estos son desaturados mediante el enzima SCD1, principal enzima involucrado en la síntesis de AG monoinsaturados en rumiantes, el cual introduce un doble enlace en la posición Δ-9 del miristoil-CoA, palmitoil-CoA y Stearoil-CoA (Ntambi y Miyazaki, 2003).

La síntesis de los AG de cadena muy larga se produce a partir del AG desaturasa 1 (FADS1) y 2 (FADS2), los cuales añaden un doble enlace en la posición Δ-5 y Δ-6 de los AGPI. Esta vía forma los ácidos araquidónico (20:4n-6), eicopentaenoico (20:5n-3) y docosahexanioco (22:6n-3) (Rodriguez-Cruz *et al.*, 2006).

7.4. **Esterificación de AG**

Los AG *de novo* y los captados de la circulación sanguínea son esterificados a glicerol-3-fosfato para la síntesis de TAG. El primer paso es la formación del ácido lisofosfatídico a partir de la adición de un ácido acil-CoA en la posición sn-1 mediante glicerol-3-fosfato aciltransferasa (GPAM). El ácido lisofosfatídico se convierte a ácido fosfatídico por la adición de otro ácido acil-CoA en la posición sn-2, catalizado por acilglicerol-3-fosfato aciltransferasa (AGPAT). El grupo fosfato en el ácido fosfatídico se retira mediante la enzima lipin ácido fosfatídico fosfohidrolasa (LPIN) para producir diacilglicerol. Finamente, los TAG se forman por la esterificación del un tercer ácido acil-CoA en la posición sn-3, catalizado por diacilglicerol aciltransferasa (DGAT) (Bernard *et al.*, 2008). Las isoformas AGPAT6, LIPIN1, DGAT1 Y DGAT2 son las principales en el tejido mamario bovino (Bionaz y Loor, 2008b).

7.5. **Secreción de la grasa láctea**

La grasa láctea es secretada en forma de glóbulos de grasa formados en la membrana del retículo endoplasmático mediante la incorporación de los TAG sintetizados. Los glóbulos de grasa son transportados a la membrana apical y liberados de la célula (Keenan and Mather, 2006). Los proteínas principales involucradas en este proceso son la butirofilin (BTN1A1), la xantina dehidrogenasa (XDH) y la adipofilin (ADPH) (Bionaz y Loor, 2008).

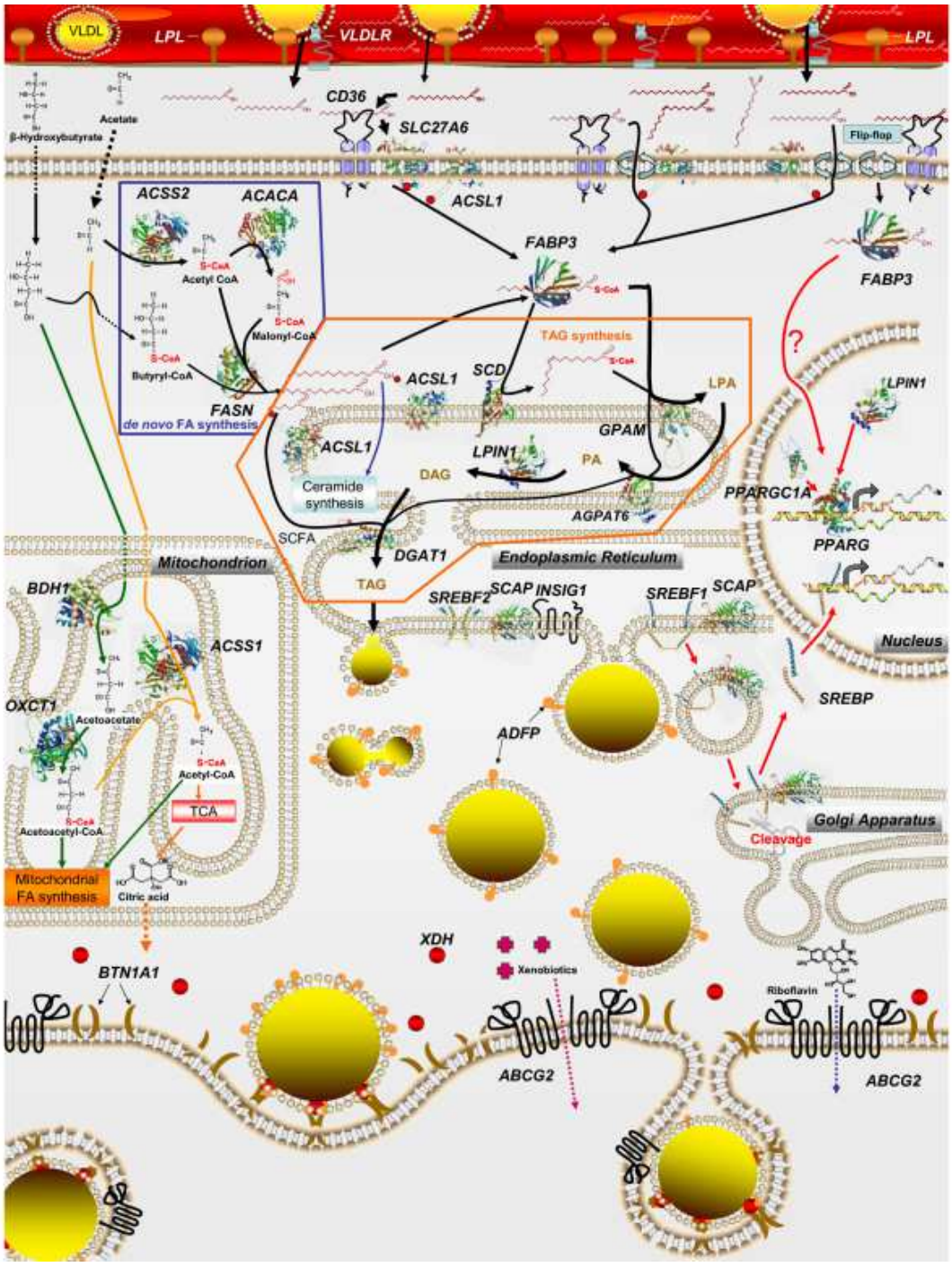

Figura 6. *Síntesis de la grasa láctea (Adaptada de Bionaz y Loor, 2008).*

7.6. **Factores de transcripción**

Los factores de transcripción son proteínas que participan en la regulación de la transcripción del ADN pero que no forman parte de la ARN polimerasa. Los factores de transcripción pueden actuar reconociendo y uniéndose a secuencias concretas de ADN, uniéndose a otros factores, o uniéndose directamente a la ARN polimerasa. Los factores de transcripción son estimulados por señales citoplasmáticas. Al ser activados adquieren la capacidad de regular la expresión génica en el núcleo, activando/inhibiendo la transcripción de diversos genes. Hasta el momento la síntesis de la grasa láctea se ha relacionado con 3 factores de transcripción: El factor de unión a elementos reguladores del esterol (SREBP), el receptor del factor de proliferación del peroxisoma activado (PPAR) y el receptor X del hígado (LXR).

7.6.1. Factor de unión a elementos reguladores del esterol (SREBP)

Se ha demostrado que SREBP1 tiene un papel central en la regulación de la síntesis de grasa láctea en la glándula mamaria de vacas lecheras (Bionaz y Loor, 2008; Wickramasinghe *et al.*, 2012).

Se han identificado 3 isoformas de SREBP, SRBP1a, SRBP1c y SRBP2. SRBP1c y SREBP2 actúan en la regulación de la síntesis de AG y colesterol, respectivamente, mientras SRBP1a está involucrado en los 2 procesos (Eberle *et al.*, 2004). El SREBP1 y 2 se encuentran en la membrana del retículo endoplasmático como precursores inactivados. Éstos son transportados al aparato de Golgi donde se activan mediante una serie de degradaciones proteolíticas antes de entrar en el núcleo y unirse a los elementos reguladores del esterol en los promotores de genes diana, como ACACA y FASN, para activar su transcripción. Para el paso de SREBP desde el retículo endoplasmático hacia el aparato de Golgi se necesita la proteína de activación de la segmentación de SREBP (SCAP). Bajo ciertas condiciones, como la presencia de esteroles, SCAP asociado a SREBP queda bloqueado en el retículo endoplasmático mediante el gen inducido por insulina (INSIG). Cuando desaparecen los esteroles, el complejo SREPB-SCAP se separa de INSIG, y SCAP escoltará a SREBP hasta el aparato de Golgi para su activación (Eberle *et al.*, 2004). Se ha demostrado que INSIG1 puede inhibir la segmentación proteolítica para la activación de SREBP sin la presencia de esteroles, particularmente si incrementa el ratio INSIG1/SCAP. Por lo tanto, parece que un incremento en la expresión de INSIG1 por si sola puede ser suficiente para inhibir la activación de SREBP. Estas evidencias podrían indicar que la

síntesis de la grasa láctea en la glándula mamaria no puede basarse únicamente en la regulación transcripcional a través de SREBP1 que probablemente está inhibida o controlada por INSIG1 (Bionaz y Loor, 2008).

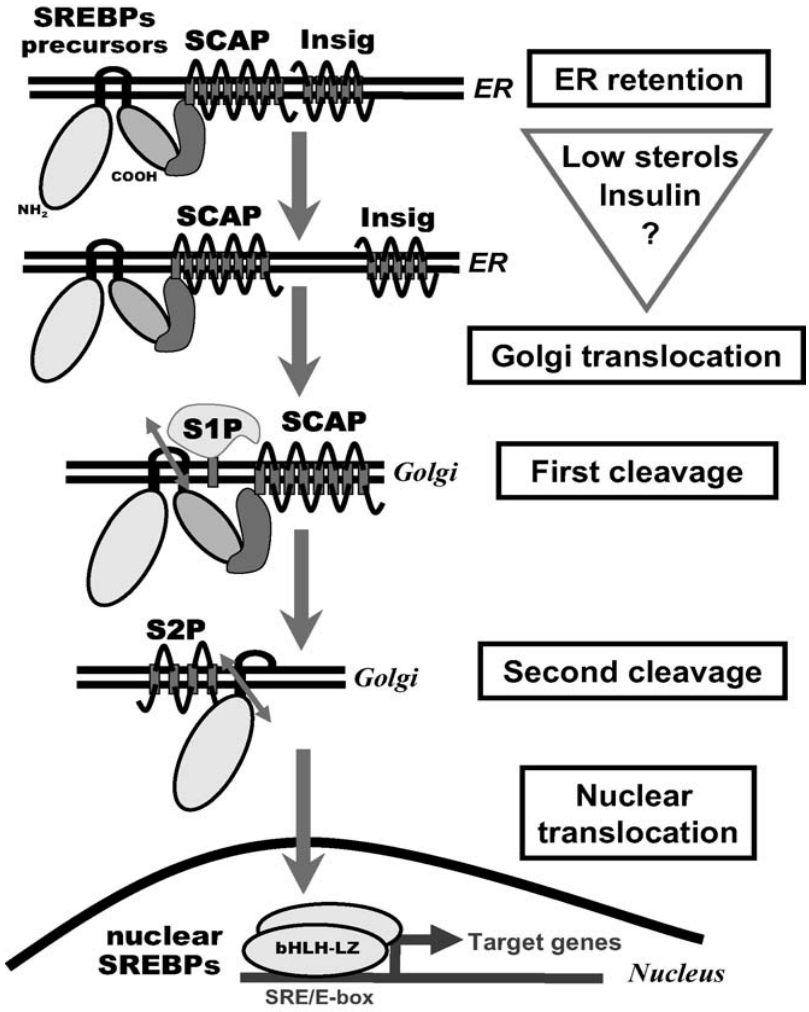

Figura 7. *Activación del factor de unión a elementos reguladores del esterol (SREBP) mediante degradaciones proteolíticas. El factor de transcripción SREBP se sintetiza a partir de los precursores inactivos ligados a la membrana del reticulo endoplasmatico. Ahí se asocia a la proteina de activación de la segmentación de SREBP (SCAP), donde interacciona con el gen inducido por insulina (INSIG). Cuando desaparecen los esteroes, la interacción entre SCAP y INSIG disminye, y el complejo SCAP-SREBP se desplazará al aparato de Golgi para su activación. En el aparato de Golgi SREBP se transforma a su forma madura después de una serie de degradaciones proteolíticas. Finalmente, la forma madura de SREBP es translocada al nucleo donde se liga a los genes diana mediante sus elementos de respuesta a los esteroles (Adaptado de Eberle et al., 2004).*

7.6.2. Receptor del factor de proliferación del peroxisoma activado (PPAR)

Hay 3 subtipos de PPAR, α,δ y γ. Los 3 subtipos regulan la expresión de genes en varios procesos fisiológicos. El subtipo PPARα actúa en la regulación de la captación, activación y oxidación de los AG (Martin *et al.*, 1997; Schoonjans *et al.*, 1995; Zhang *et al.*, 1993). El subtipo PPARδ actúa en el control de la resistencia a la insulina, el mantenimiento de la capacidad reproductiva, en el desarrollo del sistema nervioso central, y en la señalización de la transducción en el sistema nervioso central (Granneman *et al.*, 1998; Lim *et al.*, 1999; Oliver *et al.*, 2001; T. *et al.*, 1999; Yang *et al.*, 2006). El subtipo PPARγ regula los genes involucrados en la diferenciación de los adipocitos, la síntesis de AG, la inflamación y el cáncer (Chinetti *et al.*, 1998; Kallen y Lazar, 1996; Sarraf *et al.*, 1998; Tontonoz *et al.*, 1994).

La activación de PPAR se induce mediante los receptores de proliferación activados. Además, se ha observado que diferentes AG también tienen la capacidad de activarlo (Bionaz *et al.*, 2012; MacLaren *et al.*, 2011). El PPARγ es uno de los receptores nucleares más estudiado por su papel central en la regulación del metabolismo en relación a la composición y concentración de los AG de cadena larga en el ambiente celular (Desvergne *et al.*, 2006). En no rumiantes, la mayoría de AG de cadena larga, especialmente los AGPI, son ligantes naturales del PPARγ cambiando la expresión de este gen y los ratios de lipogénesis (Bensinger and Tontonoz, 2008, Berger and Moller, 2002, Desvergne *et al.*, 2006). El hecho de que los AG de cadena larga son potentes agonistas de PPARγ en no rumiantes indica que este podría ser un gen clave en el metabolismo lipídico mamario (Kadegodwa *et al.*, 2009).

La síntesis de la grasa láctea parece estar controlada al menos en parte por PPARγ en la glándula mamaria ya que se observó un aumento en la expresión de PPARγ y de varios genes involucrados en la síntesis de grasa láctea en la glándula mamaria de vacas lecheras en lactación comparado con 15 días antes del parto (Bionaz y Loor, 2008). Basándose en estos datos, Kadegowda *et al.* (2009) demostraron que células MAC-T (células epiteliales de la glándula mamaria bovina) tratadas con rosiglita zona (ROSI; agonista específico de PPARγ) incrementaban la expresión de genes relacionados con la síntesis *de novo de* AG (ACACA, FASN), la síntesis de TAG (AGPAT6, DGAT1, LPIN1), la regulación transcripcional de la síntesis lipídica (SREBP1, SREBP2) y el supresor de SREBP1, INSIG1. Y células MAC-T tratadas con C16:0 incrementaba la expresión de FABP3, FABP4, LPIN, SCD y INSIG1 ligándose directamente a PPARγ. Estos resultados sugieren que los AG de cadena larga como ligantes naturales de PPARγ podrían no sólo regular genes diana de la síntesis de la grasa láctea (ACACA, FASN, LPIN1) sino que además también afectar a factores de transcripción (SREBP1 y SREBP2).

Por lo tanto, estos resultados sugieren que SREBP1 y PPARγ tienen un papel importante en la síntesis de grasa láctea, teniendo PPARγ un papel más fundamental, ya que éste puede ser activado mediante AG de cadena larga, mientras que SREBP1 sólo se ve influenciando por el *trans-10, cis-12* CLA el cuál, al contrario de otros AG de cadena larga, inhibe su expresión. Kadegodwa *et al.* (2009) no observaron una activación de PPARγ mediante el *trans-10, cis-12* CLA y, en cambio, se observó un incremento en la expresión de los genes relacionados con la síntesis de grasa láctea después del tratamiento con ROSI. Por lo tanto, los resultados señalan que PPARγ juega un papel importante en el control de la síntesis de la grasa láctea.

En un experimento *in vivo* la activación de PPARγ preparto mediante tiazolidinadiona (TZD; agonista de PPARγ) afectó al tejido adiposo pero, aparentemente, disminuyó la producción de grasa láctea (Smith *et al.*, 2009). Estos resultados no son completamente sorprendentes considerando que el tratamiento con TZD se dio preparto, que es cuando hay grandes cantidades de PPARγ en el tejido adiposo y bajas cantidades en la glándula mamaria (Bionaz y Loor, 2008), mientras que cuando se espera un incremento de PPARγ en la glándula mamaria en el inicio de la lactación, TZD no se suplementó y la cantidad de NEFA, los cuales podrían jugar un papel en la activación de PPARγ, estaban disminuidos en los animales tratados con TZD (Smith *et al.*, 2009). Además, el tejido adiposo compite con la glándula mamaria por los sustratos lipogénicos, especialmente si la sensibilidad a la insulina es alta, como se ha demostrado que mediante la inyección de insulina en vacas se reduce la grasa láctea (Corl *et al.*, 2006). La insulina parece tener un papel esencial en la activación de PPAR, pero está puede ser crucial para algunos genes más que para otros (LPIN1 y SREBP1 *vs.* carnitina palmitoil transferasa 1A) (Bionaz *et al.*, 2012; Thering *et al.*, 2009).

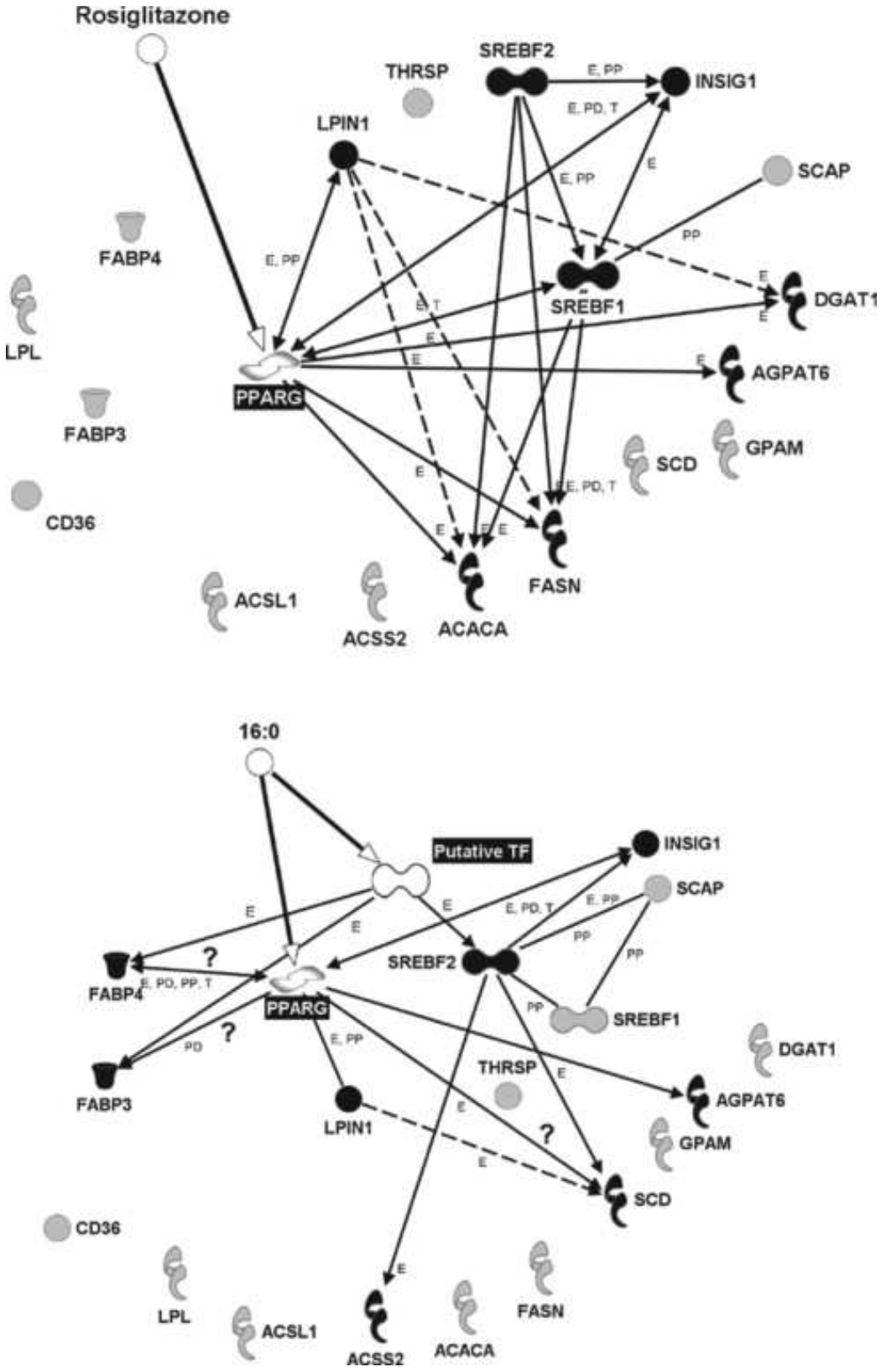

Figura 8. *Resumen de las potenciales redes de regulación entre diversos genes involucrados en la síntesis de la grasa láctea en respuesta al factor de proliferación activado gamma (PPARG) activado via rosiglita zona o ácido palmitato (C16:0) (Genes sobreregulados = negro; Genes subregulados = gris) (Adaptado de Kadegodwa et al., 2009).*

7.6.3. Receptor X del hígado (LXR)

Los receptores X del hígado (LXR) son una familia de receptores nucleares que regulan la síntesis de lípidos y controlan la homeostasis del colesterol mediante su activación a partir de oxiesteroles o agonistas sintéticos (Lehmann *et al.*, 1997; Schultz *et al.*, 2000). Se han identificado 2 miembros de esta familia: El LXRα y el LXRβ. LXRα se expresa predominantemente en tejidos activos en metabolismo lipídico: hígado, riñones, intestino delgado, bazo, tejido adiposo, y glándulas pituitaria y adrenales (Auboeuf *et al.*, 1997; Willy *et al.*, 1995). En cambio, la expresión de LXRβ está extendida a casi todos los tejidos estudiados, incluido el hígado y cerebro (Song *et al.*, 1994). Las dos isoformas regulan la transcripción de enzimas lipogénicos mediante la unión al DNA en un complejo heterodímero con el receptor X de retinoides (RXR; Willy *et al.*, 1995). El complejo LXR-RXR se une a los elementos de respuesta de LXR (LXRE) en el promotor de los genes respondedores.

El promotor de SREBP1c contiene un LXRE para LXRα y LXRβ, sugiriendo el potencial para la regulación de la transcripción de SREBP1c por LXRs (Lengi and Corl, 2010). La transcripción de SREBP1c fue estimulada a partir de oxiesteroles que activaron LXRα y LXRβ en células de hepatoma en ratas (DeBose-Boyd *et al.*, 2001). La incubación con T0901317 (T09), agonista de LXR, en preadipocitos humanos incrementó la expresión de ACACA, FASN, SCD1 y SREBP1c (Darimont *et al.*, 2006).

En ratones, la activación de LXR mediante T09 incrementaba las cantidades hepáticas de SREBP1, ACACA, FASN y GPAT, y su efecto se vio reducido en ratones knockout de SREBP1c, indicando un papel esencial de SREBP1c en la respuesta de LXR (Liang *et al.*, 2002). Repa *et al.* (2000) concluyeron que oxiesteroles derivados del colesterol activan LXR e inducen la expresión de SREBP1c en ratones a través de su promotor de LXRE. Además, SREBP1a y SREBP2 no respondían a la activación de LXR mediante T09 (Schultz *et al.*, 2000; DeBose-Boyd *et al.*, 2001). Además de incrementar la transcripción de ACACA y FASN mediante la activación de SREBP1c, LXR puede incrementar la transcripción de ACACA y FASN directamente mediante la unión a LXRE encontradas en las regiones promotores de estos genes (Joseph *et al.*, 2002; Talukdar and Hillgartner, 2006). Estos resultados sugieren que LXR puede tener un papel en la regulación de genes lipogénicos, manteniendo la expresión de algunos genes y activando otros (Oppi-Williams *et al.*, 2013).

En vacas lecheras, LXRα y SREBP1, están considerados importantes reguladores de la expresión de genes lipogénicos, y la expresión de estos incrementa durante la lactación

comparado con el tejido mamario no lactante (Harvatine and Bauman, 2007; Mani *et al.*, 2009). McFadden y Corl (2010) observaron un incremento en la síntesis de AG *de novo,* y de la cantidad de mRNA de SREBP1 y FASN en células epiteliales mamarias bovinas tratadas con T09. Estos resultados podrían indicar que LXR actúa incrementado la lipogénesis mediante SREBP1. Además, la activación de LXR incrementa la expresión de FASN, gen diana de SREBP1 y enzima esencial para la síntesis de AG *de novo,* observado también por otros autores (Magana y Osborne, 1996; Harvatine y Bauman, 2006), indicando que LXR puede unirse directamente a promotores de FASN que incluyen secuencias LXRE (Joseph *et al.*, 2002). Joseph *et al.* (2002) confirmó que la máxima inducción de FASN requiere la unión de SREBP1c y LXR a sus respectivos elementos de respuesta en la región del promotor de FASN. Igual que FASN, ACACA aumentó en células MAC-T tratadas con T09, lo que parece indicar que su promotor contiene un LXRE y la posibilidad de que esté se active por los dos mecanismos, a través de SREBP1 activado por LXRα y directamente por LXRα (Oppi-Williams *et al.*, 2013). La cantidad de mRNA de ACACA y FASN se redujo mediante knockdown SREBP1 y aumentó en T09, sugiriendo que los 2 factores de transcripción (SREBP1 y LXRα) son reguladores importantes de la lipogénesis (Oppi-Williams *et al.*, 2013). Estos resultados indican que varios genes involucrados en el metabolismo lipídico pueden ser activados directamente por LXRα y SREBP1, o indirectamente, en ausencia de LXRE en el gen diana, mediante la activación de SREBP1 por LXRα (Oppi-Williams et al, 2013)

La reducción en el mRNA de LXRα en células MAC-T no alteró la cantidad de los transcritos para proteínas involucradas en la síntesis *de novo* y el ratio de lipogénesis, como ACACA y FASN, lo que podría indicar que la expresión de los genes involucrados en la síntesis de AG se mantiene por otros factores de transcripción, como SREBP1, el cual aumenta en LXRα knockdown (Oppi-Williams *et al.*, 2013). Además, SREBP1, LPL y SCD1 aumenta cuando la cantidad de LXRα se reduce. El incremento en SREBP1 podría entonces estimular la expresión de LPL y SCD1, incrementando la cantidad de estos genes independientemente de la activación de LXRα (Oppi-Williams *et al.*, 2013).

8. GENES RELACIONADOS CON LA DEPRESIÓN DE LA GRASA LÁCTEA

Como se ha discutido, numerosos enzimas están involucrados en la síntesis de la grasa láctea. Estos enzimas son responsables de la síntesis de AG *de novo,* la activación de AG, y la captación, transporte, desaturación y síntesis de TAG (Bionaz y Loor, 2008b). Como proteínas, estos enzimas están posiblemente regulados por los 3 factores de transcripción comentados

anteriormente. Además, la teoría de la biohidrogenación se basa en la hipótesis de una inhibición de uno o más pasos en la síntesis de grasa láctea (Bauman y Griinari, 2001).

La DGL debida a los AGPI formados durante la biohidrogenación ruminal como *trans*-10, *cis*-12 CLA produce una represión coordinada de varios genes involucrados en la vía *de novo* síntesis de AG y la vía de síntesis de TAG (ACACA, FASN, GPAT y AGPAT) (Peterson *et al.,* 2003). Además, se ha visto que varios reguladores juegan un papel importante en la síntesis de grasa láctea como el SREBP-1, PPARγ y LXR (Harvatine y Bauman, 2006; Kadegowda *et al.,* 2009; McFadden y Corl, 2010).

La regulación transcripcional de los enzimas lipogénicos podría ser uno de los mecanismos que explican la DGL. Por lo tanto, diversos autores han examinado la expresión de genes lipógenicos y sus factores de transcripción en cultivos celulares o en vacas lecheras. Los estudios en vacas con infusiones o dietas con *trans*-10, *cis*-12 CLA muestran una reducción de la expresión de genes lipógenicos involucrados en la síntesis *de novo* de AG (ACACA y FASN), y la desaturación (SCD1) (Baumgard *et al.*, 2002; Peterson *et al.*, 2003; Peterson *et al.*, 2004). Además, los genes involucrados en la síntesis de TAG (GPAT y AGPAT), y en la captación y transporte de AG circulantes (LPL y FABP) disminuyen en las vacas que reciben infusiones orales o abomasales de *trans*-10, *cis*-12 CLA (Baumgard *et al.*, 2002; Peterson *et al.*, 2003).

En relación a los factores de transcripción involucrados en la DGL, se ha observado que SREBP1 disminuyó a causa de *trans*-10, *cis*-12 CLA en células epiteliales mamarias y los promotores de FASN y SCD1 contienen un SRE, indicando un papel importante en la regulación de la síntesis de la grasa láctea durante la DGL (Peterson *et al.*, 2004). La reducción de SREBP1 durante la DGL también se observó en vacas alimentadas con *trans*-10, *cis*-12 CLA o dietas que inducen la DGL (baja en fibra y alta en aceites vegetales) (Harvatine y Bauman, 2006). Además de SREBP1, PPARγ y LXR parecen estar involucrados en la regulación de los enzimas lipogénicos durante la DGL, basado en la sobrerespuesta coordinada de los genes lipogénicos cuando estos 2 factores de transcripción se activan mediante sus agonistas en células epiteliales mamarias bovinas (Kadegowda *et al.*, 2009; McFadden y Corl, 2010).

Aunque no se conoce como los factores dietarios afectan a la producción de la grasa láctea, se ha descubierto como algunos factores de transcripción pueden mediar esta respuesta. La suplementación de dietas con aceites de pescado o aceites vegetales está relacionado con la DGL y se ha relacionado con la disminución de la cantidad de mRNA de ACACA, FASN, GPAT y AGPAT (Peterson *et al.*, 2003). Las células MAC-T tratadas con *trans*-10,

cis-12 CLA redujeron la proteólisis para crear la forma de SREBP1 madura y redujeron la cantidad de mRNA de ACACA, FASN y SCD1 (Peterson *et al.*, 2004). Las dietas que inducen DGL y tratamientos con *trans*-10, *cis*-12 CLA redujeron el mRNA de SREBP1 y INSIG1 en el tejido mamario (Harvatine y Bauman, 2006). Parecido al tratamiento de CLA, el small interfering RNA (siRNA) redujo la forma madura de SREBP1, la síntesis de AG *de novo* y los niveles de mRNA de ACACA, FASN y SCD1, pero incrementó ACACA y FASN en respuesta de la activación a LXR, mientras que SREBP1 estaba disminuido, indicando el potencial de LXR en su regulación (Oppi-Williams *et al.*, 2013). Harvatine y Bauman (2006) propusieron que la reducción de SREBP1 en el tejido mamario asociado a dietas o CLA que inducen DGL se debe a la regulación directa de la transcripción de SREBP1 mediante el mismo, debido a elementos respuesta en su promotor. Además, el SREBP1 contiene un LXRE en su promotor (Lengi y Corl, 2010) y, por lo tanto, LXR puede tener un papel importante en la regulación de SREBP1 y varios genes lipogénicos que incluyen un LXRE en su promotor.

La expresión de la hormona tiroidea respondedora a SPOT14 (THRSP) disminuye en el tejido mamario de vacas tratadas mediante infusiones sanguíneas de *trans*-10, *cis*-12 CLA o en vacas alimentadas con dietas que causan la DGL (bajas en fibra y altas en aceites) (Harvatine y Bauman, 2006). Este gen parece interactuar con factores transcripcionales, entre ellos SREBP1, ya que THRSP contiene un SRE y además en el mismo estudio se observó una relación entre la expresión de THRSP y la expresión de FASN y LPL, indicando que THRSP es un potente regulador candidato de la DGL, aunque no se haya observado un papel fundamental de este gen en la síntesis de la grasa láctea (Bionaz y Loor, 2008).

En relación al FT PPARγ, aunque se ha demostrado claramente su papel en la síntesis de la grasa láctea (comentado anteriormente), Kadegodwa *et al.* (2009) no observaron una activación de PPARγ mediante el *trans-10, cis-12* CLA. Por lo tanto, los resultados señalan que PPARγ juega un papel importante en el control de la síntesis de la grasa láctea pero no sería responsable de la DGL. La misma situación sucede con el FT LXR: aunque se ha demostrado su papel en la síntesis de la grasa láctea, parece no estar relacionado con la DGL. Harvatine *et al.* (2014) evaluaron la respuesta de LXR en vacas tratadas con *trans*-10, *cis*-12 CLA o alimentadas con dietas que inducen la DGL (baja en fibra y altos contenidos de aceites) y no observaron cambios su la expresión.

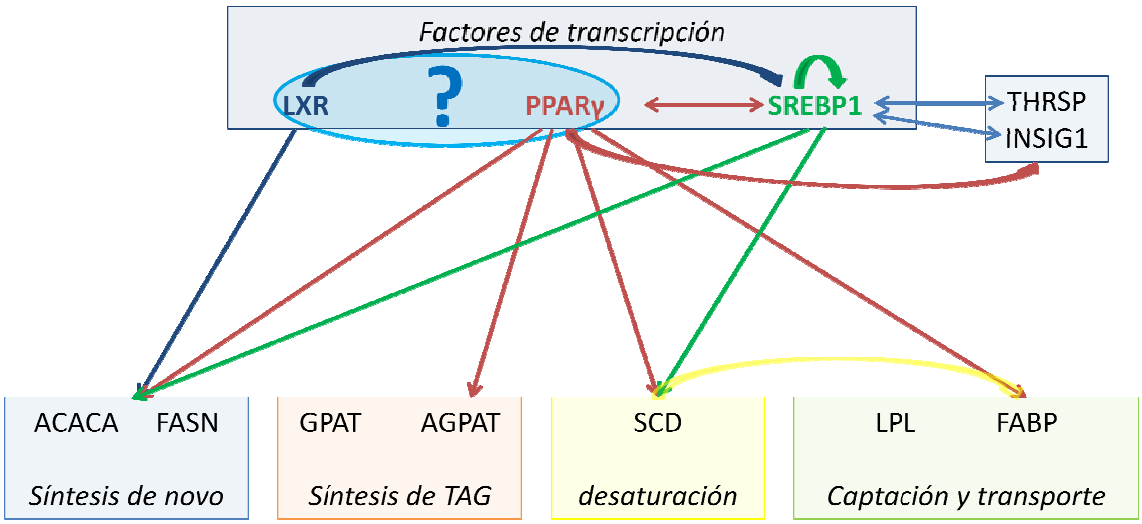

Figura 9. *Genes con la expresión disminuida en la glándula mamaria debido a dietas que inducen la depresión de la grasa láctea o debido a infusiones abomasales de trans-10, cis-12 CLA descritos hasta la fecha.*

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CAPÍTULO 2

Objetivos

1. OBJETIVO GENERAL

El objetivo general de esta tesis fue evaluar diferentes estrategias para evitar la disminución de la cantidad de la grasa láctea debida a dietas ricas en AGPI, específicamente, en dietas suplementadas con lino (fuente de ácido linolenico).

2. OBJETIVOS ESPECÍFICOS:

2.1. **Primer trabajo**

Los objetivos del primer estudio fueron: 1) Justificar las recomendaciones diarias de CLA para observar sus potenciales los efectos beneficiosos en la salud humana (búsqueda bibliográfica); 2) Determinar el consumo actual de CLA en la población (búsqueda bibliográfica); 3) Determinar el efecto de estrategias nutricionales en vacas lecheras para enriquecer la leche en CLA (metaanalisis); y 4) Calcular el incremento esperado de CLA en el consumo humano si toda la leche y productos lácteos fueran consumidos enriquecidos en CLA.

2.2. **Segundo trabajo**

El objetivo del segundo trabajo fue evaluar el efecto de 2 lipasas comerciales, un inhibidor de la lipasa y tres aceites esenciales (PTSO, Cinamaldehído y Eugenol) en la concentración de AGPI en el rumen. Especificamente los objetivos fueron: 1) Inhibir la lipolisis ruminal; 2) Incrementar la actividad lipolítica para aumentar los AGPI disponibles que pueden saturar el proceso de biohidrogenación ruminal. 3) Reducir la actividad microbiana responsables del último paso de la biohidrogenación (C18:1 a C18:0); y 4) Inhibir la via alternativa de la biohidrogención que produce los isomeros responsables de la DGL (*trans*-10 C18:1 y *trans*-10, *cis*-12 CLA).

2.3. **Tercer trabajo**

Los objetivos del tercer estudio fueron: 1) Analizar el transcriptoma de vacas lecheras resistentes o sensibles a la DGL debida a dietas suplementadas con lino; 2) Identificar vías metabólicas y genes reguladores afectados por la DGL en vacas lecheras resistentes o sensibles a la DGL; 3) Identificar genes candidatos con variaciones estructurales (SNP) que juegen un papel clave en la regulación de la DGL en vacas lecheras resistentes a la DGL.

CAPÍTULO 3

The relationship between human daily requirements of conjugated linoleic acid (CLA), the potential enrichment of milk through cow's nutrition and daily human consumption.

The relationship between human daily requirements of conjugated linoleic acid (CLA), the potential enrichment of milk through cow's nutrition and daily human consumption.

Abstract

Interest in functional foods has increased in recent years, being the enrichment of milk with conjugated linoleic acid (CLA) one of the targeted products. The objectives of this research were: a) To identify the source of current human daily recommendations (literature search); b) To determine the effect of feeding strategies on CLA concentration in milk (meta-analysis); and c) To determine current average human intake of CLA and the expected improvement if milk and milk products were consumed in a CLA enriched form (literature search). The most commonly reported intake recommendations for human are 0.8 g/d (from 0.6 to 3.0 g/d). All recommendations have been extrapolated from animal models and the few human studies reported contradictory results. We selected published papers (n = 69) where dairy cows were fed different fats and the milk fat content and fatty acid (FA) profile were reported. Treatments were categorized by source (vegetable oils, fish oils or the combination) and method of processing (raw, processed or extruded seeds, and oils). Data were analyzed using meta-analysis techniques. The combination of fish and vegetable oils resulted in the greatest increase (0.61 *vs* 1.34 g of CLA/100 g of FA), but milk fat content decreased (3.61 *vs* 3.12%). Linseed increased CLA proportion (0.61 *vs* 0.90 g of CLA/100 g of FA) without affecting milk fat content. The best processing methods to enriched milk with CLA were extruded seeds (0.57 *vs* 1.11 g of CLA/100 g of FA) and oils (0.57 *vs* 1.10 g of CLA/100 g of FA), but extruded seeds decreased milk yield (30.4 *vs* 28.9 kg/d) and oils decreased milk fat content (3.61 *vs* 3.31%). Considering the changes in CLA and milk fat content, supplementation with fish oils together with vegetable oils would be the best strategy (395 mg of *cis*-9, *trans*-11 CLA/l *vs* 188 mg of *cis*-9, *trans*-11 CLA/l; increase of 2.1 times). The estimated current average human consumption in Europe, US and Canada is 0.21 g/d, ranging from 0.06 g/d in Portugal to 0.40 g/d in Germany, well below the requirements. If we assume an increase content of 2.1 times in CLA in milk and milk products, average human consumption would increase from 0.21 to 0.46 g/day. Although there is sufficient data on feeding strategies to increase CLA content in milk, human requirements have not been well established and, based on current recommendations, they are unattainable even if all milk and milk products were consumed as CLA enriched products.

Keywords: CLA, dairy products, human health, polyunsaturated fatty acid.

Abbreviations: CTR, control; PAS, feeding fresh pasture; RA, diets enriched with rapeseed; CO, diets enriched with corn products; SOY, diets enriched with soybean; SUN, diets enriched with sunflower; LIN, diets enriched with linseed; FO, diets enriched with fish oils; FO+PO, diets enriched with fish oils plus plant oils; RS, raw supplements; PS, processed seeds; EXT, extruded seeds; OIL, oils; FA, fatty acids; PHVO, partially hydrogenated vegetable oils; VA, vaccenic acid; CLA, conjugated linoleic acid; PUFA, polyunsaturated fatty acids; FFQ, food-frequency questionnaire; DMI, dry matter intake; DIM, days in milk; TEB, terminal end buds; DM, dry matter; SEM, standard error of the mean; PC, principal component; CVD, cardiovascular disease

1. INTRODUCTION

Social perception towards dairy foods is that they contain high levels of saturated fatty acids (FA) and cholesterol, which are considered harmful to human health because they are associated with high risk for cardiovascular diseases (CVD). Cardiovascular diseases are the leading cause of death in developed societies and are associated with factors such as diet, obesity, sedentary lifestyle, diabetes, stress, and smoking, among others. Controlling the intake of cholesterol and saturated FA of animal origin has been a cornerstone dietary strategy to reduce the incidence of CVD and, therefore, the reduction in the consumption of animal products, including dairy products, has been recommended. However, several epidemiological studies have shown that the relationship between intake of saturated FA and cholesterol with atherosclerosis and CVD are inconclusive and sometimes contradictory (Hegsted and Ausman, 1988; Stamler and Shekelle, 1988; Ravnskov, 1995, 1998; Hu *et al.,* 1997; Pietinen *et al.,* 1997; Kritchevsky and Kritchevsky, 2000; McNamara, 2000; Boniface and Tefft, 2002). Elwood *et al.* (2010) conducted a metaanalysis with data from 11 prospective epidemiological studies conducted in different parts of the world which data were adjusted for confounding factors such as age, body weight index, alcohol consumption, smoking habits, social class and exercise, among others, and concluded that consumption of milk and dairy products reduces the incidence of CVD. These studies were also analyzed by other authors that reached to similar conclusions (Gibson *et al.,* 2009; Mente *et al.,* 2009). The protective properties of milk in front CVD has been associated with its content in calcium, bioactives peptides, folic acid, vitamins B6 and B12, and conjugated linoleic acid (CLA) (Hata *et al.,* 1996; Verhoeff *et al.,* 1998; Ford *et al.,* 2002; Reid *et al.,* 2002; Seppo *et al.,* 2003)

The CLA is an 18 carbons polyunsaturated FA (PUFA) with two conjugated double bonds, separated by one single bond. This FA appears to contribute positively to the health effects of milk and has attracted scientific and commercial interest (Lawson, 2001). In recent years, a large number of studies in animal models have shown that intake of CLA results in beneficial health effects. The CLA inhibits carcinogenesis, reduces atherosclerosis, is hypocholesterolemic, reduces adipose tissue mass, modulates the inflammatory and immune responses, reduces symptoms of diabetes mellitus type II, act as a growth factor in young animals, it is involved in bone formation, and it protects against end state of the disease systemic lupus erythematosus (Ip *et al.,* 1991; Kritchevsky *et al.,* 2000; Yang *et al.,* 2000; Pariza *et al.,* 2001; Whigham *et al.,* 2001; Roche *et al.,* 2002). However, epimediological studies and controlled trials in humans are inconclusive and the methodology used is often questionable (Aro *et al.,* 2000; Chajès *et al.,* 2002; Riserus *et al.,* 2002; Wolff and Precht, 2002a; Moloney *et al.,* 2004, 2007; Whigham *et al.,* 2007). Tetens (2010) concluded that the consumption of an equimolar mixture of CLA isomers *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA had no effect on the maintenance or achievement of a normal body weight, increase in lean body mass, increase in insulin sensitivity, protection of DNA, proteins and lipid from oxidative damage, or contribution to immune defenses by stimulation of production of protective antibodies in response to vaccination. In spite of that, there is a scientific interest in analyzing factors that may increase the content of CLA in the human diet. Only products derived from ruminants, especially dairy products (70% of total CLA consumed), have a significant contribution to CLA consumption in human diets (Ritzenthaler *et al.,* 2001). Consequently, enriched milk with CLA is the best strategy to increase the intake of CLA in the population.

Under natural conditions, most of the CLA is produced from the intermediates of the biohydrogenation of PUFA in the rumen. Two processes occur in the rumen metabolism of lipids: the hydrolysis of the ester bonds by microbial lipases (Dawson *et al.,* 1977); and the biohydrogenation of PUFA. The major substrates for biohydrogenation are linoleic acid (*cis*-9, *cis*-12 18:2) and α-linolenic acid (*cis*-9, *cis*-12, *cis*-15 18:3), that after a sequence of isomerizations, results in the formation and accumulation of vaccenic acid (VA) in the rumen, which is the common intermediate in the biohidrogenation of these two FA and the main source for the endogenus synthesis of *cis*-9, *trans*-11 CLA in the mammary gland (Harfoot and Hazlewood, 1988; Griinari and Bauman, 1999).

The diet given to dairy cows is the main factor affecting the content of CLA in milk and, therefore, changes in diets may allow to enriched milk with CLA. Griinari and Bauman (1999) proposed that the dietary effects could be grouped into 2 categories depending on the mechanism of action: a) Dietary factors that increase the lipid substrates for the production of CLA and VA mainly including plant oils rich in linoleic and linolenic acid, and pasture (rich in linolenic acid); b) Dietary factors that affect the bacteria involved in the process of ruminal biohydrogenation, as fish oils or diets low in fiber and high in concentrate; and c) The combination of both strategies. The combination of fish oils with plant oils results in a greater increase of CLA in milk that supplying only plant oils (AbuGhazaleh *et al.,* 2003; Whitlock *et al.,* 2002).

The objectives of this study are: 1) To justify the daily recommendations of CLA to observe its beneficial effects on human health (literature search), 2) To determine current consumption of CLA in different countries (literature search), 3) To determine the effect of feeding strategies on CLA concentration in milk (meta-analysis), and 4) To calculate the expected improvement in CLA human consumption if milk and milk products were consumed in a CLA enriched form.

2. MATERIALS AND METHODS

2.1. **Daily human recommendations and average current consumption of CLA**

To identify the source of current daily human recommendations of CLA an exhaustive bibliographic research (between 1990 to 2012) was conducted using PubMed, Web of knowlegde and Science Direct, among others.

To assess the current consumption of CLA data were collected from 9 publications containing daily intake of total CLA or *cis*-9, *trans*-11 CLA in different countries (Fritsche and Steinhart, 1998; Jiang *et al.,* 1999; Ens *et al.,* 2001; Ritzenthaler *et al.,* 2001; Fremann *et al.,* 2002; Wolff and Precht, 2002b; Laloux *et al.,* 2007; Martins *et al.,* 2007; Mushtaq *et al.,* 2010). In all these studies, the daily intake of CLA was estimated by one of the following 5 methods: 1) Recording the diet for 3 or 7 days, including all foods and drinks consumed by subjects studied, representing the average diet consumed (Jiang *et al.,* 1999; Ens *et al.,* 2001; Ritzenthaler *et al.,* 2001; Fremann *et al.,* 2002; Laloux *et al.,* 2007; Mushtaq *et al.,* 2010); 2) Recording the diet during 24 h, monthly for 7 consecutive months (Jiang *et al.,* 1999) 3)

Conducting a food-frequency questionnaire (FFQ) which displays different foods and different frequencies of consumption (Ritzenthaler *et al.,* 2001; Fremann *et al.,* 2002); 4) Summarizing data of mean food consumption obtained from national studies made in different countries (Fritsche and Steinhart, 1998; Martins *et al.,* 2007); 5) Extracting information from databases, from which an average consumption of dairy products was extracted per capita and year (1999) in the different countries analyzed. However, this method does not consider ruminant meat products or other minority sources that also represent a part of the consumption of CLA in the human diet. Therefore, results might slightly underestimate the daily CLA intake (Wolff and Precht, 2002b).

The CLA content of various foods was extracted from databases that provided this information in the different countries analyzed (Jiang *et al.,* 1999; Ens *et al.,* 2001; Ritzenthaler *et al.,* 2001; Wolff and Precht, 2002b; Laloux *et al.,* 2007); of food samples obtained from supermarkets and analyzed by gas chromatography (Fritsche and Steinhart, 1998; Ritzenthaler *et al.,* 2001; Fremann *et al.,* 2002; Martins *et al.,* 2007; Mushtaq *et al.,* 2010); or chromatographic analysis of food samples collected by the subjects tested in the study (Ritzenthaler *et al.,* 2001).

The subjects chosen in the studies were healthy (without metabolic disorders, not pregnant, not to be on a diet, not vegans or vegetarians, not with special nutritional requirements...) usually confirmed with a medical examination and blood tests. People participating in questionnaires or data collection of their diet were instructed by specialists to fill them properly. Subjects in each experiment were: 103 men (weighed and recorded their diet for 7 consecutive days in 2 periods) and 123 men (recall interviews by telephone of the diet of previous 24 hours, monthly for 7 consecutive months), subjects were 46 to 72 years older (Jiang *et al.,* 1999), 9 men and 9 women between 21 and 60 years (Mushtaq *et al.,* 2010), 530 boys and 488 girls between 3 and 14, and 672 men and 802 women over 15 years (Laloux *et al.,* 2007), 46 men and 47 women between 18 and 60 years (Ritzenthaler *et al.,* 2001), 52 female university students (Fremann *et al.,* 2002), and 4 men and 18 women, age was 31.8 ± 9.10 (Ens *et al.,* 2001).

In publications that did not include the average consumption of CLA for both sexes, a weighted average was performed in order to obtain this data (Ritzenthaler *et al.,* 2001; Laloux *et al.,* 2007), except in the case of Fritsche and Steinhart (1998) which did not include information of subjects studied and, therefore, an arithmetic mean was calculated.

In order to obtain an approximate value of the intake of *cis*-9, *trans*-11 CLA in all studies an arithmetic mean was calculated, because studies that estimated consumption by national statistical data bases did not have the number of people tested and was not possible to perform a weighted average. These studies did not have information on the standard error of mean (SEM) or standard deviation of the average consumption of CLA. In two studies (Martins *et al.,* 2007; Laloux *et al.,* 2007), intake was expressed as total CLA and in this case it was assumed that the *cis*-9, *trans*-11 CLA was 76% of total CLA (Martins *et al.,* 2007).

2.2. **Effect of feeding strategies on CLA concentration in milk**

2.2.1. Inclusion of Publications

Published papers (n = 69) that included 72 experiments published until 2012 with the objective of enhancing the FA profile in milk fat were selected. Dietary strategies used were based on supplementing the diet with plant oils from rapeseed, soybean, sunflower and linseed; supplementing the diet with fish oils, alone or mixed with plant oils; feeding fresh pasture; and supplementing the diet with corn products. Plant oil supplements were supplied as oils, calcium salts, whole seeds, micronized seeds, extruded seeds, ground seeds, meal, crushed seeds, cracked seeds, roasted seeds, corn distillers solubles and corn germ, rolled seeds or seed hulls.

Data collected from these experiments were: *cis*-9, *trans*-11 CLA or total CLA concentration and/or yield, the diet content of FA 18:1, 18:2, 18:3 and total FA or ether extract, dry matter intake (DMI), milk yield, fat content and/or fat yield, and their SEM.

Most experiments were conducted on Holstein or Friesian cows except 7: 1 experiment was conducted on Holstein and Montbeliers cows (Ferlay *et al.,* 2010); 4 experiments were conducted on Holstein and Brown Swiss cows (Collomb *et al.*, 2004; Abughazaleh *et al.,* 2004; Whitlock *et al.,* 2006; Abdelqader *et al.,* 2009); 2 experiments were conducted on Holstein and Jersey cows. In the later cases only results from Holstein cows were considered (Sol Morales *et al.,* 2000; Kliem *et al.,* 2009); and 4 experiments did not specify the breed (Dhiman *et al.,* 1999; He *et al.,* 2005; Ambrose *et al.,* 2006; Carriquiry *et al.,* 2009).

Days in milk (DIM) at the beginning of experiments were: < 100 DIM in 32 experiments (early lactation), 100-200 DIM in 31 experiments (mid-lactation), >200 DIM in 7 experiments (late-lactation) and 2 experiments did not specify DIM.

Simple regression was performed among breed, DIM and year of publication, and concentration of *cis*-9, *trans*-11 CLA to test the effect of these variables on concentration of *cis*-9, *trans*-11 CLA. Breed, DIM and year of publication had no effect on *cis*-9, *trans*-11 CLA results, therefore all data was included in one category.

To take into account differences among experiments, variable weight was calculate for each data collected from SEM of variables. Variable weights were calculated as: $1/SEM^2$. Any data with no SEM was excluded from the analysis.

2.2.2. Data processing

Of the 69 publications, 260 data from different experimental treatments were extracted. Data were encoded in three different ways:

1- According to the dietary source used in: 1) unsupplemented diet (CTR; n = 62); 2) fresh pasture (PAS; n = 18); diets enriched with: 3) rapeseed (RA; n = 13); 4) corn products (CO; n = 20); 5) soybean (SOY; n = 33); 6) sunflower (SUN; n = 16); 7) linseed (LIN; n = 59); 8) diets enriched with fish oils (FO; n = 18); and 9) diets enriched with fish oils plus plant oils (FO + PO; n = 19).

2- According to their major FA content in the supplement in: 1) unsupplemented diet (CTR; n = 62); 2) oleic acid (n = 13); 3) linoleic acid (n = 71); and 4) linolenic acid (n = 72). Group 2 included data from rapeseed products; group 3 included data from soybean, sunflower and corn products; and group 4 included data from linseed products and fresh pasture. In this analysis fish oils and fish oils + plant oil were excluded.

3- Treatments based on supplementation with plant oils were encoded by the method of presentation of the dietary supplement in: 1) unsupplemented diet (CTR; n = 62); 2) whole seeds (RS; n = 19); 3) processed seeds (PS; n = 40); 4) extruded seeds (EXT; n = 18); and 5) oils (OIL; n = 48). The PS group included calcium salts, micronized seeds, ground seeds, meal, crushed seeds, cracked seeds, roasted seeds, rolled seeds and seed hulls. For this analysis, fish oils, fish oils + plant oils, corn products and fresh pasture were excluded. Extruded seeds were separated from the rest of processed seeds, based on the literature, which indicated that this method of processing increased the CLA content in milk with higher efficiency compared with the rest of processing methods.

The C18:1, C18:2 and C18:3 FA composition of supplements and main forages was collected from publications that provided this information, and is presented in Table 1. The C18:1, C18:2, C18:3 concentrations and total FA of diets used in experiments were collected (n = 229). If experiments did not provide the total amount of FA, it was calculated following Allen (2000):

FA= - 0.98 + 1.03 x EE

where FA = total FA content in the diet (% DM), and EE = ether extract content of the diet (% DM). Experiments that provided total CLA in milk, the content of *cis*-9, *trans*-11 CLA was assumed to be 92% of total CLA (Chin *et al.,* 1992).

Most publications reported data of FA profile in milk and diet as a percentage of total FA. The amount of FA in milk and diet in grams per day were calculated from the total content of FA and the profile of foods and milk, assuming that milk fat contains 90% FA.

Table 1. *Averages of FA composition (mean ± SD, g/100 g of total FA) of supplements used in publications (including all methods of presentation).*

Supplement	N	C18:1	C18:2	C18:3
Rapeseed	4	60.0 ± 2.10	20.6 ± 2.76	8.9 ± 0.90
Corn	6	23.3 ± 5.39	51.6 ± 1.61	3.1 ± 2.67
Corn silage	14	16.1 ± 4.28	36.7 ± 8.68	8.0 ± 4.52
Fish	12	9.8 ± 1.88	1.2 ± 0.22	1.3 ± 0.87
Sunflower	8	30.3 ± 19.35	54.6 ± 17.14	0.7 ± 1.23
Linseed	16	19.0 ± 1.75	17.7 ± 4.29	52.9 ± 6.30
Soybean	15	20.3 ± 2.81	52.5 ± 2.43	6.8 ± 1.69

2.2.3. Statistical Analyses

The effect of diet and DIM on the *cis*-9, *trans*-11 CLA content in milk, regardless of the source fed, was assessed by multiple regression of *cis*-9, *trans*-11 CLA on C18:1, C18:2, C18:3, total FA and DIM. Variable weight of *cis*-9, *trans*-11 CLA were assigned to each experiment. Data were analyzed by PROC REG, and the STEPWISE procedure.

The effect of different dietary sources (CTR, PAS, RA, CO, SOY, SUN, LIN, FO and FO+PO), major FA content in the supplement (CTR, oleic acid, linoleic acid and linolenic acid) and method of presentation of plant oils (CTR, RS, PS, EXT, OIL) on DMI, performance parameters and *cis*-9, *trans*-11 CLA in milk was assessed using GLIMMIX procedure and multiple

comparisons were performed by LSMEANS adjusted with the Tukey's test. Variable weight were assigned to each response variable by experiment. Experiment was included as random effect.

Finally, the principal component (PC) analysis was performed to study correlations between response variables. A cluster analysis was performed by grouping data according their associations. The frequency of groups of treatments in each cluster was also analyzed. Data were analyzed by PROC CORR, PRINCOMP, FASTCLUS and FREQ.

All statistical analyseswere performed using the SAS program (v. 9.2., SAS Institute, Cary, NC, USA) and the level of significance was fixed at 0.05.

3. RESULTS

3.1. **Daily human recommendations**

The desing of experiments to determine the requirements of CLA in humans is difficult because the effects are mid to long term, and doses are apparently dependent on the effects expected. Therefore, animal models have been commonly used.

For the anticarcinogenic effect, the only data found are extrapolations from prescribed doses in animal models. Banni *et al.* (1999) reported a reduction in tumor incidence and total number of tumors when a 0.5 to 1% dietary CLA was added to rat diets. Ip *et al.* (1994) reported that diets containing 0.1% CLA reduced the incidence of induced mammary tumors in rats. From these data they extrapolated the recommended intake of CLA in human diets: if a rat with 350 g of body weight would have to ingest 0.015 g/d of CLA, a human of 70 kg would be an intake of 3 g/d of CLA. However, Terpstra (2001) suggested that an extrapolation based on metabolic weight would be more appropriate resulting in 0.8 g CLA/d reveal an anticarcinogenic effects in humans. Hubbard *et al.* (2003) reported that 0.1% dietary *cis*-9, *trans*-11 CLA or 0.1% dietary *trans*-10, *cis*-12 decreased the total volume of tumors in lungs of mice and decreased metastatic cell survival from both spontaneous and implanted metastasis. Ip *et al.* (1999) observed that 0.8% of *cis*-9, *trans*-11 CLA enriched butter fat or 0.8% of a mixture of CLA isomers had the same effects in rats: reduced epithelial mass, decreased size of the terminal end buts (TEB) population, suppressed proliferation of TEB cells and inhibited mammary tumor yield during the pubescence mammary gland development. In humans, only

epidemiological studies have been conducted and results are inconclusive. Only 1 study found a negative association between the intake of CLA and risk of breast cancer in postmenopausal women (Aro *et al.,* 2000). Other studies, reported no effect of CLA intake on protection against breast cancer (Chajès *et al.,* 2002, Voorrips *et al.,* 2002, McCann *et al.,* 2004). For the antiatherosclerotic effect, Kritchevsky *et al.* (2000, 2002) observed that 0.05 to 0.1% dietary CLA inhibited the development of atherosclerosis in rabbits, and a 1% dietary CLA resulted in the regression of established atherosclerosis. Other studies, in ApoE knockout mice, reported a regression of pre-established atherosclerosis with a 1% dietary CLA (Toomey *et al.,* 2006). However, Nestel *et al.* (2006) observed no effect on the severity of aortic atherosclerosis at 0.9% dietary CLA, although plasma triglyceride concentration decreased, and HDL cholesterol concentration increased. On other hand, different isomers of CLA (*cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA) had different effects in mice. Arbonés-Mainar *et al.* (2006) observed that mice fed a 1% dietary *cis*-9, *trans*-11 CLA inhibited atherosclerosis, but a 1 % of dietary *trans*-10, *cis*-12 CLA had pro-atherogenic effects. However, no differences between isomers were observed in rabbits. Diets supplemented with 0.5% *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA or a mixture, inhibited atherosclerosis in rabbits and diets supplemented with 1% *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA or a mixture resulted in a regression of established lesions in rabbits (Kritchevsky *et al.,* 2004). Using a direct extrapolation of the doses used in animals models based on metabolic weight, a human of 70 kg would require 0.6 g/d to observe some inhibitory effect on atherosclerosis, and 12 g/d to observe a regression of an established atherosclerosis lesion. For other effects there are some human studies, although results are often controversial. For reducing fat mass, Whigham *et al.* (2007) conducted a meta-analysis that included 18 studies on the effect of CLA on weight loss: 7 found a reduction in body fat (Blankson *et al.,* 2000; Mougios *et al.,* 2001; Smedman and Vessby, 2001; Gaullier *et al.,* 2004; Pinkoski *et al.,* 2006; Gaullier *et al.,* 2007; Watras *et al.,* 2007) and observed no significant effects (Atkinson *et al.,* 1999; Berven *et al.,* 2000; Kreider *et al.,* 2002; Riserus *et al.,* 2002; Petridou *et al.,* 2003; Eyjolfson *et al.,* 2004; Malpuech-Brugere *et al.,* 2004; Riserus *et al.,* 2004; Taylor *et al.,* 2006; Lambert *et al.,* 2007). Three studies investigated the effects of CLA on body weight maintenance after weight loss (Kamphuis *et al.,* 2003; Whigham *et al.,* 2004; Larsen *et al.,* 2006) and observed no effect of CLA. The doses of CLA used in these studies ranged from 1 to 6.8 g/d (mean of 3.2 g/d). For the increased insulin sensitivity, no positives effects have been observed in humans when diets contained between 3 to 4.2 g/d of CLA (Smedman and Vessby, 2001; Riserus *et al.,* 2002; Moloney *et al.,* 2004; Syvertsen *et al.,* 2007).

In summary, and despite the large variability among studies and models, an effective dose in humans to observe the anticarcinogenic effect could be 0.8 g/d, 0.6 g/d for the antiatherosclerotic effect and 3.2 g/d for the reduction of body fat. For other effects, no specific dose has been recommended.

3.2. **Average current consumption of CLA**

The dietary intake of *cis*-9, *trans*-11 CLA in different countries ranges from 56 to 400 mg/d, with a mean of 211 mg/d. When separated by sex, the average intake was 172 mg/d (range from 68 to 360 mg/d) in women, and 181 mg/d (range from 126 to 440 mg/d) in men (Table 2). Parodi (2003) suggested that ruminant-derived products contain significant levels of VA, and humans are able to transform 20% of ingested VA to *cis*-9, *trans*-11 CLA (Turpeinen *et al.,* 2002). In these conditions, it was suggested that daily intake of CLA could be multiplied by 1.4 to determine the total available CLA. Thus, when applying this factor, the average effective CLA (ingested plus endogenous) was 295 mg/d (range from 78 to 560 mg/d) for both sexes, 241 mg/d (range from 95 to 504 mg/d) for women and 253 mg/d (range from 176 to 616 mg/d) for men.

Table 2. *Daily intake (mg/d) of CLA in the different countries analyzed.*

Country	Method	Daily intake (mg/d ± SD)			Isomer CLA
		Men	Women	Both	
Portugal	DB[1b]			74	total CLA
Germany	Milk (kg/p/yr)[2c]			280	*cis*-9, *trans*-11 CLA
France	Milk (kg/p/yr)[2c]			300	*cis*-9, *trans*-11 CLA
Italy	Milk (kg/p/yr)[2c]			220	*cis*-9, *trans*-11 CLA
Holland	Milk (kg/p/yr)[2c]			230	*cis*-9, *trans*-11 CLA
Belgium/ Luxemburg	Milk (kg/p/yr)[2c]			240	*cis*-9, *trans*-11 CLA
United Kingdom	Milk (kg/p/yr)[2c]			210	*cis*-9, *trans*-11 CLA
Ireland	Milk (kg/p/yr)[2c]			380	*cis*-9, *trans*-11 CLA
Denmark	Milk (kg/p/yr)[2c]			250	*cis*-9, *trans*-11 CLA
Greece	Milk (kg/p/yr)[2c]			150	*cis*-9, *trans*-11 CLA
Spain	Milk (kg/p/yr)[2c]			140	*cis*-9, *trans*-11 CLA
Portugal	Milk (kg/p/yr)[2c]			150	*cis*-9, *trans*-11 CLA
Austria	Milk (kg/p/yr)[2c]			270	*cis*-9, *trans*-11 CLA
Finland	Milk (kg/p/yr)[2c]			360	*cis*-9, *trans*-11 CLA
Sweden	Milk (kg/p/yr)[2c]			330	*cis*-9, *trans*-11 CLA
European Union (15)	Milk (kg/p/yr)[2c]			250	*cis*-9, *trans*-11 CLA
Germany	DB[3b]	440	360	400	*cis*-9, *trans*-11 CLA
Sweden	7d-DR[4e]	160 ± 70			*cis*-9, *trans*-11 CLA
Sweden	24h-DR[4d]	160 ± 60			*cis*-9, *trans*-11 CLA
United Kingdom	7d-DR[5e]	126 ± 90	68 ± 38	98 ± 73	*cis*-9, *trans*-11 CLA
France	7d-DR[6e]	183 ± 98	170 ± 72	177	total CLA
France	7d-DR[6e]	213 ± 95	178 ± 80	194	total CLA
USA	3 d FD[7g]	193 ± 13[a]	140 ± 14[a]	166	*cis*-9, *trans*-11 CLA
USA	3 d FD[7g]	212 ± 14[a]	151 ± 14[a]	181	total CLA
USA	FFQ[7h]	151 ± 15[a]	72 ± 9[a]	111	*cis*-9, *trans*-11 CLA
USA	FFQ[7h]	197 ± 19[a]	93 ± 11[a]	144	total CLA
USA	3d-DR[7f]	133 ± 17[a]	79 ± 17[a]	106	*cis*-9, *trans*-11 CLA
USA	3d-DR[7f]	176 ± 17[a]	104 ± 17[a]	139	total CLA
Germany	7 d DR[8e]		323 ± 79		*cis*-9, *trans*-11 CLA
Germany	FFQ[8h]		246 ± 97		*cis*-9, *trans*-11 CLA
Canada	7d-DR[9e]			95 ± 41	*cis*-9, *trans*-11 CLA

[1] Martins *et al.,* 2007; [2] Wolff and Precht, 2002; [3] Fritsche and Steinhart, 1998; [4] Jiang *et al.,* 1999; [5] Mushtaq *et al.,* 2010; [6] Laloux *et al.,* 2007; [7] Ritzenthaler *et al.,* 2001; [8] Fremann *et al.,* 2002; [9] Ens *et al.,* 2001.

[a] mean (mg/d) ± SEM (Standard error of the mean).

Method = Method used to estimate daily intake of CLA. [b] Data of mean food consumption obtained from national studies; [c] Daily intake of CLA estimated from milk consumption per capita and year in each country; [d] Recorded diet during 24 h, monthly for 7 consecutive months; [e] Recorded diet during 7 consecutive days; [f] Recorded diet during 3 days, 2 days of the week and 1 day of the weekend; [g] 3d-DR with food samples collected by subjects of the study analyzed for duplicate; [h] Food frequency questionnaire.

3.3. **Effect of feeding strategies on CLA concentration in milk form dairy cows**

3.3.1. Relationship between the total supply of PUFA in the diet and cis-9, trans-11 CLA

The multiple regression model that best predicted the production of CLA in milk included linoleic acid (C18:2), linolenic acid (C18:3) and DIM (R^2 = 0.51) with a partial R^2 of 0.45, 0.05, and 0.01, respectively (Figure 1: *cis*-9, *trans*-11 CLA (g/d) = 3.0411 – (0.0052 * DIM) + (0.0121 * (linoleic acid (g/d))) + (0.0061 * (linoleic acid (g/d))).

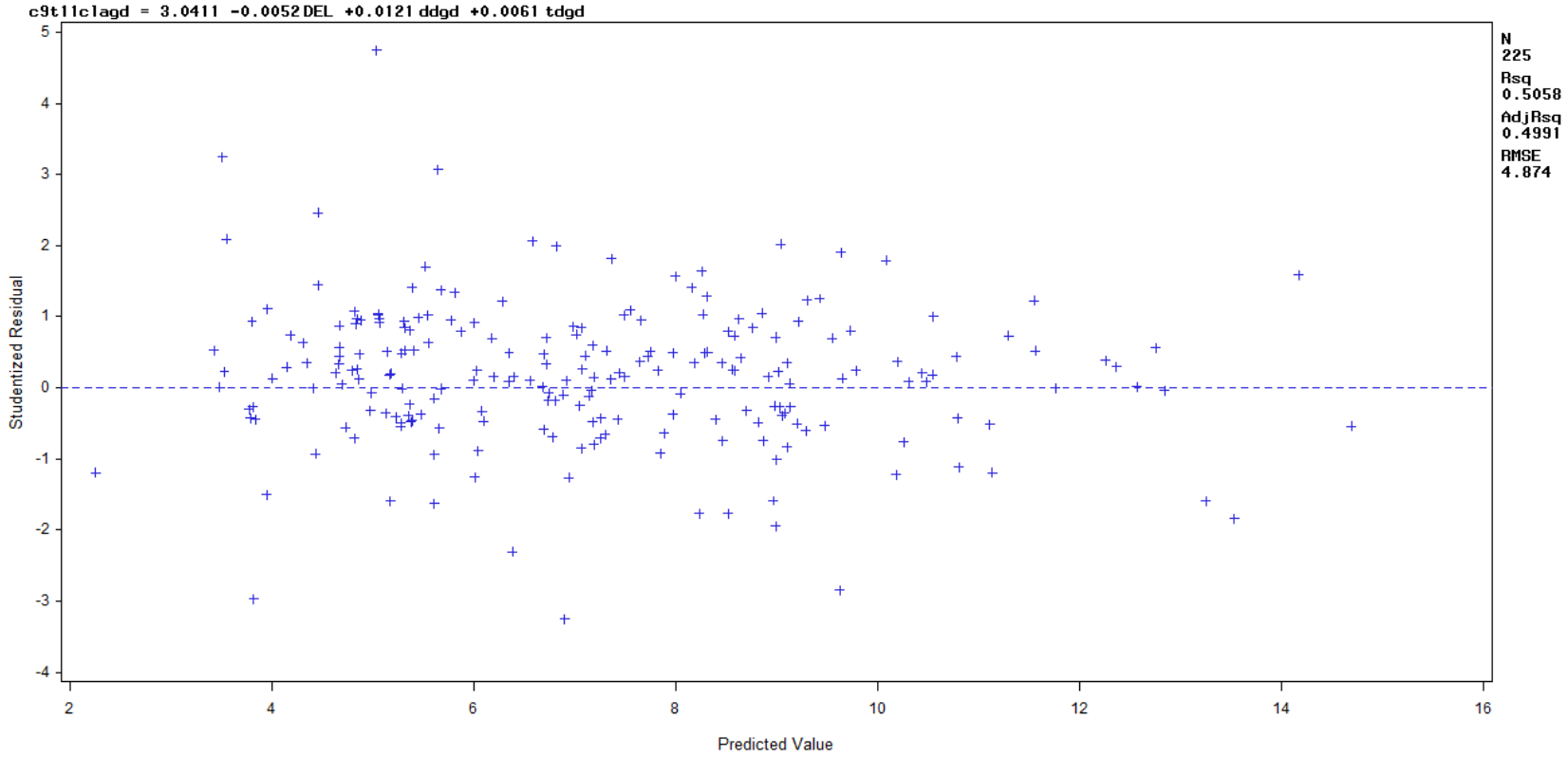

Figure 1. *Relationship between student residuals of precursors C18: 2 and C18: 3 (g/d) in the diet and, DIM and the cis-9, trans-11 CLA in milk (g/d). Equation: cis-9, trans-11 CLA (g/d) = 3.0411 – (0.0052 * DIM) + (0.0121 * (linoleic acid (g/d))) + (0.0061 * (linoleic acid (g/d))).*

3.3.2. Comparison of averages of DMI, milk yield and composition, and CLA in milk by dietary ingredient used

Averages of DMI, milk yield and composition, and content of *cis*-9, *trans*-11 CLA in milk fat in percentage of total FA, and grams per day is presented in Table 3. DMI was lower in SOY compared with CTR and LIN. Milk yield was lower in PAS compared with the other groups except SUN, and lower in SUN and LIN compared with CO. Milk yield in SOY was higher compared with SUN and LIN, and lower in LIN compared with CTR. Fat content was lower in SOY, SUN, FO and FO+PO compared with LIN, CTR and PAS. Fat yield was lower in SUN compared with CTR, LIN and CO, lower in SOY compared with CTR, and lower in PAS compared with CTR and LIN. *Cis*-9, *trans*-11 CLA content was higher in FO+PO compared with CTR, RA, CO, LIN and FO, lower in CTR compared with the others groups except CO and FO, and lower in FO compared with SUN, SOY and LIN. *Cis*-9, *trans*-11 CLA yield was higher in FO+PO compared with CTR, RA, LIN and FO, and lower in CTR and FO compared with the other groups except PAS.

Table 3. *Averages of DMI, milk yield, fat content, fat yield, cis-9, trans-11 CLA content and cis-9, trans-11 CLA yield in milk fat by dietary source used.*

	CTR[a]	PAS[a]	RA[a]	CO[a]	SOY[a]	SUN[a]	LIN[a]	FO[a]	FO + PO[a]	SEM[b]	*P*-Value
DMI, Kg/d	21.3	19.0	21.2	21.8	20.4	19.6	20.8	21.0	20.9	0.51	< 0.01
Milk yield, Kg/d	30.1^{ab}	27.0^{c}	29.6^{ab}	32.4^{a}	30.6^{ab}	28.8^{bc}	29.2^{b}	31.0^{ab}	31.4^{ab}	0.87	< 0.01
Fat, %	3.61^{a}	3.80^{a}	3.42^{ab}	3.44^{ab}	3.32^{b}	3.36^{b}	3.65^{a}	3.22^{b}	3.12^{b}	0.063	< 0.01
Fat yield, Kg/d	1.11^{a}	0.94^{ab}	1.04^{ab}	1.13^{a}	1.02^{ab}	0.94^{b}	1.07^{a}	1.03^{ab}	1.01^{ab}	0.039	< 0.01
Cis-9, *trans*-11 CLA, g/100 g FA	0.61^{c}	1.13^{ab}	0.83^{bc}	0.84^{bc}	1.00^{ab}	1.04^{ab}	0.90^{b}	0.67^{bc}	1.34^{a}	0.044	< 0.01
Cis-9, *trans*-11 CLA, g/d	5.67^{c}	8.56^{abc}	7.78^{bc}	8.75^{ab}	9.24^{ab}	10.1^{ab}	8.50^{bc}	5.91^{c}	12.4^{a}	0.399	< 0.01

[a] CTR = Control; PAS = Feeding fresh pasture; RA = Diets enriched with rapeseed; CO = Diets enriched with corn products; SOY = Diets enriched with soybean; SUN = Diets enriched with sunflower; LIN = Diets enriched with linseed; FO = Diets enriched with fish oils; FO + PO = Diets enriched with fish oils plus plant oils.

[b] Standard error of CTR group.

[a-c] Means within a row differ with treatment due to pH ($P < 0.05$).

The following additional pairs were significantly different: for variable DMI: CTR/SOY and LIN/SOY; for variable milk yield: SOY/LIN, SOY/SUN, CTR/LIN; for variable fat yield: CTR/SOY, CTR/PAS, LIN/PAS; for variable *cis*-9, *trans*-11 CLA (g/100 g FA): SUN/FO, SOY/FO, LIN/FO, CTR/RA; for variable *cis*-9, *trans*-11 CLA (g/d): LIN/FO, CTR/LIN, RA/FO, CTR/RA.

3.3.3. Comparison of averages of DMI, milk yield and composition, and CLA in milk by main source of FA

Averages of DMI, milk yield and composition, and content of *cis*-9, *trans*-11 CLA in milk fat in percentage of total FA, and grams per day is presented in Table 4. DMI was lower in diets rich in linoleic acid compared with CTR and diets rich in linolenic acid. Milk yield was lower in diets rich in linolenic acid compared with CTR and diets rich in linoleic acid. Fat content was lower in diets rich in linoleic acid compared with CTR and diets rich in linolenic acid. Fat yield was lower in diets rich in linoleic acid compared with CTR. *Cis*-9, *trans*-11 CLA content and yield was higher in diets rich in linoleic acid compared with other diets, and lower in CTR compared with other diets.

Table 4. *Averages of DMI, milk yield, fat content, fat yield, cis-9, trans-11 CLA content and cis-9, trans-11 CLA yield in milk fat by principal source of FA.*

	Control	Oleic acid	Linoleic acid	Linolenic acid	SEM[a]	*P*-Value
DMI, Kg/d	21.1[ab]	21.0[abc]	20.2[c]	20.7[b]	0.55	< 0.01
Milk yield, Kg/d	30.0[a]	30.1[ab]	30.1[a]	28.8[b]	0.92	< 0.01
Fat, %	3.63[a]	3.44[ab]	3.39[b]	3.68[a]	0.064	< 0.01
Fat yield, Kg/d	1.11[a]	1.06[ab]	1.02[b]	1.05[ab]	0.040	< 0.01
Cis-9, *trans*-11 CLA, g/100 g FA	0.62[c]	0.82[b]	1.00[a]	0.83[b]	0.044	< 0.01
Cis-9, *trans*-11 CLA, g/d	5.79[c]	7.82[b]	9.66[a]	7.54[b]	0.390	< 0.01

[a] Standard error of Control group.
[a-c] Means within a row differ with treatment due to pH ($P < 0.05$).

3.3.4. Comparison of averages of DMI, milk yield and composition, and CLA in milk by the method of presentation of plant oils

Averages of DMI, milk yield and composition, and content of *cis*-9, *trans*-11 CLA in milk fat in percentage of total FA, and grams per day is presented in Table 5. DMI was lower in OIL compared with RS and CTR, and lower in PS compared with RS. Milk yield was lower in RS and EXT compared with CTR and PS. Fat content was lower in OIL compared with CTR, RS and PS. Fat yield was lower in OIL compared with CTR. *Cis*-9, *trans*-11 CLA content was higher in PS and RS compared with CTR, and higher in EXT compared with CTR and PS, and higher in OIL

compared with CTR, RS and PS. *Cis*-9, *trans*-11 CLA yield was lower in CTR compared with other groups.

The SEM reported in Table 3, 4 and 5 were of the CTR groups and it was used as the reference value.

Table 5. *Averages of DMI, milk yield, fat content, fat yield, cis-9, trans-11 CLA content and cis-9, trans-11 CLA yield in milk fat by method of presentation of plant oils.*

	CTR[1]	RS[1]	PS[1]	EXT[1]	OIL[1]	SEM[2]	*P*-value
DMI, Kg/d	21.3	21.4	20.9	19.9	20.2	0.54	< 0.01
Milk yield, Kg/d	30.4[a]	29.3[b]	30.4[a]	28.9[b]	30.3[ab]	0.92	< 0.01
Fat, %	3.61[a]	3.65[a]	3.57[a]	3.43[ab]	3.31[b]	0.067	< 0.01
Fat yield, Kg/d	1.11	1.11	1.07	1.00	1.02	0.041	< 0.01
Cis-9, *trans*-11 CLA, g/100 g FA	0.57[c]	0.84[ab]	0.77[b]	1.11[a]	1.10[a]	0.041	< 0.01
Cis-9, *trans*-11 CLA, g/d	5.67[b]	8.52[a]	7.59[a]	9.75[a]	8.82[a]	0.399	< 0.01

[1] CTR = Control; RS = Raw supplements; PS = Processed seeds; EXT = Extruded seeds; OIL = Oils.
[2] Standard error of CTR group.
[a-c] Means within a row differ with treatment due to pH ($P < 0.05$).
The following additional pairs were significantly different: for variable DMI: RS/PS, RS/OIL, CTR/OIL; for variable fat yield: CTR/OIL; for variable *cis*-9, *trans*-11 CLA (g/100g FA): OIL/RS.

3.3.5. Principal components analysis

Pearson's correlation coefficients are presented in Table 6. Results of principal component analysis are presented in Table 7. The first PC accounted for 43% the total variance. All variables except *cis*-9, *trans*-11 CLA (g/d) and fat (%), correlated with PC1, although *cis*-9, *trans*-11 CLA (%) was not very strong. PC2 accounted for 31% the total variance, and the variables correlated were predominately *cis*-9, *trans*-11 CLA in % and g/d. No significance results of cluster analysis and frequency of groups of treatments in each cluster were obtained.

Table 6. *Correlations among variables.*

Variable	DMI (kg/d)	Milk yield (kg/d)	Fat (%)	Fat yield (kg/d)	c9t11CLA (%)
Milk yield (kg/l)	0.797				
Fat (%)	-0.114	-0.21			
Fat yield (kg/d)	0.593	0.726	0.331		
c9t11 CLA(%)	-0.273	-0.252	-0.258	-0.349	
c9t11 CLA (g/d)	0.072	0.137	-0.056	0.156	0.836

Table 7. *Results of Eigenanalysis – The Principal Components.*

	PC1	PC2	PC3	PC4	PC5
Eigenvalue	2.57	1.85	1.16	0.29	0.09
Proportion	0.43	0.31	0.19	0.05	0.02
Cumulative	0.43	0.74	0.93	0.98	0.99
Variable					
DMI, Kg/d	0.54	0.16	-0.17	0.78	-0.24
Milk, Kg/d	0.56	0.23	-0.18	-0.25	0.73
Fat, %	0.05	-0.30	0.82	0.31	0.36
Fat, Kg/d	0.54	0.06	0.37	-0.47	-0.53
Cis-9, *trans*-11 CLA, %	-0.33	0.61	0.10	0.11	0.05
Cis-9, *trans*-11 CLA, g/d	-0.04	0.67	0.34	-0.02	-0.02

4. DISCUSSION

4.1. **Daily human recommendations and average current consumption of CLA**

Daily human recommendations are not well established. Most current recommendations are derived from animals studies, and recommendations, vary depending on the beneficial effect expected and the experimental animal model used, ranging from 0.6 to 3.2 g/d. In all cases, the extrapolation of animal doses into humans based on body weight or metabolic body weight should be interpreted with the outmost caution.

Data on human research is inconsistent. In epidemiological studies on the effect of CLA in preventing cancer, mean daily intake in all subjects (cases and controls) ranged from 134 to 200 mg/d (McCann *et al.,* 2004; Voorrips *et al.*, 2002), all under current recommended CLA intake in treatment group, which may explain the lack of effects observed in these studies. For reducing fat mass and the maintenance of normal body weight, the recommended dose of CLA (3.2 g/d) is far from current average intake. Atherosclerotic effects appear to require a lower dose (0.6 g/d), but it is still well above current intakes. Therefore, there is no conclusive positive effect or recommended dose that can be suggested from human studies. Therefore, as most commonly used value is extrapolated from animal studies where CLA was used at therapeutic doses, those should be interpreting with caution. However, if these recommendations are trusted, the average current human intake of *cis*-9, *trans*-11 CLA (0.21 g/d) is well below current daily human recommendation, and although values can vary

depending on the method chosen to estimate the average intake and the reference country it has served as the basis for the development of a strong interest and a large body research on the development of CLA-enriched foods.

4.2. **Effect of feeding strategies on CLA concentration in milk from dairy cows**

Interpreting results from animal studies may be tricky, because a change in profile may not correspond necessarily with a change in daily supply. Therefore, averages of CLA have been calculated in different units (percentage of total FA and total supply in g/d) for several reasons. When results are expressed as percentage of total FA, the variation indicates a change in FA profile. However, if fat or milk yield are decreased, then total production by cows or intake by humans would not necessarily occur. On the other hand, CLA expressed as grams per day or grams of CLA per liter allows to evaluate the contribution of CLA-enriched milk to human intake and its contribution to daily recommendations.

CLA in milk is related to the availability of its precursors (oleic acid, linoleic acid and linolenic acid) from ruminal and endogenous synthesis (Bauman *et al.,* 2003; Griinari and Bauman, 1999). The relationship observed in this study had an R^2 = 0.51 and was only related to linoleic acid and linolenic acid. Therefore, there must be other factors which influence the production of CLA in milk, as individual variation (Peterson *et al.,* 2002) or changes in rumen microbial populations due to the increase in polyunsaturated FA (Jenkins, 1993; Bauman and Griinari, 2001; Bauman *et al.,* 2003), among others.

Diets rich in linolenic decreased milk yield, but fat content and fat yield was not affected. In contrast, diets rich in linoleic acid did not affect milk yield but decreased fat content and fat yield. Fat content was also decreased in diets supplemented with fish oils. All strategies studied increased content and yield of CLA except diets supplemented with fish oils. Moreover, diets enriched with linoleic acid and FO + PO were more effective that diets enriched with oleic or linolenic acid. Other studies reported that diets rich in linoleic acid were more effective in increasing milk CLA than diets rich in oleic or linolenic acid (Kelly *et al.,* 1998; Dhiman *et al.,* 2000). Other studies observed that the combination of FO + PO increased the CLA in milk more than diets supplemented only with PO (Whitlock *et al.,* 2002; AbuGhazaleh *et al.,* 2003)

Plant oils can be supplemented as free oils, protected oils, raw seeds or processed seeds. When data were grouped according to the method of presentation results indicated

that all method increased CLA. The concentration of CLA increased more when plant oils were presented as extruded seeds or oils. To achieve an increase in CLA concentration in the rumen, oils must be available to the microorganisms responsible for ruminal biohydrogenacion (Stanton *et al.,* 2003). Therefore, is logical that free oils and processed seeds will produce a greater increase of CLA. Raw and extruded seeds decreased milk yield but fat content and yield were not affected. In contrast, oils decreased fat content and fat yield but milk yield was not affected. When results were expressed in grams per day of CLA all methods of presentation resulted in an increase in CLA without differences among groups.

In summary, the best strategies to produced milk enriched with CLA were diets supplemented with plant oils rich in linoleic acid and diets supplemented with fish oils plus plant oils even though fat yield was decreased. Diets rich in linolenic acid were also a good strategy to enrich milk with CLA without affecting fat content, but milk yield decreased. Within plant oils, the best methods of presentation to enrich milk with CLA were extruded seeds or oils, although extruded seeds decreased milk yield and oils decreased fat yield. Results of the component principal analysis indicated that there was no variable that could be correlated with CLA production.

Finally, the last objective of this research was to calculate the improvement in human intake of CLA if all milk and milk products were consumed in a CLA enriched form. Based on results of the meta-analysis, dairy cows fed unsupplemented diets produce 5.7 g/d of CLA and 30.1 kg of milk/d. Normal milk contains an average of 188 mg of *cis*-9, *trans*-11 CLA/l and dairy cows fed a diet enriched with FO+PO (best strategy to increase CLA) produce 12.4 g/d of CLA and 31.4 kg of milk/d. Therefore, a CLA enriched milk contains an average of 395 mg of *cis*-9, *trans*-11 CLA/l, which means an increase of 2.1 times the content of CLA. If we apply this increase to the average human intake found in this study, current intake would increase from 0.21 g/d to 0.44 g/d of CLA, and the average of effective CLA (ingested plus endogenous) would be 0.62 g/d of CLA.

Although the values used for this calculation could vary slightly depending on the source of data, the intention is to reflect that the use of CLA-enriched dairy products in the human diet would be insufficient to achieve the recommended levels of 0.6 to 3.2 g/d reported in literature and, therefore, either the recommendations are overestimated or other

supplementation alternatives are needed. It should be also interpreted, however, that the current recommendations were established with a therapeutic objective, and intake under normal conditions are most likely lower than the therapeutic dose.

5. CONCLUSIONS

The CLA has the potential to protect against atherosclerosis, cancer, diabetes mellitus type II, to regulate the immune function and to modulate body composition, but most of these effects have been only demonstrated in animal models at therapeutic doses. From this data, doses ranging from 0.6 to 3.2 g CLA/d have been suggested. These doses are well above current average intake (0.21 g/d). Feeding polyunsaturated fatty acids and fish oils to dairy cattle has been successful in consistently increasing the content of CLA in milk being fish oils plus plant oils the best strategy increasing the CLA content in milk by 2.1 times. If all dairy products were consumed in a CLA-enriched form, current average intake would increase from 0.21 to 0.44 g/d, suggesting that recommended doses are unattainable even if all milk and milk products were consumed as CLA enriched products. Therefore, either recommendations are overestimated or other sources of CLA need to be included in human diets. The recommendations for healthy individuals have not been determined.

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7. ANNEX 1: REFERENCES OF META-ANALYSIS

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CAPÍTULO 4

Strategies to modify the biohydrogenation pathways of polyunsaturated fatty acids in the rumen

Strategies to modify the biohydrogenation pathways of polyunsaturated fatty acids in the rumen

Abstract

Two experiments were conducted to determine the effects of lipases and essential oils on rumen fermentation and apparent biohydrogenation of linoleic (LA) and linolenic (LNA) acids. In experiment 1, a 50:50 forage:concentrate diet containing linseed oil (8.3% of DM) was incubated in a batch culture of rumen fluid at 2 pH levels (6.4 and 5.6) in 2 replicated periods. Treatments were: control; lipase 1 and 2 (0.4 and 4 µl/g DM); a lipase inhibitor (0.4 and 2 mg/g DM); Oxy-propyl-thiosulfate (PTSO; 60 and 120 mg/l); Eugenol (EUG; 150 and 500 ml/l) and Cinnamaldehyde (CIN; 150 and 500 ml/l). Samples were collected to analyze ammonia-N, volatile fatty acids (VFA) and the fatty acid (FA) profile. In experiment 2, 8 continuous culture fermenters (1,320 ml) were used in 3 replicated periods (5 d of adaptation and 3 d of sampling). Fermenters were fed 95 g/d of DM of a 60:40 forage:concentrate diet containing 5% DM of linseed oil. Treatments were control, lipase 1 (4 µl/l), PTSO (90 mg/l) and CIN (250 mg/l), and 2 pH levels (6.4 and 5.6). During the last 3 d of each period, samples were taken to analyze VFA, ammonia-N and the FA profile. The low pH inhibited the apparent biohydrogenation of LA and LNA, and decreased total VFA in the two experiments. The low pH also increased the isomers responsible for milk fat depression in experiment 1 after two hours of incubation (*trans*-10, *cis*-12 CLA) and in experiment 2 (*trans*-10 C18:1). In experiment 1, Lipase 1 increased the apparent biohydrogenation of LNA and reduced the efficiency of intermediary steps of biohydrogenation of LA and LNA, but these results were not observed in experiment 2. The PTSO inhibited the apparent biohydrogenation of LA and LNA and decreased total VFA concentrations in the two experiments. Although some effects were observed in the short-term fermentation, those observed in the long-term fermentation were not relevant.

Keywords: milk fat depression, polyunsaturated fatty acid, ruminal biohydrogenation.

Abbreviations: FA, fatty acids; LA, linoleic acid; LNA, linolenic acid; CLA, conjugated linoleic acid; PUFA, polyunsaturated fatty acids; EO, essential oils; VFA, volatile fatty acids; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE,

ether extract; CTR, control; LIP1, lipase 1; LIP2, lipase 2; INL, lipase inhibitor; PTSO, Oxy-propyl-thiosulphate; EUG, eugenol; CIN, cinnamaldehyde; OM, organic matter; BCVFA, branched-chain volatile fatty acid.

1. INTRODUCTION

There is comercial interest in producing milk with a healthier fatty acids (FA) profile. One strategy for this objective is to supplement the diet of dairy cows with plant oils rich in linoleic (LA) and linolenic (LNA) acids that will increase the polyunsaturated fatty acids (PUFA) supply in the diet. The addition of plant oils rich in LA and LNA in the diet of ruminants results in the production of vaccenic acid (*trans*-11 C18:1) in the rumen and its subsequent desaturation to *cis*-9, *trans*-11 conjugated linoleic acid (CLA) in the mammary gland by the delta-9 desaturase enzyme (Griinari and Bauman, 1999). However, in certain dietary conditions, the biohydrogenation pathways are altered and FA intermediaries are produced, of which some are potent inhibitors of milk fat synthesis (Bauman and Griinari, 2001). This depression of milk fat has been linked primarily to the increase in *trans*-10 C18: 1 in the rumen, and *trans*-10, *cis*-12 CLA in the rumen and the mammary gland. This reduction in milk fat has important economic implications in dairy farms and in the planning and marketing of milk enriched with PUFA omega-3 or CLA.

The metabolism of lipids in the rumen consists of two processes: lipolysis and biohydrogenation of FA. Several factors can affect the rate of lipolysis and biohydrogenation such as diets rich in concentrate, diets supplemented with unprotected lipids, low ruminal pH, ionophores and when fish oils are added, among others (Van Nevel and Demeyer, 1996; Demeyer and Doreau, 1999; Beam *et al.,* 2000; Fuentes *et al.,* 2011, 2009). Some studies reported that inhibiting lipolysis in the rumen resulted in higher amount of PUFA available in the intestine (Van Nevel and Demeyer, 1996; Krueger *et al.,* 2009). Alternatively, increasing lipolysis increased concentrations of PUFA in the rumen, that could inhibited the biohydrogenation process due to toxic effects on bacteria in the rumen, resulting in higher production of *trans*-11 C18:1 and *cis*-9, *trans*-11 CLA (Lourenço *et al.,* 2010).

Essential oils (EO) have been shown to affect ruminal fermentation, changing volatile fatty acids (VFA) production, protein degradation, or both (Calsamiglia *et al.,* 2007). However, the effect of EO on ruminal biohydrogenation has been less studied. Lourenço *et al.* (2009) reported that EO rich in monoterpenes such as limonene and carvone resulted in the ruminal

accumulation of *cis*-9, *trans*-11 CLA, indicating the potential of plant secondary metabolites to affect ruminal biohydrogenation. Durmic *et al.* (2008) reported the ability of some plants extracts and EO to inhibit the growth and/or activity of important ruminal biohydrogenating bacteria such as *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus.* Gunal *et al.* (2013) observed a reduction in concentrations of C18:0 and *trans* C18:1 FA with some EO (Siberian fir needle oil, citronella oil, rosemary oil, sage oil) suggesting a shift in the biohydrogenation pathways. Ramos-Morales *et al.* (2013) also observed changes in the FA profile when diets supplemented with diallyl disulfide and propyl propane thiosulfinate, two soluble organosulfur compounds derived from garlic EO. These studies suggest the potential of some EO to modify lipolysis and biohydrogenation in the rumen. In contrast, Benchaar *et al.* (2006, 2007) reported no effects of EO on milk FA profile.

The objective of this study was to evaluate *in vitro* effect of several nutritional strategies to increase PUFA outflow and decrease ruminal outflow of isomers that cause milk fat depression in dairy cows. These strategies include: 1) the inhibition of ruminal lipolysis; 2) the increase in lipolysis activity to increase free PUFA in the rumen that may saturate the biohydrogenation pathways in the rumen; 3) the reduction in the microbial activity in the last step of the biohydrogenation (saturation *trans*-11 C18: 1 to C18:0); and 4) the inhibition of the alternative pathway of the biohydrogenation that produces *trans*-10 C18:1.

2. MATERIAL AND METHODS

2.1. **Experiment 1**

2.1.1. Diet and Treatments

The effects of several commercial lipases and EO on apparent biohydrogenation of LA (C18:2n-6) and LNA (C18:3n-3) and rumen fermentation were evaluated by *in vitro* incubations (Tilley and Terry, 1963) at two pH levels (6.4 and 5.6) in two replicated periods. A 50:50 forage:concentrate diet containing corn grain (29.3%), soybean (15.6%), dehydrated alfalfa (18.3%), corn silage (27.5%), linseed oil (8.4%) and a vitamin and mineral mixture (0.92%) was used. The vitamin and mineral mixture contained, per Kg of DM: 7 mg Co, 167 mg Cu, 33 mg I,

2,660 mg Mn, 27 mg Se, 4,660 mg Zn, 1,000 KIU of vitamin A, 200 KIU of vitamin D3, 1,330 mg of vitamin E, 267 g of urea, 67 g of NaCl, 33 g of sulphur and 300 g of MgO. The diet was formulated to meet or exceed current nutrient recommendations for lactating dairy cows (NRC, 2001). The chemical composition (DM basis) of the diet was: 13.7% CP, 29.0% NDF, 15.6% ADF, and 11.1% EE. The FA profile of the diet (g/100 g of total FA) was: 8.07% C16:0, 3.46% C18:0, 9.86% *cis*-9 C18:1, 0.73% *cis*-11 C18:1, 24.0% *cis*-9, *cis*-12 C18:2 (LA), and 42.6% *cis*-9, *cis*-12, *cis*-15 C18:3 (LNA).

Treatments were control (CTR); lipase 1 (LIP1) and 2 (LIP2) at 0.4 (LIP1L and LIP2L, respectively) and 4 (LIP1H and LIP2H, respectively) µl/g DM; lipase inhibitor (INL) at 0.4 (INLL) and 2 (INLH) mg/g DM; Oxy-propyl-thiosulphate (PTSO) at 60 (PTSOL) and 120 (PTSOH) mg/l; Eugenol (EUG) and Cinnamaldehyde (CIN) at 150 (EUGL and CINL, respectively) and 500 mg/l (EUGH and CINH, respectively). Treatments were tested in triplicate at each dose and pH level. Incubations were conducted using rumen fluid from 2 fistulated, lactating dairy cows fed a 60:40 forage:concentrate diet. Rumen fluid was strained through 2 layers of cheesecloth, and mixed in 1:1 proportion with phosphate-bicarbonate buffer (McDougall, 1948). The incubation was conducted in 90 ml tubes containing 50 ml of diluted fluid and 0.5 g of diet per tube. Each tube was gassed with CO_2 before sealing with rubber corks fitted with a gas release valve. Treatments PTSO, EUG and CIN were dissolved in ethanol, and a total of 0.4 ml were added to the culture fluid. Treatments with lipase, lipase inhibitor and control were also supplied with 0.4 ml of ethanol. Incubations were conducted in a water bath at 39ºC. After 2h (lipases and inhibitor lipase) or 6h (EO) incubations, pH was determinate, and samples were collected in order to analyze ammonia N and VFA. The remainder of the culture fluid was lyophilized to analyze the FA profile. Incubation times were selected based on literature. Moate *et al.* (2008) and Lee *et al.* (2008) showed a maximun lipolysis activity at 2 to 6 h of incubation. Biohydrogenation of PUFA was almost complete at 8 h of incubation (Boufäied *et al.,* 2003; Akraim *et al.,* 2006; Enjalbert *et al.,* 2003).

2.1.2. Chemical Analyses

Samples for VFA analysis were prepared as described by Jouany (1982) with modifications. A solution (1 ml) containing 2 g/l of mercuric chloride, 2 g/l of 4-methylvaleric acid as an internal standard, and 20 g/l orthophosphoric acid, was added to 4 ml of filtered fermentation fluid and frozen. Samples were centrifugated at 15,000 x *g* for 15 min, and the supernatant was analyzed by gas chromatography (model 6890, Hewlett Packard, Palo Alto,

CA) using a polyethylene glycol nitroterephthalic acid-treated capillary column (BP21, SGE, Europe Ltd., Buckinghamshire, UK). Ammonia N was analyzed by colorimetry as described by Chaney and Marbach (1962), where 4 ml of filtered fermentation fluid sample was acidified with 4 ml of 0.2 *N* HCl, and frozen. Samples were centrifugated at 15,000 x *g* for 15 min, and the supernatant was used to determinate ammonia N by spectrophotometry (Libra S21, Biochrom Technology, Cambridge, UK).

The FA profile was determined in 250 mg freeze dried sample. Transesterification was conducted as described Vlaeminck *et al.* (2014) by a base-catalyzed step followed by an acid-catalized step. Fatty acid methyl esters were extracted with hexane and analyzed by gas chromatography. Fatty acids analyses were carried out with a gas chromatograph (HP 7890A, Agilent Technologies, Diegem, Belgium) equipped with a 75-m SP-2560™ capillary column (i.d., 0.18 mm, film thickness, 0.14 µm; Supelco Analytical, Bellefonte, PA) and a flame ionization detector. A combination of two oven temperature programs was used in this study to achieve determination of most *cis* and *trans* C16:1 and C18:1 isomers according to the method of Kramer *et al.* (2008) with modifications. The first temperature program was conducted as described Vlaeminck *et al.* (2014). The second temperature program was used to separate most of the coeluting isomers: at the time of sample injection, the column temperature was 70°C, then ramped up at 50°C/min to 175°C, and maintained isothermal for 13 min, followed by a second increase at 5°C/min to 215°C, and maintained for 10 min. For both programs, inlet and detector temperatures were 250°C and 255°C, respectively. The split ratio was 100:1. The flow rate for hydrogen carrier gas was 1 ml/min. Most FA peaks were identified using quantitative mixtures of methyl ester standards (BR2 and BR3, Larodan Fine Chemicals, Malmö, Sweden; Supelco® 37, Supelco Analytical, Bellefonte, PA; PUFA-3, Matreya LLC, Pleasant Gap, PA). Fatty acids for which no standards were available commercially were identified by order of elution according to Precht *et al.* (2001) and Kramer *et al.* (2008).

2.1.3. Calculations and Statistical Analyses

Apparent biohydrogenation of LA and LNA was calculed as: $(FA_I - FA_E)/FA_I$, where FA_I is the intake of either LA or LNA and FA_E is LA or LNA in the effluent (Fuentes *et al.,* 2011).

Efficiency of biohydrogenation steps was calculed as:

1-. First step of apparent biohydrogenation of LA (*cis*-9, *cis*-12 C18:2 to *cis*-9, *trans*-11 C18:2 + *trans*-10, *cis*-12 C18:2):

$100 - (100*((\ cis\text{-}9, trans\text{-}11\ C18{:}2_E + trans\text{-}10, cis\text{-}12\ C18{:}2_E) / (LA_I - LA_E)))$

2-. Second step of apparent biohydrogenation of LNA (*cis*-9, *cis*-12, *cis*-15 C18:3 to *trans*-11, *cis*-15 C18:2):

$100 - (100*(trans\text{-}11, cis\text{-}15\ C18{:}2_E/(LNA_I - LNA_E)))$

3-. Second step of apparent biohydrogenation of LA plus third step of apparent biohydrogenation of LNA (*cis*-9, *trans*-11 C18:2 + *trans*-10, *cis*-12 C18:2 to *trans*-11 C18:1 + *trans*-10 C18:1 plus *trans*-11, *cis*-15 C18:2 to *trans*-11 C18:1 + *cis*-15 C18:1 + *trans*-15 C18:1):

$100 - ((100*((trans\text{-}10\ C18{:}1_E + trans\text{-}11\ C18{:}1_E + trans\text{-}15\ C18{:}1_E + cis\text{-}15\ C18{:}1_E) / ((LA_I + LNA_I) - LA_E - LNA_E - trans\text{-}11, cis\text{-}15\ C18{:}2_E - cis\text{-}9, trans\text{-}11\ C18{:}2_E - trans\text{-}10, cis\text{-}12\ C18{:}2_E)))$,

where nFA_I is the intake of nFA and nFA_E is nFA in the effluent.

Statistical analysis was performed using the GLIMMIX procedure and multiple comparisons was performed by LSMEANS adjusted with the Dunnett test using SAS (v. 9.2., SAS Institute, Cary, NC, USA). The model accounted for the effects treatment and pH, and the interaction of treatments with pH. The period was considered a random effect. The level of significance was determined at $P < 0.05$.

2.2. **Experiment 2**

2.2.1. Apparatus and experimental design

Eight 1,320 ml dual-flow continuous culture fermenters (Hoover *et al.,* 1976) were used in 3 replicated periods of 8 d (5 d of adaptation and 3 d for sampling) to study the effects of LIP1, PTSO and CIN on apparent biohydrogenation of LA and LNA acids. Fermenters were fed 95 g/d of DM of a 60:40 forage:concentrate diet formulated to meet or exceed current nutrient recommendations for lactating dairy cows (NRC, 2001). The diet (DM basis) consisted of corn silage (34.2%), soybean meal (16.3%), corn grain (18.3%), dehydrated alfalfa (21.4%), straw (4.28%), linseed oil (4.89%), and a vitamin and mineral mixture (0.64%). The vitamin and

mineral mixture was the same as in experiment 1. The chemical composition (DM basis) of the diet was: 16.3% CP, 31.2% NDF, 17.6% ADF, and 6.35% EE. The FA profile of the diet (g/100 g of total FA) was: 9.31% C16:0, 4.27% C18:0, 18.7% *cis*-9 C18:1, 0.84% *cis*-11 C18:1, 25.4% *cis*-9, *cis*-12 C18:2, and 39.5% *cis*-9, *cis*-12, *cis*-15 C18:3. The diet was fed every 8 h in 3 equal portions per day. Treatments were control (CTR), LIP1 (4 µl/l), PTSO (90 mg/l) and CIN (250 mg/l) at two pH levels (6.4 and 5.6). Treatments PTSO and CIN were dissolved in ethanol, and a total of 0.4 ml were added to the culture fluid. Fermenters with the CTR and LIP1 treatments were also supplied 0.4 ml ethanol. Treatments were divided in 3 fractions and dosed into fermenters 1 min before each feeding.

On the first day of each period, all fermenters were inoculated with undiluted ruminal fluid obtained from two ruminally fistulated dairy cows fed a 60:40 forage:concentrate diet. Fermentation conditions were maintained at a constant temperature of 38.5ºC and pH 6.4 ± 0.05 or 5.6 ± 0.05 depending on treatments and controlled by the infusion of 3N HCl or 5N NaOH, and monotorited by a computer and a Programmable Linear Controller (FieldPoint, National Instruments, Austin, TX). Anaerobic conditions were maintained by infusion of N_2 gas at a rate of 40 ml/min. Artificial saliva (Weller and Pilgrim, 1974) was continously infused into flasks and contained 0.4 g/l of urea to simulate recycled N. Infusion of saliva and flows of filtered liquid were set to maintain a liquid and solid dilution rates at 0.10 and 0.05 h^{-1}, respectively.

2.2.2. Sample collection

During the last 3 d of each period, 60 ml of solid and liquid effluents were collected for 30 min at 3 and 6 h after the morning feeding to analyze the FA profile and 8 ml of filtered fermenter fluid was collected at 3 h after the morning feeding to analyze the VFA and ammonia N concentrations.

During sampling days, effluent collection vessels were maintained at 4ºC to prevent microbial activity. Solid and liquid effluents were mixed and homogenized for 1 min at 24,000 rpm (Diax900, Heidolph, Nurnberg, Alemania), and 500 ml sample was removed by aspiration and frozen at -20ºC. Upon completation of each period, effluents of the 3 sampling days were composited and mixed within fermenter, and homogenized for 1 min. Subsamples were

collected for determination of total N, ammonia N and VFA. The remainder of the samples was lyophilized. Dry samples were analyzed for DM, ash, NDF, ADF, FA profile and purine contents.

Bacterial cells were obtained from fermenter flasks on the last day of each experimental period. Solid and liquid associated bacteria were isolated using a combination of several detachment procedures (Whitehouse *et al.,* 1994) selected to obtain the maximum detachment without affecting cell integrity. One hundred ml of 2 g/l methylcellulose solution and small marbles (30 of 2 mm and 15 of 4 mm diameter) were added to each fermenter and incubated in the same fermenters flasks at 39ºC, and mixed for 1 h to remove attached bacteria. After incubation, fermenters flasks were refrigerated for 24 h at 4ºC and then agitated for 1 h to dislodge loosely attached bacteria. Finally, fermenter contents were filtered through 4 layers of cheesecloth, and washed with saline solution (8.5 g/l Na Cl). Bacterial cells were isolated within 4 h by differential centrifugation at 1000 x *g* for 10 min to separate feed particles, and the supernatant was centrifuged at 20,000 x *g* for 20 min to isolate bacterial cells. Pellets were rinsed twice with saline solution and recentrifuged at 20,000 x *g* for 20 min. The final pellet was recovered with destilled water to prevent contamination of bacteria with ash. Bacterial cells were lyophilized and analyzed for DM, ash, N and purine contents. Digestion of DM, OM, NDF, ADF and CP, and flows of total, nonammonia, bacterial, and dietary N were calculated as described by Stern and Hoover (1990).

2.2.3. Chemical Analyses

Samples for VFA and ammonia N were analyzed as described in experiment 1. Transesterification was conducted as described in experiment 1. Fatty acid methyl esters were extracted with hexane and analyzed by gas chromatography as described in experiment 1 with slight modifications. Composition analyses of FA were carried out with a gas chromatograph (6890, Agilent Technologies, Hewlett Packard, Palo Alto, CA) equipped with a CP-Sil-88 column (100 x 0.25 mm i.d. x 0.2 µm; Varian Inc., Mississauga, ON). The temperature program was: 70ºC for 1 min, followed by an increase of 1ºC/min to 225 ºC, maintained for 15 min. Inlet and detector temperatures were 250°C and 255°C, respectively. The split ratio was 100:1 for ruminal fluid samples and 50:1 for diet samples. The flow rate for hydrogen carrier gas was 40 ml/min. Fatty acid peaks were identified by comparing retention times with those of the corresponding standards (Supelco 37 Component FAME Mix, *cis*-11-Octadecenoic methyl ester, *trans*-11-Octadecenoic methyl ester (Supelco Analytical, Bellefonte, PA); Linoleic acid, conjugated methyl ester (Sigma-aldrich, Sant Louis, MO).

Calculations were conducted as described in experiment 1 with slight modifications. In this experiment, FA peaks of *cis*-15 C18:1 and *trans*-15 C18:1 were not identified. Therefore, in the second step of apparent biohydrogenation of LA plus the third step of apparent biohydrogenation of LNA these isomers were omitted.

Statistical analysis was performed using the GLIMMIX procedure and multiple comparisons were performed by LSMEANS adjusted with the Tukey test using SAS (v. 9.2., SAS Institute, Cary, NC, USA). The model accounted for the effects of treatment, pH, and the interaction of treatment with pH. The period and fermenter were considered random effects. The level of significance was determined at $P < 0.05$.

3. RESULTS

3.1. **Experiment 1**

Effects of lipases and the lipase inhibitor at pH 6.4 on FA profile, apparent biohydrogenation and efficiency of the biohydrogenation steps are shown in Table 1. The C18:0 concentration was higher in treatments LIP1H, LIP2L, LIP2H and INLL compared with CTR at pH 6.4. The *trans*-10 C18:1 concentration was higher in treatment LIP1L compared with CTR at pH 6.4. The *trans*-11 C18:1; *trans*-11, *cis*-15 C18:2 and *cis*-9, *trans*-11 CLA concentrations were higher in treatments LIP1L and LIP1H compared with CTR at pH 6.4. The C18:3n-3 concentration was lower in treatments LIP1L, LIP1H, LIP2L and LIP2H compared with CTR at pH 6.4. The apparent biohydrogenation of LNA increased in treatments LIP1L, LIP1H, LIP2L and LIP2H compared with CTR at pH 6.4. The efficiency of the second step of biohydrogenation of LNA decreased in treatments LIP1L and LIP1H compared with CTR at pH 6.4. The efficiency of the second step of biohydrogenation of LA plus the third step of biohydrogenation of LNA decreased in treatments LIP1L and LIP1H compared with CTR at pH 6.4.

Table 1. *Experiment 1. Effect of lipases and a lipase inhibitor at pH 6.4 on fatty acid (FA) profile, apparent biohydrogenation and efficiency of the biohydrogenation steps.*

	Treatments[1]								
	CON	LIP1L	LIP1H	LIP2L	LIP2H	INLL	INLH	SEM[1]	*P*-Value
FA (g/100 g FA)									
C18:0	7.51	10.40	11.50*	11.80*	11.00*	11.40*	10.10	0.660	0.03
t10 C18:1	0.19	0.35*	0.34	0.24	0.25	0.13	0.13	0.033	0.01
t11 C18:1	1.49	4.84*	4.81*	2.38	2.18	1.46	1.28	0.573	0.01
t15 C18:1	0.03	0.08	0.11	0.12	0.12	0.07	0.04	0.037	0.45
c15 C18:1	0.11	0.23	0.24	0.18	0.16	0.10	0.12	0.039	0.17
t11,c15 C18:2	0.28	2.93*	2.76*	0.56	0.46	0.14	0.09	0.231	0.01
C18:2n-6	18.4	15.4	15.1	15.7	16.6	16.4	17.9	0.68	0.08
C18:3n3	33.9	23.3*	21.6*	28.2*	28.9*	30.3	29.4	0.93	0.01
c9, t11 CLA	0.27	0.70*	0.60*	0.25	0.23	0.20	0.19	0.063	0.01
t10, c12 CLA	0.02	0.06	0.06	0.02	0.02	0.00	0.01	0.015	0.10
Apparent biohydrogenation (%)									
C18:2n-6	23.2	36.0	37.0	34.8	30.9	31.7	25.5	2.82	0.08
C18:3n-3	20.4	45.2*	49.2*	33.8*	32.2*	28.8	30.9	2.22	0.01
Efficiency of biohydrogenation steps (%)									
1st C18:2[2]	94.5	91.3	92.3	96.8	96.5	97.3	96.7	1.02	0.02
2nd C18:3[3]	96.7	84.8*	86.8*	96.1	96.6	98.8	99.3	1.19	0.01
2nd C18:2 + 3rd 8:3[4]	86.8	77.3*	78.9*	86.5	86.7	90.7	91.6	2.60	0.01

[1] CON= Control diet; LIP1L = Lipase 1 at 0.4 µl/g DM; LIP1H = Lipase 1 at 4 µl/g DM; LIP2L = Lipase 2 at 0.4µl/g DM; LIP2H = Lipase 2 at 4 µl/g DM; INLL = Inhibitor lipase at 0.4 mg/g DM; INLH = Inhibitor lipase 2 mg/g DM; SEM = Standard error of the mean.

* Means within a row differ from control ($P < 0.05$).

[2] First step of apparent biohyodrogenation of LA: C18:2 c9c12 to C18:2 c9t11 + t10c12.

[3] Second step of apparent biohyodrogenation of LNA: C18:3 c9c12c15 to C18:2 t11c15.

[4] Second step of apparent biohydrogenation of LA plus third step of apparent biohydrogenation of LNA: C18:2 c9t11 + t10c12 to C18:1 t11 + t10 plus C18:2 t11c15 to C18:1 t11 + c15+t15.

Effects of lipases and the lipase inhibitor at pH 5.6 on FA profile, apparent biohydrogenation and efficiency of the biohydrogenation steps are shown in Table 2. The *trans*-15 C18:1 concentration was higher in treatment LIP1H compared with CTR at pH 5.6. The *trans*-11, *cis*-15 C18:2 and *cis*-9, *trans*-11 CLA concentrations were higher in treatment LIP1H compared with CTR at pH 5.6. The efficiency of the first step of LA was lower in treatments LIP1L and LIP1H compared with CTR at pH 5.6. The efficiency of second step of biohydrogenation of LNA was lower in treatment LIP1H compared with CTR at pH 5.6.

Table 2. *Experiment 1. Effect of lipases and lipase inhibitor at pH 5.6 on fatty acid (FA) profile, apparent biohydrogenation and efficiency of the biohydrogenation steps.*

	Treatments[1]								
	CON	LIP1L	LIP1H	LIP2L	LIP2H	INLL	INLH	SEM[1]	*P*-Value
FA (g/100 g FA)									
C18:0	8.43	9.10	10.0	10.9	9.6	9.7	9.89	0.65	0.36
t10 C18:1	0.20	0.21	0.24	0.24	0.22	0.19	0.18	0.018	0.10
t11 C18:1	0.87	1.40	1.65	1.28	1.20	0.96	0.95	0.302	0.22
t15 C18:1	0.02	0.02	0.07*	0.06	0.03	0.02	0.03	0.013	0.02
c15 C18:1	0.10	0.11	0.13	0.13	0.12	0.10	0.09	0.009	0.12
t11,c15 C18:2	0.11	1.02	1.25*	0.29	0.37	0.07	0.08	0.268	0.04
C18:2n-6	19.3	19.4	17.8	17.2	18.3	17.7	17.7	0.51	0.12
C18:3n3	32.7	28.6	28.3	29.3	30.3	32.2	31.7	1.06	0.12
c9, t11 CLA	0.08	0.19	0.26*	0.10	0.13	0.07	0.09	0.052	0.05
t10, c12 CLA	0.04	0.06	0.07	0.05	0.07	0.04	0.04	0.018	0.20
Apparent biohydrogenation (%)									
C18:2n-6	19.6	19.3	26.0	28.4	23.8	26.1	26.2	2.14	0.12
C18:3n-3	23.2	32.9	33.5	31.0	28.9	24.3	25.5	2.50	0.12
Efficiency of biohydrogenation steps (%)									
1st C18:2[2]	97.4	94.5*	94.7*	97.7	96.5	98.2	98.0	0.56	0.01
2nd C18:3[3]	98.9	92.8	91.3*	97.7	97.0	99.3	99.3	1.75	0.03
2nd C18:2 + 3rd C18:3[4]	91.9	90.0	89.0	91.1	90.9	92.2	92.5	1.75	0.16

[1] CON = Control diet; LIP1L = Lipase 1 at 0.4 µl/g DM; LIP1H = Lipase 1 at 4 µl/g DM; LIP2L = Lipase 2 at 0.4 µl/g DM; LIP2H = Lipase 2 at 4 µl/g DM; INLL = Inhibitor lipase at 0.4 mg/g DM; INLH = Inhibitor lipase 2 mg/g DM; SEM = Standard error of the mean.

* Means within a row differ from control ($P < 0.05$).

[2] First step of apparent biohyodrogenation of LA: C18:2 c9c12 to C18:2 c9t11 + t10c12.

[3] Second step of apparent biohyodrogenation of LNA: C18:3 c9c12c15 to C18:2 t11c15.

[4] Second step of apparent biohydrogenation of LA plus third step of apparent biohydrogenation of LNA: C18:2 c9t11 + t10c12 to C18:1 t1 + t10 plus C18:2 t11c15 to C18:1 t11 + c15 + t15.

The pH 5.6 reduced the C18:0, *trans*-11 C18:1, *trans*-15 C18:1, *cis*-15 C18:1, *trans*-11, *cis*-15 C18:2, *cis*-9, *trans*-11 C18:2 concentrations and increased the 18:2n-6, 18:3n-3 and *trans*-10, *cis*-12 CLA concentrations compared with pH 6.4. The pH 5.6 reduced the apparent biohydrogenation of LA and LNA compared with pH 6.4. The pH 5.6 increased the efficiency of the first step of biohydrogenation of LA, the efficiency of the second step of biohydrogenation of LNA and the efficiency of the second step of biohydrogenation of LA plus the third step of biohydrogenation of LNA compared with pH 6.4. There was an interaction between treatments and pH for *trans*-10 C18:1, *trans*-11 C18:1, *trans*-11, *cis*-15 C18:2, 18:3n-3, *cis*-9, *trans*-11 CLA concentrations, apparent biohydrogenation of LNA, efficiency of the second step of biohydrogenation of LNA, and the efficiency of the second step of biohydrogenation of LA plus the third step of biohydrogenation of LNA.

Effects of lipases and the lipase inhibitor at pH 6.4 on total and individual VFA concentrations and ammonia N concentration are shown in Table 3. Branched-chain VFA (BCVFA) concentration increased in treatments LIP1H, LIP2H and INLL compared with CTR at pH 6.4. Ammonia N concentration increased in treatment LIP1H, LIP2L and INLL compared with CTR at pH 6.4.

Table 3. *Experiment 1. Effect of lipases and lipase inhibitor at two pH levels on total and individual VFA concentrations and ammonia N.*

	Treatments[1]								
	CON	LIP1L	LIP1H	LIP2L	LIP2H	INLL	INLH	SEM[1]	*P*-Value
pH 6.4									
Total VFA (mM)	56.88	60.31	62.81	60.92	60.59	60.09	61.70	4.644	0.665
VFA, (mol/100 mol)									
Acetate	71.99	71.40	71.24	71.48	71.41	71.36	71.69	0.854	0.413
Propionate	17.64	17.52	17.39	17.24	17.39	17.22	17.24	0.310	0.213
BCVFA[2]	1.73	1.84	1.88*	1.86	1.87*	1.88*	1.84	0.077	0.073
NH_3 (mg/100 ml)	5.79	7.11	7.84*	7.34*	7.26	7.30*	7.08	0.878	0.047
pH 5.6									
Total VFA (mM)	61.1	58.3	58.9	61.0	58.2	58.2	60.1	2.51	0.94
VFA (mol/100 mol)									
Acetate	72.0	71.8	71.7	71.7	71.8	71.7	71.9	1.02	0.72
Propionate	17.2	17.1	17.1	16.9	17.0	17.0	16.9	0.30	0.65
BCVFA[2]	1.76	1.77	1.78	1.79	1.79	1.80*	1.80*	0.048	0.04
NH_3 (mg/100 ml)	6.26	6.97	6.57	7.09	6.33	6.44	6.52	0.579	0.32

[1] CON = Control diet; LIP1L = Lipase 1 at 0.4 µl/g DM; LIP1H = Lipase 1 at 4 µl/g DM; LIP2L = Lipase 2 at 0.4 µl/g DM; LIP2H = Lipase 2 at 4 µl/g DM; INLL = Inhibitor lipase at 0.4 mg/g DM; INLH = Inhibitor lipase 2 mg/g DM; SEM = Standard error of the mean.

* Means within a row differ from control ($P < 0.05$).

[2] BCVFA = Branched-chain VFA.

Effects of lipases and the lipase inhibitor at pH 5.6 on total and individual VFA concentrations and ammonia N concentration are shown in Table 3. The BCVFA concentration increased in treatments INLL and INLH compared with CTR at pH 5.6. The pH 5.6 increased acetate proportion and decreased propionate proportion, and BCVFA and ammonia N concentrations compared with pH 6.4. There were no interactions between diet and pH for total and individual VFA concentrations and ammonia N concentration.

Effects of EO at pH 6.4 on the FA profile, apparent biohydrogenation and efficiency of the biohydrogenation steps are shown in Table 4. The C18:0 concentration decreased in treatments PTSOL, PTSOH and CINH compared with CTR at pH 6.4. The *trans*-15 C18:1 and *cis*-15 C18:1 decreased in treatments PTSOL, PTSOH and CINH compared with CTR at pH 6.4. The C18:2n-6 concentration increased in treatments PTSOL and PTSOH compared with CTR at pH 6.4. The C18:3n-3 concentration increased in treatments PTSOL, PTSOH and CINH compared with CTR at pH 6.4. The *cis*-9, *trans*-11 CLA concentration decreased in treatments PTSOL and

PTSOH compared with CTR at pH 6.4. The apparent biohydrogenation of LA decreased in treatments PTSOL and PTSOH compared with CTR at pH 6.4. The apparent biohydrogenation of LNA decreased in treatments PTSOL, PTSOH and CINH compared with CTR at pH 6.4.

Table 4. *Experiment 1. Effect of essential oils at pH 6.4 on fatty acid (FA) profile, apparent biohydrogenation and efficiency of the biohydrogenation steps.*

	Treatments[1]								
	CON	PTSOL	PTSOH	EUGL	EUGH	CINL	CINH	SEM[1]	*P*-Value
FA (g/100 g FA)									
C18:0	18.8	9.68*	10.0*	16.2	15.6	15.9	11.3*	1.33	0.01
t10 C18:1	0.35	0.75	0.28	0.40	0.43	0.33	0.30	0.231	0.80
t11 C18:1	3.96	1.49	0.96	4.70	4.44	4.17	3.22	0.89	0.05
t15 C18:1	0.33	0.04*	0.02*	0.38	0.32	0.25	0.00*	0.070	0.01
c15 C18:1	0.26	0.10*	0.11*	0.29	0.25	0.23	0.12*	0.020	0.01
t11,c15 C18:2	0.82	0.38	0.11	1.24	1.14	0.90	0.77	0.234	0.08
C18:2n-6	12.2	17.3*	17.4*	12.8	12.8	12.9	13.9	1.00	0.01
C18:3n3	22.3	30.2*	31.0*	23.3	23.7	22.6	26.8*	1.27	0.01
c9, t11 CLA	0.29	0.10*	0.03*	0.44	0.36	0.37	0.28	0.033	0.01
t10, c12 CLA	0.02	0.55	0.77	0.02	0.01	0.03	0.02	0.154	0.05
Apparent biohydrogenation (%)									
C18:2n-6	49.2	28.0*	27.4*	46.7	46.9	46.3	42.3	4.16	0.01
C18:3n-3	47.5	29.1*	27.2*	45.2	44.2	47.0	36.9*	2.99	0.01
Efficiency of biohydrogenation steps (%)									
1st C18:2[2]	97.4	88.3	88.0	95.8	96.7	96.5	97.0	2.82	0.18
2nd C18:3[3]	95.9	97.1	99.0	93.6	93.8	95.4	94.8	1.61	0.29
2nd C18:2 + 3rd C18:3[4]	83.9	87.6	92.1	80.0	80.5	83.1	84.6	4.62	0.41

[1] CON = Control; PTSOL = Oxy-propyl-thiosulphate at 60 mg/l; PTSOH = Oxy-propyl-thiosulphate at 120 mg/l; EUGL = Eugenol at 150 mg/l; EUGH = Eugenol at 500 mg/l; CINL = Cinnamaldehyde at 150 mg/l; CINH = Cinnamaldehyde at 500 mg/l; SEM = Standard error of the mean.

* Means within a row differ from control ($P < 0.05$).

[2] First step of apparent biohyodrogenation of LA: C18:2 c9c12 to C18:2 c9t11+ t10c12.

[3] Second step of apparent biohyodrogenation of LNA: C18:3 c9c12c15 to C18:2 t11c15.

[4] Second step of apparent biohydrogenation of LA plus third step of apparent biohydrogenation of LNA: C18:2 c9t11 + t10c12 to C18:1 t11 + t10 plus C18:2 t11c15 to C18:1 t11 + c15 + t15.

Effects of EO at pH 5.6 on the FA profile, apparent biohydrogenation and efficiency of the biohydrogenation steps are shown in Table 5. The C18:0 concentration decreased in treatments PTSOL, EUGH, CINL and CINH compared with CTR at pH 5.6. The *trans*-10 C18:1 decreased in treatments PTSOL, PTSOH and CINH compared with CTR at pH 5.6. The *trans*-15 C18:1 concentration decreased in treatments PTSOH, CINL and CINH compared with CTR at pH 5.6. The C18:2n-6 concentration increased in treatment PTSOL compared with CTR at pH 5.6. The C18:3n-3 concentration increased in treatment PTSOH compared with CTR at pH 5.6. The *cis*-9, *trans*-11 CLA concentration increased in treatment CINH compared with CTR at pH 5.6. The *trans*-10, *cis*-12 CLA concentration increased in treatments PTSOL, PTSOH and CINH

compared with CTR at pH 5.6. The apparent biohydrogenation of LA decreased in treatment PTSOL compared with CTR at pH 5.6. The apparent biohydrogenation of LNA decreased in treatment PTSOH compared with CTR at pH 5.6. The efficiency of the first step of biohydrogenation of LA decreased in treatments PTSOL, PTSOH, CINH compared with CTR at pH 5.6.

Table 5. *Experiment 1. Effect of essential oils at pH 5.6 on fatty acid (FA) profile, apparent biohydrogenation and efficiency of the biohydrogenation steps.*

	Treatments[1]								
	CON	PTSOL	PTSOH	EUGL	EUGH	CINL	CINH	SEM[1]	*P*-Value
FA (g/100g FA)									
C18:0	11.6	9.48*	10.1	10.5	9.91*	9.50*	9.35*	0.33	0.03
t10 C18:1	0.56	0.11*	0.08*	0.56	0.43	0.54	0.17*	0.069	0.01
t11 C18:1	1.90	0.83	0.91	1.85	1.77	1.77	1.20	0.401	0.10
t15 C18:1	0.08	0.02	0.01*	0.04	0.02	0.00*	0.00*	0.021	0.04
c15 C18:1	0.15	0.11	0.09	0.14	0.09	0.09	0.09	0.018	0.17
t11,c15 C18:2	0.34	0.07	0.06	0.36	0.42	0.44	0.29	0.155	0.15
C18:2n-6	16.2	18.6*	17.6	16.2	16.9	16.8	16.8	0.37	0.03
C18:3n3	28.7	31.2	31.4*	30.3	31.0	29.3	30.4	0.91	0.07
c9,t11 CLA	0.11	0.02	0.03	0.14	0.15	0.22	0.49*	0.061	0.02
t10,c12 CLA	0.08	0.81*	0.78*	0.11	0.08	0.24	0.28*	0.071	0.01
Apparent biohydrogenation (%)									
C18:2n-6	32.5	22.5*	26.8	32.5	29.7	30.0	30.0	1.53	0.03
C18:3n-3	32.6	26.8	26.1*	28.9	27.2	31.1	28.5	2.15	0.07
Efficiency of biohydrogenation steps (%)									
1st C18:2[2]	97.6	84.5*	87.4*	96.8	96.8	93.6	89.3*	1.26	0.01
2nd C18:3[3]	97.5	99.4	99.4	97.0	96.2	96.6	97.4	1.38	0.20
2nd C18:2 + 3rd C18:3[4]	87.2	93.2	93.4	86.7	86.9	87.7	91.9	2.80	0.06

[1] CON = Control; PTSOL = Oxy-propyl-thiosulphate at 60 mg/l; PTSOH = Oxy-propyl-thiosulphate at 120 mg/l; EUGL = Eugenol at 150 mg/l; EUGH = Eugenol at 500 mg/l; CINL = Cinnamaldehyde at 150 mg/l; CINH = Cinnamaldehyde at 500 mg/l; SEM = Standard error of the mean.

* Means within a row differ from control ($P < 0.05$).

[2] First step of apparent biohyodrogenation of LA: C18:2 c9c12 to C18:2 c9t11+ t10c12.

[3] Second step of apparent biohyodrogenation of LNA: C18:3 c9c12c15 to C18:2 t11c15.

[4] Second step of apparent biohydrogenation of LA plus third step of apparent biohydrogenation of LNA: C18:2 c9t11 + t10c12 to C18:1 t11 + t10 plus C18:2 t11c15 to C18:1 t11 + c15 + t15.

The pH 5.6 decreased the C18:0, *trans*-11 C18:1, *trans*-15 C18:1, *cis*-15 C18:1, *trans*-11, *cis*-15 C18:2, *cis*-9, *trans*-11 CLA concentrations and increased C18:2n-6, C18:3n-3 concentrations compared with pH 6.4. The pH 5.6 decreased the apparent biohydrogenation of LA and LNA and increased the efficiency of the second step of biohydrogenation of LNA and the efficiency of the second step of biohydrogenation of LA plus the third step of biohydrogenation of LNA compared with pH 6.4.

There was an interaction between treatments and pH for C18:0, *trans*-15 C18:1, *cis*-15 C18:1, C18:3n-3, *cis*-9, *trans*-11 CLA concentrations, and apparent biohydrogenation of LNA.

Effects of EO at pH 6.4 on total and individual VFA concentrations and ammonia N concentration are shown in Table 6. Total VFA concentration decreased in treatments PTSOL, PTSOH and CINH compared with CTR at pH 6.4. Acetate proportion increased in treatment PTSOH compared with CTR at pH 6.4. The BCVFA concentration decreased in treatment PTSOL compared with CTR at pH 6.4. Ammonia N concentration decreased in treatments PTSOL and PTSOH compared with CTR at pH 6.4.

Table 6. *Experiment 1. Effect of essential oils at two pH levels on total and individual VFA and ammonia N concentrations.*

	Treatments[1]								
	CON	PTSOL	PTSOH	EUGL	EUGH	CINL	CINH	SEM[1]	P-Value
pH 6.4									
Total VFA (mM)	72.5	53.9*	50.3*	72.9	70.7	72.01	64.1*	4.84	0.01
VFA, mol/100 mol									
Acetate	70.7	72.3	72.9*	70.6	70.5	70.9	71.0	0.80	0.02
Propionate	16.7	17.1	17.2	17.0	16.5	17.0	16.7	0.58	0.12
BCVFA[2]	1.84	1.63*	1.68	1.85	1.96	1.82	1.95	0.05	0.01
NH_3, mg/100 ml	9.05	5.66*	4.99*	8.23	9.18	7.85	9.61	1.671	0.01
pH 5.6									
Total VFA (mM)	64.8	53.2*	50.8*	64.5	54.8*	61.0	56.4*	3.41	0.01
VFA (mol/100 mol)									
Acetate	69.8	73.1*	73.0*	69.8	70.2	70.1	72.0*	0.84	0.01
Propionate	16.6	17.1*	17.1*	16.7	17.0	17.1*	17.3*	0.37	0.01
BCVFA[2]	1.97	1.67*	1.70*	2.00	1.95	1.95	1.73*	0.086	0.01
NH_3 (mg/100 ml)	8.69	4.96*	4.63*	8.25	7.88*	8.41	6.48*	0.824	0.01

[1] CON = Control; PTSOL = Oxy-propyl-thiosulphate at 60 mg/l; PTSOH = Oxy-propyl-thiosulphate at 120 mg/l; EUGL = Eugenol at 150 mg/l; EUGH = Eugenol at 500 mg/l; CINL = Cinnamaldehyde at 150mg/l; CINH = Cinnamaldehyde at 500mg/l; SEM = Standard error of the mean.

* Means within a row differ from control ($P < 0.05$).

[2] BCVFA = Branched-chain VFA.

Effects of EO at pH 5.6 on total and individual VFA concentrations and ammonia N concentration are shown in Table 6. Total VFA concentration decreased in treatments PTSOL, PTSOH, EUGH and CINH compared with CTR at pH 5.6. Acetate proportion increased in treatments PTSOL, PTSOH and CINH compared with CTR at pH 5.6. Propionate proportion increased in treatments PTSOL, PTSOH, CINL and CINH compared with CTR at pH 5.6. The BCVFA concentration decreased in treatments PTSOL, PTSOH and CINH compared with CTR at pH 5.6. Ammonia N concentration decreased in treatments PTSOL, PTSOH, EUGH and CINH compared with CTR at pH 5.6. The pH 5.6 decreased total VFA and ammonia N concentrations compared with pH 6.4. There was an interaction between treatments and pH for total VFA and BCVFA concentrations.

3.2. **Experiment 2**

Effects of treatments on the FA profile, apparent biohydrogenation and efficiency of the biohydrogenation steps at 3 h postfeeding are shown in Table 7. Treatment PTSO decreased C18:0 concentration and increased C18:2n-6 and C18:3n-3 concentrations, and decreased apparent biohydrogenation of LA and LNA compared with others treatments. Low pH decreased C18:0, *trans*-11 C18:1, *cis*-9, *trans*-11 CLA concentrations, apparent biohydrogenation of LA and LNA, and efficiency of the second step of biohydrogenation of LA plus the third step of biohydrogenation of LNA, and increased C18:2 n-6, C18:3n-3 and *trans*-10, *cis*-12 CLA concentrations.

Effects of treatments on the FA profile, apparent biohydrogenation and efficiency of the biohydrogenation steps at 6 h postfeeding are shown in Table 8. Treatment PTSO decreased C18:0 concentration compared with other treatments and decreased *trans*-10 C18:1 concentration compared with treatments LIP1 and CIN. Treatment PTSO increased C18:2n-6 and C18:3n-3 compared with other treatments. Treatment PTSO decreased the apparent biohydrogenation of LA and LNA. Low pH decreased C18:0, *trans*-11 C18:1 and *cis*-9, *trans*-11 CLA concentrations. Low pH increased *trans*-10 C18:1, C18:2n-6, C18:3n-3 and *trans*-10, *cis*-12 CLA concentrations. Low pH decreased the apparent biohydrogenation of LA and LNA and the efficiency of the second step of apparent biohydrogenation of LA plus the third step of apparent biohydrogenation of LNA.

Effects of treatments on the FA profile, apparent biohydrogenation and efficiency of the biohydrogenation steps in effluents are shown in Table 9. Treatment PTSO decreased C18:0 concentration and apparent biohydrogenation of LA and LNA and increased C18:2n-6 and C18:3n-3 concentrations compared with other treatments. Low pH decreased C18:0, *trans*-11 C18:1 and *cis*-9, *trans*-11 CLA concentrations and increased *trans*-10 C18:1, C18:2n-6 and C18:3n-3 concentrations. Low pH decreased the apparent biohydrogenation of LA and LNA.

Effects of treatments on total and individual VFA concentrations are shown in Table 10. Treatment PTSO decreased total VFA and BCVFA concentrations, and the propionate proportion, and increased the acetate to propionate ratio and the acetate proportion compared with other treatments. Low pH decreased total VFA concentrations, the acetate to propionate ratio, and the acetate proportion, and increased the propionate proportion and BCVFA concentration. The PTSO decreased true digestibility of DM and OM compared with other treatments. Low pH decreased true digestibility of OM, NDF and ADF compared with pH

6.4 (Table 11). Ammonia N flow increased in PTSO compared with other treatments. Bacterial N flow decreased in PTSO compared with LIP1. Low pH decreased ammonia N concentration, ammonia and bacterial N flow and CP degradation and increased nonammonia and dietary N flows (Table 12).

Table 8. *Experiment 2. Effect of treatments on fatty acid (FA) profile, apparent biohydrogenation and efficiency of the biohydrogenation steps at 6 h postfeeding and two pH levels.*

	Treatments[1]								SEM[1]	*P*-Value[2]		
	CON		LIP1		PTSO		CIN					
	6.4	5.6	6.4	5.6	6.4	5.6	6.4	5.6		T	P	T*P
FA (g/100 g FA)												
C18:0	34.3^{a}	17.9^{ab}	34.1^{a}	17.7^{ab}	7.4^{b}	4.6^{b}	30.5^{a}	16.0^{ab}	4.54	0.01	0.01	0.33
t10 C18:1	4.57^{bc}	23.3^{abc}	8.99^{abc}	27.5^{ab}	5.95^{abc}	1.30^{c}	10.4^{abc}	28.1^{a}	4.19	0.02	0.01	0.06
t11 C18:1	6.16^{ab}	3.15^{ab}	4.53^{ab}	3.26^{ab}	6.06^{ab}	0.02^{b}	7.46^{a}	2.03^{ab}	1.270	0.18	0.02	0.12
t11,c15 C18:2	3.89^{ab}	9.63^{ab}	4.33^{ab}	9.32^{ab}	13.8^{a}	1.13^{b}	4.49^{ab}	9.26^{ab}	2.12	0.98	0.65	0.01
C18:2n-6	2.26^{c}	6.32^{bc}	2.44^{c}	5.94^{bc}	11.0^{b}	24.2^{a}	2.34^{c}	6.73^{bc}	1.52	0.01	0.01	0.02
C18:3n3	2.13^{b}	5.69^{b}	2.91^{b}	5.47^{b}	15.8^{b}	33.8^{a}	2.47^{b}	5.93^{b}	3.23	0.01	0.02	0.07
c9,t11 CLA	0.25^{abc}	0.08^{bc}	0.20^{abc}	0.07^{bc}	0.42^{a}	0.02^{c}	0.30^{ab}	0.07^{bc}	0.045	0.27	0.01	0.05
t10,c12 CLA	0.18	0.58	0.15	0.65	0.39	0.20	0.11	0.70	0.182	0.92	0.04	0.21
Apparent biohydrogenation (%)												
C18:2n-6	91.1^{a}	75.1^{ab}	90.4^{a}	76.6^{ab}	56.6^{b}	4.7^{c}	90.8^{a}	73.5^{ab}	6.01	0.01	0.01	0.02
C18:3n-3	94.6^{a}	85.6^{a}	92.6^{a}	86.1^{a}	59.9^{a}	14.3^{b}	93.7^{a}	85.0^{a}	8.19	0.01	0.02	0.07
Efficiency of biohydrogenation steps (%)												
1st C18:2[3]	98.1	96.5	98.5	96.1	94.5	98.6	98.2	95.4	2.98	0.99	0.76	0.64
2nd C18:3[4]	89.5^{a}	71.2^{ab}	88.2^{a}	72.3^{ab}	33.3^{b}	87.2^{a}	87.8^{a}	71.8^{ab}	7.21	0.05	0.86	0.01
2nd C18:2 + 3rd C18:3[5]	81.9	37.7	74.6	28.8	52.3	79.1	66.0	25.5	10.18	0.29	0.01	0.02

[1] CON = Control; LIP1 = Lipase 1 at 4 µl/l; PTSO = Oxy-propyl-thiosulphate at 90 mg/l; CIN = Cinnamaldehyde at 150 mg/l; SEM = Standard error of the mean.

[2] T = Treatment; P = pH; T*P = Treatment by pH interaction.

[abc] Means within a row differ with treatment due to pH (P<0.05).

[3] First step of apparent biohyodrogenation of LA: C18:2 c9c12 to C18:2 c9t11 + t10c12.

[4] Second step of apparent biohyodrogenation of LNA: C18:3 c9c12c15 to C18:2 t11c15.

[5] Second step of apparent biohydrogenation of LA plus third step of apparent biohydrogenation of LNA: C18:2 c9t11 + t10c12 to C18:1 t11 + t10 plus C18:2 t11c15 to C18:1 t11 + c15+t15.

Table 9. *Experiment 2. Effect of treatments on fatty acid (FA) profile, apparent biohydrogenation and efficiency of the biohydrogenation steps in the effluent at two pH levels.*

	Treatments[1]								SEM[1]	*P*-Value[2]		
	CON		LIP1		PTSO		CIN					
	6.4	5.6	6.4	5.6	6.4	5.6	6.4	5.6		T	P	T*P
FA (g/100 g FA)												
C18:0	32.4^{a}	16.5^{ab}	33.1^{a}	16.2^{ab}	7.13^{b}	5.11^{b}	29.8^{a}	15.9^{ab}	3.81	0.01	0.01	0.18
t10 C18:1	4.51	22.3	7.74	17.8	6.32	1.44	10.6	24.1	4.76	0.10	0.03	0.17
t11 C18:1	5.88^{ab}	2.48^{ab}	5.22^{ab}	1.75^{ab}	5.27^{ab}	0.22^{b}	6.32^{a}	1.65^{ab}	1.121	0.37	0.01	0.75
t11,c15 C18:2	5.60^{ab}	9.52^{ab}	5.70^{ab}	10.2^{ab}	14.0^{a}	1.37^{b}	5.59^{ab}	9.36^{ab}	1.97	0.99	0.94	0.01
C18:2n-6	2.14^{d}	7.45^{bcd}	2.15^{d}	8.90^{bc}	12.0^{b}	23.5^{a}	3.11^{cd}	7.47^{bcd}	1.24	0.01	0.01	0.04
C18:3n3	1.90^{c}	6.45^{bc}	2.57^{c}	8.20^{bc}	16.9^{b}	34.7^{a}	3.20^{c}	6.51^{bc}	2.66	0.01	0.01	0.04
c9,t11 CLA	1.00	0.13	0.50	0.13	0.62	0.04	1.03	0.11	0.194	0.35	0.01	0.42
t10,c12 CLA	0.97	1.61	0.90	2.01	0.94	0.21	0.81	2.34	0.452	0.19	0.08	0.14
Apparent biohydrogenation (%)												
C18:2n-6	91.6^{a}	70.6^{abc}	91.5^{a}	64.9^{bc}	52.6^{c}	7.4^{d}	87.7^{ab}	70.6^{abc}	4.88	0.01	0.01	0.04
C18:3n-3	95.2^{a}	83.7^{ab}	93.5^{a}	79.2^{ab}	57.1^{b}	12.0^{c}	91.9^{a}	83.5^{ab}	6.75	0.01	0.01	0.04
Efficiency of biohydrogenation steps (%)												
1st C18:2[3]	91.5	90.3	93.9	85.4	89.1	88.1	91.7	85.3	3.64	0.90	0.14	0.67
2nd C18:3[4]	84.9^{a}	71.0^{ab}	84.7^{a}	65.5^{ab}	32.7^{b}	78.2^{a}	84.5^{a}	71.1^{ab}	7.46	0.04	0.96	0.01
2nd C18:2 + 3rd C18:3[5]	79.8	36.0	76.7	54.3	45.2	69.1	64.9	33.0	11.83	0.57	0.07	0.06

[1] CON = Control; LIP1 = Lipase 1 at 4 µl/l; PTSO = Oxy-propyl-thiosulphate at 90 mg/l; CIN = Cinnamaldehyde at 150 mg/l; SEM = Standard error of the mean.

[2] T = Treatment; P = pH; T*P = Treatment by pH interaction.

[abc] Means within a row differ with treatment due to pH (P<0.05).

[3] First step of apparent biohyodrogenation of LA: C18:2 c9c12 to C18:2 c9t11 + t10c12.

[4] Second step of apparent biohyodrogenation of LNA: C18:3 c9c12c15 to C18:2 t11c15.

[5] Second step of apparent biohydrogenation of LA plus third step of apparent biohydrogenation of LNA: C18:2 c9t11 + t10c12 to C18:1 t11 + t10 plus C18:2 t11c15 to C18:1 t11 + c15+t15.

Table 10. *Experiment 2. Effect of treatments on total and individual VFA concentrations in the effluent at two pH levels.*

	Treatments[1]								SEM[1]	*P*-Value[2]		
	CON		LIP1		PTSO		CIN					
	6.4	5.6	6.4	5.6	6.4	5.6	6.4	5.6		T	P	T*P
Total VFA (mM)	116.0^{a}	87.6^{b}	112.8^{a}	75.6^{b}	87.1^{b}	46.5^{c}	112.2^{a}	85.2^{b}	5.40	0.01	0.01	0.22
VFA (mol/100 mol)												
Acetate	52.7^{bc}	42.0^{e}	53.2^{ab}	43.5^{de}	58.4^{a}	55.6^{ab}	49.8^{bcd}	45.9^{cde}	2.25	0.01	0.01	0.01
Propionate	25.6^{bc}	31.7^{a}	25.5^{bc}	31.0^{ab}	19.4^{d}	21.1^{cd}	26.8^{ab}	27.6^{ab}	1.76	0.01	0.01	0.08
Acetate/Propionate	2.08^{bcd}	1.34^{d}	2.27^{bc}	1.40^{cd}	3.22^{a}	2.67^{ab}	1.85^{bcd}	1.70^{cd}	0.268	0.01	0.01	0.13
BCVFA[3]	4.66^{c}	11.0^{a}	5.16^{bc}	11.4^{a}	5.93^{bc}	6.78^{b}	5.68^{bc}	10.1^{a}	0.73	0.01	0.01	0.01

[1] CON = control; LIP1 = Lipase 1 at 4 µl/l; PTSO = Oxy-propyl-thiosulphate at 90 mg/l; CIN = Cinnamaldehyde at 150 mg/l; SEM = Standard error of the mean.

[2] T = Treatment; P = pH; T*P = Treatment by pH interaction.

[abc] Means within a row differ with treatment due to pH ($P < 0.05$).

[3] BCVFA = Branched-chain VFA.

Table 11. *Experiment 2. Effect of treatments on DM, OM, NDF, and ADF digestion in a dual-flow continuous culture at two pH levels.*

	Treatments[1]								SEM[1]	P-Value[2]		
	CON		LIP1		PTSO		CIN					
	6.4	5.6	6.4	5.6	6.4	5.6	6.4	5.6		T	P	T*P
True digestibility (%)												
DM	47.5^{ab}	44.7^{ab}	48.3^{a}	45.5^{ab}	38.1^{ab}	33.7^{b}	49.0^{a}	45.3^{ab}	2.81	0.01	0.12	0.95
OM	34.5^{a}	31.4^{a}	35.5^{a}	29.3^{ab}	27.3^{ab}	16.9^{b}	34.0^{a}	27.2^{ab}	2.77	0.01	0.02	0.54
NDF	29.0^{a}	4.57^{abc}	29.4^{a}	3.85^{abc}	11.1^{abc}	1.60^{bc}	24.3^{ab}	-10.2^{c}	5.96	0.06	0.01	0.07
ADF	37.5^{a}	10.5^{ab}	35.3^{a}	10.1^{ab}	10.7^{ab}	-0.99^{ab}	33.2^{a}	-9.62^{b}	7.75	0.07	0.01	0.25

[1] CON = Control; LIP1 = Lipase 1 at 4 µl/l; PTSO = Oxy-propyl-thiosulphate at 90 mg/l; CIN = Cinnamaldehyde at 150 mg/l; SEM = Standard error of the mean.

[2] T = Treatment; P = pH; T*P = Treatment by pH interaction.

[abc] Means within a row differ with treatment due to pH ($P< 0.05$).

Table 12. *Experiment 2. Effect of treatments on N metabolism of rumen microbes in a dual-flow continuous culture at two pH levels.*

	Treatments[1]								SEM[1]	*P*-Value[2]		
	CON		LIP1		PTSO		CIN					
	6.4	5.6	6.4	5.6	6.4	5.6	6.4	5.6		T	P	T*P
N-NH_3 (mg/100mg)	14.4[a]	3.96[c]	13.6[a]	3.18[c]	13.0[ab]	13.8[a]	15.0[a]	4.20[bc]	2.17	0.04	0.01	0.01
N flow (g/d)												
Total	2.74	2.73	2.72	2.66	2.72	2.75	2.86	2.68	0.298	0.67	0.24	0.44
Ammonia	0.46[a]	0.13[b]	0.43[a]	0.10[b]	0.42[a]	0.44[a]	0.48[a]	0.13[b]	0.069	0.04	0.01	0.02
Non-ammonia	2.28	2.60	2.29	2.56	2.30	2.31	2.37	2.55	0.267	0.18	0.01	0.18
Bacterial	1.04	0.84	1.20	0.98	0.83	0.55	0.94	0.79	0.119	0.04	0.04	0.94
Dietary	1.21	1.79	1.10	1.59	1.48	1.73	1.45	1.73	0.306	0.20	0.01	0.44
CP degradation (%)	60.2	41.3	64.0	47.6	51.5	43.2	52.5	43.1	10.0	0.20	0.01	0.44
EMPS[3]	34.0	28.8	37.5	36.8	33.6	49.1	30.7	33.0	6.98	0.31	0.58	0.38

[1] CON = Control; LIP1: Lipase 1 at 4 µl/l; PTSO = Oxy-propyl-thiosulphate at 90 mg/l; CIN = Cinnamaldehyde at 150 mg/l; SEM = Standard error of the mean.

[2] T = Treatment; P = pH; T*P = Treatment by pH interaction.

[abc] Means within a row differ with treatment due to pH ($P < 0.05$).

[3] EMPS = Efficiency of microbial protein synthesis (g Bacterial N/Kg organic matter truly digested).

4. DISCUSSION

In general, low pH caused an inhibition of the apparent biohydrogenation of LA and LNA, and increased the efficiency of the intermediary steps of biohydrogenation, resulting in a decrease in intermediary FA concentrations and an increase in the LA and LNA concentrations in the final FA profile. Low pH decreased *trans*-11 C18: 1 and *cis*-9, *trans*-11 CLA concentrations but increased *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA concentrations, which are responsible for milk fat depression. Low pH affected ruminal fermentation reflected by a reduction in total VFA, although this effect was not observed in Experiment 1 in treatments LIP1, LIP2 and INL, probably because these treatments were only incubated for 2 h compared to the 6 h performed in experiment 1 with EO or 8 days in experiment 2. This shows that the low pH had negative effects at longer incubation times. Similar effects on the effect of pH on the intermediaries of the biohydrogenation and VFA were observed by Fuentes *et al.* (2009, 2011).

Results indicate that LIP1, regardless of dose, increased the apparent biohydrogenation of LNA as expected, which is reflected by the reduction in LNA concentration and the increase in stearic acid (C18:0) concentration. The increase in the lipolytic activity produces an increase of the PUFA available for the biohydrogenation at 2 h of incubation. Lourenço *et al.* (2010) indicated that this increase of PUFA can saturate the ruminal microbial activity responsible for the intermediary steps of biohydrogenation. Therefore, the increase in PUFA intermediates due to the effects of LIP1 can be attributed to the increased lipolytic activity that could inhibit the whole biohydrogenation process. The increase in PUFA available in rumen can increase the flow of *trans*-11 C18:1 and *cis*-9, *trans*-11 CLA from the rumen to the mammary gland and therefore increase the concentration of *cis*-9, *trans*-11 CLA in milk fat and obtain milk enriched with CLA. However, according to the objectives of this study, LIP1 did not decrease the *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA concentrations, therefore no effect was observed that would prevent milk fat depression. In contrast, these effects were not observed in Experiment 2, suggesting that the effect of LIP1 was lost in long term fermentation periods.

Cinnamaldehyde at dose of 500 mg/l inhibited the biohydrogenation of LNA, which can increase the ruminal flow of omega-3 and decrease the flow of C18:0, but no effect was observed in *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA. These results are consistent with the results obtained by Lourenço *et al.* (2008) where cinnamaldehyde at 500 mg/l in *in vitro* dual-flow continuous culture fermenters inhibited the apparent biohydrogenation of LA and LNA. However, Lourenço *et al.* (2008) concluded that cinnamaldehyde caused a change of the

biohydrogenation to the alternative pathway resulting in an increase in *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA isomers, which was not observed in this study. In contrast, in Experiment 2 no effect was observed in the FA profile due to the addition of cinnamaldehyde, which is consistent with the results of Benchaar and Chouinard (2009) who tested a dose of 1 g/d in Holstein cows *in vivo* experiment, which would indicate that the effects of cinnamaldehyde on the FA profile are lost over time.

The PTSO inhibited biohydrogenation of LA and LNA and the efficiency of the first step of the biohydrogenation of LA, which may increase ruminal flow of omega-6 and omega-3, and decrease the flow of saturated fat to the small intestine. Treatments PTSO and CIN decreased total VFA, which indicates that rumen fermentation was inhibited. This inhibition has been reported for cinnamaldehyde at doses of 3000 mg/l in *in vitro* Telley and Terry fermentations (Busquet *et al*., 2006), while a dose of 312 mg/l (Busquet *et al*., 2005) did not affect total VFA concentration. In Experiment 1, treatment CIN at dose of 500 mg/l, decreased total VFA but not at dose of 150 mg/l in Experiment 1 or at doses of 250 mg/l in Experiment 2. In experiment 1, PTSOL and PTSOH (60 mg/l and 120 mg/l, respectively) decreased total VFA concentration indicating an effect in normal ruminal fermentation. Busquet *et al.* (2005) reported that doses of 312 mg/l of garlic oil did not affect total VFA in *in vitro* Telley and Terry fermentation while doses of 3000 mg/l decreased total VFA (Busquet *et al*., 2006). Foskolos *et al.* (2015) reported doses between 50-100 mg/l of PTSO did not affect total VFA while doses of 150 mg/l decreased total VFA in *in vitro* dual-flow continuous culture fermenters suggesting that PTSO had a stronger antimicrobial activity than garlic oil. Therefore, the inhibition of apparent biohydrogenation of LA and LNA observed when including PTSO diet can be attributed to the reduction of microbial activity indicated by a decrease of total VFA concentration, it cannot demonstrate that inhibition of apparent biohydrogenation of LA and LNA are due to the inclusion of PTSO but because of its collateral effects.

5. CONCLUSIONS

Low pH reduced the apparent biohydrogenation of LA and LNA *in vitro* Telley and Terry fermentations and in dual-flow continuous culture fermenters, and increased the isomer *trans*-10 C18:1 in dual-flow continuous culture fermenteres, the main isomer responsible to MFD produced in the rumen by the alternative pathway of biohydrogenation, as expected.

The addition of LIP1 increased the apparent biohydrogenation of LNA in experiment 1 suggesting an increased kipolytic activity. LIP1 also reduced the efficiency of intermediary steps of biohydrogenation of LA and LNA in experiment 1 probably due to the inhibitory effect of the high PUFA concentrations resulting in an increase of FA intermediates. In experiment 2 these effecta were not observed, suggesting an adaptation of microbial population to LIP1.

The PTSO inhibited the apparent biohydrogenation of LA and LNA and decreased total VFA concentrations in the two experiments, but the magnitude of the effect was pH-dependent. The addition of cinnamaldehyde and PTSO, in experiment 1, inhibited the apparent biohydrogenation of LNA, although PTSO also increases the concentration of *trans*-10, *cis*-12 CLA, but not of *trans*-10 C18:1 which is the one mostly accumulates in the rumen. In experiment 2, the addition of PTSO inhibited the apparent biohydrogenation of LA and LNA. Furthermore the inclusion of these two EO decreased total VFA concentration in experiment 1 and in experiment 2 (only PTSO) indicating that normal rumen fermentation was altered. In none of these treatments there was a decrease in the *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA isomers responsible for milk fat depression.

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CAPÍTULO 5

Transcriptome profile in cows resistant or sensitive to milk fat depression

Transcriptome profile in cows resistant or sensitive to milk fat depression

Abstract

Feeding linseed to dairy cows results in milk fat depression (MFD), but there is a wide range of sensitivity among cows. The objectives of this study were: 1) To compare the mRNA expression of transcripts expressed in milk somatic cells in cows resistant or sensitive to MFD; and 2) to identify metabolic pathways and transcription factors affected by MFD in resistant or sensitive cows under different dietary conditions (no fat supplemented or linseed rich-diet). Four cows were selected from a dairy farm after a switch from a control diet to a linseed-rich diet. Among them, two cows (R-MFD) were resistant to MFD having high milk fat content in both control (CTR) (4.06%) and linseed-rich diet (LIN) (3.90%); and two cows (S-MFD) were sensitive to MFD decreasing milk fat content after the change into the LIN diet (3.87 to 2.52 %). Fresh milk samples were collected from each cow the week before and two weeks after the diet change. Transcriptome and SNP discovery analysis were performed using RNA-Sequencing technology with a HiSeq2000 platform. Differential expression analysis between S-MFD and R-MFD cows allowed to detect a large number of differentially expressed genes in both diets, CTR (n = 1,316) and LIN (n = 1,888). In addition, 816 genes were differentially expressed between CTR and LIN diets in R-MFD cows whereas only 43 genes were identified in S-MFD cows. Pathway analysis allowed to detect around 100 differently expressed pathways in R-MFD cows fed the LIN diet compared with R-MFD fed the CTR diet, in R-MFD fed the CTR diet compared with S-MFD cows fed the CTR diet and in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet. Only 13 pathways were differently expressed in S-MFD cows fed the LIN diet compared with S-MFD cows fed the CTR diet. Results suggests that R-MFD cows could be activating a compensatory mechanism to increase the fatty acid synthesis in linseed-rich diets. Results showed expression changes in genes and pathways related to fatty acid biosynthesis such as VDR/RXR Activation, Prolactin Signaling, PPARα/RXRα Activation, LXR/RXR Activation, JAK/Stat Signaling, TR/RXR Activation, ERK/MAPK Signaling, EIF2 Signaling and PTEN Signaling. Analysis of key gene regulators also allowed to detect a large number of differently expressed genes (27 to 294) in the different comparisons. Analysis of SNP discovery showed 641 SNP only in R-MFD cows and 1024 only in S-MFD cows among differently expressed genes in all comparisons. Finally, 15 genes were detected as differently expressed genes, key gene regulators and harboring SNP in R-MFD fed the LIN diet compared with S-MFD fed the LIN. Among them, MTOR and PDK1 seemed to be involved in milk fat

synthesis. However, these genes were overexpressed in S-MFD cows fed the LIN diet compared with R-MFD cows fed the LIN diet, and SNPs also were in S-MFD cows.

Keywords: Milk fat depression, milk fat synthesis, RNA-Sequencing, SNP discovery

Abbreviations**:** PUFA, polyunsaturated fatty acids; MFD, milk fat depression; FA, fatty acid; CLA, conjugated linoleic acid; ACACA, acetyl-CoA carboxylase; FASN, fatty acid synthase; GPAT, Glycerol-3-phosphate acyltransferase; AGPAT, Acylglycerol-3-phosphate acyltransferase; SREBP1, Sterol regulatory element binding protein 1; PPARG, peroxisome proliferator-activated receptor gamma; LXR, liver X receptor; LIN, linseed diet; CTR, Control diet; R-MFD, resistant to milk fat depression; S-MFD, sensitive to milk fat depression; FC, fold change; RPKM, reads per kilobase per million mapped reads; IPA, Ingenuity Pathway Analysis; LPL, lipoprotein lipase; VLDLR, very-low density lipoprotein receptor; CD36, CD36 molecule (thrombospondin receptor); SLC27A, solute carrier family 27 (fatty acid transporter); FABP, fatty acid binding protein; ACSS, acyl-CoA synthetase short-chain family; ACSL, acyl-CoA synthetase long-chain family; SCD, stearoyl-CoA desaturase; FADS, fatty acid desaturase; LIPIN, lipin phosphatidic acid phosphorilase; DGAT, diacylglycerol acyltransferase; BTN, Butyrophilin; XDH, xanthine dehydrogenase; ADFP, adipophilin; SCAP, SREBP cleavage activating protein; INSIG, insulin induced gene; THRSP, thyroid hormone responsive SPOT14; DROSHA, drosha, ribonuclease type III; PRKAG3, protein kinase, AMP-activated, gamma 3 non-catalytic subunit; TFF3, trefoil factor 3 (intestinal); LCN2, lipocalin 2; IL37, interleukin 37; SMO, smoothened, frizzled class receptor; MKL2, MKL/myocardin-like 2; FOXO3, forkhead box O3; MGAT5, mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase; TCF12, transcription factor 12; PRLR, prolactin receptor; NTRK1, neurotrophic tyrosine kinase, receptor, type 1; IL17A, interleukin 17A; AGR2, anterior gradient 2, protein disulphide isomerase family member; VIPR1, vasoactive intestinal peptide receptor 1; HDAC9, histone deacetylase 9; UPF2, UPF2 regulator of nonsense transcripts homolog (yeast); PLA2G10, phospholipase A2, group X; HNF1A, HNF1 homeobox A; PROC, protein C (inactivator of coagulation factors Va and VIIIa) CSF2, colony stimulating factor 2 (granulocyte-macrophage); ACVR1C, activin A receptor, type IC; DNASE1L3, deoxyribonuclease I-like 3; CD82, CD82 molecule; EGFR, epidermal growth factor receptor; FOXA1, forkhead box A1; PDE4D, phosphodiesterase 4D, cAMP-specific; INHBA, inhibin, beta A; CCL2, chemokine (C-C motif) ligand 2; FOXM1, forkhead box M1; SIN3A, SIN3 transcription regulator family member A; DCN, decorin; RNASE1, ribonuclease, RNase A family, 1 (pancreatic); PAX5, paired box 5; PTGS2/COX-2, cyclooxygenase-2; MTOR, mammalian target of rapamycin; Akt,

Serine/threonine kinase; ACLY, ATP-citrate lyase; PDPK1, phosphoinositide-dependent kinase 1; RICTOR, rapamycin-insensitive companion of mTOR.

1. INTRODUCTION

The production of omega-3-enriched milk is generally achieved by feeding oilseeds rich in these fatty acids (FA), namely linseed. This production has two main problems: linseed is an expansive ingredient, and feeding high polyunsaturated FA (PUFA) usually results in milk fat depression (MFD) which may have important economic implications due to milk price penalties. Diet-induced MFD are classified in tow types: One involves diets with high levels of concentrate and low fiber, and the second type involves diets with high PUFA oils (plant and fish oils). Although usually these two types occur independently, the situation worsens when high production dairy farms (usually with diets rich in concentrate) introduce PUFA to produce omega-3-enriched milk. Under these dietary conditions, an alternative biohydrogenation pathway occurs, and the FA intermediaries produced are potent inhibitors of milk fat synthesis. Specifically, MFD in these conditions has been linked primarily to the increase in *trans*-10 C18:1 in the rumen and the subsequent desaturation to *trans*-10, *cis*-12 conjugated linoleic acid (CLA) in the mammary gland by the delta-9 desaturase enzyme (Bauman and Griinari, 2001).

Free oils in the rumen produce changes in bacterial populations (Jenkins, 1993). To avoid the effects of high PUFA oils in the rumen, processing methods to protect oils, as an extruded seeds, or full fats seeds were analyzed (Chouinard *et al.*, 1998; 2001). However, nutritional strategies such as protected oils, as full-fat seeds or meal, also produce MFD (Bauman and Griinari, 2001). Supplementing diets with additives to avoid the production of *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA in *in vitro* batch fermentation and dual-flow continuous culture fermenters had no effects (Siurana *et al.,* 2013). Therefore, it is necessary to study alternative ways to avoid MFD.

A careful review of milk fat content of individual cows fed linseed revealed that certain cows were able to maintain milk fat content, suggesting that these cows could be resistant to MFD. In recent years, nutrigenomics is playing an important role in the understanding the impact of nutrition on physiological processes. Diets that induce MFD represent a good example of the role of nutrigenomics in understanding nutrition effects on physiological changes in the mammary gland in MFD conditions (Bauman *et al.*, 2011). The inhibition of milk

fat synthesis resulting from ruminal biohydrogenation of FA such a *trans*-10*, cis*-12 CLA in diets that induce MFD have been related to a coordinated repression of several genes involved in *de novo* FA synthesis and triglyceride synthesis such as acetyl-CoA carboxylase (ACACA), Fatty acid synthase (FASN), Glycerol-3-phosphate acyltransferase (GPAT) and Acylglycerol-3-phosphate acyltransferase (AGPAT) (Peterson *et al.,* 2003). Moreover, some regulators play an important role in the regulation of fat synthesis such as sterol regulatory element binding protein 1 (SREBP1), peroxisome proliferator-activated receptor gamma (PPARG) and liver X receptor (LXR) (Harvatine and Bauman, 2006; Kadegowda *et al.,* 2009; McFadden and Corl, 2010).

With this evidence, the aims of this study were: 1) To compare the mRNA expression of transcripts expressed in milk somatic cells of cows resistant or sensitive to MFD; 2) to identify metabolic pathways and transcription factors affected by MFD in resistant or sensitive cows under different dietary conditions (no fat supplemented or linseed rich-diet). 3) To identify target genes containing SNPs that plays a key role in the regulation of MFD in cows resistant or sensitive to MFD.

2. MATERIALS AND METHODS

2.1. **Animals and diets**

This experiment was conducted in a 800 Holstein commercial dairy farm in Girona (Spain). From October 2012 to January 2013, cows were fed a diet containing linseed (LIN, linseed diet cointaining triticale silage (20.4%), brewers grains (8.6%), corn silage (10.2%), rapassed meal (12.9%), extruded linseed (6.1%), corn grain (32.1%), soybean meal (3%), barley grain (2.6%) and a vitamin and mineral mixture (3.9%)). Cows were then switched to a diet with no linseed from February 2013 to June 2013 (CTR, Control diet containing triticale silage (20.4%), brewers grains (8.6%), corn silage (10.2%), rapassed meal (12.9%), fat (1.7%), corn grain (33.8%), soybean meal (6%), barley grain (2.6%) and a vitamin and mineral mixture (3.9%). Milk samples were taken monthly from all cows and submitted to the milk official control laboratory (Allic, Cabrils, Spain) for milk fat, protein, lactose and milk somatic cells analysis. Results were used to selected 4 cows: two cows where milk fat content was high for at least four consecutive controls in the LIN (3.90%) and CTR (4.06%) diets (resistant to milk fat depression = R-MFD); and two cows where fat content was for at least four consecutive controls high in the CTR diet (3.87%) but low in the LIN diet (2.54%; sensitive to milk fat depression = S-MFD). Selected cows were submitted to treatments for two periods of 21 days.

In period one cows were fed the CTR diet, and in period two cows were fed the LIN diet. At the end of each period fresh milk samples were collected, for extraction of mRNA from milk somatic cells.

2.2. **Sampling and analysis**

Milk samples were collected and processed following the protocol decribed by Wickramasinghe *et al.* (2012). Milk samples were obtained by hand milking two quarters (50 ml from each quarter) of each of the 4 selected cows before the morning milking, stored in ice and transported to the laboratory within 4 hours to conduct RNA extraction of milk somatic cells. A representative milk sample (50 ml) of the morning milking was also collected to analyze the FA profile. The FA profile analysis was conducted in the official control laboratory (Allic, Cabrils, Spain) by gas chromatography.

2.3. **RNA extraction**

RNA extraction from milk somatic cells was conducted following the protocol described by Wickramasinghe *et al.* (2012). Milk somatic cells were pelleted by adding 50µl of 0.5 M EDTA to 50 ml of fresh milk and centrifuged at 1800 rpm at 4ºC for 10 min. The pellet was washed with 10 ml of PBS at pH 7.2 and 0.5 mM EDTA and filtered through an sterile cheesecloth to remove any debris. Milk cells were then centrifuged again at 1800 rpm, 4ºC for 10 min. The supernatant was decanted, and RNA was extracted using the Trizol method (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The purified total RNA was treated with Turbo DNase (Invitrogen, Carlsbad, CA). Quality of the total RNA was evaluated using the RNA Integrity Number (RIN) in the Agilent Bioanalyzer 2100. The RIN values ranged from 6.6 to 8.8 (Table 1).

Table 1. *RNA concentration (ng/µl) and RNA Integrity Number (RIN) of samples analyzed .*

Sample	diet	% milk fat	RNA Concentration (ng/µl)	RIN[1]
616	linseed	3.51	145	7.30
616-2	control	4.22	77	8.80
9274	linseed	4.29	133	7.10
9274-2	control	3.90	74	8.70
9412	linseed	2.89	95	8.10
9412-2	control	4.04	13	8.40
9413	linseed	2.16	76	6.60
9413-2	control	3.71	21	7.70

[1]RNA Integrity Number.

2.4. **RNA-Sequencing**

The mRNA was purified, fragmented, and converted to cDNA, as described by Cánovas *et al.* (2010). Adapters were ligated to the ends of double-stranded cDNA and PCR amplified to create libraries. These procedures were executed with TruSeq RNA Sample Preparation kit (Illumina, San Diego, CA).

Sequencing was conducted by Illumina HiSeq2000 (Illumina, San Diego, CA) that yielded 100 bp pairend sequences. Quality of reads obtained was checked with FastQC sofware (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) as descibed Cánovas *et al.* (2014). Raw reads that passed the quality filter threshold were mapped using Tophat 2.0.7 (Trapnell *et al.*, 2009) and Bowtie2 2.0.6 (Langmead, 2010) to identify known and novel splice junctions and to generate read alignments for each sample. The Bos taurus genome 4.6.1. waas used as the reference genome, and genomic annotations were obtained from Illumina database (http://support.illumina.com/sequencing/sequencing_software/igenome.ilmn) in general feature format 3. The inner distance between mate pairs used was 50 bp and the rest of the parameters were used with the default values. The transcript isoform level and gene level counts were calculated and reads per kilobase per million mapped reads (RPKM) normalized using Cufflinks 2.0.2 software (Trapnell *et al.*, 2010). To select expressed genes, a threshold of RPKM ≥ 0.2, p-value < 0.01 and fold change (FC) > 2 was used. Ingenuity Pathway Analysis (IPA, Qiagen, Silicon Valley, CA) was used to conduct functional analysis to identify metabolic pathways and key gene regulators involved in FA synthesis and lipid metabolism that explain the observed phenotype.

2.5. **SNP Discovery**

A SNP detection analysis was performed using sequencing reads from the 4 cows to determine putative polymorphisms in genes involved in the FA and lipid metabolism. The SNP detection was performed as described by Cánovas *et al.* (2010), considering the same criteria and quality filters to select only true SNP.

3. RESULTS AND DISCUSSION

3.1. **Performance and FA analysis**

The four cows at the start of the experiment were in 241-264 days in milk. Results of yield milk (kg/d), fat content (%), protein content (%) and FA profile are presented in Table 2.

Table 2. *Performance and fatty acid profile of S-MFD cows and R-MFD cows fed the CTR diet or the LIN diet.*

	Treatments[1]							
	R-MFD		S-MFD			P-value[2]		
	CTR	LIN	CTR	LIN	SEM[3]	T	D	T*D
DIM[4]	254-262		264-241					
Milk yield (kg/d)	36.9	42.4	28.2	39.9	3.48	0.148	0.007	0.275
Fat (%)	4.06[a]	3.90[a]	3.87[a]	2.52[b]	0.31	0.036	0.006	0.025
Protein (%)	3.12	3.03	3.65	3.35	0.125	0.004	0.086	0.307
FA profile (g/100g total FA)								
Total saturated	64.8	61.3	58.9	54.9	3.90	0.347	0.225	0.910
Total unsaturated	35.2	38.6	41.1	45.1	3.90	0.347	0.225	0.910
Total monounsaturated	27.3	29.0	31.9	34.8	3.03	0.302	0.378	0.776
Total polyunsaturated	5.18	6.08	6.47	6.91	0.728	0.411	0.022	0.151
Total omega-3	0.34[b]	0.96[a]	0.61[b]	1.00[a]	0.034	0.085	0.001	0.002
C18:0	13.3[b]	17.8[a]	11.6[b]	10.8[b]	0.34	0.006	0.034	0.016
Trans-10 C18:1	0.54	0.85	0.66	0.84	0.252	0.871	0.332	0.785
Trans-11 C18:1	2.04	1.98	2.58	3.06	1.036	0.633	0.327	0.233
C18:1	22.0	23.9	25.3	26.2	1.61	0.293	0.347	0.694
Total *trans*-C18:2	0.74	1.26	0.97	1.60	0.239	0.391	0.105	0.748
Cis-9, *trans*-11 CLA	0.63	0.62	0.89	0.70	0.286	0.695	0.613	0.648
cis-9, *cis*-12, *cis*-15 C18:3	0.30[b]	0.88[a]	0.43[b]	0.83[a]	0.044	0.531	0.001	0.038
De novo synthesis	36.6	31.5	33.8	32.8	3.01	0.881	0.091	0.173
Uptake FA	60.3	64.8	62.6	63.8	2.90	0.883	0.087	0.202

[1]R-MFD = Resistant milk fat depression cows; S-MFD = Sensitive milk fat depression cows; CTR = Control diet; LIN = Linseed diet.
[2]T = Treatment = R-MFD or S-MFD cows; D = diet; T*D = treatment by diet interaction.
[3]Standar error of mean.
[4]Days in milk.
[ab] Means within a row differ with treatment due to diet($P < 0.05$).

3.2. **RNA-Seq expression analysis**

An average of 72 sequence reads were obtained for each sample. In all samples, 80-85% of the reads were categorized as mapped to the bovine reference sequence (Table 3). Approximately, 90% of total annoted *Bos taurus* genes were represented in the samples (24,881 genes out of a total of 27,368).

Table 3. *Total reads, and mapped reads of samples analyzed.*

Sample	diet	total reads (n)	mapped reads	% mapped reads
616	linseed	66,447,127	56,089,476	84.4
616-2	control	68,871,588	58,295,198	84.6
9274	linseed	66,261,238	56,194,156	84.8
9274-2	control	71,354,503	60,249,942	84.4
9412	linseed	77,788,874	65,275,316	83.9
9412-2	control	74,157,347	61,930,670	83.5
9413	linseed	79,326,575	68,098,358	85.8
9413-2	control	77,979,928	65,499,148	84.0

3.3. **Expression analysis**

Differential expression analysis was conducted to identify differently expressed genes between R-MFD cows and S-MFD cows fed the CTR or LIN diet (RPKM ≥ 0.2, p-value < 0.01 and FC > 2). Four different comparisons were conducted: 1) S-MFD cows fed the CTR diet *vs.* S-MFD cows fed the LIN diet; 2) R-MFD cows fed the CTR diet *vs.* R-MFD cows fed the LIN diet; 3) S-MFD cows fed the CTR diet *vs.* R-MFD cows fed the CTR diet; and 4) S-MFD cows fed the LIN diet *vs.* R-MFD cows fed the LIN diet.

Differential expression analysis between R-MFD and R-MFD cows allowed to detect a large number of differentially expressed genes in both diets, CTR (n = 1316) and LIN (n = 1888). In addition, 816 genes were differentially expressed between CTR and LIN diets in R-MFD cows whereas only 43 genes were identified in S-MFD cows. This 43 genes were overexpressed in S-MFD cows fed the LIN diet. In contrast, the R-MFD cows fed the LIN diet compared with R-MFD cows fed the CTR diet had 72% of genes downexpressed, the R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet had 80% of genes downexpressed, and the R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet had 65% of genes downexpressed (Figure 1). The R-MFD cows fed the CTR diet *vs.* R-MFD cows fed the LIN diet had a large number of differentially expressed genes compared with the S-MFD cows fed the CTR diet *vs.* S-MFD cows fed the LIN diet. These results indicate that R-MFD cows could be activating a compensatory mechanism to increase the FA synthesis in linseed-rich diets.

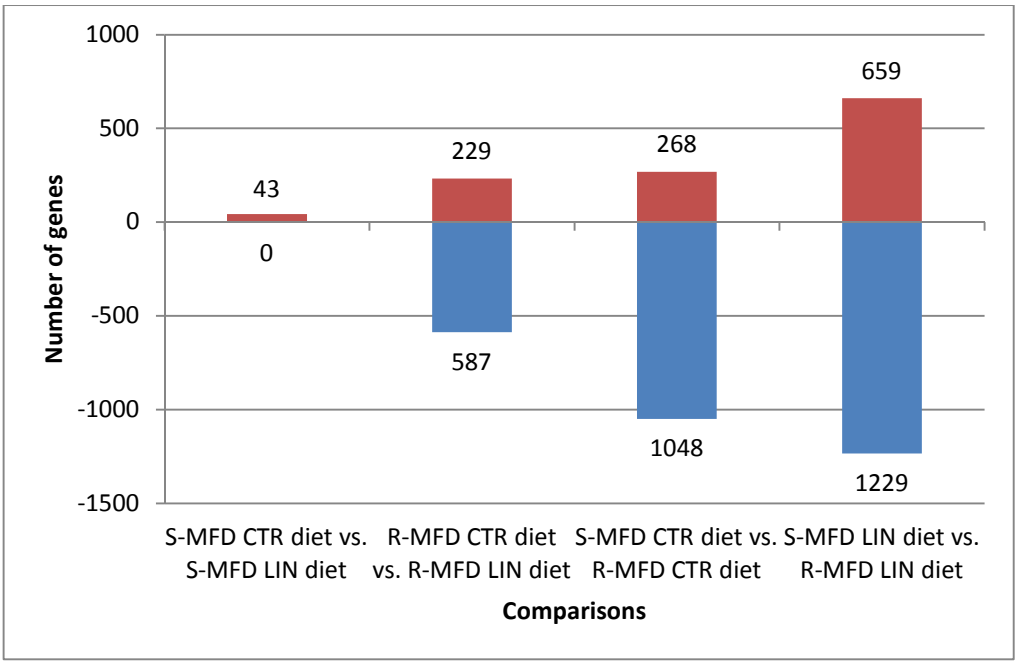

Figure 1. *Number of genes down-(blue) and overexpressed (red) between R-MFD and S-MFD with or without a linseed-rich diet.*

In particular, the expression of genes involved in milk fat synthesis in the mammary gland of dairy cows described in the literature was analyzed. Milk fat synthesis and their genes involved consists in the following processes: *de novo* FA synthesis (ACACA, FASN); FA uptake from blood (lipoprtein lipase, LPL; very-low density lipoprotein receptor, VLDLR; CD36 molecule (thrombospondin receptor), CD36; solute carrier family 27 (fatty acid transporter), SLC27A); intracellular transport (fatty acid binding protein, FABP); FA activation (acyl-CoA synthetase short-chain family, ACSS; acyl-CoA synthetase long-chain family, ACSL); desaturation (stearoyl-CoA desaturase, SCD; fatty acid desaturase, FADS); synthesis of TAG (GPAT; AGPAT; lipin phosphatidic acid phosphorilase, LPIN; diacylglycerol acyltransferase, DGAT); lipid droplet formation (Butyrophilin, BTN; xanthine dehydrogenase, XDH; adipophilin, ADFP); and regulation of transcription (SREBP; SREBP cleavage activating protein, SCAP; insulin induced gene, INSIG; thyroid hormone responsive SPOT14, THRSP; PPAR; LXR) (Bionaz and Loor, 2008; Wickramasinghe *et al.*, 2012; Oppi-Williams *et al.*, 2013).

In this study the R-MFD cows fed the LIN diet compared with R-MFD cows fed the CTR diet resulted in the downexpression of ACACA (FC=-2.38), and LPIN1 (FC=-2.3), and the overexpression of FABP3 (FC=3.6), AGPAT6 (FC=1.99), DGAT2 (FC=2.14) and BTN1A1 (FC=4.02). The S-MFD cows fed the LIN diet compared with S-MFD cows fed the CTR diet resulted in an overexpression of GPAT (FC=4.39) and BTN1A1 (FC=4.02). The R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet resulted in the overexpression of LPL (FC=2.7),

FABP7 (FC=20.11), ACSL6 (FC=3.03), ACSL1 (FC=3.18) and PPARG (FC=2.24), and the downexpression of FASN (FC=-5.09), SLC27A5 (FC=-5.36), SLC27A6 (FC=-8.40), FABP3 (FC=-2.98), GPAT (FC=-2.6), AGPAT2 (FC=-2.34), LPIN2 (FC=-2.65), DGAT2 (FC=-5.7), BTN1A1 (FC=-8.84) and BTN2A1 (FC=-3.18). The R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet resulted in the overexpression of SLC27A1 (FC=2.53), FABP7 (FC=20), ACSL6 (FC=2.7), FADS6 (FC=3.15) and LXRA (FC=2.2), and the downexpression of ACSS2 (FC=-2.43), ACACA (FC=-2.96), FASN (FC=-8.55), SLC27A6 (FC=-6.19), SLC27A5 (FC=-4.19), GPAT (FC=-10.83), BTN1A1 (FC=-13.66) and XDH (FC=-3.42)

Genes involved in *de novo* FA synthesis (ACACA and FASN), desaturation (SCD), TAG synthesis (GPAT and AGPAT) and uptake and transport of FA (LPL, FABP) were downexpressed in diets that induced MFD or after the abomasal infusions of *trans*-10, *cis*-12 in dairy cows (Baumgard *et al.*, 2002; Peterson *et al.*, 2003; Peterson *et al.*, 2004). In this study, the R-MFD cows fed the LIN diet compared with R-MFD cows fed the CTR diet, the gen ACACA involved in *de novo* FA synthesis was downexpressed as described in the literature, but genes involved in transport, TAG synthesis and lipid droplet formation (FABP3, AGPAT6, DGAT2 and BTN1A1) were overexpressed in contrast to what is described in the literature in diets that induce MFD or after the abomasal infusions of *trans*-10, *cis*-12 CLA, where GPAT, AGPAT and FABP are downexpressed (Baumgard *et al.*, 2002; Peterson *et al.*, 2003; Peterson *et al.*, 2004). Therefore, it seems that the overexpression of these genes could counterbalance the downexpression of ACACA and explain the resistance to MFD in R-MFD cows. In the S-MFD cows fed the LIN diet compared with the S-MFD cows fed the CTR diet, only GPAT and BTN1A1 were overexpressed.

The R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet, had an overexpression of genes involved in FA transport and activation (LPL, FABP7, ACSL6 and ACSL1) and the transcription factor PPARG. Kadegodwa *et al.* (2009) reported an overexpression of genes involved in milk fat synthesis when bovine mammary epithelial cells were treated with rosiglitazone (specific agonist of PPARG) indicating that PPARG could play an important role in milk fat synthesis, whereas no activation of PPARG was observed when bovine mammary epithelial cells were treated with *trans*-10, *cis*-12 CLA, indicating that this gene is not involved in MFD. The gene FABP7 was 20 times overexpressed in R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet, but FABP3 was downexpressed, which is the isoform more commonly referred involved in intracellular transport of FA in bovine mammary gland (Bionaz and Loor, 2008). Genes involved in *de novo* FA synthesis (FASN), transport

(SLC27A), TAG synthesis (GPAT, AGPAT2, LPIN2, DGAT2) and lipid droplet (BTN1A1, BTN2A1) were downexpressed in R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet. In this comparison can be observed that the expression of the genes involved in milk fat synthesis is very different between S-MFD and R-MFD cows fed the CTR diet, suggesting that this animals under normal conditions (without LIN) have different genes expressed and different isoforms of genes involved in milk fat synthesis, and these differences could explain the resistance to MFD in R-MFD cows.

The R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet had an overexpression of genes involved in transport, long-chain FA activation, long-chain FA desaturation (SLC27A1, FABP7, ACSL6, FADS6), and transcription factor (LXRα). The gen FABP7 was 20 times overexpressed in R-MFD cows. The transcription factor LXRα was related as a important regulator in milk fat synthesis. Oppi-Williams *et al.* (2013) reported that genes involved in milk fat synthesis (ACACA, FASN and SREBP1) were overexpressed during incubation of bovine mammary epithelial cells with T0901317, an agonist of LXRα, indicating a pivotal role in the regulation of transcription of genes involved in milk fat synthesis. However, Harvatine *et al.* (2014) analyzed the response of LXR in cows treated with *trans*-10, *cis*-12 CLA or in cows fed diets that induced MFD (low fiber and high oil) and no changes in expression of LXR was observed, indicating that this transcription factor, although it has a role in milk fat synthesis, it is not related with MFD. In this study, no changes in expression of LXRα was observed in S-MFD or R-MFD cows fed the LIN diet compared with S-MFD cows or R-MFD cows fed the CTR diet, which would also suggest that LXRα is not related with MFD. However, the R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet, LXRα in R-MFD cows was overexpressed, indicating that this transcription factor could be involved in the resistance to MFD, although this gene was no related to MFD. In contrast, genes involved in *de novo* FA synthesis (ACACA and FASN), short-chain FA activation (ACSS2), TAG synthesis (GPAT), and lipid droplet formation (BTN1A1 and XDH) were downexpressed in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet.

Results from de comparison between R-MFD cows fed the LIN and S-MFD cows fed the LIN diet, help in the understanding of how R-MFD cows can avoid MFD in presence of LIN at the same time that explain the differences between S-MFD and R-MFD cows. Results seem to indicate that genes involved in uptake, transport, activation and desaturation of long-chain FA could participate in the resistance mechanism to MFD, but other genes such as *de novo*

synthesis (ACACA and FASN), short-chain FA activation (ACSS2) and lipid droplet formation (BTN1A1 and XDH) were overexpressed in S-MFD cows.

Results suggest that complex networks of genes are involved in milk fat synthesis and in the response to diets that induced MFD. The R-MFD cows overexpressed different isoforms of genes involved in milk fat synthesis such as FABP7, ACSL6 and FADS6 instead of the isoforms described in milk fat synthesis in the mammary gland of dairy cows as FABP3, ACSL1 and FADS1 or 2 (Bionaz and Loor, 2008).

3.4. **Pathway analysis**

In order to identify the significant metabolic pathways represented by the list of differentially expressed genes generated for each comparison, a pathway analysis was conducted using the IPA software (Qiagen, Silicon Valley, CA). The R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet had 117 differently expressed metabolic pathways ($P < 0.01$). These metabolic pathways were involved in the immune and inflammatory system, in development and growth processes, and in lipid metabolism and FA synthesis. The complete list of significant pathways ($P < 0.01$), and their type of regulation are presented in Annex 1, Table A1. The R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet had 105 differentially expressed metabolic pathways. The complete list of significant pathways ($P < 0.01$), and their type of regulation are presented in Annex 1, Table A2. The R-MFD cows fed the LIN diet compared with R-MFD cows fed the CTR diet had 104 differentially expressed metabolic pathways, which can explain the effect of linseed-rich diet in R-MFD cows. The complete list of significant pathways ($P < 0.01$), and their type of regulation are presented in Annex 1, Table A3. The S-MFD cows fed the LIN diet compared with S-MFD cows fed the CTR diet had only 13 differentially expressed metabolic pathways. These results indicate that S-MFD cows had different response against linseed-rich diet compared with R-MFD cows. The complete list of significant pathways ($P < 0.05$), and their type of regulation are presented in Annex 1, Table A4. The top 10 differently expressed pathways of the R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet are presented in Figure 2. Pathways related to FA biosynthesis such as VDR/RXR Activation, Prolactin Signaling, PPARα/RXRα Activation, LXR/RXR Activation, JAK/Stat Signaling, TR/RXR Activation, ERK/MAPK Signaling, EIF2 Signaling and PTEN Signaling had different expression in some of the comparisons.

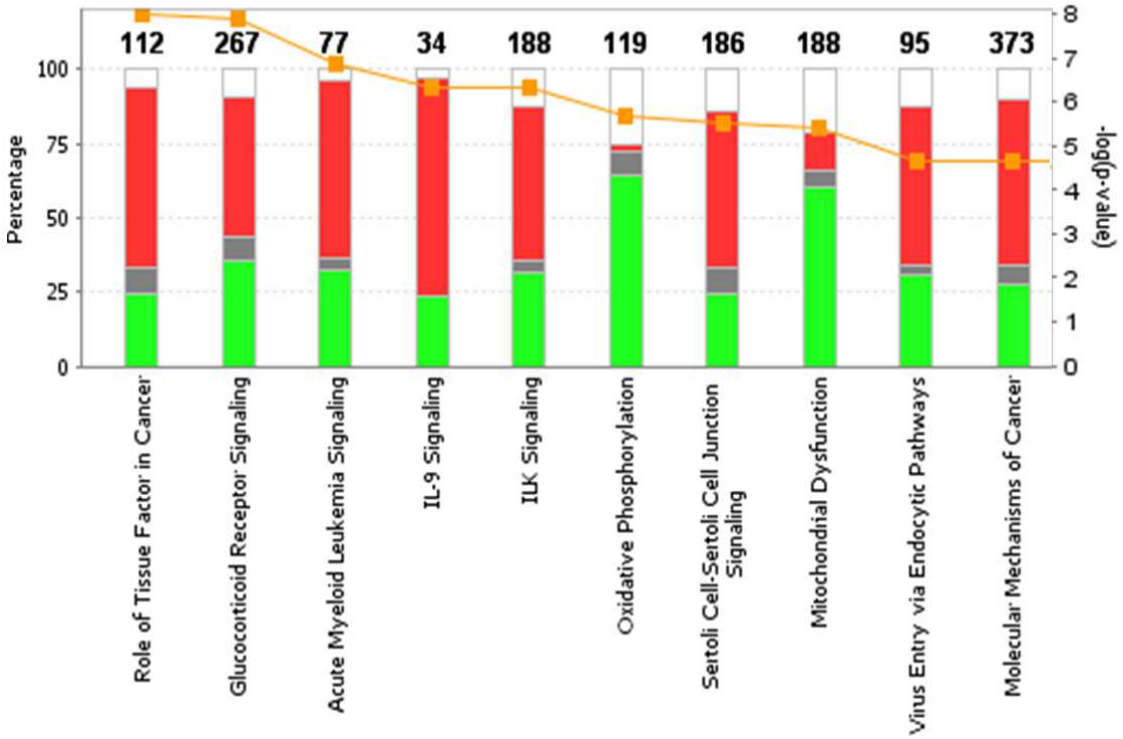

Figure 2. *Top 10 differentially expressed pathways in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet. Percentage of genes downexpressed (red), overexpressed (green), and no change (grey).*

3.5. **Identification of key gene regulators**

IPA software was used to identify the genes that can be acting as transcription factors or key gene regulators with the evidence contributing to the list of differentially expressed genes in each of the four comparison conducted in this study.

Key gene regulators were selected with a threshold of FC > 2. In S-MFD cows fed the LIN diet compared with S-MFD cows fed the CTR diet, 27 key gene regulators were identify. Of these genes, 18 were overexpressed and 9 were downexpressed. The complete list of key gene regulators (FC > 2) and their regulated genes are presented in Annex 1, Table A5. The R-MFD cows fed the LIN diet compared with R-MFD cows fed the CTR diet had 122 key gene regulators, where 60 genes were downexpressed and 62 genes overexpressed. The complete list of key gene regulators (FC > 2), and their regulated genes are presented in Annex 1, Table A6. The R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet had 294 key gene regulators, where 255 were downexpressed and 39 overexpressed. The complete list of key gene regulators (FC > 2), and their regulated genes are presented in Annex 1, Table A7. The R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet had 266 key gene

regulators, where 205 were downexpressed and 61 overexpressed. The complete list of key gene regulators (FC > 2), and their regulated genes are presented in Annex 1, Table A8. The R-MFD cows had 87% and 77% of key gene regulators downexpressed compared with S-MFD cows fed the CTR diet or the LIN diet, respectively. These results indicate that R-MFD cows fed the CTR diet have a basal different expression compared with S-MFD cows, and R-MFD cows have different expression responses against linseed enrich-diets compared with S-MFD cows, which may be responsible for MFD resistance.

The top five overexpressed key regulators in R-MFD cows fed the LIN diet compared with R-MFD cows fed the CTR diet were drosha, ribonuclease type III (DROSHA), protein kinase, AMP-activated, gamma 3 non-catalytic subunit (PRKAG3), trefoil factor 3 (intestinal) (TFF3), lipocalin 2 (LCN2), and interleukin 37 (IL37); and the top five downexpressed key regulators were smoothened, frizzled class receptor (SMO), MKL/myocardin-like 2 (MKL2), forkhead box O3 (FOXO3), mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase (MGAT5), and transcription factor 12 (TCF12). The top five overexpressed key regulators in S-MFD cows fed the LIN diet compared with S-MFD cows fed the CTR diet were prolactin receptor (PRLR), neurotrophic tyrosine kinase receptor type 1 (NTRK1), interleukin 17A (IL17A), anterior gradient 2, protein disulphide isomerase family member (AGR2), and vasoactive intestinal peptide receptor 1 (VIPR1); and the top five downexpressed key regulators were histone deacetylase 9 (HDAC9), UPF2 regulator of nonsense transcripts homolog (yeast) (UPF2), phospholipase A2, group X (PLA2G10), HNF1 homeobox A (HNF1A), and protein C (inactivator of coagulation factors Va and VIIIa) (PROC). The top five overexpressed key regulators in R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet were PROC, IL17A, colony stimulating factor 2 (granulocyte-macrophage) (CSF2), activin A receptor, type IC (ACVR1C), and deoxyribonuclease I-like 3 (DNASE1L3); and the top five downexpressed key regulators were CD82 molecule (CD82), UPF2, epidermal growth factor receptor (EGFR), forkhead box A1 (FOXA1), phosphodiesterase 4D, and cAMP-specific (PDE4D). The top five overexpressed key regulators in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet were DROSHA, PROC, CSF2, inhibin, beta A (INHBA), and chemokine (C-C motif) ligand 2 (CCL2); and the top five downexpressed key regulators were forkhead box M1 (FOXM1), SIN3 transcription regulator family member A (SIN3A), decorin (DCN), ribonuclease, RNase A family, 1 (pancreatic) (RNASE1), and paired box 5 (PAX5) (Table 4).

Among these key gene regulators, DROSHA, involved in diverse RNA maturation and decay pathways in eukaryotic and prokaryotic cells (Fortin *et al.*, 2002), and the core nuclease that executes the initiation step of microRNA (miRNA) processing in the nucleus (Lee *et al.*, 2003), was overexpressed in R-MFD cows fed the LIN diet compared with R-MFD cows fed the CTR diet and in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet, suggesting that this key gene regulator changes its expression in presence of linseed only in R-MFD cows.

The key gene regulator PROC encodes a vitamin K-dependent plasma glycoprotein. The encoded protein is cleaved to its activated form by the thrombin-thrombomodulin complex. This activated form contains a serine protease domain and functions in degradation of the activated forms of coagulation factors V and VIII. Mutations in this gene have been associated with thrombophilia due to protein C deficiency, neonatal purpura fulminans, and recurrent venous thrombosis (provided by RefSeq, Dec 2009). The PROC was overexpressed in R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet and in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet, and downexpressed in S-MFD cows fed the LIN diet compared with S-MFD cows fed the CTR diet. This results suggests that this key gene regulator was overexpressed in R-MFD cows with or without linseed compared with S-MFD cows. In contrast, S-MFD cows had a downexpression with linseed, whereas in R-MFD cows no observed change in expression of this gene by the inclusion of linseed, suggesting that the gen PROC may be involved in MFD and in the resistance to MFD.

The CSF2 encodes a cytokine that controls the production, differentiation and function of granulocytes and macrophages (provided by RefSeq, Jul 2008). This key gene regulator was overexpressed in R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet, and in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet. Therefore, it seems that this gene is overexpressed only in R-MFD cows with or without linseed, suggesting a possible role in the resistance to MFD.

The UPF2, encodes a protein that is part of a post-splicing multiprotein complex involved in both mRNA nuclear export and mRNA surveillance (provided by RefSeq, Jul 2008). The UPF2 was downexpressed in R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet, and in S-MFD cows fed the LIN diet compared with S-MFD cows fed the CTR diet, suggesting that this gene was downexpressed in R-MFD cows, but decreased its expression in presence of linseed only in S-MFD cows. Then, in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet, no changes in its expression was observed.

The IL17A, encoded a proinflammatory cytokine produced by activated T cells. This cytokine regulates the activities of NF-kappaB and mitogen-activated protein kinases. This cytokine can stimulate the expression of IL6 and cyclooxygenase-2 (PTGS2/COX-2), as well as enhance the production of nitric oxide (provided by RefSeq, Jul 2008). This gene had an overexpression in R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet, and in S-MFD cows fed the LIN diet compared with S-MFD cows fed the CTR diet, suggesting that this gene was overexpressed in R-MFD cows, but increase its expression in presence of linseed in S-MFD cows. The opposite situation to the UPF2 gene.

Table 4. *Top five key gene regulators down (5) and up (5) regulated in the four comparisons.*

KGR[1]	FC[2]	Regulated Genes (10)
R-MFD fed the LIN diet compared with R-MFD fed the CTR diet		
DROSHA	4723.37	KRAS, THBS1
PRKAG3	302.718	ABCA1, ATP6V1A, CRYBG3, ITGA6, JUNB, MAP4, NRIP1, POLK, REV3L, RPL13A
TFF3	11.787	CDH1, CLDN1, FOS
LCN2	5.535	ANLN, CCL5, CDH1, CKAP2, CXCL2, IL1A, IL1B, TWIST1
IL37	5.515	IL1A, IL1B
TCF12	-4.528	AZGP1, CDH1, IL7R NR4A3, PTPRCAP
MGAT5	-5.378	CXCR2, ITGB1, TWIST1
FOXO3	-6.338	APAF1, CDH1, CLDN1, CXCL8, FOS, FOXO3, IER3, JUNB, MAP1LC3A, MKI67
MKL2	-6.608	CCL5, G0S2, ITGA6, ITGB1, LTF, S100A9
SMO	-6.803	ALCAM, BCL2, LIMD2, NRCAM,NRP2, VCAN
R-MFD fed the CTR diet compared with S-MFD fed the CTR diet		
PROC	22.629	NFKB2, NOS2, SERPINE1, THBD, THBS1, TJP1, TNFAIP3, VCAM1
IL17A	10.846	ACTA2, CCL17, CD274, CYR61, DEFB4A/DEFB4B, EREG, FAS, HSPB8, IL16, ITPR1
CSF2	9.543	ATXN1, BCL2L11, BCL3, BIRC3, C4A/C4B, C5AR1, CARD11, CD1A, CD28, CD69
ACVR1C	9.002	CDKN1A, JUNB, SERPINE1
DNASE1L3	6.041	CDKN1A
PDE4D	-24.642	BCL2L11, PLAT
FOXA1	-27.638	ACTA2, AQP3, BMP2, COL181A, EFHD1, ERBB3, MGP, NES, RORC, S100A2
EGFR	-45.696	ACTA2, BCL2L11, BCL6, BTC, CAV1, CDKN1A, COL6A1, COL6A2, CRYAB, DSC2
UPF2	-190.72	CYFIP2, DSP, KANK1, TNFAIP3, TSPAN12
CD82	-235.403	BCAR1, DCN
S-MFD fed the LIN diet compared with S-MFD fed the CTR diet		
PRLR	238.881	SLC34A2
NTRK1	64.772	NR4A1
IL17A	17.491	CXCL2, PTGS2
AGR2	4.967	MUC1
VIPR1	4.839	CXCL2
PROC	-2.623	CXCL2, NR4A1
HNF1A	-2.687	FBP2, FOLR1, LBP, PAH, PIGR
PLA2G10	-3.939	PTGS2
UPF2	-409.194	PTGS2
HDAC9	-1242.560	NR4A1
R-MFD fed the LIN diet compared with S-MFD fed the LIN diet		
DROSHA	2930.234	KRAS, THBS1
PROC	65.916	NR4A1, SERPINE1, THBD, THBS1, TJP1, TNF, TNFAIP3, VCAM1
CSF2	9.846	ABCA1, AKR1A1, ANLN, BCL2L1, BCL3, CCL3, CD38, CD86, CENPH, CISH
INHBA	7.861	ACVR1B, AGO1, ANKRD1, BCL2L1, BMP2, CALB1, CCR2, CDH1, CHD7, COX6A1
CCL2	6.175	ABCA1, CCL5, CCR2, CD86, FOLR2, INHBA, MKI67, MMP1, NOTCH1, SERPINE1
PAX5	-32.188	BLK, CD19, CD22, CSF2RA, FCER2, IGJ, IGLL1/IGLL5, MET, POU2AF1, PTPRF
RNASE1	-34.244	CCL24, CCL3, CCL5, CCL8, FCER2, OSM, TNF
DCN	-41.198	CDH1, CDKN1C, FBN1, ITGAV, KIT, MET, MMP1, MMP13, SDC1, SERPINE1
SIN3A	-326.801	ALDH6A1, BCL6, CCNG2, GADD45B, GSTP1, HOXA9, KLF10, KLF6, REST, SYT4
FOXM1	-7777.011	CAV1, CDH1, CENPB, FLT1, LEF1, PECAM1, SNAI1, TJP1, TOP2A, VCAN

[1]Key gene regulators

[2]Fold change

3.6. **Identification of genes harboring SNP**

Between 25,000 to 34,000 SNP were identified in each of the four cows analyzed. Of these, 6,700-7,300 SNP were identified in R-MFD cows, associated with the differentially expressed genes and key gene regulators lists in the three comparisons involving R-MFD cows. In S-MFD cows, 6,900-8,900 SNP were identified associated with of differentially expressed genes and key gene regulators lists in the three comparisons involving S-MFD cows. Among SNP identified associated with the list of differentially expressed genes and key genes regulators, 641 SNP were identified only in R-MFD cows and 1024 only in S-MFD cows (Table 5).

Table 5. *SNPs in R-MFD cows and S-MFD cows.*

Sample	Treatment	Total SNP	Total SNP in differentially expressed genes and Key genes Regulators	Specific Treatment SNP[1]
616	R-MFD	26365	6770	641
9274	R-MFD	28657	7312	
9412	S-MFD	33816	8971	1024
9413	S-MFD	25855	6946	

[1] SNP in differentially expressed genes and Key Genes Regulators only in animals R-MFD or S-MFD

The SNP identified as specific for R-MFD or S-MFD cows were classified according to the type of function (Table 6). In R-MFD cows, 63% of the SNP were intron-variant, 12% were synonymous codon, 7% were utr-variant-3-prime and the remaining 15% were downstream-variant-500B, missense, nc-transcript-variant, upstream-variant-2KB or utr-variant-5-prime. In S-MFD cows, 65% were intron-variant, 13% synonymous codon, 9% utr-variant-3-prime and the remaining 14% were missense, nc-transcript-variant, upstream-variant-2KB, utr-variant-5-prime, downstream-variant-500B, splice-acceptor-variant or stop-gained.

Table 6. *SNPs in differentially expressed genes and key gene regulators only in animals R-MFD or S-MFD classify by their function type.*

Function Type	R-MFD	S-MFD
Downstream-variant-500B	8 (1.25%)	8 (0.78%)
Intron-variant	425 (63.3%)	663 (64.7%)
Missense	39 (6.08%)	79 (7.71%)
Nc-transcript-variant	35 (5.46%)	21 (2.05%)
Synonymous codon	78 (12.2%)	130 (12.7%)
Upstream-variant-2KB	7 (1.09%)	19 (1.85%)
Utr-variant-3-prime	46 (7.18%)	89 (8.69%)
Utr-variant-5-prime	3 (0.47%)	8 (0.78%)
Intron-variant, upstream-variant-2KB		2 (0.19%)
Splice-acceptor-variant		1 (0.10%)
Stop-gained		1 (0.10%)
Upstream-variant-2KB, intron-variant		2 (0.19%)
Upstream-variant-2KB, utr-variant-5-prime		1 (0.10%)

3.7. **Identification of target genes differentially expressed, key genes regulators and contained SNP**

To identify the genes that could be involved in the synthesis of FA and lipid metabolism in the different comparisons conducted in this study, the genes that were differentially expressed (RPKM ≥ 0.2, p-value < 0.01 and FC > 2), key gene regulators and contained SNP in the two cows R-MFD or S-MFD at the same time were selected. These genes in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet would explain differences between S-MFD and R-MFD cows, and at the same time the effect of diet (Figure 3). The selected genes where used to check differences among the other comparisons.

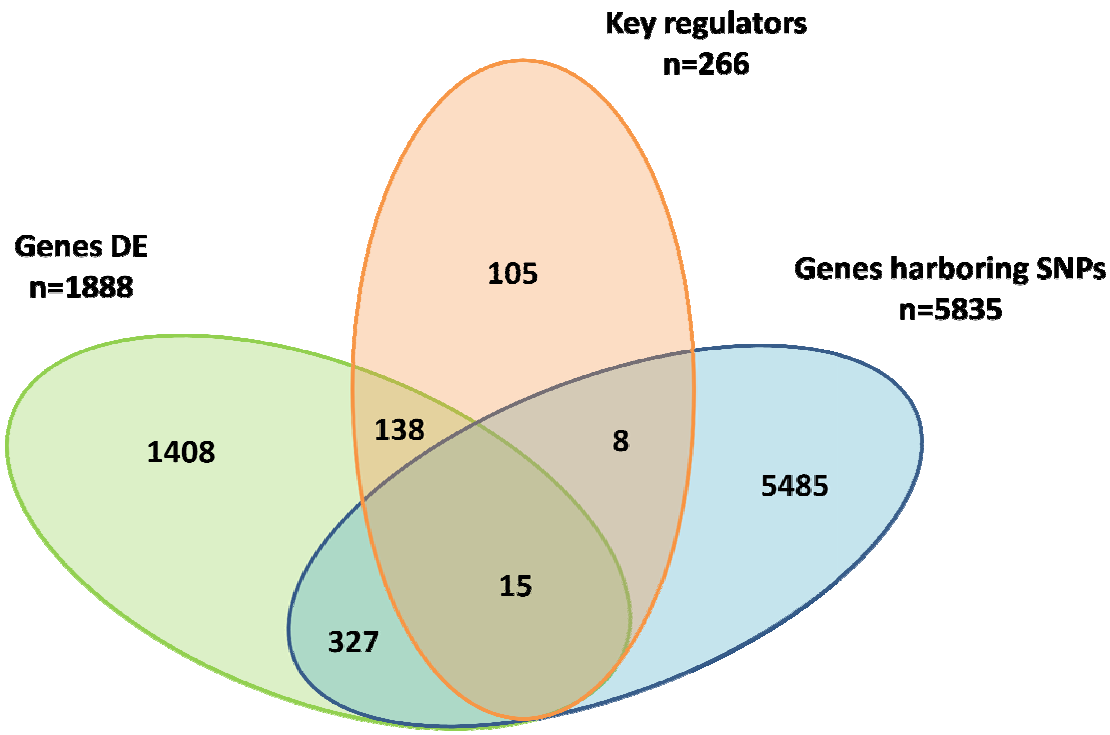

Figure 3. *Number of differentially expressed genes, key gene regulators, and genes harboring SNPs in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet.*

The R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet had 1888 differentially expressed genes, 266 key gene regulators and 5835 genes containing SNP. Of these, 15 genes were differentially expressed, were key gene regulators and contained SNP in the two R-MFD or S-MFD cows. These genes and their SNP are presented in Table 7.

Table 7. *Genes containing SNP associated with differentially expressed genes and key gene regulators in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet.*

Cow[1]	Gen	FC[2]	Chr.[3]	Chr. Position	SNP ID	Allele Change	Function type
S	APBB1	-3.36	15	45561080	rs207812421	C/T	synonymous-codon
S	APBB1	-3.36	15	45561104	rs209491693	T/C	synonymous-codon
S	APBB1	-3.36	15	45569813	rs210165374	G/C	missense
S	APBB1	-3.36	15	45569330	rs210595896	C/T	synonymous-codon
S	APBB1	-3.36	15	45569819	rs211146069	T/C	synonymous-codon
S	APBB1	-3.36	15	45576807	rs41255144	C/T	intron-variant
S	CD38	-2.31	6	115589806	rs109641719	C/T	intron-variant
S	CD38	-2.31	6	115591914	rs134955750	G/A	intron-variant
R	CD38	-2.31	6	115595101	rs136147162	G/C	utr-variant-3-prime
S	CD38	-2.31	6	115592674	rs43434904	C/T	intron-variant
S	CD38	-2.31	6	115589816	rs43434912	G/A	intron-variant
S	EREG	-9.50	6	92346668	rs42580620	G/A	intron-variant
R	FLT1	3.55	12	31554406	rs109247749	A/G	synonymous-codon
R	FLT1	3.55	12	31489476	rs111027111	C/T	intron-variant
R	FLT1	3.55	12	31483700	rs133983660	A/G	intron-variant
R	FLT1	3.55	12	31485999	rs136560138	G/C	synonymous-codon
R	FLT1	3.55	12	31528552	rs137508649	A/G	synonymous-codon
R	FLT1	3.55	12	31484135	rs207631114	G/C	intron-variant
R	FLT1	3.55	12	31483177	rs209090694	T/C	intron-variant
R	FLT1	3.55	12	31483898	rs209547908	A/G	intron-variant
R	FLT1	3.55	12	31483715	rs210883339	G/C	intron-variant
R	FLT1	3.55	12	31483473	rs211512991	T/C	intron-variant
S	ITGB4	-4.12	19	57146058	rs41926899	G/A	missense
S	ITGB4	-4.12	19	57157390	rs41927658	C/G	synonymous-codon
S	MTOR	-2.18	16	39224720	rs208757293	T/A	synonymous-codon
S	MTOR	-2.18	16	39235739	rs211448695	G/A	intron-variant
S	MTOR	-2.18	16	39231288	rs211677647	C/T	intron-variant
S	NFATC2	-6.51	13	80208242	rs137043317	T/C	synonymous-codon
S	NOTCH1	-2.47	11	107708189	rs110163085	C/G	intron-variant
R	NOTCH1	-2.47	11	107686385	rs133307736	A/G	intron-variant
S	NOTCH1	-2.47	11	107685263	rs207760072	A/G	intron-variant
S	NOTCH1	-2.47	11	107698629	rs211580903	G/A	synonymous-codon
S	NOTCH1	-2.47	11	107679366	rs378232535	C/T	missense
R	NOTCH2	-2.51	3	25061073	rs135438495	T/C	synonymous-codon
S	PDPK1	-3.58	25	2633350	rs208965123	T/C	intron-variant
S	PROM1	-17.9	6	115755844	rs110069470	A/G	synonymous-codon
S	PROM1	-17.9	6	115732932	rs42165955	A/C	missense
S	RICTOR	-2.90	20	37609145	rs41940570	T/C	utr-variant-3-prime
S	RICTOR	-2.90	20	37610024	rs41940571	A/G	utr-variant-3-prime
R	TGFBR3	-3.90	3	54795884	rs110491344	A/G	utr-variant-3-prime
R	TGFBR3	-3.90	3	54796526	rs134330950	T/C	utr-variant-3-prime
R	TGFBR3	-3.90	3	54798328	rs379514543	A/C	utr-variant-3-prime
R	WWC1	-7.19	7	82262781	rs108980081	C/T	utr-variant-3-prime
S	ZNF217	-2.06	13	82061022	rs134599263	A/G	intron-variant

[1]Cow: S = sensitive milk fat depression cows; R = resistant to milk fat depression cows

[2]Fold change

[3]Chromosome

Of the genes selected, mammalian target of rapamycin (MTOR) plays a central role in enhancing protein synthesis and cell growth (Wullschleger *et al.*, 2006). Moreover, recent studies have demonstrated that MTOR is related to lipid biosynthesis by controlling SREBP1 expression (Yecies *et al.*, 2011; Li *et al.*, 2010; 2011). Li *et al.* (2014) observed a positive feedback-loop regulation between SREBP1 and mTOR signaling pathways in dairy cow mammary epithelial cells. The SREBP is known to play an important role in the regulation of milk fat synthesis by activation of sterol responsive element containing genes such as ACACA and FASN involved in *de novo* FA shyntesis (Bionaz and Loor, 2008).

On the other hand, Portmann *et al.* (2008) reported that the activation of *de novo* FA shynthesis by Serine/threonine kinase (Akt) requires MTOR function. Akt plays a role in glycolisis and FA bioshyntesis by activation of ATP-citrate lyase (ACLY) and FASN. ACLY converts cytosolic citrate into acetyl-CoA and oxaloacetate to lipid biosynthesis. Activation of SREBP and Akt-dependent induction of lipid biosynthesis requires the activity of MTORC1. Moreover, Phosphoinositide-dependent kinase 1 (PDPK1), a selected gen, is related to phosphorylation and activation of Akt. Moreover, Akt is suggested to be downstream of mTORC2, which contains mTOR, rapamycin-insensitive companion of mTOR (RICTOR), a selected gen, and GβL (Sarbassov *et al.*, 2005) (Figure 4). However, MTOR, PDK1 and RICTOR were overexpressed in S-MFD cows fed the LIN diet compared with R-MFD cows fed the LIN diet and SNP were also in S-MFD cows.

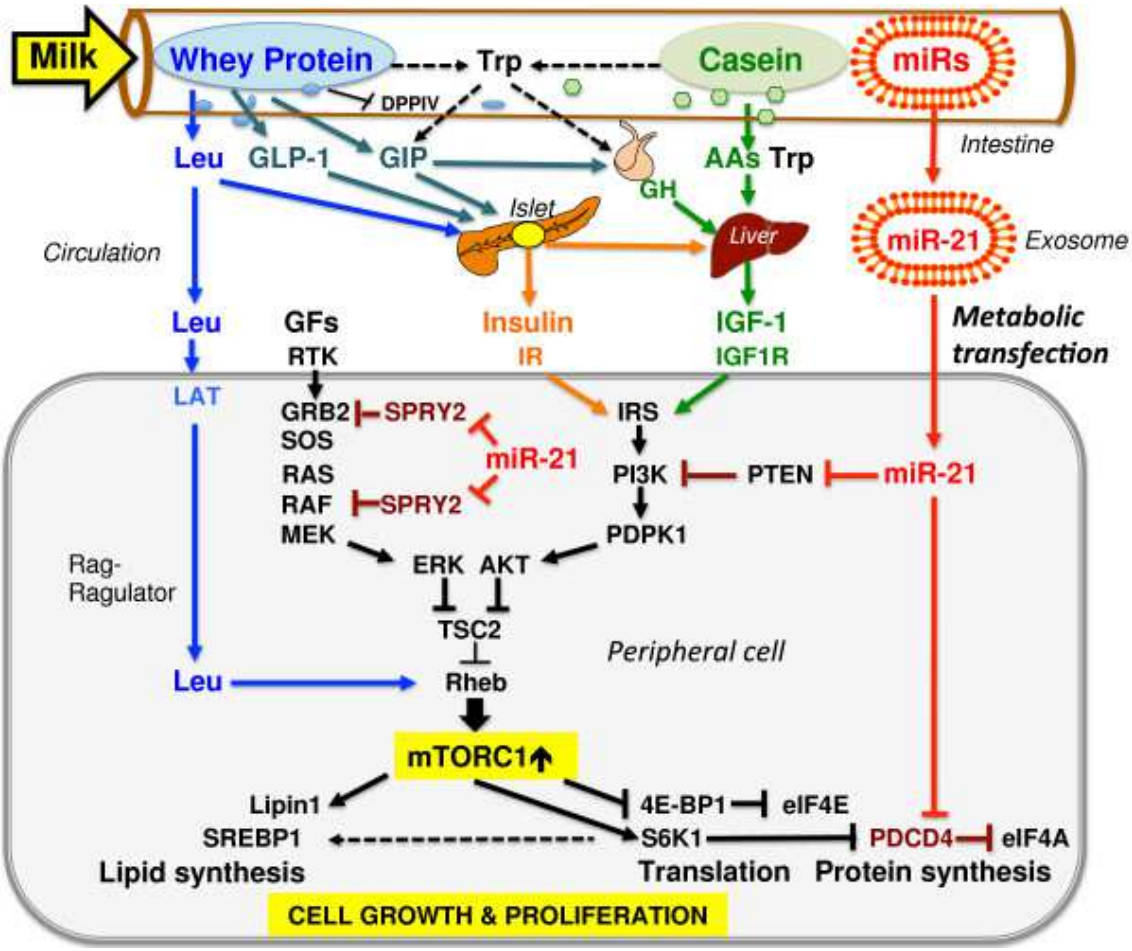

Figure 4. *Synoptic model of amino acid- and exosomal miR-mediated signaling of milk for the activation of mTORC1-mediated postnatal growth. Whey protein (WP)-derived leucine, isoleucine and valine stimulate insulin synthesis. WP (especially Trp) induces the incretrin GIP, which will further enhance insulin synthesis. Peptide fragments of WP hydrolysis competitively inhibit DPPIV, thereby extending GIP bioactivity. GIP via GIP-R on somatotroph pituitary cells stimulates the synthesis of GH, which up-regulates hepatic IGF-1-synthesis, further augmented by insulin and Trp. Leucine derived from milk proteins increases GLP-1. Increased insulin/IGF-1 signaling via inhibition of TSC2 activates mTORC1. WP-derived leucine via Rag/Ragulator interaction promotes the activation of mTORC1. mTORC1 stimulates lipid synthesis by phosphorylation of lipin1 and activation of S6K1, which enhance lipogenesis. Milk is hypothesized to operate as an exosome-driven miR transfection system of metabolism to increase mTORC1-driven anabolic reactions of the milk recipient. Especially, milk exosomes containing miR-21 may enhance mTORC1 signaling by suppression of tumor suppressor proteins PTEN, Sprouty and PDCD4. Adapted from Melnik et al.* (*2013).*

4. CONCLUSION

Differential expression analysis allowed to detect a large number of differentially expressed genes in all the comparisons conducted, except in the LIN *vs.* the CTR diet in S-MFD cows. These results suggest that R-MFD cows could be activating a compensatory mechanism to increase the FA synthesis in linseed-rich diets.

Genes involved in milk fat synthesis (*de novo* FA synthesis, desauration, FA activation, uptake, transport, TAG synthesis, lipid droplet formation and transcription factors) had changes in their expression in the different situations analyzed. These genes had different expression and different isoforms in control and linseed diets between S-MFD and R-MFD cows, and these differences could explain the resistance to MFD.

Pathway and key gene regulators analysis allowed to detect a large number of pathways and key gene regulators differently expressed between diets and cows. These metabolic pathways and key gene regulators are involved in immune and inflammatory system, development and growth processes, lipid metabolism and FA synthesis.

Analysis of SNP discovery showed 641 SNP only in R-MFD cows and 1024 only in S-MFD cows among differentially expressed genes in all comparisons. Finally, 15 genes were detected as differentially expressed genes, key gene regulators and harboring SNP in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet. Among them, MTOR and PDK1 seem to be involved in milk fat synthesis. However, these genes were overexpressed in S-MFD cows and SNP were also in S-MFD cows.

Results suggest that more complicated networks are involved in milk fat synthesis and in the response to diets that induced MFD. The R-MFD cows had a higher expression of different isoforms of genes involved in milk fat synthesis compared with S-MFD cows, which could explain the resistance to MFD.

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6. ANNEX 1

Table A1. *Significant pathways (P < 0.05), and their type of regulation observed in S-MFD cows fed the LIN diet compared with S-MFD cows fed the CTR diet.*

Pathways	**Nº genes**	**% genes differentially expressed**	**Regulation**
Eicosanoid Signaling	81	70	↑
Glucocorticoid Receptor Signaling	267	90	↓
Tyrosine Biosynthesis IV	7	40	↑
Corticotropin Releasing Hormone Signaling	121	85	↓
Phenylalanine Degradation I (Aerobic)	11	37	↑
LXR/RXR Activation	128	80	↑
Calcium Transport I	13	65	↑
Prostanoid Biosynthesis	15	55	↑
Extrinsic Prothrombin Activation Pathway	18	82	↓
Fatty Acid α-oxidation	19	75	↓
Acutate Phase Response Signaling	171	88	↓
Granulocyte Adhesion and Diapedesis	179	75	↑
ILK Signaling	188	90	↓
CDP-diacylglycerol Biosynthesis I	27	50	↑

Table A2. *Significant pathways (P < 0.01), and their type of regulation observed in R-MFD cows fed the LIN diet compared with R-MFD cows fed the CTR diet.*

Pathways	**Nº genes**	**% genes differentially expressed**	**Regulation**
PI3K Signaling in B Lymphocytes	133	87	↓
Glucocorticoid Receptor Signaling	267	90	↑
T Cell Recpector Signaling	102	91	↓
B Cell Receptor Signaling	181	87	↓
IL-8 Signaling	187	92	↓
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Artritis	304	86	↓
Role of NFAT in Regulation of the Immune Response	177	82	↓
Docosahexaenoic Acid (DHA) Signaling	45	84	↓
IGF-1 Signaling	99	95	↓
JAK/Stat Signaling	72	97	↓
RANK Signaling in Osteoclasts	89	94	↓
Prolactin Signaling	76	91	↓
CD28 Signaling in T Helper Cells	123	81	↑
Role of Tissue Factor in Cancer	112	93	↓
iCOS-iCOSL Signaling in T Helper Cells	113	80	↓
fMLP Signaling in Neutrophils	115	88	↓
3-phophoinositide Biosynthesis	168	80	↓
ILK Signaling	188	89	↓
GDNF Family Ligand-Receptor Intactions	71	88	↓
Renin-Angiotensin Signaling	118	87	↓
Melonocyte Development and Pigmentation Signaling	86	92	↓
Netrin Signaling	44	81	↓
Myc Mediated Apoptosis Signaling	58	100	↓
FcyRIIB Signaling in B Lymphocytes	45	85	↓
UVA-Induced MAPK Signaling	89	92	↓
FLT3 Signaling in Hematopoietic Progenitor Cells	74	93	↓
Cardiac Hypertrophy Signaling	232	87	↓
Insulin Receptor Signaling	140	88	↓
RhoA Signaling	124	90	↓
TR/RXR Activation	92	84	↓
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	127	80	↓
IL-4 Signaling	78	85	↓
Virus Entry via Endocytic Pathways	95	87	↓
Leulocite Extravasation Signaling	204	88	↓
Systemic Lupus Erythematosis Signaling	224	63	↑
Role of Osteoblasts, Osteoclasts and Chondiocytes in Rheumatoid Arthritis	225	88	↓
Regulation if IL-2 Expression in Activated and Anergic T Lymphocytes	81	88	↓
Regulation if eIF4 and p7056K Signaling	150	90	↑
Molecular Mechanisms of Cancer	373	90	↓
Ovarian Cancer Signaling	133	90	↓
IL-6 Signaling	116	92	↓
ERK/MAPK Signaling	191	88	↓
Endometrial Cancer Signaling	54	93	↓
Gαq Signaling	155	86	↓
Prostate Cancer Signaling	86	90	↓

Superpathway of Inositol Phosphate Compounds	215	80	↓
Non-Small Cell Lung Cancer Signaling	71	89	↓
Role of IL-17A in Arthritis	56	88	↑
Neurotrophin/TRK Signaling	72	88	↓
Cholecystokinin/Gastrin-mediated Signaling	106	88	↓
HGF Signaling	108	94	↓
Pancreatic Adenocarcinoma Signaling	108	94	↓
Melanoma Signaling	44	94	↓
Aryl Hydrocarbon Receptor Signaling	146	86	↓
Phospholipase C Signaling	247	81	=
Chronic Myeloid Leukemia Signaling	93	97	↓
EIF2 Signaling	188	87	↑
SAPK/JNK Signaling	95	90	↓
NGF Signaling	113	92	↓
3-phosphoinositide Degradation	151	81	↓
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	133	81	↓
D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	133	81	↓
Role of JAK1 and JAK3 in yc Cytokine Signaling	63	92	↓
GM-CSF Signaling	63	95	↓
Huntington's Disease Signaling	236	88	↓
Signaling by Rho Family GTPases	236	90	↓
Telomerase Signaling	99	93	↓
Role of JAK2 in Hormone-like Cytokine Signaling	35	81	↓
Ephin A Signaling	50	90	↓
Breast Cancer Regulation by Stathmin1	197	90	↓
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	23	90	↑
HMGB1 Signaling	120	93	↑
PTEN Signaling	120	90	↓
Ceramide Signaling	84	90	↓
Dendritic Cell Maturation	179	75	↑
Paxillin Signaling	102	93	↓
Protein Kinase A Signaling	398	86	↓
Hepatic Fibrosis/Hepatic Stellate Cell Activation	201	85	↑
CDK5 Signaling	103	88	↓
CNTF Signaling	52	90	↓
PKC0 Signaling in T Lymphocytes	122	80	↓
ErbB Signaling	86	92	↓
UVB-Induced MAPK Signaling	53	93	↓
April Mediated Signaling	38	100	↑
Germ Cell-Stertoli Cell Junction Signaling	163	92	↓
Erythropoietin Signaling	70	93	↓
P7056K Signaling	125	92	↓
IL-17A Signaling in Gastric Cells	25	94	↑
IL-3 Signaling	71	94	↓
PEDF Signaling	71	100	↓
Endothelin-1 Signaling	186	81	↓
Role of NFAT in Cardiac Hypertrophy	186	88	↓
Rac Signaling	107	93	↓
IL-2 Signaling	55	94	↓
IL-17 Signaling	72	92	↓
Clathrin-mediated Endocytosis Signaling	188	88	↓
B Cell Activating Factor Signaling	40	95	↑
DNA Double-Strand Break Repair by Homologous Recombination	14	100	↓

Lymphotoxin β Receptor Signaling	56	88	↓
LPS-stimulated MAPK Signaling	73	100	↓
Growth Hormone Signaling	73	88	↓
Renal Cell Carcinoma Signaling	73	90	↓
P2Y Purigenic Receptor Signaling Pathway	129	88	↓
NF-κB Activation by Viruses	74	94	↓

Table A3. *Significant pathways (P < 0.01), and their type of regulation observed in R-MFD cows fed the CTR diet compared with S-MFD cows fed the CTR diet.*

Pathways	**Nº genes**	**% genes differentially expressed**	**Regulation**
Tec Kinase Signaling	161	87	↓
Leukocyte Extracasation Signaling	204	85	↓
ILK Signaling	188	85	↓
Role of Tissue Factor in Cancer	112	92	↓
Hepatic Fibrosis/Hepatic Stellate Cell Activation	201	85	↓
Granulocyte Adhesion and Diapedesis	179	70	↓
VDR/RXR Activation	79	92	↓
Virus Entry via Endocytic Pathways	95	88	↓
HER-2 Signaling in Breast Cancer	76	95	↓
Reelin Signaling in Neurons	79	94	↓
Neuregulin Signaling	93	87	↓
Signaling by Rho Family GTPases	236	90	↓
Stertoli Cell-Stertoli Cell Junction Signaling	186	85	↓
Molecular Mechanisms of Cancer	373	90	↓
TGF-β Signnaling	87	98	↓
Thrombin Signaling	197	88	↓
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	304	85	↓
Epithelial Adherens Junction Signaling	148	87	↓
NF-κB Signaling	173	90	↓
Paxillin Signaling	102	93	↓
IL-8 Signaling	187	92	=
Germ Cell-Stertoli Cell Junction Signaling	163	93	↓
Oncostatin M Signaling	34	100	↓
Integrin Signaling	202	91	↓
Ephin Receptor Signaling	177	90	↓
Agranulocyte Adhesion and Diapedesis	190	70	↓
Semaphorin Signaling in Neurons	53	95	=
HGF Signaling	108	94	↓
HMGB1 Signaling	120	94	↓
Macropinocytosis Signaling	68	95	↓
Glioma Invasiveness Signaling	58	95	↓
Erythropoietin Signaling	70	93	↓
Inhibition of Matrix Metalloproteases	40	80	↓
Chronic Myeloid Leukemia Signaling	93	98	↓
Tight Junction Signaling	167	88	↓
Gαq Signaling	155	87	↓
Cholecystokinin/Gastrin-mediated Signaling	106	88	↓
Rac Signaling	107	94	↓
PTEN Signaling	120	92	↓
NF-κB Activation by Viruses	74	95	↓
Sperm Motility	135	75	↓
IL-9 Signaling	34	95	↓
Prolactin Signaling	76	93	↓
Acutate Myeloid Leukemia Signaling	77	95	↓
Axonal Guidance Signaling	439	87	↓
Phospholipase C Signaling	247	81	↓
Dendritic Cell Mturation	179	75	↓
RhoGDI Signaling	179	87	↓
Lymphotoxin β Receptor Signaling	56	90	↓

IL-15 Signaling	67	98	↓
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	127	80	↓
eNOS Signaling	154	80	↓
B Cell Receptor Signaling	181	87	↓
Death Receptor Signaling	92	90	↓
PPARα/RXRα Activation	184	80	↓
Colorectal Cancer Metastasis Signaling	241	87	↓
Thrombopoietin Signaling	59	90	↓
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	186	87	↓
TNFR2 Signaling	29	95	↓
IL-3 Signaling	71	94	↓
TNFR1 Signaling	49	95	↓
CXCR4 Signaling	160	88	↓
Ephrin A Signaling	50	90	↓
Role of NFAT in Regulation of the Immune Response	177	80	↓
ERK/MAPK Signaling	191	90	↓
ErbB Signaling	86	93	↓
G-Protein Coupled Receptor Signaling	264	87	↓
Glioblastoma Multiforme Signaling	151	90	↓
Fcy Receptor-mediated Phagocytosis in Macrophages and Monocytes	99	90	↓
Role of JAK1 and JAK3 in yc Cytokine Signaling	63	90	↓
Sphingosine-1-phosphate Signaling	113	87	↓
TREM1 Signaling	76	80	↓
Apoptosis Signaling	89	92	↓
Breast Cancer Regulation by Stathmin1	197	88	↑
IL-22 Signaling	24	90	↓
HIF1α Signaling	104	87	↓
TWEAK Signaling	34	95	↓
Factors Promoting Cardiogenesis in Vertebrates	92	88	↓
Gα12/13 Signaling	118	90	↓
Role of IL-17A in Arthritis	56	87	↓
Actin Nucleation by ARP-WASP Complex	56	98	=
Angiopoietin Signaling	68	90	↓
Role of JAK family kinases in IL-6-type Cytokine Signaling	25	95	↓
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	81	87	↓
Coagulation System	35	87	↓
Actin Cytoskeleton Signaling	221	88	↓
CCR3 Signaling in Eosinophils	122	88	=
Glucocorticoid Receptor Signaling	267	90	↓
PEDF Signaling	71	100	↓
NRF2-mediated Oxidative Stress Response	180	90	↑
Atherosclerosis Signaling	124	80	↓
IL-10 Signaling	72	87	↓
T Helper Cell Differentation	72	80	↓
JAK/Stat Signaling	72	95	↓
IL-15 Production	27	90	↓
P53 Signaling	98	95	↓
Human Embryonic Stem Cell Pluripotency	139	80	↓
LPS-stimulated MAPK Signaling	73	98	↓
Notch Signaling	38	87	↓

iCOS-iCOSL Signaling in T Helper Cells	113	80	↓
CCR5 Signaling in Macrophages	74	80	=
FLT3 Signaling in Hematopoietic Progenitor Cells	74	93	↓
Bladder Cancer Signaling	87	85	↓
LXR/RXR Activation	128	80	↓
Inhibition of Angiogenesis by TSP1	39	80	↓

Table A4. *Significant pathways (P < 0.01), and their type of regulation observed in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet.*

Pathways	**Nº genes**	**% genes differentially expressed**	**Regulation**
Role of Tissue Factor in Cancer	112	90	↓
Glucocorticoid Receptor Signaling	267	87	↓
Acutate Myeloid Leukemia Signaling	77	93	↓
IL-9 Signaling	34	95	↓
ILK Signaling	188	87	↓
Oxidative Phosphorylation	119	75	↑
Stertoli Cell-Stertoli Cell Junction Signaling	186	87	↓
Mitochondrial Dysfunction	188	80	↑
Virus Entry via Endocytic Pathways	95	87	↓
Molecular Mechanisms of Cancer	373	90	↓
Leukocyte Extravasation Signaling	204	90	↓
Tec Kinase Signaling	161	87	↓
HMGB1 Signaling	120	90	↓
FLT3 Signaling in Hematopoietic Progenitor Cells	74	93	↓
IL-15 Signaling	67	98	↓
Germ Cell-Stertoli Cell Junction Signaling	163	90	↓
Neuregulin Signaling	93	87	↓
Paxillin Signaling	102	93	↓
Reelin Signaling in Neurons	79	98	↓
JAK/Stat Signaling	72	95	↓
PTEN Signaling	120	90	↓
Epithelial Adherens Junction Signaling	148	87	↓
Integrin Signaling	202	90	↓
Axonl Guidance Signaling	439	85	↓
Oncostatin M Signaling	34	100	↓
IL-8 Signaling	187	90	↓
Chronic Myeloid Leukemia Signaling	93	98	↓
GM-CSF Signaling	63	98	↓
VDR/RXR Activation	79	90	↓
NF-κB Signaling	173	87	↓
RAR Activation	183	90	↓
Ephrin A Signaling	50	90	↓
Actin Cytoskeleton Signaling	221	87	↓
Colorectal Cancer Metastasis Signaling	241	88	↓
UVA-Induced MAPK Signaling	89	93	↓
Acutate Phase Response Signaling	171	87	↓
Prolactin Signaling	76	90	↓
Estrogen Receptor Signaling	127	94	↑
ERK/MAPK Signaling	191	88	↓
eNOS Signaling	154	81	↓
ErbB Signaling	86	93	↓
Erythropietin Signaling	70	93	↓
Regulation of the Epithelial-Mesenchymal Transition Pathway	184	90	↓
TGF-β Signaling	87	93	↓
PEDF Signaling	71	98	↓
Tight Junction Signaling	167	90	↓
Ephrin Receptor Signaling	177	90	↓

Lymphotoxin β Receptor Signaling	56	90	↓
VEGF Signaling	97	90	↓
Granulocute Adhesion and Diapedesis	179	75	↓
B Cell Receptor Signaling	181	88	↓
IGF-1 Signaling	99	93	↓
Glioma Invasiveness Signaling	58	95	↓
NF-κB Activation by Viruses	74	95	↓
STAT3 Pathway	74	90	↓
HGF Signaling	108	94	↓
Hereditary Breast Cancer Signaling	117	90	↓
Hepatic Fibrosis/Hepatic Stellate Cell Activation	201	87	↓
Renin-Angiotensin Signaling	118	87	↓
Melanoma Signaling	44	94	↓
Aryl Hydrocarbon Receptor Signaling	146	87	↓
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	304	87	↓
Angiopoietin Signaling	68	90	↓
T Cell Receptor Signaling	102	93	↓
Semaphoin Signaling in Neurons	53	88	↓
Melanocyte Development and Pigmentation Signaling	86	88	↓
Insulin Receptor Signaling	140	87	↓
Endometrial Cancer Signaling	54	93	↓
Androgen Signaling	114	90	↓
NRF2-mediated Oxidative Stress Response	180	90	↑
Ovarian Cancer Signaling	133	88	↓
PI3K Signaling in B Lymphocytes	133	88	↓
Non-Small Cell Lung Cancer Signaling	71	88	↓
Role of JAK1 and JAK3 in yc Cytokine Signaling	63	93	↓
Rac Signaling	107	94	↓
Role of IL-17A in Arthritis	56	88	↓
FAK Signaling	90	90	↓
PPARα/RXRα Activation	184	85	↓
Renal Cell Carcinoma Signaling	73	93	↓
ErbB2-ErbB3 Signaling	57	90	↓
PAK Signaling	91	93	↓
TR/RXR Activation	92	82	↓
Thrombin Signaling	197	90	↓
Coagulation System	35	93	↓
Thrombopoietin Signaling	59	88	↓
TREM1 Signaling	76	80	↓
Agranulocyte Adhesion and Diapedesis	190	75	↓
Macropinocytosis Signaling	68	94	↓
iCOS-iCOSL Signaling in T Helper Cells	113	81	↓
Cardiac Hypertrophy Signaling	232	87	↓
Aldosterone Signaling in Ephitelial Cells	162	88	↓
IL-4 Signaling	78	85	↓
Docosahexaeboic Acid (DHA) Signaling	45	85	↓
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	23	90	↑
IL-3 Signaling	71	95	↓
GDNF Family Ligand-Receptor Interactions	71	90	↓
p53 Signaling	98	93	↓
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	127	80	↓
Regulation of IL-2 Expression in Activated and Anergic T	81	92	↓

Lymphocytes			
RhoGDI Signaling	179	87	↓
Actin Nucleation by ARP-WASP Complex	56	93	↑
Death Receptor Signaling	92	88	↓
Inhibition of Matrix Metalloproteases	40	87	↓
Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	18	88	↑
Role of JAK family kinases in IL-6-type Cytokine Signling	25	100	↓
Glutathione Redox Reactions I	25	65	↑
Nitric Oxide Signaling in the Cardiovascular System	112	80	↓
Estrogen-Dependent Breast Cancer Signaling	66	90	↓
CCR3 Signaling in Eosinophils	122	87	↓
Antioxidant Action of Vitamin C	103	85	↓
Protein kinase A Signaling	398	87	↓
Antiproliferative Role of Somatostatin Receptor 2	67	87	↓
HER-2 Signaling in Breast Cancer	76	95	↓
HIF 1α Signaling	104	90	↓
Mouse Embryonic Stem Cell Pluripotency	95	92	↓
RhoA Signaling	124	88	↓
Prostate Cancer Signaling	86	90	↓

Table A5. *Key gene regulators (FC>2) and their regulated genes (10) in S-MFD cows fed the LIN diet compared with S-MFD cows fed the CTR diet.*

Transcription factor/Regulator	**FC**	**(n)**	**Regulated Genes (10)**
PRLR	38.881	1	SLC34A2
NTRK1	64.772	1	NR4A1
IL17A	17.491	2	CXCL2, PTGS2
AGR2	4.967	1	MUC1
VIPR1	4.839	1	CXCL2
IL17F	4.141	2	CXCL2, PTGS2
PROK1	3.603	1	PTGS2
NR4A1	3.526	2	CIDEA, FBP2
PLA2G2F	3.486	1	PTGS2
IL6	3.152	7	ABCG2, CXCL2, FGG, LBP, MUC1, PTGS2, SCGB2A2
ELF5	2.723	1	CRISP3
WNT5A	2.491	2	CXCL2, PTGS2
ABCC4	2.295	1	ABCG2
CSF2	2.292	3	CXCL2, NR4A1, PTGS2
PGR	2.266	2	ALOX15, PTGS2
HPSE	2.135	1	PTGS2
IL22	2.104	3	CXCL2, LBP, MUC1
FFAR4	2.090	1	PTGS2
HDC	-2.066	1	PTGS2
AMH	-2.273	1	CXCL2
COCH	-2.331	1	CXCL2
CDH4	-2.513	2	PTGS2
PROC	-2.623	2	CXCL2, NR4A1
HNF1A	-2.687	5	FBP2, FOLR1, LBP, PAH, PIGR
PLA2G10	-3.939	1	PTGS2
UPF2	-409.194	1	PTGS2
HDAC9	-1242.560	1	NR4A1

Table A6. *Key gene regulators (FC>2) and their regulated genes (10) in R-MFD cows fed the LIN diet compared with R-MFD cows fed the CTR diet.*

Transcription factor/Regulator	FC	(n)	Regulated Genes (10)
DROSHA	4723.37	2	KRAS, THBS1
PRKAG3	302.718	13	ABCA1, ATP6V1A, CRYBG3, ITGA6, JUNB, MAP4, NRIP1, POLK, REV3L, RPL13A
TFF3	11.787	3	CDH1, CLDN1, FOS
LCN2	5.535	8	ANLN, CCL5, CDH1, CKAP2, CXCL2, IL1A, IL1B, TWIST1
IL37	5.515	2	IL1A, IL1B
GATA1	4.921	13	ABCC4, BCL2, CCL5, IKZF1, IL6R, IL7R, ITGA6, JUNB, MITF, NFE2
CXCL2	4.795	4	CCL5, CXCL2, CXCR2, IL1B
MUC1	4.524	4	ABCC1, CDH1, CXCL8, MKI67
INHBA	4.508	13	AGO1, BCL2, CCR2, CDH1, CXCL8, INHBA, JUNB, KRAS, NAV3, NR4A2
PTHLH	4.385	5	BCL2, CXCL8, FOS, FOSL2, ITGA6
AZGP1	4.334	6	AZGP1, CDH1, DSP, HK2, KRT19, MTOR
PLA2G2A	4.059	2	CD69, CXCL8
RAMP3	3.819	1	FOS
PRKG1	3.698	4	ALOX15, BCL2, FOS, THBS1
CD69	3.547	4	CCL5, CD69, IL1B, S100A9
CXCL12	3.541	16	ATRX, BCL2, CCL5, CCNT1, CD69, CDH1, CXCL8, FOS, GOPC, HIPK3
KCNIP3	3.313	3	EDEM1, FOS, FOSL2
TWIST1	3.297	12	ALCAM, ASPM, BCL2, BMI1, CDH1, CSN2, CXCL8, FOS, IL1B, LALBA
WISP2	3.282	8	CDH1, CLDN1, DSP, LAMB3, MUC1, TFF3, TGFBR1, TWIST1
PROK2	3.279	3	BCL2, FOS, IL1B
TWIST2	3.249	5	BMI1, CDH1, IL1B, MAF, TWIST1
SLC1A2	3.168	1	FOS
IL1B	3.143	56	ALOX15, BCL2, CCL5, CCR2, CCRL2, CPB2, CXCL2, CXCL8, DUSP1, EDN1
F2	3.07	25	B4GALT1, CALD1, CASP2, CCL8, CITED4, CXCL8, DUSP1, EDN1, EDNRB, EHD4
KLK5	3.065	3	CXCL2, IL1B, S100A9
ALOX15	2.893	3	ALOX15, BCL2, IL1B
PGF	2.785	5	CXCL8, EDN1, IL1B, NRP2
CMTM8	2.772	1	CDH1
CCL5	2.659	12	AHR, ALCAM, CCL5, CCRL2, CXCL2, CXCL8, DUSP1, FOS, IER2, IL1B
IL6	2.632	47	ABCA1, ABCC1, AHR, BATF, BCL2, BMI1, CCL5, CCR2, CDH1, CEACAM1
PTX3	2.581	6	CCL5,CCR2, CXCL2, CXCL8, FOS, IL1B
CXCL8	2.579	8	BCL2, CD69, CXCL2, CXCL8, CXCR2, IL1B, MTPN, TGFBR1
EPO	2.544	16	BCL2, CD52, EDN1, FOS, GCC2, ITGB1, JUNB, KLHL24, KMT2A, LY6E
IL12A	2.536	5	BCL2, CCL5, IL1A, IL1B, IRF9
EGFR	2.467	21	BCL2, CCL5, CDH1, CXCL8, DAG1, DUSP1, E2F3, FOS, FOSL2, GZMB
IL1A	2.385	5	BCL2, CCL5, CCL8, CCR2, CXCL2, CXCL8, FOS, GNB4, IL1A, IL1B
CORT	2.385	16	CCL5, CCR2, IL1A, IL1B, MIF
ERBB4	2.382	7	BRCA1, CDH1, CSN2, DUSP1, GZMH, LALBA, THBS1
SELE	2.381	2	FOS, IL1B
FOS	2.379	30	ABCC1, AFF4, B4GALT1, CALU, CDH1, CDON, CXCL8, EDN1, EEA1, FOS
HCAR2	2.341	3	IL1B, IQGAP3, KIF20A
TF	2.313	3	CCL5, CXCL8, CYBRD1
CSF2	2.299	31	ABCA1, ANLN, APAF1, BCL2, CD69, CEACAM1, CXCL2, CXCL8, EDN1, FFAR2

CDKN2A	2.269	20	APAF1, BARD1, BCL2, BMI1, CCL5, CXCR2, DCTN4, DUSP1, E2F3, GFRA2
OSCAR	2.234	2	BCL2, CXCL8
CEBPE	2.194	4	BCL2, CSF3R, LTF, NCF1
GAL	2.177	3	BCL2, CSN2, FOS
RARRES2	2.174	3	CCL5, CXCL2, IL1B
DUSP1	2.163	7	BCL2, CXCL2, CXCL8, DUSP1, IL1A, IL1B, ISG20
CNTFR	2.146	2	CXCL8, IL1A
FADD	2.145	7	ARID5A, CXCL2, CXCL8, FOS, JUNB, LY6E, RC3H2
DGAT2	2.144	2	ACACA, DGAT2
IL4	2.136	53	AHR, ALOX15, BBX, BCL2, CCL5, CCL8, CD58, CD69, CLEC4G, CPOX
SOX10	2.108	4	EDNRB, FOS, GZMB, MITF
EDN1	2.102	15	ATF6, BCL2, CDH1, CXCL8, EDN1, EDNRB, FOS, ITGB1, JUNB, MITF
LY6E	2.086	2	CXCL8, IL1B
NUPR1	2.077	36	API5, ARHGAP11A, ASPM, ATP6V0A1, ATP8B1, BRCA1, CASC5, CASP2, CXCL8, FLVCR1
MIF	2.059	6	CXCL2, CXCL8, DUSP1, FOS, GZMB, IL1B
SLC2A4	2.04	3	ACACA, GCK, LNPEP
DOK3	2.024	1	NFATC2
VIPR2	2.009	2	JUNB, MAF
ZNF580	2.006	1	CXCL8
SSTR2	-2.000	3	BCL2, FOS, GCK
DOCK8	-2.018	7	ARID5A, BBX, EDN1, INHBA, ISG20, RBL1, TNFSF8
ABCC1	-2.023	2	BCL2, NCF1
RC3H1	-2.031	4	CCL5, CXCL2, IL1A, IL1B
TNFSF10	-2.045	7	BCL2, CXCL8, DPYD, FADD, FOS, IRF9, TNFSF10
TLR5	-2.054	5	CCL5, CXCL2, CXCL8, IL1A, IL1B
ITGB1	-2.064	11	ABCC1, BCL2, CDH1, CSN2, CXCL8, DAG1, FOS, IL1B, ITGAV, JUNB
BANF1	-2.067	1	S100A9
EPAS1	-2.131	10	ACACA, CCR2, CXCL2, CYBRD1, DMXL1, EDN1, FOS, ITGAV, MIF, TNFSF15
PTGER2	-2.248	12	ASPM, BRCA1, CDH1, CXCL8, CXCR2, IL1A, IL1B, KIF18B, KIF20A, MASTL
PTPN11	-2.264	9	CSF3R, CSN2, CXCL8, FOS, LTF, NF1, PTPN11, TP53I11, ZMAT3
SNAI2	-2.284	6	BMI1, CDH1, CLDN1, DSP, ITGB1, MUC1
IFNLR1	-2.32	6	CCL5, CXCL2, IL1A, IL1B, ITGAV, THBS1
CYFIP2	-2.329	1	KRAS
CCR2	-2.333	5	CCL5, CCR2, CXCL2, IL1A, IL1B
SIN3A	-2.345	5	ALDH6A1, BCL2, MAP4, REST, S100A9
ANKS1A	-2.346	1	FOS
BMI1	-2.389	5	BMI1, CDH1, DLC1, HK2, IKZF1
MTOR	-2.400	13	ACACA, AKAP13, BCL2, CDH1, FADD, MTOR, MUC1, OAS1, PRKAR2A, RPL32
IFNAR1	-2.433	7	CCL5, CXCL2, IL1A, IL1B, ITGAV, OAS1, THBS1
ACKR2	-2.439	5	CCL5, IL1A, IL1B, ISG20, OAS1
IL6R	-2.444	8	ABCC1, CCL5, CXCL2, CXCL8, GAL, IL1B, IL6R, TNFRSF11A
EGR3	-2.449	4	CBLB, LMO7, NF1, PTPN11
DCN	-2.466	6	CDH1, DUSP1, IL1B, ITGAV, ITGB1, THBS1
TCF4	-2.479	5	EDN1, MITF, NCOR2, TCF7L2, VCAN
NUP153	-2.521	1	SENP1
OCLN	-2.523	1	CLDN1
ETV1	-2.528	3	BRCA1, MITF, TWIST1
KLF13	-2.576	2	CCL5, GYPC
STIM1	-2.661	2	NFATC2, NFATC3
APLN	-2.678	4	BCL2, FOS, NCF1, TESC

MKI67	-2.723	1	CDH1
KRAS	-2.778	25	ABCA1, ABCC4, AHR, BCL2, BMI1, CASP2, CDH1, CXCL8, FOS, GZMK
MAPK9	-2.790	17	ACACA, BCL2, BDP1, CXCL8, EDN1, FOS, FOSL2, IER2, IL1B, IRF9
NFASC	-2.824	2	GLDN, NRCAM
TNFSF11	-2.879	23	AKAP13, BCL2, CCL5, CSN2, CXCL8, DUSP1, DUSP16, FOS, IER3, IL1A
ITGAV	-2.896	4	BCL2, CDH1, FOS, ITGB1
ADAM10	-3.135	9	ATRX, CCR2, FOS, GNAQ, GZMH, HIP1, HIP1K, INHBA, S100A9
NCOA2	-3.141	5	AHR, CCL5, HCAR2, IL1A, IL1B
E2F3	-3.201	6	BMI1, E2F3, MCM10, NPAT, RBL1, TOP2B
MTPN	-3.213	8	BCL2, EDN1, FADD, FOS, LTB, MIF, MTPN, TNFSF10
ESR1	-3.218	28	BCL2, BRCA1, C1QTNF6, CD69, CDH1, CSN2, CXCL8, EDN1, FOLR1, FOS
NFATC3	-3.289	7	BCL2, CD69, EDN1, FOS, NUPR1, PPP3R1, STIM1
NCOR2	-3.421	5	BCL2, CD69, CXCL8, FOS, IL1B
ERN1	-3.493	5	CDH1, CXCL2, DGAT2, SLC7A1, TCN1
PTPRJ	-3.513	9	ARID5A, BBX, CD69, CXCL2, EDN1, FOS, IL1A, INHBA, TNFSF8
NFATC2	-3.582	11	ABCA1, BATF, BBX, EDN1, IKZF1, IL9, INHBA, ISG20, PLD1, PPP3R1
BCL2	-3.594	7	ABCC1, BCL2, CXCL8, FOS, IL1B, ITGB1, RBL1
COCH	-3.621	2	CXCL2, IL1B
MITF	-3.647	15	ALCAM, BCL2, CXCL8, DAPK1, EDNRB, FOS, GNPTAB, GZMB, IL6R, ITPKB
PELI2	-3.781	2	CXCL2, CXCL8
ZNF423	-3.829	2	BRCA1, CD79B
MYCN	-4.289	15	ABCC1, BMI1, CCNT1, DUSP2, EEF1D, FAU, FKBP9, HK2, INHBA, ITGB1
RASGRF1	-4.309	2	FOS, KRAS
GRHL2	-4.464	3	BCL2, CDH1, NRP2
TCF12	-4.528	5	AZGP1, CDH1, IL7R NR4A3, PTPRCAP
MGAT5	-5.378	3	CXCR2, ITGB1, TWIST1
FOXO3	-6.338	12	APAF1, CDH1, CLDN1, CXCL8, FOS, FOXO3, IER3, JUNB, MAP1LC3A, MKI67
MKL2	-6.608	6	CCL5, G0S2, ITGA6, ITGB1, LTF, S100A9
SMO	-6.803	6	ALCAM, BCL2, LIMD2, NRCAM,NRP2, VCAN

Table A7. *Key gene regulators (FC>2) and their regulated genes (10) in R-MFD cows fed the CTR diet compared with S-MFD cows fed a CTR diet.*

Transcription factor/Regulator	FC	(n)	Regulated Genes (10)
PROC	22.629	8	NFKB2, NOS2, SERPINE1, THBD, THBS1, TJP1, TNFAIP3, VCAM1
IL17A	10.846	23	ACTA2, CCL17, CD274, CYR61, DEFB4A/DEFB4B, EREG, FAS, HSPB8, IL16, ITPR1
CSF2	9.543	49	ATXN1, BCL2L11, BCL3, BIRC3, C4A/C4B, C5AR1, CARD11, CD1A, CD28, CD69
ACVR1C	9.002	3	CDKN1A, JUNB, SERPINE1
DNASE1L3	6.041	1	CDKN1A
FLT1	5.952	7	CDKN1A, EPAS1, MARCKS, NOTCH1, RNF125, THBS1, VWF
MMP2	5.733	7	BMP2, CDKN1A, COL18A1, ITGA3, MMP1, OCLN, TJP1
RPL22	3.91	2	BCL2L11, CDKN1A
COL4A2	3.753	1	FAS
INHBA	3.674	17	ACTA2, ACVR1, ACVR1B, ACVR2B, ANKRD1, BMP2, CALB1, CDKN1A, CYP11A1, EREG
HNF4A	3.366	123	ABCA6, ACSL1, ACTA2, ACVR1, AKAP13, AQP3, AQP9, BCAR1, BCL6, BTN2A1
FABP4	3.322	4	MSR1, NOS2, PPARG, SOCS3
NOS2	3.266	15	AZGP1, CDKN1A, CLEC4D, COL4A1, CSF3R, CYCS, FAS, FASN, FOXO1, GPD1
CD9	3.128	4	ITGA2, ITGA3, TJP1, VEGFA
NR1H4	2.976	12	ACTA2, DDAH1, FABP7, FASN, HK3, NDRG2, PPARG, S1PR1, SDC1, SLC27A5
LGALS3	2.794	14	CDKN1A, COL4A1, ITPR3, KRT18, KRT19, LMNB1, MGST1, MSR1, MUC1, MOS2
LPL	2.7	5	LPL, MSR1, THBS1, VCAM1, VEGFA
NME1	2.68	4	CDKN1A, L1CAM, MET, SERPINE1
TXN	2.621	6	CDKN1A, MS4A1, NOS2, SELL, TXN, VEGFA
TXNRD1	2.585	2	FAS, TNFRSF25
SERPINF1	2.584	9	CRYAB, G0S2, LOXL1, NOS2, OCLN, PPARG, SOX10, THBS1, TJP1
NQO1	2.52	7	BIRC3, C5AR1, CDKN1A, CSF3R, KIT, NOS2, VCAM1
TMSB10/ TMSB4X	2.478	2	FAS, TMSB10/TMSB4X
TLR7	2.437	14	CALCA, CDKN1A, DMXL2, ETS2, FAIM3, GPR84, IDO1, PLAT, PTX3, RSAD2
FBXO32	2.355	12	ALDH1A3, BCL2L11, CADM1, CSRP2, HOOK1, ISYNA1, ITGA3, KRT19, LBP, LEF1
IL33	2.293	8	CCL17, CD69, CXCR2, DEFB4A/DEFB4B, GATA3, MSR1, PLCG1, ZC3H12A
ETV1	2.264	4	MMP1, MMP13, TERT, TMPRSS2
PPARG	2.237	46	ABCG2, ACSL1, ACTA2, AGPAT2, AQP3, BCL6, CA2, CAV1, CCL17, CDKN1A
FABP5	2.213	7	DEFB4A/DEFB4B, FABP3, ISG20, PPARG, RORC, SOCS3, VEGFA
ITGB2	2.196	7	ACTA2, CD69, GADD45B, GATA3, HES1, VCAM1, VEGFA
CTSS	2.193	4	BCL2L11, COL4A1, COL4A2, FAS
TCF7L2	2.191	11	AQP3, AQP9, CDKN1A, EPCAM, ERBB3, LAMC2, LBH, LEF1, MMP1, SDC2
ACVR1	2.184	8	ALPL, AMHR2, CDKN1A, CSRP2, DCN, LEF1, MOSPD2, NOV

ABLIM2	2.16	1	TAGLN
LGALS1	2.079	6	ACTA2, CD69, CDKN1A, IL2RA, PTX3, SERPINE1
PTGER2	2.071	14	CLEC4D, CLEC6A, CXCR2, EMR1, IL1R2, NFKBID, NOS2, PXN, SERPINE1, THBS1
MITF	2.012	18	ACSL1, DCSTAMP, ESRP1, GM2A, GPR137B, HPGD, ITGA3, KIAA1598, KIT, MET
EDN1	2.009	24	ACTA2, ADAM19, CALCRL, COL4A1, CYP11A1, EREG, ITGA2, ITGB3, JUNB, MMP1
APOE	2.003	28	ACTA2, BCAM, BMP4, C5AR1, C5AR2, COL18A1, CYR61, DCSTAMP, EMR1, F2RL1
LMTK3	-2.012	1	HSPB8
CD4	-2.015	7	CD79A, CXCR5, FAS, ID3, IL2RA, MME, NOS2
PTPRH	-2.031	1	CD69
CXCL12	-2.033	21	ACTA2, AXL, BCL3, BMP2, CA2, CD69, CDKN1C, DOCK9, GSR, IL2RA
CFB	-2.037	4	C4A/C4B, C5AR1, TGFB2, THBD
SERPINH1	-2.04	2	COL4A2, SERPINE1
IL12A	-2.04	6	FAS, ICOS, NOS2, SOCS3, STAT4, VCAM1
SOX7	-2.041	7	EVPL, GRHL3, IRF6, KRT17, KRT7, OVOL1, PPL
NR4A1	-2.055	25	BCL2L11, COL6A1, COL6A2, DFFA, EHF, EPCAM, FASN, G0S2, GPD1, IL16
SPTAN1	-2.058	3	CDKN1A, ITGA3, ITGB3
PRKAA1	-2.061	9	ACTA2, CA2, CDKN1A, DDAH1, IGFBP4, MGST1, NCF2, NDRG2, NFIB
BRAF	-2.062	7	BCL2L11, BIRC3, CDKN1A, EPAS1, MMP1, RND3, THBS1
DYRK2	-2.062	1	TERT
KDR	-2.066	5	CREBBP, EPHB3, MESDC2, NOTCH1, TJP1
KMT2D	-2.092	18	CA4, CDCA7, DUSP2, FABP3, FAM117A, GPR56, GPRC5C, LAMB3, LOXL1, NT5E
MIA	-2.099	3	ITGB3, PLAT, SPARC
RORA	-2.099	15	ACSL6, CCL24, CH25H, CXCR2, FABP3, FASN, GADD45B, HPGD, KCNK5, LPIN2
STAT5A	-2.107	22	BCL6, BIRC3, CDKN1A, EPAS1, FAS, FASN, FOSL2, GADD45G, IL2RA, KLF10
STXBP1	-2.119	1	STX11
GLIS2	-2.131	5	LTBP2, MGP, SERPINE1, SNAI1, SPARC
SIT1	-2.134	2	CD5, CD69
TCF4	-2.139	7	AMHR2, CDKN1A, CDKN1C, LEF1, MMP1, NOS2, TERT
FOXO1	-2.174	42	AQP9, ATP6V0D2, BACH2, BCL2L11, BIRC3, CA2, CD79B, CDKN1A, CDKN1C, CYCS
NFATC2	-2.176	21	BATF, CDKN1A, CRYAB, DAB2, FAS, GATA3, ICOS, IFIT2, IL2RA, INHBA
EGR3	-2.196	7	APBB1, IL2RA, LMO7, NOTCH1, RORC, SOCS3, ZNF292
PECAM1	-2.202	3	IL2RA, SELL, THBS1
ABLIM3	-2.206	1	TAGLN
LYN	-2.21	12	BCL2L11, C5AR1, CD19, CD22, CDKN1A, CXCR5, CYCS, ITPR1, NFKB2, SOCS3
PARM1	-2.225	2	BMP2, BMP4
ETS1	-2.245	34	BCL11A, BMP4, CAV1, CD79A, CD79B,CDKN1A, ETS1, ETS2, FLT1, HPGD

SIN3A	-2.246	6	BCL6, E2F2, GADD45B, HOXA9, KLF10, TMEM71
ZAP70	-2.258	6	BCL2L11, CD69, ITPR1, LYPD3, NFE2, QPCT
SMAD3	-2.269	37	ACTA2, NKRD1, BCL2L11, BMP2, CD79A, CDKN1A, COL6A1, CYP11A1, EREG, GADD45B
CELA2A	-2.291	2	CDKN1A, SLPI
ICAM3	-2.312	1	PPARG
MDK	-2.331	3	ACTA2, PLAT, TAGLN
SMAD1	-2.332	7	ACTA2, ALPL, BTG2, CDKN1A, COL4A1, GADD45B, VEGFA
NRG1	-2.339	22	BDH2, CAV1, CDC42EP1, CDKN1A, CYR61, ERBB3, GPX3, GRB7, GRIN2C, HES1
TNFRSF1B	-2.347	13	CD69, EFNA1, FAS, IL2R, LBP, MMP1, MMP13, MSR1, NOS2, SELL
IL15	-2.354	38	AQP3, BCL2L11, CCL17, CD274, CD28, CD5, CD69, DHRS4, ETS1, FAS
STAT5B	-2.365	24	AGPAT2, ANKRD1, BCL6, BIRC3, CDKN1A, DGAT2, EBF1, FAS, GCK, GPD1
STAT4	-2.37	25	ACAP1, ASPH, BCL2L11, BCL3, CCNL2, FYB, GADD45G, GATA3, HIST2H2AA3/HIST2H2AA4, IFIT2
LIMO2	-2.431	2	GATA3, KIT
FOXO3	-2.446	23	ATP6V0D2, BCL2L11, CDKN1A, CDKN1C, CYR61, DEFB4A/DEFB4B, FASN, FOXO1, FOXO4, GAB…
CXCL8	-2.448	10	CD69, COL18A1, CXCR2, DEFB4A/DEFB4B, FAS, IL2RA, SELL, TPM1, VCAM1, ZFP36
PTPRJ	-2.448	7	ARID5A, CD69, INHBA, MET, PLAT, SLA, ZC3H12A
TRPV4	-2.448	4	FAS, SOCS3, THBS1, VCAM1
TET2	-2.453	5	BCL2L11, BCL3, CLEC4D, FOSL2, MMP13
TNFRSF8	-2.461	4	CDKN1A, FAS, JUNB, TNFRSF25
RUNX2	-2.486	17	ACTA2, ALPL, BMP2, C4A/C4B, CDKN1A, DPYSL3, GADD45B, IL20RB, ITGB4, LEF1
ABCG2	-2.489	2	ABCG2, GSR
PTK2B	-2.49	3	BCAR1, MMP13, TERT
FOS	-2.493	61	ALDH1A3, AQP3, BCL2L11, CA2, CADM1, CASZ1, COBLL1, COL18A1, COL6A1, CREBBP
ELN	-2.493	2	MMP1, MMP12
HDAC4	-2.504	16	CALB1, CDKN1A, CYFIP2, FGF13, HOMER2, KLF2, MMP13, NOS2, RGS2, SERPINB2
CTLA4	-2.511	5	CD69, GATA3, ICOS, IDO1, IL2RA
CAV2	-2.528	1	CAV1
TNFSF11	-2.543	31	AKAP13, AQP9, ATP6V0D2, BMP4, CA2, CALCA, CD1A, CDKN1A, CKB, DAB2
TFAP2C	-2.552	8	CDKN1A, CYP11A1, ELF5, HIST1H1C, KRT8, SEMA3B, THBSI, TNFAIP3
FGF2	-2.554	57	ACTA2, AQP3, BCL6, BMP4, BTG2, CALB1, CAV1, CDKN1A, CRYAB, CTSL
TIMP3	-2.561	3	FAS, KIT, LAMC2
LHCGR	-2.563	5	CYP11A1, IGFBP4, IL1R2, SULT2A1, TNFAIP6
FOXO4	-2.564	8	BCL6, CDKN1A, FASN, GADD45B, OVOL1, RICTOR, SERPINE1, VEGFA
PML	-2.573	10	CDKN1A, FAS, FASN, GPAT, HADHB, JUP, LPL, NQO1, TNFAIP3, TXN
ARID5B	-2.591	2	ACTA2, TAGLN
ROR1	-2.6	3	KRT19, SNAI1, TJP1

EPAS1	-2.611	24	ABCG2, AXL, BIRC3, CA9, CAV1, CKB, DTX1, FASN, FLT1, GADD45B
PRKD2	-2.611	2	DCSTAMP, ITGA2
CREBBP	-2.615	17	ADORA2A, CDKN1A, CYP11A1, CYR61, GRIN2C, HAS2, IL2RA, KLF2, LPL, NOS2
ERG	-2.62	23	ARMC12, BIRC3, CDKN2D, COL11A2, ETS1, FAM174B, FLT1, GUCY1B3, HPGD, LEF1
VEGFA	-2.622	37	ACSL1, BMP4, CAV1, CCRL2, CDKN1A, CREBBP, CSTB, CYR61, E2F2, ETS1
CD69	-2.639	5	CD69, IL2RA, RORC, S1PR1, TGFB2
SNAI1	-2.649	16	CDKN1A, CLDN3, CLDN4, CLDN7, HPGD, ITGA2, KRT17, KRT18, LEF1, MUC1
TNFRSF1A	-2.675	17	BIRC3, CDKN1A, EFNA1, FLT1, GATA3, LBP, MMP1, MMP13, NOS2, NQO1
NFKB2	-2.691	7	BCL2L11, BIRC3, BMP2, CDKN1A, CXCR5, NFKB2, VCAM1
JADE1	-2.695	1	CDKN1A
LIF	-2.697	22	ACTA2, BATF, BMP2, CALCA, CDKN1A, CYP11A1, E2F2, EREG, FGG, HES1
PRKD1	-2.701	6	CD5, IL2RA, LPL, MMP1, TNFAIP3, VCAM1
IRF1	-2.732	15	CDKN1A, IDO1, IFIT2, LTB, NFE2, NOS2, OAS1, PIGR, PML, RSAD2
TNFAIP6	-2.737	2	SPINT2, TNFAIP6
PLAU	-2.748	9	C5AR1, CYR61, FCGR1A, MMP1, MMP12, OAS1, SERPINE1, SLPI, SNAI1
ZNF217	-2.773	7	ERBB3, FRK, GPRC5A, KRT18, MPZL2, PLAT, RGS20
SPARC	-2.807	13	CABYR, CLDN4, GPD1, HES1, LEF1, LOXL4, NES, NOTCH1, SERPINE1, SLC7A11
CBX7	-2.814	4	ANKRD1, BMP2, CBX4, FER1L6
RBP1	-2.814	2	GCK, PPARG
TREM1	-2.823	34	ABL2, ARRDC4, ASPH, CCL17, CCRL2, CD1A, CD274, DCSTAMP, DEFB4A/DEFB4B, ETS2
NOX1	-2.824	4	EPAS1, FLT1, NOS2, VEGFA
CDKN1A	-2.828	16	ACTA2, CDKN1A, CPA3, CRYAB, CYR61, FAS, INHBA, ITGB3, KRT18, LIMA1
CD28	-2.859	32	ALKBH1, CD274, CD69, CH25H, CREM, CYCS, FYB, GATA3, GUCY1B3, HIST1H2AC
RGS2	-2.867	3	LPL, PLAT, PPARG
DDR2	-2.873	2	MMP1, MMP13
MYD88	-2.899	28	ACSL1, BATF, BCL3, CCNL2, CCRL2, CD274, CH25H, CYR61, DEFB4A/DEFB4B, ETS2
JUNB	-2.935	18	BCL3, CAV1, CD274, FASN, HES1, HPGD, KNHBA, ITGB3, ITGB4, JUNB
SAMSN1	-2.979	13	ARID5A, BATF, DAB2, IFIT2, INHBA, ISG20, PLAT, PML, RSAD2, SDC1
ACVR2B	-2.981	1	CDKN1A
EPHB4	-3.001	7	BMP2, BMP4, GATA3, IGFBP4, KIT, STAT5B, TGFB2
TNFAIP3	-3.006	6	BIRC3, FAS, NOS2, SOCS3, TNFAIP3, VCAM1
THY1	-3.064	5	AXL, ETS1, MMP13, NES, VEGFA
PRKCE	-3.088	10	ACTA2, CAV1, CDKN1A, FCGR1A, IL2RA, JUNB, NOS2, PRKCE, VCAM1, VEGFA
CD70	-3.096	4	CD69, FAS, IL2RA, SELL
PBX1	-3.098	6	BMP4, CDKN1A, DCN, FABP7, GCK, NCF2

KCNK9	-3.119	4	CALB1, DKK3, GADD45B, LYPD3
ID3	-3.125	6	BLK, CD79A, CDKN1A, GPR56, ID3, IGLL1/IGLL5
EREG	-3.133	4	CALB1, EREG, HAS2, TNFAIP6
RET	-3.163	12	CALB1, DCN, DUSP8, MET, MMP1, NOV, PTPN13, SDK1, SELL, TACC3
SOCS3	-3.228	10	CDKN1A, FCGR1A, IFIT2, ISG20, NOS2, OAS1, SGCA, SOCS3, STAT5A, TFF3
FYB	-3.269	2	CD69, IL2RA
SPDEF	-3.276	16	BIRC3, CDKN1A, CLDN7, COL4A1, COL4A2, COL6A1, COL6A2, DKK3, FGFR1, ITGA3
FGF1	-3.3	16	CDKN1A, CYR61, FAM101B, GADD45G, HAS2, LTB, NOTCH1, NQO1, PRICKLE1, TGFB2
LMNB1	-3.303	7	AEBP1, CRYAB, FBN1, MGP, MGST1, S100A13, TGFB2
ITGA2	-3.345	3	DCN, ITGA2, MMP1
FAS	-3.359	53	ACTA2, BCL11A, BIRC3, CCRL2, CD22, CDKN1A, COL18A1, COL4A1, COL4A2, COL6A1
TXNIP	-3.366	6	BCL6, CDKN1A, FASN, G0S2, LPL, VEGFA
ADORA2A	-3.369	11	CCL17, CD28, CKB, COL6A1, DGAT2, NAPB, NOS2, OCLN, SPARC, TJP1
SBNO2	-3.404	1	DCSTAMP
TGFB1I1	-3.409	4	CDKN1A, KRT18, KRT19, SERPINE1
FBN1	-3.413	7	COL4A2, CYR61, FBN1, INHBA, MMP1, SERPINE1, TIMP3
ID1	-3.416	6	CDKN1A, DTX1, DTX4, GATA3, PROM1, SNAI1
DDR1	-3.424	3	CDKN1A, MMP1, VCAM1
KIT	-3.478	9	ADORA3, BCL2L11, CPA3, IL2RA, KIT, OSM, PSTPIP2, SOCS3, TJP1
KRT17	-3.48	4	CCL17, CCL24, CXCR2, MMP13
TLR9	-3.501	16	ARRDC4, CCL17, CD274, CD69, CPM, FAIM3, FAS, GADD45B, IDO1, IFIT2
NOTCH1	-3.523	33	ACTA2, ADAM19, ANKRD1, BCL6, BIRC3, BMP2, CDCA7, CDKN1A, CDKN1C, CYR61
SFRP1	-3.535	6	ACTA2, EPCAM, GATA3, KRT18, MMP1, SDC2
TCF7	-3.558	8	BCL11B, GATA3, MET, NES, PPARG, RORC, SDC2, TCF7
FGF13	-3.558	3	CDKN1A, MMP13, VEGFA
RND3	-3.581	3	CDKN1A, HES1, NOTCH1
NR4A3	-3.587	6	BIRC3, COL6A1, COL6A2, FOXO1, NQO1, TGFB3
MYLPF	-3.615	1	ACTA2
TGFA	-3.621	17	BIRC3, CDKN1A, EREG, FOSL2, GJB2, IGFBP4, JUNB, LAMC2, MMP13, NES
GATA3	-3.628	24	BATF, BCL11A, BCL11B, CDKN1A, DTX1, EBF1, EVPL, GATA3, GRHL3, ICOS
FOSL2	-3.632	7	FAS, HPGD, ITGB4, MMP1, MMP13, NOS2, SERPINB2
FHL2	-3.688	7	ACTA2, BCAR1, BCL2L11, CYP11A1, FHL2, S1PR1, TAGLN
BCL6	-3.719	21	BACH2, BCL3, BCL6, CD19, CD69, CD79B, CDKN1A, CXCR5, DNAJB11, FAS
KLF10	-3.773	3	CDKN1A, GATA3, SERPINE1
LTBP1	-3.843	4	BCL2L11, GATA3, SERPINE1, VWF
AMH	-3.846	4	ACVR1, BTG2, CDKN1A, CYP11A1
FAM3B	-3.97	7	CAV1, CDKN1A, DCN, IGFBP4, KRT19, RNASE1, SPARC
KCNIP3	-4.003	3	CREM, FOSL2, TNFAIP3

PGR	-4.009	24	ACSL1, AKAP13, ALDH1A3, ALOX15, BCL6, BIRC3, CCL8, CDKN1A, CDKN1C, EPAS1
MC1R	-4.022	2	NR4A3, SDC2
PTGES	-4.029	6	CDKN1A, ERBB3, FLT1, ITGB4, NOS2, PPARG
IL9R	-4.042	3	BCL3, JUNB, SELL
AQP5	-4.085	3	CLDN3, CLDN7, OCLN
RARG	-4.099	5	BMP2, COL4A1, CRYAB, HAS2, THBD
SOD2	-4.122	9	CDKN1A, ICOS, INPP1, JADE2, MLXIP, MMP1, MMP13, SERPINE1, SLC19A2
CALCA	-4.127	12	AKAP13, CALCA, CREM, FLT1, JUNB, LIMA1, MME, NDRG2, NOS2, PHLDB2
CD200	-4.146	3	IDO1, MET, NOS2
PER1	-4.156	3	BCL2L11, DBP, PER1
TEAD4	-4.189	6	BMP4, FGFR1, FLT1, GATA3, JUNB, VEGFA
ABLIM1	-4.21	1	TAGLN
KLF2	-4.252	28	BCL3, BMP4, CD1A, CDKN1A, COL4A1, EFNA1, EPAS1, FLT1, ID3, ITGB3
BMP7	-4.253	11	ACTA2, ACVR1, BMP4, CDKN1A, FASN, IGFBP4, MMP13, PPARG, SERPINE1, SOCS3
PRDM5	-4.267	13	DKK3, EBF1, ETS2, GADD45B, GCK, NLRC5, NOTCH1, OVOL1, PHC1, PML
PRKAA2	-4.346	12	ACTA2, CA2, CDKN1A, DDAH1, FASN, IGFBP4, MGST1, NCF2, NDRG2, NFIB
HAS1	-4.347	1	HAS2
ACTA2	-4.352	2	ACTA2, MET
NR3C2	-4.394	14	CACNA1H, CNKSR3, CNN3, CYFIP2, GPX3, NDRG2, PTPRCAP, RGS2, RRAD, SERPINE1
ETS2	-4.405	14	BMP4, CD79A, CDKN1A, FLT1, HPGD, IL2RA, JUNB, KRT18, MMP13, MSR1
PRKAG3	-4.422	17	ACSL6, ACVR1, ANKRD1, ANXA8/ANXA8L1, ASPH, BTG2, ELL2, FGFR1, GADD45G, GPX3
CREM	-4.44	13	ADORA3, BTG2, CH25H, CREM, CSRNP1, GADD45B, JUNB, MIDN, NFKBID, NOS2
HXA7	-4.441	4	CALCRL, GPR56, MSI2, PTPRCAP
MET	-4.457	10	AQP3, ARID5A, CDKN1A, DAB2, INHBA, MMP13, MUC1, SLA, SOCS3, VEGFA
WISP2	-4.464	12	DSP, GATA3, JUP, KRT18, KRT8, LAMB3, LAMC2, MUC1, SERPINE1, SPARC
GJA1	-4.467	9	CD69, CDKN1A, FOXO1, GJB2, IL2RA, NOS2, NOV, THBS1, VEGFA
FCGR3A/ FCGR3B	-4.542	2	FCGR3A/FCGR3B, SELL
PAX5	-4.593	11	BLK, CD19, CD22, CD79A, IGJ, IGLL1/IGLL5, MET, POU2AF1, PTPRF, SDC1
DTX1	-4.63	5	LY6E, SNAI1, TGFB2, TNFSF9, VEGFA
BMPR1B	-4.653	4	CDKN1A, ID3, PPARG, SERPINE1
TRPV5	-4.733	2	CALB1, TRPV6
ABCB4	-4.747	7	ACTA2, COL4A1, MMP13, NFKB2, SERPINE1, SPARC, TGFB2
GH1	-4.832	25	ACSL1, ACVR1, BMP2, BMP4, CALB1, CD19, COL18A1, ERBB3, FASN, FGG
KRT18	-4.856	1	KRT7
TERT	-4.937	20	ALDH1A3, BTBD11, CAV1, CDKN1A, EREG, ETS1, IFIT2, IGFBP4,

			JUP, KRT19
ITGB4	-4.975	2	SPARC, VEGFA
OSM	-4.99	62	ANXA3, AQP9, BTC, CADM4, CCL24, CDKN1A, CH25H, CLDN4, COL6A1, CPM
MGAT3	-5.016	4	ITGA2, ITGA3, PROM1, SNAI1
ERBB4	-5.017	12	ACTA2, ADAM19, BTG2, CADM1, CDKN1A, IGFBP4, LALBA, SLPI, SOCS3
FGFR2	-5.026	15	BMP4, CA2, CA4, CDKN1A, COL4A1, COL4A2, DAB2, FAS, HES1, MRAS
TGFB3	-5.033	27	ACTA2, ADAM19, CDKN1A, CYR61, ETS1, F2RL1, FOSL2, ITGB3, LEF1, LTBP2
FASN	-5.087	4	FASN, FOXO1, GPAT, MET
POU2AF1	-5.115	8	BCL6, CD79A, CD79B, CXCR5, ID3, PLCG1, SPIB, TNFRSF13C
ALOX15	-5.131	5	ALOX15, NOS2, PPARG, RGS2, VCAM1
HR	-5.218	6	COL6A1, FGF13, GPD1L, IL1R2, LEF1, NAPSA
HIC1	-5.315	13	ACTA2, CA2, CDKN1A, CDKN1C, FHL2, INHBA, ITPR1, MMP12, NOV, SLC7A11
CFTR	-5.35	10	CA4, CPA3, FAS, GPX2, GSR, HPGD, ITPR1, NOS2, SOCS3, TNFAIP3
DCLK1	-5.35	2	NOTCH1, SNAI1
THBS1	-5.371	5	ADCYAP1R1, CD69, TGFB2, THBS1, VEGFA
BMP2	-5.45	29	ACTA2, ATP2C2, BMP2, BMP4, BTC, CALB1, CALCA, CDKN1A, CREB3L1, FGFR1
SNAI2	-5.531	10	DSP, HPGD, ITGA3, ITGB4, JUP, KRT18, L1CAM, MUC1, NES, SOX10
GDF10	-5.583	1	SERPINE1
CAV1	-5.648	23	BCL2L11, CAV1, CDKN1A, CLDN7, FAS, FOLR1, GIPC2, IDO1, KRT18, KRT19
ETV4	-5.684	6	ACTA2, CAV1, GGT1, HPGD, JUP, KRT5
SMO	-5.694	9	COL4A2, EPCAM, FAS, KRT5, LEF1, LIMD2, TGFB3, TNIK, VCAM1
DGAT2	-5.697	2	DGAT2, GPAT
CTF1	-5.735	6	FASN, JUNB, LBP, MMP1, PPARG, SOCS3
CD79A	-5.748	2	CD79A, IL2RA
ITGB6	-5.811	3	MMP12, PTHLH, SERPINE1
CHRM3	-5.861	3	CYR61, ITGA2, ITGA3
AZGP1	-6.067	4	AZGP1, BMP4, DSP, KRT19
AEBP1	-6.121	2	NOS2, PPARG
ACKR1	-6.125	4	CXCR2, NINJ1, TNFAIP3, VCAM1
MXD1	-6.166	3	BCL6, MXD1, TERT
GPR87	-6.232	1	PROM1
ERBB3	-6.275	16	ACTA2, ADAM19, ADAM9, CDKN1A, COL6A1, ERBB3, FBN1, HAS2, IER2, SLC4A7
LRP2	-6.315	2	BMP4, DAB2
KRT8	-6.379	3	ATP4B, KRT18, KRT19
APBB1	-6.42	4	ACTA2, HES1, TAGLN, TPM1
COL18A1	-6.622	13	EFNA1, ETS1, F2RL1, FGFR1, ID3, ITGB3, JUNB, MMP1, NOS2, SERPINE1
TP63	-6.696	38	ALDH1A3, BCL2L11, CA4, CDK18, CDKN1A, CLDN3, COL4A1, CYR61, FAS, FASN
EBF1	-6.723	9	BCL6, CD19, CD79A, CD79B, EBF1, FOXO1, GATA3, IGLL1/IGLL5, LEF1

KRT14	-6.73	9	DSC2, GJB2, KRT15, KRT17, KRT18, KRT5 KRT8, MMP1, MMP13
FGFR3	-6.759	6	BMP4, CDKN1A, CDKN2D, HES1, LTB, SPARC
F2RL1	-6.833	10	ACTA2, CAV1, CYR61, F2RL1, FAS, ITGB4, NOS2, RARG, SERPINB2, VEGFA
PLAT	-7.093	4	INHBA, MMP1, NOS2, SERPINE1
EHF	-7.314	12	BMP4, EHF, EREG, GRHL3, MET, MMP1, MUC1, PLAT, SCEL, SERPINE1
SLC2A4	-7.316	3	GCK, LNPEP, VAMP2
HOXA10	-7.362	23	ALPL, BCL2L11, CDKN1A, CSF3R, DBP, FLT1, IRX3, ITGB3, KLF10, KRT15
FOXG1	-7.424	4	BCL2L11, CDKN1A, GCK, SERPINE1
ZBTB7C	-7.645	1	CDKN1A
mir-223	-7.718	19	ACTA2, C5AR1, CA4, CARD11, LEF1, LPL, MCTP2, MMP13, MMP25, MREG
GRHL2	-7.73	6	BMP2, CLDN4, ERBB3, OCLN, SEMA3B, TERT
CSF3R	-7.771	3	CSF3R, FCGR1A, LTF
CLDN7	-7.787	19	ABLIM1, ANXA3, HIST2H2AA3/HIST2H2AA4, IFI44, ITPR3, LGR4, LIMA1, MT1A, MUC1, MUC13
SDC2	-7.968	3	ACTA2, CDKN1A, TGFBR3
NKX3-1	-7.982	6	BTG2, CA2, CA8, ETS1, PTPRK, SDC1
HOXA9	-8.029	25	AKAP13, BCL2L11, CALB1, CALCRL, CPB2, CREM, EPS8, ETS2, GPR56, GTF2B
VCAM1	-8.126	2	ABCG2, VCAM1
TGFB2	-8.128	15	ACTA2, BMP4, FAS, HAS2, MLLT3, NOS2, PTAFR, PTHLH, SELL, SERPINE1
CYR61	-8.41	9	BCL2LL11, CDKN1A, CYR61, ITGA2, ITGA3, ITGB3, MMP1, SERPINE1, VEGFA
NFIB	-8.522	6	CDK18, KIAA1462, KRT8, NT5E, PLEKHF1, STEAP4
RIPK4	-8.711	3	GRHL3, IRF6, OVOL1
TFAP2A	-8.743	11	BIRC3, CDKN1A, CYP11A1, DCLK1, EREG, GEM, KRT5, PPARG, TERT, TPM1
HOXC9	-8.757	3	BMP4, MMP13, TIMP3
DSP	-8.811	4	DSC2, JUP, LPL, PPARG
RORC	-9.145	12	ACSL6, CD28, GADD45B, HPGD, KCNK5, LPIN2, NAPSA, PPP1R3C, RORC, SPARC
NR2F2	-9.543	5	LPL, LTF, NR2F2, TFAP2A, VEGFA
FOLR1	-9.661	9	ACVR1, BASP1, BIRC3, CAV1, EPCAM, GIMAP1, GPAT, LAMB2, ST3GAL3
GATA1	-10.03	22	BCL2L11, BTG2, CDKN2D, CNN3, CPA3, ETS1, FYB, GATA3, GUCY1B3, ITGB3
DLL4	-12.625	6	DTX1, FLT1, GATA3, HES1, NR2F2, RORC
F2	-12.693	36	ACTA2, BCL3, BIRC3, CCL8, CDC42EP1, CDKN1A, COL4A1, CYR61, DKK3, ELL2
ZBTB8A	-12.715	1	CDKN1A
PAEP	-13.401	2	GATA3, VEGFA
HES6	-13.822	2	CDKN1A, MMP13, VEGFA
MSX2	-13.875	8	ABCG2, ALPL, AQP3, BMP4, LEF1, PPARG, TGFB2, TGFB3
HOXC6	-14.299	5	APBB1, DCN, MME, NFKB2, VCAM1
RNASE1	-15.541	5	CCL24, CCL8, IL16, IL2RA, OSM

TFAP2B	-15.774	3	EPAS1, KIT, TERT
DCN	-17.523	15	CDKN1A, CDKN1C, FBN1, KIT, MET, MMP1, MMP13, NOS2, S1PR1, SCD1
HOXA5	-19.495	5	CDKN1A, GADD45B, IER2, KLF10, TGFB3
PDE4D	-24.642	2	BCL2L11, PLAT
FOXA1	-27.638	16	ACTA2, AQP3, BMP2, COL181A, EFHD1, ERBB3, MGP, NES, RORC, S100A2
EGFR	-45.696	49	ACTA2, BCL2L11, BCL6, BTC, CAV1, CDKN1A, COL6A1, COL6A2, CRYAB, DSC2
UPF2	-190.72	5	CYFIP2, DSP, KANK1, TNFAIP3, TSPAN12
CD82	-235.403	2	BCAR1, DCN

Table A8. *Key gene regulators (FC>2) and their regulated genes (10) in R-MFD cows fed the LIN diet compared with S-MFD cows fed the LIN diet.*

Transcription factor/Regulator	FC	(n)	Regulated Genes (10)
DROSHA	2930.234	2	KRAS, THBS1
PROC	65.916	8	NR4A1, SERPINE1, THBD, THBS1, TJP1, TNF, TNFAIP3, VCAM1
CSF2	9.846	57	ABCA1, AKR1A1, ANLN, BCL2L1, BCL3, CCL3, CD38, CD86, CENPH, CISH
INHBA	7.861	22	ACVR1B, AGO1, ANKRD1, BCL2L1, BMP2, CALB1, CCR2, CDH1, CHD7, COX6A1
CCL2	6.175	12	ABCA1, CCL5, CCR2, CD86, FOLR2, INHBA, MKI67, MMP1, NOTCH1, SERPINE1
GNLY	5.167	2	CCL5, TNF
MB	4.82	3	EPAS1, VCAM1, VEGFA
MMP12	4.47	4	CCL5, CSF2, CXCR2, MMP13
IFNB1	4.437	29	BCL2L1, CCL3, CCL5, CD38, CD86, CH25H, CMPK2, CRYAB, FBN1, GBP7
APOB	3.91	5	ACACA, FASN, PSMA7, PSMB7, PSME2
APOE	3.886	27	ABCA1, ARHGEF12, BCAM, C5AR2, CCL5, CD86, COX6B1, CYR61, EMR1, F2RL1
IL27	3.664	16	AHR, CCL3, CCL5, CD86, CSF2, EMR1, GATA3, HPGD, IL9, LAG3
LGALS1	3.62	10	AHR, FIS1, FN1, HBEGF, KRAS, LAT, LGALS1, MAF, PTX3, SERPINE1
TXN	3.595	7	CD38, CD86, MS4A1, NFKB1, TNF, TXN, VEGFA
FLT1	3.555	7	EPAS1, MARCKS, NOTCH1, TCF4, THBS1, UBE2H, VWF
SATB2	3.467	3	AUTS2, BCL11B, PERP
CTSD	3.433	2	ABCA1, TXN
HNF4A	3.353	197	ABCA6, ACBD6, ACOX2, AKAP13, ANKHD1/ANKHD1-EIF4EBP3, AQP3, AQP9, ATP6V1F, AVPI1, B9D2
FTL	3.127	2	PSENEN, TFRC
TNFRSF6B	2.984	4	CSF2, TNF, TNFRSF6B, VCAM1
LIMS2	2.955	3	GJA1, PARVA, TJP1
IFNG	2.912	167	ABCA1, ABCA6, AGRN, AHR, ALDH1A3, ALOX15, ANKS1A, AQP9, ARAP2, ARL6IP5
LGALS3	2.898	17	CCR2, CTSF, ENPP2, FN1, ITGA6, ITGAV, ITPR3, KRAS, KRT19, LMNB1
S100A8	2.885	6	BCL2L1, MMP1, MMP13, S100,A8, TNF, VCAM1
GPX1	2.774	7	EMR1, FADD, GPX2, GPX4, TNF, TXN, VCAM1
CYBA	2.682	4	EPAS1, SERPINE1, TNF, VEGFA
S100A4	2.669	9	CCL24, CCL5, CDH1, FN1, MMP13, OCLN, SNAI1, THBS1, VCAM1
IL4	2.635	107	ADAM19, AHR, ALOX15, APRT, BBX, BCAT2, BCL2L1, BCL3, BCL6, CA2
CLEC4G	2.598	4	CDK6, PRF1, RUNX3, TNF
CD9	2.547	6	ITGA2, ITGA3, ITGA6, TJP1, TNF, VEGFA
SERPINF1	2.532	9	BCL2L1, CRYAB, CYBA, NEFH, NFKB1, OCLN, SOX10, THBS1, TJP1
PLA2G5	2.464	3	PLA2G5, VEGFA, VEGFB
FABP5	2.458	5	DEFB4A/DEFB4B, PDPK1, RORC, SOCS3, VEGFA
NUPR1	2.442	68	AGRN, ANK3, API5, ARHGEF26, ASPM, ATP8B1, AVPI1, B3GAT3, BCL9, BRCA1
TNF	2.426	223	ABCA1, ABCG2, ABTB2, ACACA, AEBP1, ALCAM, ALDH1A3,

			AMPD3, AQP3, AQP9
DOK2	2.395	2	DOK2, TNF
CD209	2.343	3	CCL3, CD86, TNF
TNFRSF17	2.333	3	BCL2L1, CD86, CSF2
GPI	2.326	5	CAV1, CDH1, FN1, SNAI1, STEAP4
VIM	2.309	3	CDH1, ITGB4, RAB25
POMC	2.308	16	FOSL1, FOSL2, GNAQ, MITF, NCOA2, NR4A1, NR4A2, NR4A3, PMCH, PRF1
ETHE1	2.293	2	PERP, TP53I3
ITGB2	2.285	11	CCL3, CD86, CSF2, FN1, GADD45B, GATA3, HES1, ITGA4, TNF, VCAM1
AKR1C3	2.284	2	PROS1, TMPRSS2
SLAMF7	2.258	2	FLT3LG, TNF
S100A9	2.235	6	BACE2, MMP1, MMP13, S100A8, TNF, VCAM1
IGFBP2	2.23	7	CADM1, DDAH1, FN1, ITGA6, MMP13, NEDD9, NUP210
EEF1D	2.218	2	CDH1, ITGA4
MMP2	2.201	8	BCL2L1, BMP2, CSF2RB, FN1, ITGA3, MMP1, OCLN, TJP1
NR1H3	2.199	12	ABCA1, ACACA, CCL17, CCL24, CCL5, FASN, GCK, GPAT, MYLIP, NR1H3
NAB2	2.196	5	BCAR1, FLT1, FN1, NAB2, RRAD
LGALS3BP	2.195	3	FN1, MMP13, TNF
IL7	2.19	27	AHR, BCL2L1, CBLB, CCL3, CD19, CD2, CD86, CD8B, CDK6, CISH
GRN	2.173	3	MMP13, TNF, VEGFA
CDKN2A	2.121	30	BTG2, CAPG, CCL24, CCL5, CXCL6, CXCR2, CXCR5, DCTN4, DDR1, EBF1
BSG	2.072	5	ABCG2, CDH1, MMP1, TJP1, TNF
PHB2	2.063	5	BMP2, CDKN1C, GJA1, SOCS3, TXN
CCL1	2.056	3	CCL3, ITGA2, SERPINE1
mir-221	2.046	5	CDKN1C, KIT, PIK3R1, STAT5A, TIMP3
CEBPE	2.04	5	BCL2L1, CDK6, LTF, SERPINB2, TNF
RPSA	2.037	4	CCL5, CSF2, ITGA6, TNF
TGFB1	1.708	248	ABCA1, ABCG2, ADAM10, ADAM19, AGR2, AHR, ALOX15, AMOTL2, ANKRD1, ANXA8/ANXA8L1
PPARG	1.691	63	ABCA1, ABCG2, ACACA, ACOT8, AQP3, ATP5O, BCL6, BRCA1, CA2, CAV1
TWIST1	1.597	28	ALCAM, ASPM, CDH1, CELSR1, CLDN4, COL6A1, DCN, EMP3, ENPP3, ETS1
USF2	1.595	15	ABCA1, CD2, FASN, FMR1, GATA3, GCK, IGF2R, LGALS3BP, OXT, PIGR
RARA	1.48	20	ABCA1, ACACA, ADAM10, CALB1, CD38, CLMN, CRABP2, CXCR5, FOLR2, ITGA4
ADA	1.424	7	ADORA3, CCL17, CCL24, MMP12, SERPINE1, TNF, VEGFA
CEBPA	1.322	58	ACACA, ARHGEF5, ARL6IP5, ASL, ASNS, BTG2, CA2, CD19, COX4I1, CRABP2
RIPK2	1.312	15	ABCA1, ACACA, CCL5, CD86, FASN, GPR84, MEFV, NR1H3, PDE4B, RASGRP1
RHOA	1.285	12	ANLN, CDH1, FN1, MMP1, MMP13, NFKB1, OCLN, RND3, ROCK2, SDC2
MYC	1.009	113	ACACA, ALCAM, ASNS, BCL2L1, BCL6, BRCA1, CAV1, CCNG2, CD19, CD86

SREBP1	-1.004	24	AARS, ABCA1, ACACA, ACSS2, ATOX1, BCL2L1, CCL17, COL6A1, EHHADH, ELOVL7
PTEN	-1.005	59	ABCC4, ACACA, ATP5I, BCL3, BTG2, CA2, CA8, CBX4, CCL5, CDK6
NR1H2	-1.137	9	ABCA1, ACACA, CCL5, FASN, GCK, NR1H3, SULT2A1, TNF, VEGFA
LIPE	-1.154	20	ABCA1, ACSS2, ANKRD1, BCL6, CA2, CLK1, ELL, FASN, FBP2, GJA1
PPARA	-1.168	51	ABCA1, ACACA, ACOT8, ACSS2, AQP3, AQP9, ASL, ASNS, CCNT1, CIDEA
SYVN1	-1.199	26	ABCC4, AHR, AMOTL2, ATP1B3, CYB561, DAG1, DPYD, EPHA2, ERN1, FASN
JUNB	-1.212	19	BCL3, CAV1, CSF2, FASN, FN1, FOSL1, HES1, INHBA, ITGAV, ITGB4
ACLY	-1.437	4	ACACA, ACSS2, FASN, GPAT
INSIG1	-1.493	13	ABCA1, ACACA, ACSS2, CCL17, CCL5, CXCL6, FASN, GPAT, PCYT2, PLA2G7
IGF1	-1.526	47	ACACA, ACSS2, BCL2L1, BMP2, BRCA1, CCL5, CCNG2, CDH1, CISH, CXCL6
FOXO4	-1.597	10	ACACA, BCL2L1, BCL6, CCNG2, FASN, GADD45B, OVOL1, RICTOR, SERPINE1, VEGFA
TP53	-1.627	159	ADD3, ALOX15, AMOTL2, ANLN, ANXA8/ANXA8L1, API5, ASL, ASNS, ASPM, ATG2B
FOS	-1.718	88	ADNP, AFF4, ALDH1A3, ANK3, AQP3, ASL, B4GALT1, BCL9, CA2, CADM1
MAPK9	-1.834	22	ACACA, BDP1, CAV1, FASN, FOSL1, FOSL2, GADD45B, ITGB4, LGALS3BP, LMO7
RXRA	-1.841	40	ABCA1, ABCG2, ACACA, ASNS, BRCA1, CALB1, CCL17, CD38, CIDEA, CLMN
FOXO1	-1.922	46	ACACA, AFF3, ANLN, AQP9, ASPM, ATP5J, ATP6V0B, ATP6V0D2, BACH2, BANK1
REL	-1.927	23	AHR, BCL2L1, BCL3, CD86, CDH1, CREM, CSF2, FCER2, FN1, FOXO3
STIM1	-2.005	4	GSTO1, NFATC2, NFATC3, TNF
EIF2AK2	-2.019	11	ERAP2, IFIT1, LGALS3BP, NEDD9, OAS1, PSMB10, SAMHD1, SOCS3, TNF, TNFAIP3
LYN	-2.02	13	CCL5, CD19, CD22, CD86, CSF2, CXCR5, CYCS, MITF,NFKB1, SOCS3
TOP2A	-2.033	2	KMT2A, TOP2A
SPARC	-2.057	21	ABCC4, BRCA1, CDH1, CLDN4, CTSF, FN1, HES1, LEF1, LOXL4, NES
CD86	-2.058	8	BCL2L1, CCL3, CD86, FCER2, HOMER2, TNF, TNFAIP3, TNFRSF11A
ZNF217	-2.062	11	AGR2, ANK3, ERBB3, FRK, GPRC5A, MPZL2, MYCBP2, PLAT, RGS20, ST6GAL1
MNT	-2.064	6	BCL6, CCNG2, GADD45B, KLF10, KLF6, TNF
HSPD1	-2.074	5	GATA3, NFATC2, SOCS3, TNF, VCAM1
SPON2	-2.106	2	NFKB1, TNF
ITGA6	-2.112	5	ENPP2, ITGA2, ITGA3, ITGB4, VEGFA
TJA1	-2.114	2	HBB, TNF
MAP3K3	-2.127	5	BMP2, HAS2, TGFB2, TGFBR3, TNFAIP3
SMARCD3	-2.137	4	ALCAM, ITGA3, MGP MITF
TCF7L2	-2.141	11	AQP3, AQP9, EHHADH, EPCAM, ERBB3, FBP2, LAMC2, LEF1, MMP1, SDC2
NR1D1	-2.147	5	EHHADH, MEF2C, NUPR1, RORC, ULK1
STAT5A	-2.164	27	BCL2L1, BCL6, CDH1, CDK6, CISH, EPAS1, FASN, FOSB, FOSL2, GZMK
MTOR	-2.179	30	ACACA, AKAP13, BCL2L1, CD86, CDH1, COX4I1, ERCC1, FADD,

			FASN, FN1
SPDEF	-2.182	17	CDH1, CLDN7, COL6A1, COL6A2, FGFR1, ITGA3, ITGA6, LAMB2, LASP1, LEF1
TCF7	-2.192	8	BC11B, GATA3, LAT, MET, NES, RORC, SDC2, TCF7
IL2	-2.225	77	ACVR1B, AHR, BCL2L1, BCL6, BMP2, CCL3, CCL5, CCNG2, CCR2, CD2
FOSL1	-2.226	12	CXCL6, FOSB, FOSL1, MET, MMP1, MMP13, MT2A, SERPINB2, SERPINE1, SNAI1
mir-223	-2.229	22	ABCA1, ALCAM, AMPD3, DNAJC6, EGR3, FAM20C, FNIP2, GALC, GPR65, LEF1
IL6R	-2.244	12	CCL3, CCL5, CXCL6, FN1, GAL, IL6R, MMP13, SOCS3, TNF, TNFRSF11A
PLAUR	-2.276	6	CCL5, CDH1, ITGA6, ITGAV, SNAI1, TNF
DLL4	-2.278	7	EPHB4, FLT1, GATA3, HES1, MAF, NR2F2, RORC
MTPN	-2.293	12	BCL2L1, FADD, FN1, MTPN, NFKB1, PLAT, S100A8, SERPINE1, TAGLN, TGFB2
IDH1	-2.298	9	CDH1, CLDN3, CLDN7, CREB3L1, EDEM1, FASN, FN1, RAB25, TGFB2
CD38	-2.315	26	ASNS, BCL6, CD86, CISH, CPEB3, CSF2RB, EIF4EBP1, GBP7, GCAT, GLIPR2
STAT5B	-2.332	25	ANKRD1, BCL2L1, BCL6, CDH1, CDK6, CIDEA, CISH, COX7A1, EBF1, GCK
NKX3-1	-2.365	11	BTG2, CA2, CA8, ETS1, ETV3, FES, FOXO3, PIK3R1, PTPRK, SDC1
HBEGF	-2.385	8	BMP2, CDH1, EREG, GJA1, MUC1, NES, REST, VEGFA
DOCK8	-2.385	9	BBX, CMPK2, IFIT2, INHBA, PLAT, RBL1, PSAD2, TCF4, TNF
LMNB1	-2.387	8	AEBP1, CRYAB, FBN1, GJA1, HBEGF, MGP, MGST1, TGFB2
SOCS3	-2.388	11	ABCA1, CD86, FCGR1A, IFIT1, IFIT2, OAS1, SGCA, SOCS3, STAT5A, TNF
KLF13	-2.39	7	BCL2L1, BMP2, CCL5, FECH, GYPC, HBB, KLF9
ROR1	-2.405	4	CDH1, KRT19, SNAI1, TJP1
MITF	-2.442	29	ALCAM, AMDHD2, CCNG2, DAPK1, DSTYK, ESRP1, FN1, HPGD, HPS4, IL6R
PRKCE	-2.449	9	CAV1, CDH1, CDON, CSF2, FCGR1A, NFKB1, TNF, VCAM1, VEGFA
NOTCH1	-2.467	32	ADAM19, ANKRD1, BCL2L1, BCL6, BMP2, CDH1, CDKN1C, CYR61, ERBB3, FABP7
TERT	-2.474	21	ALDH1A3, ASNS, CALD1, CAV1, EREG, ETS1, FRMD6, IFIT1, IFIT2, ITGAV
CALCA	-2.481	16	ADD3, AKAP13, ANK3, CD86, CREM, CYBA, FLT1, IL9, LRBA, MKI67
NOTCH2	-2.511	4	FCER2, HES1, SDC2, TNF
TCF3	-2.515	36	AATF, ANLN, AZGP1, BCL6, CA2, CD8B, CDH1, CDK6, CGREF1, DAB2IP
BRCA1	-2.524	22	ANXA8/ANXA8L1, BACH1, BRCA1, CCL5, CTSF, ENPP2, GADD45B, HBB, IFIT1, IFIT2
PIK3R1	-2.58	10	BCL2L1, CCL3, MITF, MKI67, PIK3R1, SERPINE1, TNF, VCAM1, VEGFA
NFKB1	-2.584	31	ANKRD1, BCL2L1, BCL3, BLK, BMP2, BTG2, CCL5, CD86, CSF2, CSF2RA
CREBBP	-2.592	20	ASL, CCL5, CYR61, EGR3, EHHADH, FOSB, HAS2, HBB, IL23R, NR4A1
TGFB3	-2.603	30	ADAM19, CDH1, CYR61, EEF1D, ETS1, F2RL1, FN1, FOSB, FOSL2, GJA1
HDAC4	-2.628	15	CALB1, CYFIP2, FOSL1, HDAC4, HOMER2, MEF2C, NR4A1, NR4A2,

			RGS2, SERPINB2
RARG	-2.653	7	ABCA1, BMP2, CRABP2, CRYAB, FOLR2, HAS2, THBD
ROCK2	-2.668	6	DSC2, DSP, PPL, S100A8, SCEL, SERPINE1
SASH1	-2.671	10	BBX, CMPK2, IFIT2, INHBA, MET, PLAT, RBL1, RSAD2, TCF4, TNF
PLCG1	-2.693	3	PECAM1, SERPINE1, THBS1
ITGA4	-2.696	3	ITGA4, TNF, TNFAIP3
HLTF	-2.743	2	HBB, SERPINE1
BCL6	-2.759	24	ALCAM, BACH2, BCL2L1, BCL3, BCL6, CCL3, CD19, CD2, CXCR5, FCER2
BRAF	-2.761	7	BMF, CCR2, CDH1, EPAS1, MMP1, RND3, THBS1
FN1	-2.769	27	CCL5, CDH1, CDK6, CSF2, EPB41L1, FERMT2, FN1, GFRA2, ITGA4, ITGAV
AHR	-2.771	43	ABCB6, ABCC4, ABCG2, ADAM19, AKR1A1, ATP2B1, BTG2, CADM4, CBR3, CCL5
MEF2C	-2.773	6	GJA1, KNJ2, MEF2C, MMP13, NFATC2, TPM1
SMAD3	-2.781	33	ANKRD1, BMP2, CCNG2, CDH1, COL6A1, DAPK1, EREG, GADD45B, GATA3, HAS2
CXCL13	-2.799	3	CXCR5, MMP1, NFATC3
ADAM10	-2.813	13	ATRX, BRAF, CCR2, CRYAB, DCLK1, FABP7, GNAQ, GZMH, HIP1, HIPK1
VDR	-2.818	14	BRCA1, CALB1, CDH1, CSF2, FGG, PLCG1, SLC34A2, SULT2A1, TCF3, TGFB2
RBL1	-2.822	9	HES1, MET, MTOR, NOTCH1, RBL1, THBS1, TNF, UXT, VEGFB
KRT17	-2.833	7	CCL17, CCL24, CCL5, CXCL6, CXCR2, MMP13, TNF
IGFBP3	-2.843	4	BCL2L1, MMP13, SDC2, SERPINE1
OSM	-2.893	84	ABCA1, ABCC4, AHR, AMPD3, AQP9, ARHGEF12, ASNS, ATP13A3, ATP2B4, BTC
RICTOR	-2.899	39	ATP5D, ATP5J, ATP5J2, ATP5O, ATP6V0B, ATP6V0D2, ATP6V1A, ATP6V1E2, ATP6V1F, ATP6V1G1
LIF	-2.939	21	ACACA, BCL2L1, BMP2, CDH1, CISH, EREG, FGG, FN1, GAL, HBEGF
F2	-2.965	39	B4GALT1, BCL3, CALD1, CBR3, CCL8, CDC42EP1, CYR61, DAAM1, DHRS9, EHD4
BMP4	-2.986	19	BMP2, CDH1, CYR61, GJA1, HES1, ITGA4, KIT, KLF10, L1CAM, LEF1
POU5F1	-2.99	31	AHR, BRCA1, CDH1, CLDN7, COBLL1, CRABP2, DSP, ECSIT, EPCAM, EPHA2
NEDD9	-2.994	6	BMP2, ERRFI1, ITGA2, KIT, PLAC8, SERPINE1
KRAS	-3.004	47	ABCA1, ABCC4, AGRN, AHR, ANXA8/ANXA8L1, AOX1, BCL2L1, CAV1, CDH1, CRYAB
CCR2	-3.023	11	CCL17, CCL24, CCL5, CCR2, CD86, CSF2, CXCL6, FN1, MMP13, SLPI
TNFRSF1B	-3.025	11	BCL2L1, CSF2, LBP, MMP1, MMP13, NFKB1, SERPINE1, SOCS3, TNF, TNFAIP3
LCN2	-3.042	9	ANLN, CAPG, CCL17, CCL5, CDH1, CXCL6, KIF23, TINAGL1, TNF
NRIP1	-3.047	9	ACACA, CCNG2, CIDEA, COX7A1, FASN, HADHB, HAS2, PTX3, TNF
MC1R	-3.074	5	NR4A1, NR4A2, NR4A3, SDC2, TNF
LTF	-3.084	5	CCL5, CD86, MMP13, PDPK1, TNF
CREM	-3.09	18	AANAT, ABCA1, ADORA3, BTG2, CH25H, CREM, CSF2, ERRFI1, FRMD6, GADD45B
FGF2	-3.115	59	AQP3, BCL2L1, BCL6, BTG2, CALB1, CAV1, CDH1, CRYAB, CTSL, CYR61
TIMP3	-3.138	3	ADAM10, KIT, LAMC2

IL6	-3.139	84	ABCA1, ABCG2, AHR, BCL2L1, BCL3, BCL6, BMP2, BTG2, CCL24, CCL5
TAF4B	-3.16	7	FN1, IGJ, INHBA, ITGA6, MMP13, TAF4B, TNFAIP3
ITGAV	-3.222	6	BCL2L1, CDH1, FN1, MMP1, PTHLH, SERPINE1
GATA3	-3.235	25	BCL11B, BCL2L1, BMF, CCL5, CDH1, CRABP2, EBF1, EVPL, GATA3, GRHL3
ERN1	-3.271	17	AP1M2, CDH1, CLDN7, EPCAM, ETS1, FASN, ITGB6, LPCAT2, MPZL2, PROS1
IRF4	-3.299	16	BCL6, CSF2, DNASE2, ERBB3, FCER2, FLT1, GATA3, IL23R, IL9, IRF4
FGF1	-3.301	20	AHR, CCL3, CDH1, CYR61, GSTP1, HAS2, NOTCH1, NR1H3, NR4A2, PRICKLE1
VEGFA	-3.334	42	ATP5J2, ATP5O, BCAT2, CAV1, COX5B, COX6B1, CREBBP, CST3, CSTB, CYR61
APBB1	-3.358	3	HES1, TAGLN, TPM1
COL18A1	-3.386	15	BCL2L1, EPHB4, ETS1, F2RL1, FGFR1, FN1, ITGAV, MMP1, PECAM1, SERPINE1
IKZF1	-3.393	21	ADAM19, BCL2L1, BCL6, CDK6, DNAJC6, DOCK9, EBF1, FGFR1, HES1, IGLL1/IGLL5
TFAP2C	-3.394	11	ALCAM, ANLN, CDH1, EGR3, ELF5, GZMH, KRT8, MAGI3, SEMA3B, THBS1
IFNAR1	-3.406	16	CCL17, CCL24, CCL5, CH25H, CMPK2, CXCL6, IFIT2, ITGAV, OAS1, PLAT
TGFBR1	-3.413	9	AHR, CD86, CDH1, FN1, HAS2, MMP13, NFATC2, SERPINE1, TAGLN
ETS1	-3.456	32	B4GALT5, BRCA1, CAV1, CCL5, CSF2, EEF1D, ETS1, FLT1, FN1, GZMK
CIDEA	-3.522	2	TNF, XDH
PDPK1	-3.578	6	CAV1, CST3, SERPINE1, SNAI1, TNF, XIST
FOSB	-3.592	10	FOLR2, FOSB, FOSL1, INHBA, MMP1, MMP13, MT2A, SERPINB2, SERPINE1, SMAD3
SNAI1	-3.639	18	CDH1, CLDN3, CLDN4, CLDN7, ERCC1, FN1, FOSL1, HPGD, ITGA2, KRT17
PTX3	-3.668	6	CCL5, CCR2, EGR3, GATA3, NFKB1, TNF
SPIB	-3.672	13	CCR2, CD86, EPCAM, GEM, IRF4, KDM6B, KIT, KLF6, LILRB3, LRRFIP1
THBD	-3.761	3	PECAM1, THBD, TNF
ITGA3	-3.78	3	FN1, ITGA2, SERPINE1
TGFA	-3.831	19	BCL2L1, CCL5, CSF2, EREG, ERRFI1, FOSL2, GSTP1, LAMC2, MMP13, NES
TGFBR3	-3.898	3	CDH1, SERPINE1, SNAI1
CTF1	-3.981	5	FASN, LBP, MMP1, SOCS3, TNF
RET	-4.005	14	CALB1, CSF2, DCN, DUSP8, GJA1, ITGA6, MET, MMP1, NOV, PTPN13
KCNIP2	-4.012	3	AANAT, CREM, FOSB
DOR1	-4.05	4	BCL2L1, CDH1, MMP1, VCAM1
AZGP1	-4.063	6	AZGP1, BCL2L1, CDH1, DSP, KRT19, MTOR
KDR	-4.067	7	CD38, CREBBP, EPHB3, NME1, NME2, NOTCH1, TJP1
CFTR	-4.085	14	ABCA1, ASNS, CAPG, CCL5, GPX2, GSR, HPGD, NFKB1, PLA2G5, S100A8
ITGB4	-4.117	3	CCL5, ENPP2, VEGFA
TLR5	-4.218	6	CCL5, CD86, CSF2, DEFB4A/DEFB4B, MMP1, TNF

EMP2	-4.291	2	CAV1, ITGA6
EPAS1	-4.506	21	ABCG2, ACACA, CAV1, CCR2, DECR2, DMXL1, FASN, FLT1, FN1, GADD45B
RORC	-4.554	18	ACSL6, ASNS, CCL5, CISH, CSF2, ELOVL7, GADD45B, GSTP1, HPGD, IL23R
LOXL2	-4.594	4	CDH1, FN1, OCLN, PECAM1
TCF12	-4.643	13	AATF, ACOT8, AZGP1, CDH1, FN1, GATA3, GJA1, IL10RA, LRBA, NOTCH1
FOSL2	-4.822	7	ABCA1, FOLR2, FSL1, ITGB4, MMP1, MMP13, SERPINB2
DLC1	-4.84	3	CDH1, CDK6, SERPINE1
NR4A1	-4.921	24	ACACA, CIDEA, COL6A1, COL6A2, CRABP2, CST7, DFFA, EHF, EHHADH, ENO3
LY75	-4.925	2	CD86, FCER2
GJA1	-4.94	6	CDH1, GJA1, NOV, THBS1, TNF, VEGFA
EBF1	-4.945	7	BCL6, CD19, EBF1, GATA3, IGLL1/IGLL5, LEF1, TCF3
ETV4	-4.992	9	B4GALT1, B4GALT5, CAV1, CDH1, FN1, GGT1, HPGD, JUP, KRT5
KRT14	-5.112	7	DSC2, GJA1, KRT17, KRT5, KRT8, MMP1, MMP13
FGFR3	-5.139	5	BCL2L1, CCL3, HES1, MAF, PECAM1
HR	-5.155	7	COL6A1, DOK2, FADD, GJA1, LEF1, UBR2, VDR
TPM1	-5.212	2	CALD1, TPM1
ALOX15	-5.27	5	ALOX15, FN1, RGS2, TNF, VCAM1
TEAD4	-5.288	7	FGFR1, FLT1, GATA3, NOTCH2, SMAD1, TFAP2C, VEGFA
TNFSF11	-5.334	36	AKAP13, AQP9, ATP6V0D2, BCL2L1, CA2, CCL5, DOK2, DUSP16, EMR1, ETS1
CDH1	-5.361	14	CCL17, CDH1, EIF4BP1, ERBB3, FOSL1, JUP, KRT19, KRT7, MMP1, MTOR
ESR1	-5.375	53	ABCG2, AQP9, BMP2, BRCA1, CA2, CALB1, CAV1, CCNG2, CDH1, CELSR2
EGR3	-5.399	9	APBB1, BC2L1, CBLB, LMO7, NAB2, NOTCH1, RORC, SOCS3, ZNF292
ZMIZ1	-5.533	2	HES1, SERPINE1
TCF4	-5.64	9	AMHR2, CDKN1C, FN1, LEF1, MITF, MMP1, NCOR2, SSTR2, VCAN
TNFRSF11A	-5.695	7	AKAP13, BCL2L1, FN1, MME, SNAI1, TNFRSF11A, VCAM1
MOG	-5.788	7	CCL5, CCR2, CD86, CSF2, PECAM1, TNF, VCAM1
MET	-5.791	10	AQP3, BMF, CDH1, FN1, HBEGF, INHBA, MMP13, MUC1, SOCS3, VEGFA
AQP5	-5.864	3	CLDN3, CLDN7, OCLN
PRKD1	-5.947	6	CD2, MMP1, NR4A1, TNF, TNFAIP3, VCAM1
NR4A2	-5.978	10	COL6A1, COL6A2, COX6A1, EIF4EBP2, IL23R, MMP1, MMP13, PTX3, SMAD3, TNF
MYCN	-6.039	23	AMOTL2, CAV1, CCNG2, CCNT1, CRIM1, DPYSL3, EEF1D, FBN1, FN1, FRMD6
RUNX2	-6.051	18	BMP2, CCR2, CDH1, DPYSL3, FN1, FRA10AC1, GADD45B, ITGB4, LEF1, MKI67
CAV1	-6.272	19	CAV1, CLDN7, FOLR1, GIPC2, KRT19, KRT7, KRT8, MMP1, NOTCH1, NR4A1
NFATC2	-6.511	26	ABCA1, BBX, CISH, CMPK2, CRYAB, CSF2, EGR3, ENPP2, FCER2, GATA3
MZB1	-6.596	2	CD86, TJP1
MKL2	-6.707	12	BCL2L1, CCL5, CXCL6, CYBA, ETV6, ITGA6, LTF, MAPK13, S100A8,

			SERPINB2
IRF6	-6.985	7	ENPP2, GRHL3, NLRP3, NR4A2, OVOL1, P2RX4, TJP1
DCLK1	-7.038	3	KRAS, NOTCH1, SNAI1
CLDN7	-7.066	18	ABLIM1, FOSL1, ITPR3, LAMTOR1, LGR4, MT1A, MT2A, MUC1, MUC13, NCOA7
KIT	-7.153	13	ADORA3, BCL2L1, CCR2, CISH, CSF2, CSF2RB, KIT, MITF, OSM, RAB32
WWC1	-7.189	3	BMP2, CDH1, OCLN
PLAT	-7.308	5	FN1, INHBA, MMP1, SERPINE1, TNF
PRKAA2	-7.372	15	CA2, CDH1, COX4I1, DDAH1, DOCK4, ERN1, FASN, FN1, FOXO3, MGST1
SNAI2	-7.691	13	CDH1, DSP, FN1, HPGD, ITGA3, ITGB4, JUP, L1CAM, MEF2C, MUC1
EHF	-7.716	16	CRABP2, EHF, EREG, FOLH1, GRHL3, MET, MMP1, MUC1, NOTCH2, PLAT
ACE2	-7.778	3	FN1, SERPINE1, TNF
TP63	-7.88	53	AGR2, AHR, ALDH1A3, ATG2B, BCL2L1, BRAF, BRCA1, CCNT1, CDK18, CDK6
PRDM5	-7.935	13	EBF1, FBXO33, GADD45B, GCK, IL6R, NCOA7, NOTCH1, NOTCH2, OVOL1, PHC1
FGFR1	-8.04	10	EIF1AX, EIF4EBP1, EIF4EBP2, FGFR1, HAS2, HES1, MMP1, NOTCH1, NR1H3, PDPK1
VCAM1	-8.091	2	ABCG2, VCAM1
TFAP2A	-8.289	12	ABCA1, ALCAM, CDH1, DCLK1, EREG, GEM, HIPK3, KRT5, MT2A, STRA13
FASN	-8.555	4	FASN, GPAT, MET, TNF
ERBB3	-8.696	14	ADAM19, CCNG2, COL6A1, ERBB3, FBN1, GZMH, HAS2, HBEGF, SLC4A7, SLPI
F2RL1	-8.768	10	CAV1, CSF2, CYR61, F2RL1, HBEGF, ITGB4, RARG, SERPINB2, TNF, VEGFA
EPHB4	-8.769	7	BMP2, CSF2RA, GATA3, KIT, STAT5B, TGFB2, TGFBR1
FOXO3	-8.785	22	ATP6V0D2, CCNG2, CDH1, CDKN1C, CYR61, DEFB4A/DEFB4B, EIF4BP1, FASN, FOSB, FOXO3
GATA2	-8.802	11	CCL5, ETS1, ETV6, GATA3, IKZF1, KIT, MEF2C, PECAM1, TFRC, TMPRSS2
MUC1	-8.887	6	BCL2L1, CDH1, MKI67, MMP13, SNAI1, TNF
ABCG2	-9.116	2	ABCG2, GSR
RIPK4	-9.185	3	GRHL3, IRF6, OVOL1
EREG	-9.496	5	CALB1, EREG, HAS2, HBEGF, TNF
BMP2	-9.51	31	ATP2C2, BC2L1, BMP2, BPIFB3, BTC, CALB1, CREB3L1, FGFR1, FN1, FOSB
PTHLH	-9.542	10	BCL2L1, FN1, FOSL2, ITGA6, LEF1, MMP13, RGS2, SERPINE1, VDR, VEGFA
EFEMP1	-10.06	2	CDH1, VEGFA
HOXA9	-10.934	22	ADD3, AKAP13, CALB1, CBX6, CCL3, CDH1, CISH, CREM, GPR56, GTPBP1
FBN1	-11.117	6	CYR61, FBN1, INHBA, MMP1, SERPINE1, TIMP3
NR3C2	-11.408	15	BCL2L1, CACNA1H, CYFIP2, FN1, KLF9, LYST, NDRG2, NR4A2, RGS2, RRAD
PDE4D	-11.408	2	MITF, PLAT
LBP	-11.722	4	CXCR2, IFIT1, LBP, TNF

HBB	-11.91	3	PECAM1, SERPINE1, VCAM1
PGR	-12.372	26	AKAP13, ALDH1A3, ALOX15, BLCL2L1, BCL6, CALD1, CCL8, CDKN1C, EIF1AX, EPAS1
ITGB6	-12.582	4	CDH1, MMP12, PTHLH, SERPINE1
SLIT2	-12.673	3	BCL2L1, CDH1, SNAI1
TGFB2	-15.754	18	ABCA1, AHR, FN1, HAS2, HBEGF, NFATC2, PECAM1, PTAFR, PTHLH, SERPINE1
CYR61	-17.113	9	BCL2L1, CYR61, ITGA2, ITGA3, ITGAV, MMP1, NR4A1, SERPINE1, VEGFA
EGFR	-17.165	54	BCL2L1, BCL6, BTC, CAV1, CCL5, CCNG2, CDH1, CDK6, CLK1, COL6A1
PROM1	-17.889	2	DSG2, NR4A2
THBS1	-18.44	5	FN1, TGFB2, THBS1, TNF, VEGFA
PPARGC1A	-26.285	18	ACACA, ATP5J2, ATP5O, BCAT2, CIDEA, COX4I1, COX5B, CYCS, FASN, ME1
LTBP1	-27.834	7	BCL2L1, BMF, GATA3, IL9, SERPINE1, SMAD3, VWF
GRHL2	-31.024	6	BMP2, CDH1, CLDN4, ERBB3, OCLN, SEMA3B
PAX5	-32.188	11	BLK, CD19, CD22, CSF2RA, FCER2, IGJ, IGLL1/IGLL5, MET, POU2AF1, PTPRF
RNASE1	-34.244	7	CCL24, CCL3, CCL5, CCL8, FCER2, OSM, TNF
DCN	-41.198	16	CDH1, CDKN1C, FBN1, ITGAV, KIT, MET, MMP1, MMP13, SDC1, SERPINE1
SIN3A	-326.801	10	ALDH6A1, BCL6, CCNG2, GADD45B, GSTP1, HOXA9, KLF10, KLF6, REST, SYT4
FOXM1	-7777.011	11	CAV1, CDH1, CENPB, FLT1, LEF1, PECAM1, SNAI1, TJP1, TOP2A, VCAN

CAPÍTULO 6

Discusión general

1. DISCUSION GENERAL

En los últimos años, ha aumentado considerablemente el interés por conocer la relación entre la dieta y la salud. Se ha demostrado que muchos alimentos tradicionales como las frutas, las verduras, el pescado y la leche contienen componentes que resultan beneficiosos para nuestro organismo. Como consecuencia de esta situación, surgen los alimentos funcionales que son elaborados no sólo por sus características nutricionales sino también para cumplir una función específica como puede ser mejorar la salud y reducir el riesgo de contraer enfermedades. Dentro de este contexto, aparecen las leches enriquecidas en AG omega-3 y ácido conjugado linoleico (CLA), que en la actualidad a tomado gran importancia por sus efectos beneficiosos relacionados con la salud humana.

El motivo de esta tesis surge a partir del interés de granjas lecheras comerciales en producir una leche enriquecida en AG omega-3 y CLA de forma natural. Esta tesis fue fruto de un proyecto CDTI en colaboración con la empresa lechera catalana ATO. Esta empresa está compuesta por siete granjas lecheras en Catalunya de entre 800 y 1500 vacas, las cuales suplementaban sus dietas con lino extrusionado con el objetivo de producir una leche enriquecida en AG omega-3 y CLA. Esta alimentación provocaba una disminución de la cantidad de la grasa láctea hasta porcentajes tan bajos como 2,5, lo que suponía al granjero penalizaciones por no cumplir los mínimos requisitos de garantía de composición, además del gasto adicional que supone incluir lino en la ración, una leguminosa más cara que la colza y la soja.

A partir de la idea general de obtener una leche enriquecida en AG omega-3 y CLA surgió el motivo del primer estudio de esta tesis, con la intención de evaluar la importancia del CLA en la salud humana y la necesidad de invertir, tanto científica como comercialmente, en la producción de alimentos enriquecidos en CLA. Nos centramos en el CLA ya que en nuestra opinión la necesidad de aumentar el consumo de AG omega-3 en la dieta humana está ampliamente justificada. Los AG omega-3 disminuyen la incidencia de enfermedades cardiovasculares y además es importante mantener una relación entro los AG omega-6 y los omega-3 para observar estos efectos (Kris-Etherton *et al.,* 2002). Los AG omega-6 se consumen en exceso en las dietas modernas, y diversos estudios en nutrición demuestran que las dietas occidentales, y más aún la típica estadounidense, pueden tener proporciones de 10:1, cuando las recomendaciones para prevenir enfermedades cardiovasculares son de 4:1 (Kris-Etherton *et al.,* 2002).

Para observar la importancia del CLA en la salud humana se realizó una revisión bibliográfica para revisar sus supuestos efectos beneficiosos e identificar unas recomendaciones diarias establecidas, ya que si supuestamente somos deficientes y la percepción en la sociedad es que es necesario aumentar nuestro consumo, y por lo tanto crear productos enriquecidos en CLA, lógicamente se asume implícitamente que han de existir unas recomendaciones establecidas.

En los últimos años un gran número de estudios en modelos animales han demostrado que consumir CLA resulta en diferentes efectos beneficiosos, entre ellos, la reducción de la carcinogénesis, de la aterosclerosis, del colesterol, de la ganancia de tejido adiposo y, de la diabetes mellitus tipo II y la modulación de la respuesta inmunitaria e inflamatoria, entre otros (Belury, 2002; Parodi, 2004; Pariza *et al.*, 2001). Sin embargo, los estudios epidemiológicos en humanos son inconcluyentes (Aro *et al.,* 2000; Chajès *et al.,* 2002; Riserus *et al.,* 2002; Moloney *et al.,* 2004; 2007; Whigham *et al.,* 2007). La opinión de la European Food Safety Authority (EFSA) de 2010 concluyó que el consumo de una mezcla equimolar de los isómeros *cis*-9, *trans*-11 CLA y *trans*-10, *cis*-12 CLA no tenia efectos en el mantenimiento o mejora del peso corporal normal, en incrementar la masa muscular, en incrementar la sensibilidad a la insulina, en la protección frente al daño oxidativo del DNA, proteínas y lípidos, o en la contribución de las defensas inmunitarias en respuesta a la vacunación (Tetens, 2010).

Además de no parecer tan claros sus efectos en humanos, sorprendentemente, los resultados de este estudio parecen indicar que no existe un consenso en la dosis necesaria de CLA para observar sus efectos beneficiosos. Para su efecto anticancerígeno las únicas dosis encontradas en la bibliografía son extrapoladas de modelos animales. Uno de los estudios realizados en ratas observó que las dietas que contenían 0,1% de CLA reducían la incidencia de tumores mamarios (Ip *et al.,* 1994). Con estos datos los autores realizaron una extrapolación a los humanos partiendo de que si una rata de 350 g de peso vivo necesita 0.015 g/d de CLA, un humano de 70 kg tendría que ingerir 3 g/d. Además, Terpstra (2001) sugirió que una extrapolación basada en el peso metabólico sería más correcta, lo cual resultaría en una dosis de 0.8 g/d de CLA al día. A parte de estas dosis extrapoladas a humanos, no hay ninguna cita que confirme estas dosis para humanos. Al contrario, los pocos estudios que hay son inconcluyentes, y sólo un estudio encontró una asociación negativa entre el consumo de CLA y el riesgo de padecer cáncer de mama en mujeres postmenopáusicas (Aro *et al.*, 2000). Otros estudios no encontraron ningún efecto del CLA en la protección contra el cáncer de mama (Chajès *et al.,* 2002, Voorrips *et al.,* 2002, McCann *et al.,* 2004). McCann *et al.* (2004) defendió

que en los estudios en humanos las dosis son mucho más bajas que las utilizadas en modelos animales, y en este estudio las dosis estaban entre 134 y 161 mg/d, claramente inferiores a los 3 g/d citados por Ip *et al.* (1994) o los 0,8 g/d citados por Terpstra (2001). El hecho es que no podemos saber si realmente dosis superiores en humanos son suficientes o simplemente si el CLA en humanos es anticancerigeno. Es indudable que estos efectos son a largo plazo y difíciles de comprobar, pero esta dificultad no puede justificar extrapolaciones de modelos animales para establecer recomendaciones.

El efecto antiaterosclerotico es el siguiente más descrito donde diversos estudios en conejos y ratones han demostrado que consumir CLA disminuye el riesgo del desarrollo y de la regresión de la aterosclerosis (Kritchevsky *et al.,* 2000; 2002; Arbonés-Mainar *et al.,* 2006). Kritchevsky *et al.* (2004) demostraron que dietas suplementadas con 0.5% y 1% del isómero *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA o una mezcla de ambos, inhibía la aterosclerosis y resultaban en una regresión de lesiones establecidas en conejos, respectivamente. Si usamos las mismas fórmulas que Trepsta (2001) para realizar la extrapolación a humanos basándonos en el peso metabólico estas dosis resultan en 0.6 g/d para observar algún efecto inhibitorio de la aterosclerosis y 12 g/d para observar la regresión de lesiones establecidas. Pero igual que para el efecto anticancerigeno no hay evidencias suficientes para decir que el CLA es antiaterosclerotico en humanos y, por supuesto, tampoco es posible definir una dosis establecida en humanos. Para los demás efectos atribuidos al CLA la información es mucho más limitada, lo que no nos ha dado pie a analizar supuestas dosis necesarias.

Otro de los efectos estudiados y del cual sí hay información en humanos es para reducir la grasa corporal y para conservar el peso corporal después de una pérdida de peso. En este contexto, Whigham *et al.* (2007) realizó un metaanalisis que englobaba 18 estudios que analizaron el efecto del CLA en la pérdida de peso: en 7 encontraron una reducción de la grasa corporal, y los demás no obtuvieron resultados significativos. Aunque podamos decir que hay alguna evidencia de este efecto, la dosis media utilizada en estos estudios es de 3.2 g/d de CLA, muy por debajo del consumo actual, y que sólo podría cumplirse mediante suplementos sintéticos. Por otro lado, este efecto está relacionado principalmente con el isómero *trans*-10, *cis*-12 CLA que no se ha relacionado con los otros efectos beneficiosos atribuidos mayoritariamente al isómero *cis*-9, *trans*-11 CLA. Además, en mi opinión, este efecto tiene fines estéticos, en la mayoría de los casos, y no de salud, por lo que no se consideraría como un efecto beneficioso para la salud humana.

Con todas estas evidencias, parece claro que no existen unas recomendaciones diarias de CLA en humanos y que las únicas dosis utilizadas son extrapoladas de modelos animales con toda las dudas, que, al menos a nuestro parecer, genera.

Del primer estudio también se calculó que el consumo actual de *cis*-9, *trans*-11 CLA es de 211 mg/d y si consideramos que los humanos convertimos parte del *trans*-11 C18:1 a *cis*-9, *trans*-11 CLA (Turpeinen *et al.,* 2002), la suma del *cis*-9, *trans*-11 CLA ingerido más el endógeno sería de 295 mg/d.

Si cogemos como valor de referencia la dosis de 0.8 g/d, que parece la más establecida, está claro que somos deficitarios. Pero la primera discusión que genera esta afirmación es si, con los efectos beneficiosos esperados, vagamente demostrados, podemos hablar de "deficitarios". En mi opinión, el consumo diario de fuentes adicionales de CLA no parece, en ningún caso, algo necesario, básicamente por la poca evidencia científica de estos efectos en humanos. Con la excepción del supuesto efecto anticancerigeno que si realmente fuera demostrado tendría su importancia en la salud humana, los demás efectos pueden prevenirse, en la mayoría de los casos, llevando una vida saludable y, por lo tanto, podemos hablar de unas recomendaciones pero en ningún caso necesidades. La segunda discusión es si la dosis de 0.8 g/d o las otras dosis encontradas en este estudio pueden ser creíbles. En nuestra opinión una extrapolación directa desde modelos animales genera muchas dudas al respecto y no hay ninguna base en humanos que las corrobore, partiendo de las diferencias que puedan existir entre una rata o un conejo y un humano, que creo que son más que evidentes, está claro el por qué de la duda de estas dosis.

En consecuencia, existe una clara necesidad de verificar los efectos del CLA en humanos y de establecer unas recomendaciones diarias de CLA, si las hubiera, y los efectos que se podrían observar con estas recomendaciones.

Por otro lado, en el área de nutrición animal se han estado invirtiendo muchos recursos para investigar las diferentes estrategias nutricionales que incrementen la cantidad de CLA en la leche y sus derivados, principalmente, ya que los productos de rumiantes (leche y carne) son la principal fuente de CLA. Consecuentemente enriquecer estos productos con CLA sería la mejor estrategia para aumentar el consumo de CLA de forma natural.

En esta tesis las estrategias en nutrición animal analizadas han demostrado ser efectivas en incrementar el contenido de CLA en la leche de vaca. Las estrategias más eficaces en aumentar el contenido de CLA en la leche fueron la suplementación con aceites de pescado

más aceites vegetales y las dietas suplementadas con aceites vegetales ricos en linoleico y linolenico. La leche derivada de vacas lecheras alimentadas con dietas suplementadas con aceites de pescado más aceites vegetales producirían una leche con un contenido de CLA de 395 mg de *cis*-9, *trans*-11 CLA/l lo que significa un incremento de 2,1 veces comparado con la leche producida por vacas lecheras con una dieta control (188 mg/l).

Aunque está claro que es posible aumentar el contenido de CLA mediante estrategias nutricionales, no se puede valorar si éste incremento es suficiente para cumplir unas recomendaciones diarias de CLA en la salud humana o sería necesario incluir fuentes sintéticas, debido a la falta de información sobre sus posibles efectos en la salud humana y las recomendaciones para observarlos.

Todas estas evidencias hacen cuestionar si es necesario el esfuerzo y dinero que se ha invertido en ciencia animal dentro del contexto de enriquecer productos con CLA, ya que parece que la base científica sobre los efectos saludables del CLA en humanos es inconcluyente. En esta tesis se seleccionaron 69 artículos con estos y, en nuestro parecer, parece desproporcionado este número de artículos y la inversión económica y el esfuerzo de personal que genera con el objetivo de aportar un alimento enriquecido con un CLA cuya necesidad real en humanos es cuestionable. Entonces la pregunta es ¿Por qué? En este punto, a nuestro parecer, juega un papel importante el marketing comercial de estos productos, que está en aumento en las últimas décadas incidiendo en la gente en las malas costumbres alimentarias y como mejorarlas, que hace que la gente no se cuestione simplemente si tiene una buena alimentación o no (algo necesario para conservar la buena salud) sino que se cuestione problemas y enfermedades, y confíe en tratamientos que tal vez no sean ni necesarios ni efectivos. Esta presión hace que aunque los efectos beneficiosos del CLA no estén demostrados científicamente, la gente crea en necesidad de aumentar su consumo de CLA, ya que el marketing ha influenciado esta decisión en el consumidor. El problema es que la comunidad científica, indirectamente, ha seguido esos pasos, ya que aunque no sea un problema "real" la sociedad pide respuestas y hay que darlas.

Aunque el primer estudio produce dudas de la necesidad de enriquecer la leche con CLA, la realidad es que las granjas comerciales están invirtiendo en estrategias nutricionales y manejo para producir este tipo de leche. Por lo tanto, hay que buscar soluciones a los efectos colaterales de la producción de una leche enriquecida en omega-3 y CLA, principalmente, la disminución de la cantidad de la grasa láctea.

Como se ha visto a lo largo de esta tesis, los isómeros responsables de la DGL son los isómeros *trans*-10 C18:1 y *trans*-10, *cis*-12 CLA. Estos se producen en el rumen (*trans*-10 C18:1) o derivan de él en la glándula mamaria (*trans*-10, *cis*-12 CLA). Por lo tanto el objetivo principal del segundo experimento de esta tesis era inhibir la producción de estos 2 isómeros en el rumen para disminuir la cantidad de ellos en la glándula mamaria. Se probaron 2 lipasas, un inhibidor de lipasas y tres aceites esenciales (PTSO, Cinnamaldehido y Eugenol) a 2 pH diferentes 6,4 y 5,6. Los resultados mostraron que una disminución del pH inhibia la aparente biohidrogenación del ácido linoleico (LA) y linolenico (LNA), e incrementó la eficiencia de los pasos intermedios de la biohidrogenación. La disminución del pH también causó un incremento en las concentraciones de *trans*-10 C18:1 y *trans*-10, *cis*-12 CLA. Todos estos efectos del pH eran esperables (Fuentes *et al.,* 2009, 2011). Los resultados del experimento de fermentación *in vitro* sugerían que el tratamiento lipasa 1, incrementaba la biohidrogenación aparente del ácido linolénico y, en consecuencia, aumentaba los ácidos grasos poliinsaturados (AGPI) intermediarios de la biohodrogenación. Estos efectos podrían explicarse por la hipótesis propuesta por Lourenço *et al.* (2010) que indicaron que un aumento en la actividad lipolítica puede provocar una saturación del sistema de biohidrogenación al existir más AGPI disponibles. Pero estos efectos no se observaron en fermentadores de doble flujo continuo y tampoco se observó una disminución de los isómeros *trans*-10 C18:1 y *trans*-10, *cis*-12 CLA en ninguno de los 2 experiemntos *in vitro* que era el objetivo principal de este estudio.

Respecto a los aceites esenciales, la inclusión de PTSO y Cinnamaldehido en fermentaciones *in vitro* inhibió la biohidrogenación aparente del LNA, lo que resultó en un aumento de AG omega-3 y un cambio del perfil hacia uno más insaturado, que debería ser más beneficiosos para la salud humana pero ninguno de los dos aceites esenciales mostraron efectos importantes en los isómeros *trans*-10 C18:1 y *trans*-10, *cis*-12 CLA. El tratamiento con Cinnamaldehido no tuvo efectos en los fermentadores de doble flujo continuo, lo que concordaría con los resultados por Benchaar y Chouinard (2009) que probaron una dosis de 1 g/d en vacas lecheras, lo que podría indicar que sus efectos se pierden a largo tiempo.

El aceite esencial PTSO fue el único que mostró efectos en fermentadores de doble flujo continuo, donde inhibió la aparente biohidrogenación del LA y LNA, lo que representa un cambio en el perfil hacia uno más insaturado, pero disminuyó la concentración de AG volátiles, lo que indica una alteración en la fermentación ruminal. Esta afectación de la fermentación ruminal podría ser la causa de la inhibición de la biohidrogenación aparente, por lo que los efectos del PTSO no son claros. Aunque el efecto negativo del PTSO sobre la fermentación

ruminal pordría atribuirse a la dosis, esta fue seleccionada en base a los experimentos realizados en nuestro grupo con anterioridad, dónde no se habían observado estos efectos (Busquet *et al.*, 2005; Foskolos *et al.*, 2015). Aunque en este experimento no queden claros los efectos del PTSO en la biohidrogenación, un trabajo realizado en nuestro grupo (Foskolos, 2012) demostró cambios en el perfil de los AG de la leche de vaca hacia uno más sin afectación de la fermentación ruminal.

Por lo tanto, parece que algún tratamiento en este estudio (lipasa 1 y PTSO) podrían tener un efecto en el cambio del perfil de AG, y en el caso del PTSO parece que podría tener un efecto beneficioso cambiando la relación entre AG saturados e insaturados de la leche. Pero en relación al objetivo de inhibir la vía alternativa de la biohidrogenación para disminuir la concentración de los isómeros responsables de la DGL no se observo ningún efecto positivo.

Al observar que mediante estrategias nutricionales no éramos capaces de evitar la DGL, se replanteó la dirección de la tesis para profundizar en el entendimiento de la DGL, dónde la bibliografía indicaba que ésta estaba relacionada con la represión coordinada de varios genes involucrados en la síntesis de la grasa láctea mediante los isómeros producidos en la vía alternativa de la biohidrogenación, principalmente a través del isómero*trans*-10, *cis*-12 CLA. Pero a la vez, analizando los controles lecheros mensuales en las granjas sometidas a una dieta suplementada con lino extrusionado relacionada con este proyecto, observamos que podíamos diferenciar cierto grupo de animales que bajo la dieta suplementada con lino no disminuían la cantidad de la grasa láctea (Figura 1). Como se puede observar en este gráfico la respuesta a la suplementación de lino es muy variable, debido a la variación individual de los animales. Pero en ciertas situaciones, como ésta, cabe la posibilidad de que esta falta de respuesta al efecto reductor de la grasa láctea tenga un origen genético, en cuyo caso abriría la puerta a seleccionar animales resistentes a la depresión de la grasa láctea.

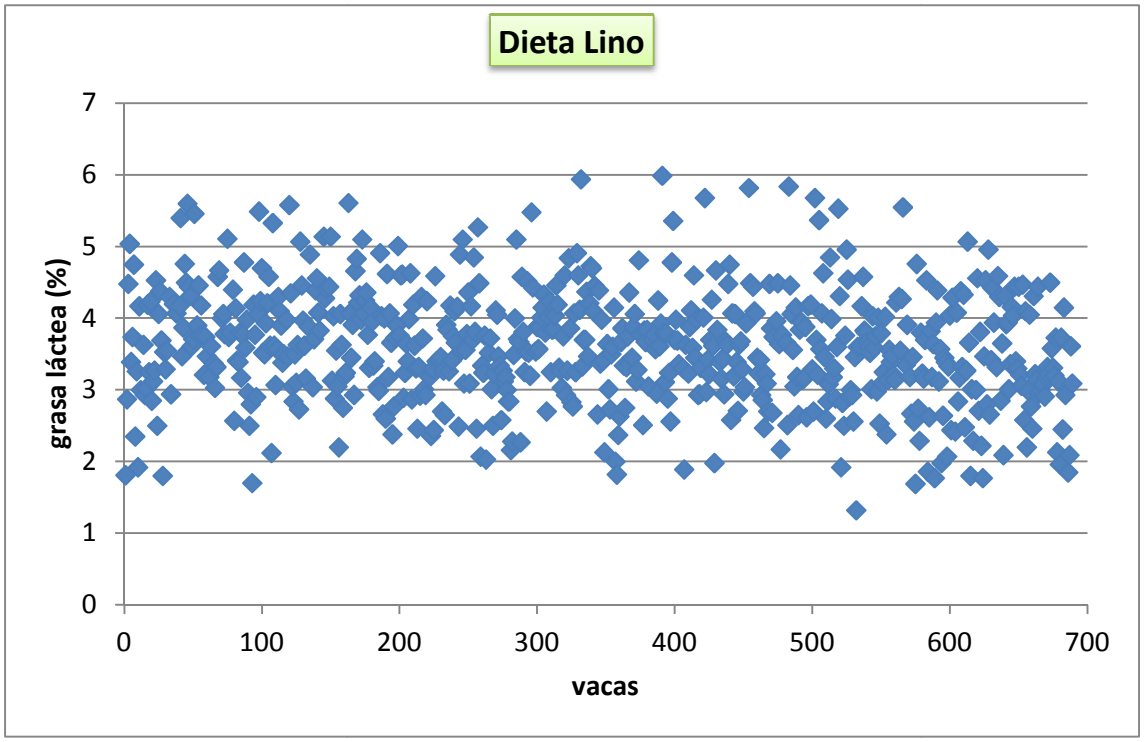

Figura 1. *Contenido de grasa láctea en porcentaje en la leche de vacas lecheras que recibían una dieta suplementada con lino.*

Con estas evidencias se realizó el tercer estudio de esta tesis, para estudiar la respuesta al lino en el transcriptoma de vacas lecheras resistentes (sin disminución de la grasa láctea con una dieta suplementada con lino; RestDGL) o sensibles (disminución de la grasa láctea con una dieta suplementada con lino; SenDGL) a la DGL. Los resultados de este experimento mostraron un gran número de genes diferencialmente expresados entre las vacas RestDGL y las vacas SenDGL, y entres las dietas control y lino, excepto cuando se compararon las vacas SenDGL con una dieta control y una dieta suplementada con lino. La gran diferencia entre el número de genes diferencialmente expresados entre los animales RestDGL y los animales SenDGL cuando se compararon las dos dietas, sugiere que los animales RestDLG podrían activar un mecanismo compensatorio para incrementar la síntesis de grasa láctea ante dietas suplementadas con lino. Los genes hasta ahora identificados e implicados en la síntesis y la depresión de la grasa lácea (Bionaz y Loor, 2008; Wickramasinghe *et al.,* 2012; Bauman *et al.,* 2011) también mostraron cambios en su expresión en nuestro estudio, pero ningún cambio deja claro qué genes podrían estar relacionados con la resistencia a la DGL, ya que los resultados muestran diferencias en la expresión de genes y diferentes isoformas de las descritas en situaciones de DGL. Estos podrían estar relacionados con la resistencia a la DGL pero también muestran estas diferencias en las condiciones normales de la DGL (SEnDGL con dieta control *vs.* SenDGL con dieta lino). Estos resultados sugieren que existen redes más complejas de genes que están

involucradas en el control de la síntesis de la grasa láctea y en la respuesta de estas vías metabólicas a las dietas que inducen la DGL. Además, las vacas RestDGL parecen tener una respuesta diferente a la descrita en la síntesis y en la depresión de la grasa láctea, que indicaría que hay otros genes diferentes a los descritos para las dos situaciones anteriores que podrían estar relacionados. Los resultados del análisis de las vías metabólicas, los genes reguladores claves y los SNP entre las vacas SenDGL y RestDGL alimentadas con ambas dietas también mostraron un gran número de vías metabólicas y genes reguladores diferencialmente expresados entre estos animales y las diferentes dietas, y un gran número de SNP en ambos grupos de animales, RestDGL y SenDGL.

Los resultados de este experimento sugieren la posibilidad de seleccionar animales resistentes a la DGL, ya que parece que, aunque no podamos decir claro que genes o redes formarían parte del control de la síntesis de la grasa láctea, la respuesta en situaciones de DGL o la respuesta de los animales resistentes a la DGL, es diferente entre los animales SenDGL y RestDGL, y entre las dietas control y lino. Para seleccionar estos animales, el primer paso sería profundizar en los resultados de este mismo estudio con el objetivo de seleccionar los SNP candidatos y comprobarlo mediante el genotipado de una población independiente. Además, en este experimento se demuestran redes más complejas de las descritas en la bibliografía de genes involucrados en el control de la síntesis de la grasa láctea, en la DGL y en el mecanismo de la resistencia a la DGL que tendríamos que llega a entender, y deben ser la motivación de futuros estudios en esta área.

Este estudio nos demuestra la complejidad de relacionar toda la información que proporciona este tipo de estudios de nutrifisiogenómica, al menos desde la perspectiva de la nutrición, pero nos abre la mente a la capacidad de respuesta génica a los cambios en la nutrición y el potencial de estas técnicas genéticas para entender la nutrición y su manipulación, y poder solucionar problemas que hasta ahora sólo se han enfocado des del punto de vista nutricional.

La nutrifisiogenómica fue descrita por Loor *et al.* (2013), y se utiliza para enfatizar la estrecha relación entre la nutrición y la fisiología mediante el estudio de la expresión de mRNA en diferentes tejidos durante las adaptaciones a diferentes condiciones fisiológicas, como alrededor del parto, o cambios nutricionales, fisiológicas y patológicas. Durante el periodo de transición en vacas lecheras, y como en la mayoría de mamíferos, varios órganos claves (ejemplo: hígado, tejido adiposo, glándula mamaria) trabajan coordinadamente e integrando diferentes niveles de regulación (ejemplo: DNA, mRNA, proteínas, metabolitos) para permitir

al animal ajustarse a las demandas fisiológicas de la lactación. Por lo tanto, la nutrifisiogenómica es el termino más apropiado para describir la investigación integrante en vacas lecheras que depende de técnicas genéticas (Loor *et al.,* 2013). La transcriptómica estudia la información genética de las proteínas, y los cambios en la expresión de mRNA que tienen una mayor influencia en las adaptaciones fisiológicas.

En 1995, se describió por primera vez la técnica de microarrays para estudiar la expresión de RNA a gran escala (Schena *et al.,* 1995). Esto supuso un cambio revolucionario para estudiar las vías y funciones de las adaptaciones en células y tejidos. Desde el conocimiento de estas técnicas se han realizado muchos esfuerzos para desarrollar microarrays específicos de diversas funciones y especies (ver revisión: Loor *et al.,* 2013).

Por otro lado, las nuevas tecnologías en la área de transcriptómica están ganando terreno sustituyendo a los microoarrays. El uso de la genómica en bovino ha aumentado considerablemente en la última década, particularmente en el campo de la expresión de mRNA (Figura 2). La mayoría de estudios publicados hasta ahora han utilizado la técnica de microarrays, y hay pocos artículos que hayan utilizado la técnica de RNA sequencing para estudiar el transcriptoma completo de vacas lecheras. Sin embargo, la técnica RNA sequencing parece que pronto será la herramienta de oro para el análisis transcriptómico (Loor *et al.,* 2013). La principal ventaja de la técnica de RNA sequencing comparado con los microarrays es el estudio del transcriptoma completo, splice variants, cadenas cortas de RNA y transcritos desconocidos hasta el momento.

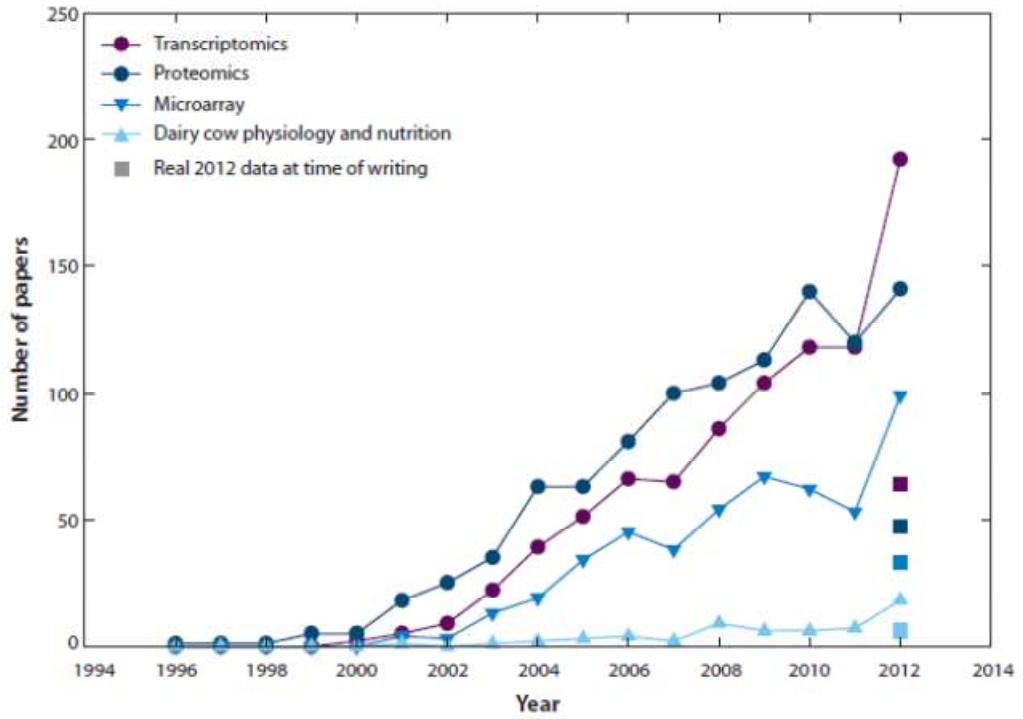

Figura 2. *Número de articulos cientificos encontrados en PubMed que utilizan técnicas de transcriptomica, proteomica, o microarrays para estudiar la especie bovina. (Adaptada de Loor et al., 2013).*

La evolución de las técnicas de transcriptómica, el conocimiento del genoma de la especie bovina y el considerable abaratamiento de estas técnicas en los últimos años, hace posible utilizarlas para estudiar los cambios metabólicos que sufren las vacas a las adaptaciones fisiológicas y a los cambios en nutrición, abriendo un amplio campo de investigación.

Loor *et al*. (2013) describieron sólo 7 artículos hasta el año 2012 que utilizaban la técnica RNA sequencing enfocada a la nutrición, estado fisiológico, estado immune, y reproducción en vacas lecheras (Huang y Khatib, 2010; Wickramasinghe *et al.,* 2011; 2012; Walsh *et al.,* 2012; Huang *et al.,* 2012; McCabe *et al.,* 2012; Baldwin *et al.,* 2012). Hasta la fecha (septiembre, 2015) sólo 3 artículos más han utilizado está técnica para analizar la expresión de RNA en glándula mamaria o leche de vacas lecheras (Cánovas *et al.,* 2014; Cui *et al.,* 2014; Cánovas *et al.,* 2013).

Las dietas que causan la DGL representan un claro ejemplo de nutrigenómica, donde los AG intermediarios formados en la biohidrogenación ruminal actúan produciendo una represión coordinada de varios genes clave involucrados en la síntesis de la grasa láctea (Bauman *et al.,* 2011) pero todos los estudios realizados hasta la fecha solo han utilizado técnicas de microarrays y seleccionando genes clave, ningún estudio ha analizado el

transcriptoma completo con este objetivo. Esto ha dificultado la discusión de el tercer estudio de esta tesis pero a la vez genera la importancia de seguir en este camino y utilizar técnicas más complejas genéticas que las utilizadas hasta la fecha para intentar entender los cambios relacionados con la DGL y la resistencia de la DGL para el objetivo específico de esta tesis, pero que es aplicable a un amplio rango de investigación. En nuestro conocimiento sólo hay 3 grupos importantes que se estén dedicando a la integración de técnicas génicas en el conocimiento de la fisiología (Loor *et al*.), nutrición (Bauman *et al*.) y con un concepto más general como especie bovina (Medrano *et al.*) en vacas lecheras y aún hay poca información. De ahí la gran importancia que está tomando en el sector científico el término nutrifisogenómica ya que juntando estos tres campos podemos ser capaces de entender límites encontrados en las áreas de nutrición y fisiología que pueden dar la posibilidad de aplicar técnicas a la práctica para mejorar el manejo y producción en granjas de vacas lecheras.

En esta tesis se ha cuestionado la necesidad de generar más estrategias para incrementar el contenido de CLA en la leche mientras no se demuestre su necesidad real en la dieta humana, y seguir investigando en este camino no tiene mucho sentido. Si es cierta la deficiencia en ácidos omega-3, que la fuente importante de ellos son el pescado azul y el consumo de éste es la manera de consumir unas cantidades suficientes. Aunque la incorporación de lino en la dieta del vacuno lechero puede ser una estrategia para mejorar esta situación aunque provoque la DGL. Así, sí que tiene sentido intentar evitar la DGL, además de que esta puede producirse en otras circunstancias como dietas altas en concentrado y bajas en fibra (Bauman *et al.,* 2001) típicas de granjas de alta producción. Y por otro lado, los amplios campos donde podrían aplicarse estas técnicas génicas en el entendimiento de la fisiología bovina y en la adaptación a cambios nutricionales, el tercer estudio abre un amplio camino hacia la integración de estas áreas y la necesidad de crear grupos de investigación en colaboración con expertos en ambas áreas para poder entender y interpretar toda la información que genera este tipo de estudios.

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CAPÍTULO 7

Conclusiones

CONCLUSIONES GENERALES

Los estudios realizados en la presente tesis permiten concluir:

1. Se han sugerido diversos efectos beneficiosos para la salud humana relacionados con el consumo de CLA, como ser antiaterosclerotico, anticancerigeno, modulador de la composición de la grasa corporal, entre otros. Pero los estudios en humanos son inconcluyentes y no hay recomendaciones establecidas para estos efectos.
2. Diversas estrategias nutricionales en vacas lecheras, como la suplementación con aceites vegetales y aceites de pescado, han demostrado incrementar efectivamente el contenido de CLA en la grasa láctea. Aunque este incremento es importante no se puede valorar si es suficiente para llegar a unas recomendaciones diarias en humanos para observar sus supuestos efectos.
3. La adición de lipasas, un inhibidor de lipasa y tres aceites esenciales (PTSO, Cinamaldehido y Eugenol) en fermentación *in vitro* y en fermentadores de doble flujo continuo no tuvo ningún efecto en la disminución de la concentración de los isómeros *trans*-10 C18:1 y *trans*-10, *cis*-12 CLA, responsables de la disminución de la grasa láctea.
4. El estudio de transcriptoma de vacas resistentes o sensibles a la depresión de la grasa láctea demostró un gran número de genes y vías metabólicas diferencialmente expresados entre los animales resistentes y sensibles a la depresión de la grasa láctea en ambas dietas (control y lino) sugiriendo redes más complejas de genes involucradas en la síntesis de la grasa láctea y en el mecanismo de la resistencia a la depresión de la grasa láctea.
5. Los resultados del estudio de transcriptomica en vacas lecheras sensibles o resistentes a la depresión de la grasa láctea sugiere la posibilidad de seleccionar animales resistentes a la depresión de la grasa láctea, aunque es necesario profundizar en los resultados y en el entendimiento de los mecanismos responsables de la resistencia a la depresión de la grasa láctea para elegir SNPs candidatos para poder utilizar en un posterior genotipado de una población y llegar a seleccionar animales resistentes a la depresión de la grasa láctea.