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## SUPPLEMENT ARTICLE

## Dietary effects of linseed on fatty acid composition of milk and on liver, adipose and mammary gland metabolism of periparturient dairy cows

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### Summary

During the transition period in dairy cows, drastic adaptations within and between key tissues and cell types occur in a coordinated manner to support late gestation, the synthesis of large quantities of milk and metabolic homeostasis. The start of lactation coincides with an increase of triacylglycerols in the liver, which has been associated with several economically important diseases in dairy cows (i.e. hepatic lipidosis, mastitis). The polyunsaturated fatty acids have been used to improve liver metabolism and immune function in the mammary gland. Therefore, the effects of dietary linseed supplementation on milk quality and liver, adipose and mammary gland metabolism of periparturient dairy cows were studied in 14 cows that were randomly assigned to control or linseed supplementation. Animals were treated from 3 weeks antepartum until 6 weeks post-partum. Linseed did not modify dry matter intake, but increased milk yield and lactose yield, and decreased milk fat concentration, which coincided with lower proportion of C16 and higher proportions of stearic acid, conjugated linoleic acid and  $\alpha$ -linolenic acid in milk fat. Linseed supplementation did not significantly change the expression of key lipid metabolism genes in liver and adipose tissues, except of glucose transporter 2 (*GLUT2*) in liver, which was increased in cows supplemented with linseed, suggesting that more glucose was secreted and probably available for lactose synthesis compared with cows fed control diet. Large adaptations of transcription occurred in the mammary gland when dairy cows were supplemented with linseed. The main affected functional modules were related to energy metabolism, cell proliferation and remodelling, as well as the immune system response.

**Keywords** lactating dairy cows, polyunsaturated fatty acids, transcriptome

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### Introduction

Although increasing the proportion of polyunsaturated fatty acids (PUFAs) in milk is limited due to extensive biohydrogenation of dietary PUFAs in the rumen, supplementing linseed to dairy cattle increased the proportion of 18:2, cis-9, trans-11 (CLAc9t11) in milk (Glasser et al., 2008), while protected linseed oil has been used to increase 18:3, cis-9, cis-12, cis-15 (ALA) in milk (Van Vuuren et al., 2010). The PUFAs are well known to reduce lipid accumulation, upregulate fatty acid (FA) oxidation in liver and skeletal muscle, and increase total body glycogen storage in rodents (Jump and Clarke, 1999). The PUFAs also affect lipid metabolism in dairy cattle, thereby having important implications during the

transition period from pregnancy to lactation. During the transition period, drastic adaptations within and between key tissues and cell types occur in a coordinated manner to support late gestation, the synthesis of large quantities of milk and metabolic homeostasis (Finucane et al., 2008; Carriquiry et al., 2009; Castaneda-Gutierrez et al., 2009). Hormonally controlled mobilization of body fat reserves at the onset of lactation results in the release of non-esterified fatty acids (NEFA; Castaneda-Gutierrez et al., 2009), which are transported via the blood to the udder where they are re-esterified into triacylglycerols (TAG) and secreted in the milk, or to the liver where they are oxidized to CO<sub>2</sub>, converted to ketone bodies or re-esterified into TAG (Drackley, 1999). Excessive accumulation of TAG in the liver could result in hepatic lipidosis,

which impairs hepatic gluconeogenic capacity (Drackley, 1999). In periparturient dairy cows, PUFAs have been used in attempts to increase the functional food value of dairy products and to improve liver metabolism and function (Carriquiry *et al.*, 2009).

Linseed, which is a good source of ALA, is shown to increase liver concentration of glycogen before calving and decrease liver concentration of TAG after calving, indicating that feeding a source of n-3 FA around calving could reduce TAG accumulation in the liver, thereby preventing hepatic lipidosis in transition dairy cows (Petit *et al.*, 2007). In monolayer cultures of bovine hepatocytes, ALA appeared to be one of the most beneficial FAs because its addition to medium resulted in decreased TAG concentrations and one of the highest rates of gluconeogenesis compared with other PUFAs (Mashek and Grummer, 2003).

Furthermore, human and animal studies have provided evidence that PUFAs from plant or fish oil are important modulators of immune reactions (Calder *et al.*, 2002), with additional implications for the periparturient dairy cow. The increased nutrient demand of the periparturient cow in combination with the reduced dry matter intake (DMI) may contribute to compromised host defence, particularly as related to the regulation of inappropriate inflammatory response (Sordillo *et al.*, 2010). As a consequence, dairy cows are more susceptible to several economically important diseases such as metritis and mastitis (Sordillo *et al.*, 2010). For the mammary gland, it has been suggested that dietary n-3 FA can affect cellular immunity (Lessard *et al.*, 2003). They observed that 5 days after calving, the lymphocyte proliferative response of cows allocated to linseed treatment was reduced. We previously reported that enriched unsaturated FA (UFA) diets in mid-lactation cows may affect immune functions of the mammary gland and thus modify the susceptibility to mastitis (Mach *et al.*, 2011a). In addition, recent functional genomics, based on quantitative real-time polymerase chain reaction (qRT-PCR; Bauman *et al.*, 2008; Harvatine *et al.*, 2009; Kadegowda *et al.*, 2009) or microarray analysis (Mach *et al.*, 2011a,b), described the effect of dietary UFA on the expression of a large number of candidate genes in the mammary gland. However, little is known about the influence of supplemental PUFAs, especially ALA and their biohydrogenation intermediates, on the incidence of hepatic lipidosis during the transition period of dairy cows and the overall mammary gland health and integrity. Therefore, the objective of this study was to identify the effect of linseed supplementation on (i) milk FA composition and (ii) FA metabolism in liver, adipose and mammary gland tissue in

periparturient cows through the use of transcriptomics and bioinformatics tools.

## Materials and methods

### Animals and diets

Fourteen Holstein-Friesian cows (four first parity, two second parity, two third parity and six older cows) were used in a complete randomized block design structure. Cows were paired in six blocks on the basis of similarity in parity, expected date of calving and milk performance in the previous lactation (in order of priority). Cows within each block were randomly allocated to one of two treatment groups: (i) 'linseed' or (ii) 'control'. The dietary linseed treatment consisted of a basal diet supplemented with a concentrate mixture including 13% of extruded linseed on a dry matter (DM) basis, whereas cows in the control group were supplemented with a concentrate mixture without linseed (Tables 1 and 2). Linseed was chosen because it is rich in ALA. Concentrate mixture was fed with a concentrate dispenser (Table 1).

From 3 weeks before calving until calving, all cows received *ad libitum* a forage mixture that consisted of corn silage, wilted grass silage, wheat straw and solvent-extracted soya bean meal, and a pre-calving concentrate mixture (Table 1). The daily pre-calving concentrate allowance was increased gradually from 0 kg on day 21 to up to 2 kg at day 1. After calving, all cows received *ad libitum* (refusal weight of at least 10%) a forage mixture that consisted of corn silage, wilted grass silage, grass seed straw, solvent-extracted soya bean meal and formaldehyde-treated soya bean meal (Mervobest<sup>TM</sup>; Agrifirm, Meppel, the Netherlands), as well as a post-calving concentrate. The daily allowance of this post-calving concentrate was increased with 0.5 kg/day from 2 kg on day of calving up to 9 kg on day 16. This level of concentrate was maintained until the end of the experimental period. The concentrate mixture was fed individually using transponder concentrate dispensers. Daily, fresh forage mixtures were prepared using a self-propelled mixer wagon equipped with a cutter loader system and an electronic weighing unit. The formulations of the forage mixtures were adjusted twice a week for changes in DM content of the roughages. The forage mixtures were supplied in feed weighing troughs with transponder-controlled access gates (Insentec, Marknesse, the Netherlands), which were continuously accessible except during milking and when refusals were removed and fresh forage was supplied. Daily, between 10:30 and 11:00 AM, feed refusals were removed from the troughs and a fresh forage mixture

**Table 1** Ingredient of the experimental diets and the concentrates offered in the basal ration (The experimental diets included concentrate mixture, corn silage, grass silage and pasture)

Ingredient	Dietary treatments			
	Control		Linseed supplementation	
	Pre-calving	Post-calving	Pre-calving	Post-calving
Ingredients feed mixture, % of DM				
Wilted grass silage	30.9	51.1	30.9	51.1
Corn silage	30.2	33.2	30.2	33.2
Grass seed straw		7.3		7.3
Wheat straw	30.3		30.3	
Soya bean meal solvent extract	7.2	4.2	7.2	4.2
Soya bean meal formaldehyde treated	–	2.9	–	2.9
Vitamin and mineral premix	0.9*	0.5†	0.9*	0.5†
Magnesium oxide	0.5	–	0.5	–
Salt	–	0.2	–	0.2
Ingredients' concentrate mixture, % of DM				
Linseed			13.1	13.0
Corn	36.9	33.3	36.6	35.2
Wheat	16.4	18.7	18.4	19.9
Soya bean meal		10.7		2.7
Corn gluten feed		9.7		11.5
Palm kernel expeller	10.0	7.3		
Soya bean meal, formaldehyde treated	11.7	6.7	9.9	9.2
Cane molasses	2.5	3.0	3.1	3.0
Palm oil	2.7	3.0		
Citrus pulp	7.9	2.2	7.3	
Canola meal	3.6	2.0		2.0
Canola meal, formaldehyde treated	3.9		7.1	
Soy hulls	0.2		0.3	
Calcium carbonate		1.0		1.2
Sodium chloride		0.5		0.5
Urea		0.5		0.5
Monocalcium phosphate		0.1		
Magnesium oxide	0.3		0.2	
Ammonium chloride	2.6		2.6	

\*Dry cow mineral and vitamin premix providing 10.8 mg Cu, 10 mg Zn, 12 mg Mn, 0.12 mg Co, 0.32 mg I, 0.14 mg Se, 2500 IU vitamin A, 500 IU vitamin D3 and 40 IU vitamin E/kg DM of feed mixture.

†Lactating cow mineral and vitamin premix providing 6 mg Cu, 12.5 mg Zn, 15 mg Mn, 0.09 mg Co, 0.6 mg I, 0.17 mg Se, 2500 IU vitamin A, 500 IU vitamin D3 and 7 IU vitamin E/kg DM of feed mixture.

was supplied. The cows had unrestricted access to fresh drinking water. Experimental treatments started 3 weeks before the expected calving date (week 3) and lasted until 6 weeks after calving (week 6). The experiment was carried out between 9 February and 15 June 2009 at the Wageningen UR dairy research farm 'Waiboerhoeve' at Lelystad, the Netherlands. All

**Table 2** Chemical composition (% of DM, except where indicated else) and fatty acid composition of the experimental diets and the concentrates offered in the basal ration (The experimental diets included concentrate, corn silage, grass silage and pasture)

Component	Dietary treatments			
	Control		Linseed supplementation	
	Pre-calving	Post-calving	Pre-calving	Post-calving
DM, % of fresh weight	49.6	50.3	49.1	50.4
Ash	8.7	8.0	8.6	7.9
CP	13.9	17.6	13.9	17.3
Crude fat	3.1	4.5	3.2	5.2
NDF	47.2	32.9	46.5	31.7
ADF	29.1	18.3	28.7	17.7
Starch	11.3	21.6	12.1	22.5
Sugars	4.8	7.3	4.8	7.1
DOM	63.9	75.1	64.2	75.0
NEL (MJ/kg DM)	5.8	7.2	5.9	7.3
DVE	6.5	10.6	6.6	10.5
Fatty acid composition, g/100 g of fatty acids				
C4:0	0.0	0.0	0.0	0.0
C6:0	0.0	0.0	0.0	0.0
C8:0	0.0	0.1	0.0	0.0
C10:0	0.0	0.1	0.0	0.0
C11:0	0.0	0.0	0.0	0.0
C12:0	0.7	1.9	0.6	0.6
C14:0	0.5	0.9	0.4	0.4
14:1, <i>cis</i> -9	0.0	0.0	0.0	0.0
C15:0	0.1	0.1	0.1	0.1
C16:0	16.6	17.8	16.4	15.4
16:1, <i>cis</i> -9	0.2	0.2	0.2	0.2
C17:0	0.1	0.1	0.1	0.1
17:1, <i>cis</i> -9	0.0	0.0	0.0	0.0
C18:0	2.5	2.6	2.6	2.6
18:1, <i>trans</i> -9	0.1	0.1	0.1	0.1
18:1, <i>cis</i> -9	16.6	16.6	16.6	17.0
18:2, <i>cis</i> -9, <i>cis</i> -12	37.4	37.5	37.6	36.3
18:3, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	23.6	24.8	23.7	25.7

NEL, net energy lactation.

experimental protocols and interventions were approved by the Ethical Committee on Animal Experiments of Wageningen UR Livestock Research.

### Sample collection and measurements

Specific details regarding feed sampling, feed analysis and calculation of feeding values are described in the study by Zom *et al.* (2012). Milk yield was automatically recorded at each milking. Weekly, milk samples of each cow were taken at four consecutive milkings (two AM milkings and two PM milkings). Both AM milk samples were pooled to one composite sample; PM samples were also pooled. The composite AM and

PM milk samples were analysed for fat, protein and lactose content by Qlip (Zutphen, the Netherlands), using a Foss Milko-Scan<sup>®</sup> infrared automatic analyser (Foss Electric, Hillerød, Denmark). Further, in weeks +1, +2, +3 and +6, extra milk samples were collected at the AM milking and frozen at  $-20^{\circ}\text{C}$  until analysis for FA profile by gas chromatography. Specific details regarding the analysis of FAs in milk and feedstuffs are described in Mach et al. (2011a).

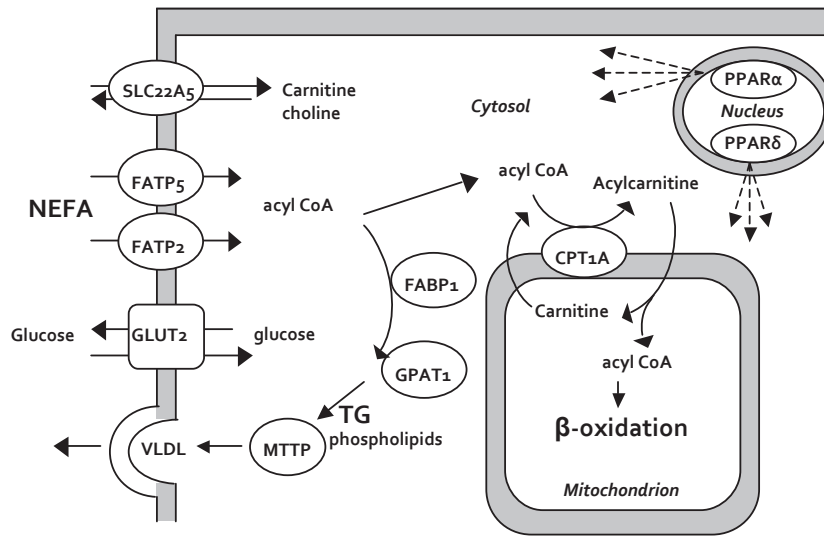
During days  $-21$ ,  $1$ ,  $4$ ,  $7$ ,  $10$ ,  $21$  and  $42$  after calving, blood samples were taken by jugular venipuncture. Blood samples to quantify glucose, NEFA and beta-hydroxy butyric acid (BHBA) were collected and processed as described elsewhere (Zom et al., 2012). Liver and adipose tissue biopsies were performed on Mondays in weeks  $-3$ ,  $+1$ ,  $+3$  and  $+6$  post-partum. During the biopsies, the cows were in a standing position, fixed in a headlock feed barrier. Liver biopsies were performed as explained by Zom et al. (2012). Approximately  $2\text{ g}$  of liver tissue in total was collected. Adipose tissue was sampled from the tail region, between the ischium (pin bone) and coccygeal vertebrae. An incision was made and  $5\text{ g}$  of adipose tissue was dissected. Incisions were sutured with staples and protected with a film dressing spray (Opsite, Smit and Nephew, Hoofddorp, the Netherlands). The concentrations of TAG in liver tissue were determined using enzymatic photometric analysis using a triglyceride test kit (Triglycerides Liquicolor Mono; Instruchemie, Delfzijl, the Netherlands) and photospectrometer (Humanalyzer 3000; Human Diagnostics, Wiesbaden, Germany). The samples were thawed, homogenized and extracted according to the prescriptions of the test kit. The photometric absorption at a wavelength of  $505\text{ nm}$  by quinoneimine was measured with photospectrometric analysis to estimate the concentration of TAG.

On the last day of the experimental period (week +6), mammary gland biopsies were carried out before the afternoon milking as described by Mach et al. (2011a). Liver, adipose and mammary gland tissue samples were collected into cryogenic, sterile, DNA-, DNAase- and RNase-free vials (Cryo.S<sup>®</sup>; Greiner Bio-one, Frickenhausen, Germany) and immediately were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA extraction.

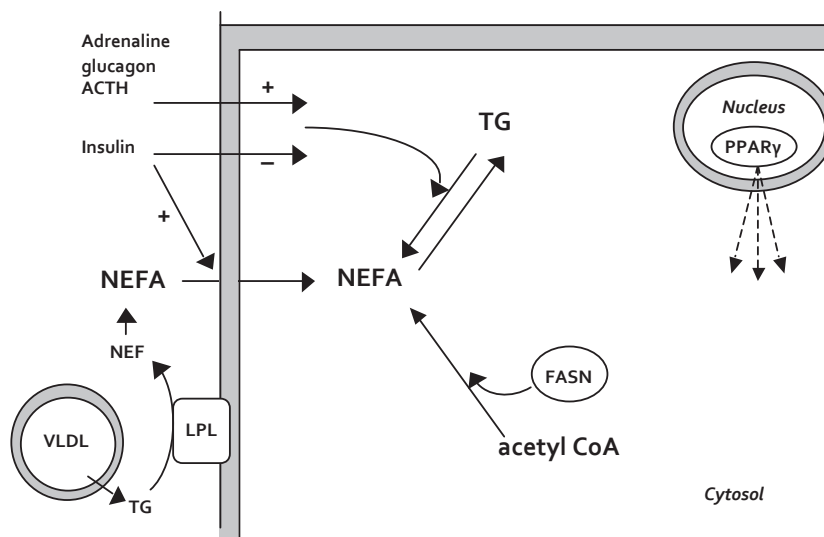
#### RNA isolation, quantitative real-time PCR, microarray data analysis

Total RNA from liver, adipose and mammary gland tissue was isolated using TRIzol reagent (Invitrogen, Breda, the Netherlands), following the manufacturer's

instructions. The isolated RNA was subjected to an on-column DNase treatment to eliminate DNA contamination (Nucleospin RNA II kit; Machery-Nagel, Düren, Germany). The resulting RNA purity and concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Isogen, Maarssen, the Netherlands), and the RNA quality was assessed using the BioAnalyzer 2100 (Agilent Technologies, Amsterdam, the Netherlands). Samples presented an average RIN of  $8.03 \pm 0.35$ . In the liver, changes in mRNA abundance of glucose transporter 2 (*GLUT2*), carnitine palmitoyltransferase I (*CPT1A*), glycerol-3-phosphate acyltransferase (*GPAT1*), solute carrier family 22 member 5 (*SLC22A5*), microsomal triglyceride transfer protein (*MTTP*), fatty acid transport protein 2 and 5 (*FATP2*, or *FATP5*), fatty acid-binding protein 1 (*FABP1*), peroxisome proliferator-activated receptor  $\alpha$  (*PPAR\alpha*) and peroxisome proliferator-activated receptor  $\delta$  (*PPAR\delta*) were measured using qRT-PCR assays (Fig. 1). Additionally, fatty acid synthase (*FASN*), lipoprotein lipase (*LPL*) and peroxisome proliferator-activated receptor  $\gamma$  (*PPAR\gamma*) were analysed in adipose tissue (Fig. 2). Briefly, first-strand cDNA synthesis was performed from  $1\text{ }\mu\text{g}$  of the isolated total RNA using Superscript III reverse transcriptase (200 units; Invitrogen), dNTPs ( $0.5\text{ mM}$ ; Roche Diagnostics, Almere, the Netherlands) and random hexamer primers ( $250\text{ ng}$ ; Roche Diagnostics) in a volume of  $20\text{ }\mu\text{l}$  for  $1\text{ h}$  at  $50^{\circ}\text{C}$  according to the manufacturer's protocol (Invitrogen). Templates were amplified after a pre-incubation for  $10\text{ min}$  at  $95^{\circ}\text{C}$ , followed by amplification for 40 cycles ( $10\text{ s}$  at  $95^{\circ}\text{C}$ ,  $5\text{ s}$  at  $60^{\circ}\text{C}$ ,  $5\text{ s}$  at  $72^{\circ}\text{C}$ ) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) by using Sensimix SYBR Low Rox kit (Bioline, London, UK). The gene-specific primers used in the liver and adipose tissues are reported in the Table S1. All reactions revealed a single product as determined by melting curve analysis. Quantitative measurement was performed by establishing a linear amplification curve from 10-fold serial dilutions of cDNAs for corresponding genes, and efficiencies of the used sets of primers were calculated to be at least 95%. Values were calculated according to the comparative CT method using  $\beta$ -actin (*ACTB*) as the housekeeping genes (HKG). The HKG was selected based on previous qPCR studies in liver of periparturient dairy cows (Rhoads et al., 2003; Janovick-Guretzky et al., 2007). Janovick-Guretzky et al. (2007) analysed eight HKG ( $\beta$ -actin, glyceraldehyde-3-phosphate dehydrogenase,  $\beta$ -glucuronidase, peptidylprolyl isomerase A, polyubiquitin, ribosomal protein S9, ribosomal protein L32 and 18S ribosomal RNA) in 91 liver RNA samples.



**Fig. 1** Fat metabolism in hepatocytes. Metabolic pathways important for fat metabolism in hepatocytes, showing the function of the genes analysed by qPCR in hepatic tissue. *PPAR $\alpha$* , peroxisome proliferator-activated receptor alpha; *PPAR $\delta$* , peroxisome proliferator-activated receptor delta; *GPAT1*, glycerol-3-phosphate acyltransferase 1; *MTP*, microsomal triglyceride transfer protein; *SLC22A5*, organic cation transporter (also known as OCTN2); *CPT1A*, carnitine palmitoyltransferase 1A; *FATP2*, fatty acid transport protein 2; *FATP5*, fatty acid transport protein 5; *FABP1*, fatty acid-binding protein 1; *GLUT2*, glucose transporter 2. Peroxisome proliferator-activated receptors (*PPAR*) are ligand-activated nuclear transcription factors that can be activated by non-esterified fatty acids (NEFA) and their derivatives, thereby regulating the transcription of other genes. *PPAR $\alpha$*  is involved in gene expression of genes involved in lipid oxidation and ketogenesis, and *PPAR $\delta$*  stimulates lipid oxidation and cell proliferation. *GPAT1* is located on the mitochondrial outer membrane that binds NEFA to glycerol, creating phospholipids or TG. *MTP* supports TG transport for VLDL assembly. Carnitine and other organic cations are transported into the cytosol by *SLC22A5* transporter. Carnitine is needed to transport NEFA into the mitochondrion, using *CPT1A* transporter on the mitochondrial membrane. *FATP2* and *FATP5* function as membrane transporters for long-chain fatty acid (FA), activating them to acyl-CoA. *FABP1* is a cytoplasmic protein that binds NEFA. *GLUT2* is a bidirectional glucose transporter located in the plasma membrane, with low glucose affinity and high transport capacity.



**Fig. 2** Fat metabolism in adipose tissue. Metabolic pathways important for fat metabolism in adipose tissue, showing the function of the genes analysed by qPCR. *PPAR $\gamma$* , peroxisome proliferator-activated receptor gamma; *LPL*, lipoprotein lipase; *FASN*, fatty acid synthase. *FASN* is needed in fatty acid (FA) synthesis, while *LPL* supports the uptake of FA from VLDL in the bloodstream. The *PPAR $\gamma$*  promotes adipocyte differentiation to enhance blood glucose uptake.

Initial analyses indicated that mRNA for ribosomal protein S9 (*RPS9*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and  $\beta$ -actin were the top three genes most stably expressed across several physiological and diet-related experimental conditions for dairy cows, making them a good HKG in liver quantitative PCR experiments.

The RNA of each biopsy of mammary gland was amplified, biotin-labelled and hybridized to single-dye Affymetrix GeneChip<sup>®</sup> Bovine Genome Array (#900493) by ServiceXS (Leiden, the Netherlands), as described in the users' manual (Affymetrix GeneChip<sup>®</sup> Expression Analysis Technical Manual, Santa Clara, CA, USA). A total of 14 one-colour arrays were prepared, one array per RNA sample. Details on transcriptome data acquisition, quality control, summarization and normalization are described in Mach *et al.* (2011a). As many of the original annotations for the Affymetrix GeneChip<sup>®</sup> Bovine have been found to be erroneous (Gautier *et al.*, 2004), a custom chip definition file (CDF; Bovine\_Bt\_REFSEQ version 14.0.0), available at <http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/14.0.0/refseq.asp> was used to re-annotate the probes to new probe sets. Therefore, the original probe set definitions were discarded, and all probes were recomposed into new probe sets by mapping each probe via their sequence to unique genes available in Refseq genomics resources. Because these custom CDFs are based on the latest genomic knowledge, the newly defined probe sets perform better and allow for more reliable comparison of gene expression. Also, as genes are uniquely represented in a custom CDF, bias towards genes represented by multiple probe sets is avoided in gene set enrichment (de Leeuw *et al.*, 2008). This resulted in gene expression values for 14 291 known genes with unique identifiers from 24 128 transcripts. All microarray experiment data are MIAME compliant and have been deposited in Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>; accession numbers: GSE24200).

### Statistical analysis

Feed intake, milk yield and composition, as well as blood parameters, TAG concentration in wet liver tissue and gene expression in the liver and adipose tissues were analysed using a mixed-effects linear model that accounted for the random effect of each cow nested within treatment and the fixed categorical effects of dietary treatment, time and the interaction

between dietary treatment and time. Time entered the model as a repeated measure using a compound symmetry variance-covariance structure. These statistical analyses were performed with SAS (release 9.1; SAS Inst., Cary, NC, USA). Means separation was performed at each time point for significant treatment per time interaction using a sliced-effect multiple comparison approach with a Tukey-Kramer adjustment. When differences between time points were significant ( $p < 0.05$ ), Tukey's test was used to compare means. Least squares means ( $\pm$ SEM) are reported throughout the tests. A treatment difference of  $p \leq 0.05$  was considered significant, and  $0.05 < p \leq 0.10$  was considered a trend.

Gene expression in the mammary gland was analysed as described above but without the time effect (as there were no repeated measures). The microarray ANOVA (MAANOVA) package of R (Language and Environment for Statistical Computing, Foundation for Statistical Computing, Vienna, Austria, <http://www.R-project.org>) programming language was used for the analysis. The p-values were corrected for multiple testing using a false discovery rate (FDR) method, which provides an estimate of the fraction of false discoveries among the significant terms (Bunger *et al.*, 2007). The differentially expressed genes were identified using a FDR < 15% ( $q$ -value < 0.15). Additionally, the gene set enrichment analysis (GSEA) approach was applied to relate changes in gene expression to functional changes. To evaluate this degree of 'enrichment', the GSEA method calculates an Enrichment Score (ES), a weighted Kolmogorov-Smirnov-like statistic that evaluates whether the members of the pathway are randomly distributed or found at the extremes (top or bottom) of the list. If genes in a pathway rank at the top of the new list, that is, they are overrepresented at the top, and then the ES will be close to 1. Conversely, if the ES = -1, then genes are overrepresented at the bottom of our data. A perfect agreement is reached if ES = 1 for the upregulated genes and ES = -1 for the downregulated genes. A normalized ES (NES) takes into account the number of genes in the pathway. A positive NES indicates that the list of genes is enriched at the 'top' of the ordered list, and a negative NES indicates that the list in question is enriched at the 'bottom'. Gene set size filter considered a minimum of 15 and a maximum of 500 genes, and the number of permutation was set to 1000. Lastly, there is an adjustment for multiple hypothesis testing (Subramanian *et al.*,

2005, 2007). Gene sets were considered significantly enriched at a FDR < 0.05.

## Results

**Feed intake, BCS, BW and milk yield and composition**  
Total DMI was not affected by linseed supplementation and averaged  $12.9 \pm 0.65$  kg/day before calving and  $17.5 \pm 0.65$  kg/day after calving (Table 3). Furthermore, intake of crude protein, intestinal digestible protein (IDP) and net energy lactation (NEL) was not affected by linseed supplementation (Table 3). However, the DMI, IDP and NEL ( $p < 0.001$ ) decreased from week -4 to week -1 and then increased from week -1 through week +6 relative to parturition. As expected, a significant interaction of treatment with time was observed for crude fat intake (Table 3). Crude fat intake was significantly greater in the cows fed with linseed from day 0 to week +6 post-partum relative to those fed control diet.

Milk yield was 10% greater ( $p < 0.05$ ) when supplementing dairy cows with linseed ( $45.1 \pm 1.0$  kg/day) relative to the cows fed the control diet ( $40.8 \pm 1.0$  kg/day; Table 4). Milk fat and protein yield did not differ between diets ( $1.84 \pm 0.07$  and  $1.36 \pm 0.05$  kg/day respectively). However, lactose yield was greater ( $p < 0.05$ ) for cows supplemented with linseed ( $2.08 \pm 0.05$  kg/day) than for cows fed the control diet ( $1.85 \pm 0.05$  kg/day). In contrast, the total milk fat percentage was over 8% lower ( $4.29$  vs.  $4.63 \pm 0.11\%$ ;  $p < 0.05$ ) for cows supplemented with linseed than for cows fed the control diet. Additionally, throughout the supplementation of dietary linseed, the proportion of 16:0 FA decreased ( $p < 0.001$ ), and the proportion of 18:0 FA, CLAc9t11, ALA and therefore PUFAs in the milk increased ( $p < 0.001$ ; Table 5). Furthermore, the desaturation ratio of C18 was significantly lower in cows supplemented with linseed in relation to the cows fed the control diet.

### Plasma metabolites and liver TAG

The effect of linseed supplementation on periparturient blood metabolites is shown in Fig. 3 and Table S2. Plasma glucose and NEFA concentration were not different among treatments during pre-partum or post-partum periods. However, plasma BHBA and NEFA concentrations increased steadily ( $p < 0.01$ ) from week -3 through the end of week +1 post-partum. Interestingly, throughout supplementation of linseed, the plasma BHBA decreased ( $p < 0.05$ ). Total hepatic TAG revealed no differences between treatments (Table 6).

### Hepatic and adipose tissue mRNA abundance

To determine the effects of linseed supplementation on the expression of key genes in lipid metabolism in liver (Fig. 1) and adipose tissue (Fig. 2), biopsies of the liver and adipose tissues were taken in week -3, week +1, week +4 and week +6 and analysed by qRT-PCR. The relative mRNA abundance of hepatic and adipose tissue genes is presented in Table 6. In liver tissue, expression of *GPAT1* and *FABP1* was lower ( $p < 0.05$ ) in cows supplemented with linseed relative to those fed control diet. However, the differences between treatments were already significant from the start of the experiment for both *GPAT1* and *FABP1*. A significant dietary treatment per time interaction was observed ( $p < 0.05$ ) on *GLUT2*. Expression of *GLUT2* mRNA increased gradually from week -3 through week +1 post-partum in cows supplemented with linseed diet, whereas that of control animals reduced. The expression of *MTTP*, *SLC22A5*, *CPT1A*, *PPAR $\alpha$* , *PPAR $\delta$* , *FATP2* or *FATP5* was not affected by dietary treatments.

In adipose tissue, no effect of supplemental linseed on *FASN*, *LPL* and *PPAR $\gamma$*  mRNA expression was observed, although expression of *FASN* and *LPL* mRNA reduced strongly ( $p < 0.05$ ) from week -3 through week +1 (Table 6).

### Gene functional clustering in the mammary gland

Applying a statistical cut-off of FDR  $q$ -values < 0.15, we did not find genes differentially expressed in the mammary gland tissue between dietary treatments. However, several GSEA categories (Fig. 4) were significantly overrepresented during linseed supplementation. Linseed supplementation mainly increased cell cycle, together with cell assembly and organization, cell signalling and development of immune system functions. One of the most induced subfunctions during linseed supplementation was the major histocompatibility complex, particularly the class I, the T-cell receptor signalling pathway and the antigen processing and presentation signalling pathways. From a metabolic standpoint, linseed supplementation decreases expression of genes involved in nutrient metabolism, with the lipid and carbohydrate metabolism being the most important. Among lipid metabolism, glycerolipid metabolism and linolenic acid metabolism were the most inhibited, whereas among carbohydrate metabolism, propionate metabolism.

Nutrient metabolism is highly relevant as it plays a central role in milk fat composition. Therefore, a



**Table 3** Effect of dietary supplementation of linseed on dry matter and nutrient intake, body condition score and live weight in dairy cows (kg/day unless stated else)

Item*	Feed supplementation														p-value							
	Control														Linseed supplementation							
	Weeks peripartum																					
	-3	-2	-1	1	2	3	4	5	6	-3	-2	-1	1	2	3	4	5	6	SEM	T	W	T*W
Dry matter	13.4	12.3	12.2	15.6	18.9	20.1	21.1	21.4	20.5	13.6	13.3	12.6	15.5	18.6	20.3	22.3	22.5	23.2	0.84	0.43	0.001	0.78
Concentrate	0.2	0.7	1.4	3.3	6.3	8.1	8.3	8.4	8.4	0.1	0.9	1.5	3.3	6.0	8.1	8.3	8.3	8.4	0.24	0.93	0.001	0.99
Feed mixture†	13.2	11.6	10.8	12.3	12.6	12.9	12.8	13.0	12.1	13.7	12.4	11.1	12.2	12.6	12.2	14.0	14.2	14.8	0.76	0.37	0.003	0.71
Crude fat	0.37	0.38	0.41	0.64	0.83	0.93	0.97	0.98	0.95	0.37	0.40	0.41	0.69	0.95	1.11	1.20	1.21	1.23	0.032	0.003	0.001	0.001
Crude protein	1.65	1.71	1.81	2.65	3.30	3.63	3.80	3.76	3.61	1.64	1.81	1.77	2.62	3.18	3.56	3.87	3.98	3.97	0.136	0.77	0.001	0.67
IDP (kg/day)‡	1.32	1.15	1.08	1.23	1.26	1.19	1.28	1.30	1.20	1.35	1.24	1.10	1.22	1.25	1.22	1.40	1.42	1.46	0.076	0.37	0.003	0.71
NEL (MJ/day)	76.8	71.4	77.7	101.0	131.3	146.3	153.2	155.9	153.0	75.6	76.8	74.3	108.3	134.9	149.7	164.2	164.9	167.8	4.86	0.16	0.001	0.53
BCS§	3.4	3.5	3.2	3.2	3.1	3.2	2.8	2.9	3.0	3.4	3.5	3.4	3.4	3.4	2.8	2.6	2.7	2.5	0.18	0.61	0.001	0.44
BW (kg)	715	724	719	669	654	649	642	637	634	758	753	774	687	676	669	669	671	669	24.5	0.001	0.001	0.96

T, treatment effect; W, effect of time; IDP, intestinal digestible protein; NEL, net energy/lactation.

\*included *n* = 14 cows.

†From roughage intake control stations.

‡IDP, according to DVE/OEB system.

§1 = thin to 5 = obese (Wildman *et al.*, 1982).

**Table 4** Effect of dietary supplementation of linseed on milk yield, and yields of milk components of dairy cows

Item*	Dietary treatments												SEM	p-value		
	Control						Linseed supplementation							T	W	T*W
	Weeks post-partum		Weeks post-partum		Weeks post-partum		Weeks post-partum		Weeks post-partum		Weeks post-partum					
1	2	3	4	5	6	1	2	3	4	5	6					
Milk yield (kg/day)	27.9	38.4	42.7	44.9	45.5	45.6	34.1	41.4	45.8	49.0	50.5	49.8	2.43	0.01	0.001	0.96
Fat concentration (%)	5.64	5.19	4.74	4.47	3.88	3.97	5.67	4.64	4.13	4.03	3.85	3.67	0.251	0.03	0.001	0.52
Fat yield (kg/day)	1.52	1.97	1.99	2.11	1.64	1.80	1.65	1.88	1.89	1.95	1.93	1.76	0.179	0.96	0.23	0.72
Protein concentration (%)	4.42	3.64	3.24	3.12	3.05	3.02	4.03	3.32	3.13	2.99	2.97	2.94	0.140	0.22	0.001	0.76
Protein yield (kg/day)	1.17	1.37	1.35	1.48	1.33	1.36	1.14	1.35	1.43	1.47	1.51	1.44	0.091	0.44	0.07	0.89
Lactose concentration (%)	4.39	4.57	4.64	4.68	4.52	4.61	4.37	4.58	4.70	4.71	4.68	4.74	0.051	0.32	0.001	0.51
Lactose yield (kg/day)	1.19	1.72	1.93	2.22	1.96	2.09	1.46	1.86	2.17	2.29	2.41	2.35	0.113	0.05	0.001	0.57
FPCM (kg/day)†	33.8	43.9	45.3	46.2	41.0	43.1	43.1	42.4	46.1	49.0	48.1	46.4	2.89	0.03	0.08	0.48

T, treatment effect; W, effect of time.

\*Included  $n = 14$  cow.

†Fat (4.0%) and protein (3.3%) corrected milk.

specific examination of the genes involved with nutrient metabolism pathways overrepresented by linseed supplementation was conducted. Results revealed a total of 28 genes involved in lipid metabolism, among them the acyl-CoA synthetase short-chain family member 1 (*ACSS2*), 1-acyl-sn-glycerol-3-phosphate acyltransferase 2 (*AGPAT2*), fatty acid desaturase 2 (*FADS2*), diglyceride acyltransferase 1 (*DGAT1*) and *LPL*.

## Discussion

### Feed intake, milk production and composition

Our study suggests that supplementing 1.0 kg of linseed/day to periparturient dairy cows does not affect the DMI (kg/day). There is little information available to evaluate the effect of supplemental linseed on DMI of cows during periparturient period. However, one experiment carried out with linseed supplementation for dairy cows from 6 weeks before the expected date of parturition until 4 weeks of lactation did not show a difference between cows fed linseed and those fed either control or saturated lipids (Petit et al., 2007). The amount of linseed supplemented ranged from 3.3% to 11.0% of DM in pre-partum and post-partum diets respectively. In contrast to their study, we found that the average daily milk yield was significantly greater for cows receiving linseed, which is in line with studies where linseed was fed to mid-lactation dairy cows (Petit, 2002; Petit et al., 2004; Bu et al.,

2007; Mach et al., 2011a). The increase in milk yield with linseed was associated with a greater yield of lactose in milk, consistent with the role of lactose in the regulation of milk osmolarity (Cant et al., 1993). The reduction in milk fat percentage observed in cows supplemented with linseed is most likely the result of a dilution effect. Additionally, supplementation with linseed increased the proportion of PUFAs, ALA and CLAc9t11 in milk, whereas C12 and C16 FAs were reduced. This improved the nutritional quality aspects of dairy milk. These results were in agreement with data reported for early lactating cows fed 3.3% (Petit et al., 2007) or 10% of whole linseed (Petit et al., 2002).

### Metabolites in plasma

The lower BHBA concentration in cows supplemented with linseed suggests that cows fed control diet may have had greater lipolysis of adipose tissue than those fed linseed. According to Mashek et al. (2005), infusion of linseed oil reduced BHBA concentrations. However, liver TAG concentration was similar for both diets, as reported elsewhere (Petit et al., 2007; Andersen et al., 2008; Ballou et al., 2009). Therefore, it is suggested that supplementing linseed during the periparturient period, at a level not to influence DMI, does not ameliorate hepatic lipodosis. It is possible that the lack of changes in blood metabolites from linseed

**Table 5** Effect of dietary supplementation of linseed on fatty acid profile of milk of dairy cows

Fatty acid*	Dietary treatments								SEM	p-value		
	Control				Linseed supplementation					T	W	T*W
	Weeks post-partum											
	1	2	3	6	1	2	3	6				
	g/100 g total fatty acids											
4:0	2.32	1.89	1.69	1.62	2.34	2.27	1.86	2.15	0.161	0.01	0.04	0.38
6:0	1.96	1.95	1.91	1.78	1.91	1.97	2.00	2.17	0.085	0.07	0.96	0.05
8:0	1.22	1.25	1.27	1.23	1.15	1.17	1.34	1.48	0.067	0.39	0.06	0.66
10:0	2.53	2.52	2.65	2.56	2.26	2.28	2.76	3.19	0.183	0.67	0.03	0.07
12:0	2.81	2.94	3.32	3.41	2.37	2.34	2.84	3.45	0.210	0.01	0.007	0.48
14:0	9.70	9.58	9.78	10.07	8.76	8.95	9.51	10.84	0.440	0.40	0.03	0.27
14:1, <i>cis</i> -9	0.59	0.68	0.78	0.83	0.55	0.61	0.65	0.78	0.040	0.03	<0.001	0.77
15:0	0.66	0.74	0.91	0.86	0.67	0.78	0.83	0.93	0.070	0.89	0.02	0.80
16:0	29.90	30.17	30.27	30.42	27.46	26.77	25.51	25.76	0.781	<0.001	0.72	0.36
16:1, <i>cis</i> -9	2.33	2.40	2.46	2.44	2.38	2.28	2.11	1.85	0.147	0.02	0.50	0.18
17:0	0.65	0.60	0.60	0.58	0.72	0.63	0.61	0.56	0.020	0.13	0.001	0.21
17:1, <i>cis</i> -9	0.32	0.31	0.31	0.29	0.35	0.31	0.29	0.22	0.025	0.01	0.39	0.21
18:0	12.48	11.27	10.15	10.19	13.64	12.47	12.74	11.97	0.550	0.001	0.007	0.57
18:1, <i>trans</i> -9	0.12	0.14	0.15	0.16	0.12	0.17	0.18	0.19	0.012	0.009	0.005	0.57
18:1, <i>cis</i> -9	24.12	24.65	24.25	24.03	25.69	24.45	24.43	22.40	1.321	0.80	0.47	0.64
18:2, <i>cis</i> -9, <i>cis</i> -12	1.44	1.69	1.67	1.78	1.61	1.81	1.83	1.89	0.091	0.03	0.01	0.97
18:2, <i>cis</i> -9, <i>trans</i> -11	0.28	0.34	0.40	0.35	0.32	0.51	0.61	0.56	0.043	0.001	0.003	0.22
18:3, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	0.38	0.38	0.31	0.30	0.66	0.85	0.83	0.81	0.089	0.001	0.55	0.27
SCFA†	21.13	20.81	21.40	21.50	19.34	19.59	20.96	24.06	1.021	0.82	0.06	0.13
C16‡	32.23	32.58	32.74	32.86	29.85	29.06	27.63	27.62	0.769	0.001	0.58	0.18
SFA§	64.27	62.91	62.55	62.76	61.28	59.63	60.00	62.50	1.335	0.62	0.56	0.68
MUFA¶	27.50	28.20	27.97	27.77	29.12	28.85	27.68	25.45	1.369	0.93	0.54	0.55
PUFAs**	2.10	2.42	2.38	2.45	2.59	3.18	3.29	3.26	0.180	0.001	0.01	0.63
Δ9-desaturase indices††												
14:1, <i>cis</i> -9	0.06	0.07	0.08	0.08	0.06	0.07	0.07	0.07	0.005	0.34	0.20	0.50
C16:1, <i>cis</i> -9	0.07	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.005	0.71	0.82	0.63
C17:1, <i>cis</i> -9	0.33	0.34	0.33	0.34	0.33	0.33	0.32	0.29	0.014	0.07	0.31	0.34
C18:1, <i>cis</i> -9	0.66	0.69	0.70	0.70	0.65	0.67	0.66	0.66	0.015	0.01	0.36	0.42

T, treatment effect; W, effect of time; PUFAs, polyunsaturated fatty acids.

\*Included n = 14 cows.

†Short- and medium-chain fatty acids Σ (C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C14:1 c9).

‡C16 fatty acids Σ (C16:0, C16:1 c9).

§Saturated fatty acids Σ (C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0).

¶Mono-unsaturated fatty acids Σ (C14:1 c9, C16:1 c9, C17:1 c9, C18:1 c9, C18:1 t9).

\*\*Poly-unsaturated fatty acids Σ (C18:2 c9c12, CLAc9,t11, C18:3 n-3).

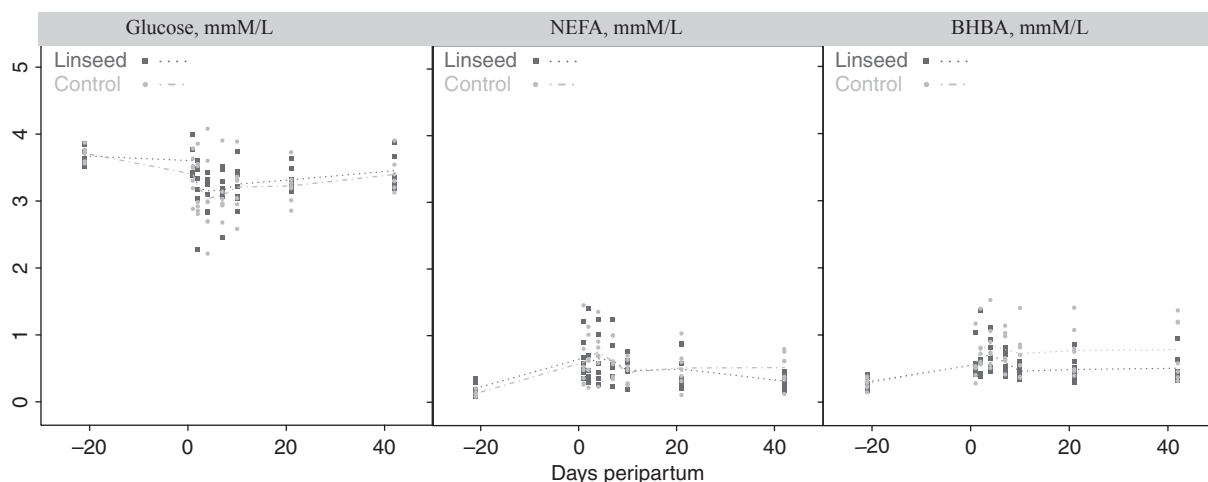
††Δ9-desaturase indices are calculated as the ratio of the Δ9-desaturase product divided by the sum of the Δ9-desaturase product and substrate.

supplements is related to the low degree to which ALA is absorbed by the intestine.

### Hepatic and adipose tissue mRNA abundance

It is likely that nutritional regulation involves transcriptomic adaptations not only in liver tissue but also in adipose tissue. Therefore, we attempted to simultaneously determine the effects of linseed supplementation on the expression of key lipogenic genes in the adipose and liver tissue, with the aim of deci-

phering their involvement in TAG accumulation in the liver. However, the gene expression was similar among dietary treatments, suggesting that supplementing linseed to periparturient cows did not strongly influence key metabolic genes in the hepatic and adipose tissue. Similar to the present study, neither blood metabolites, nor TAG concentration or key hepatic gene expression were affected by supplementing early lactating dairy cows with calcium salts of n-6 FA (Castaneda-Gutierrez et al., 2009), n-3 FA (Carriquiry et al., 2009) or fish oil (Ballou et al.,



**Fig. 3** Effect of supplementing linseed on plasma glucose, non-esterified fatty acids (NEFAs) and *beta*-hydroxybutyric acid (BHBA) concentrations (mm) of dairy cows.

**Table 6** Effect of dietary supplementation of linseed on key gene expression in the liver and adipose tissues of dairy cows

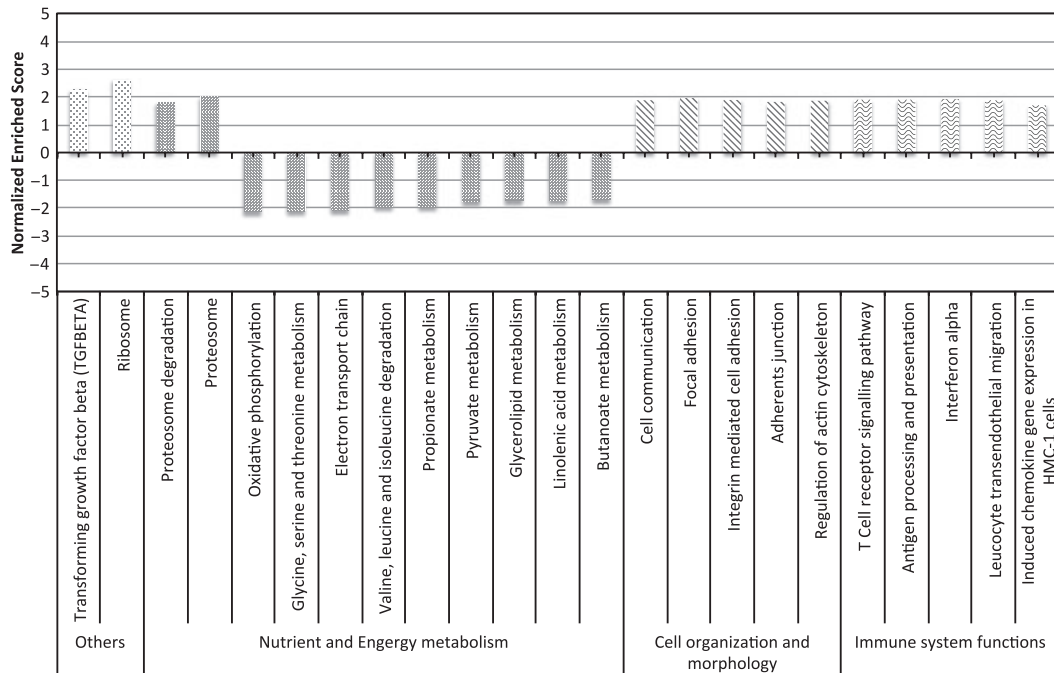
Gene symbol*	Dietary treatments								SEM	p-value		
	Control				Linseed supplementation					T	W	T*W
	Weeks peripartum											
	-3	1	3	6	-3	1	3	6				
	Ratio to ACTB											
Liver												
<i>MTTP</i>	0.110	0.117	0.113	0.072	0.097	0.163	0.087	0.096	0.0149	0.53	0.01	0.11
<i>PPAR<math>\alpha</math></i>	0.026	0.022	0.022	0.022	0.016	0.023	0.0138	0.020	0.0040	0.28	0.72	0.47
<i>PPAR<math>\delta</math></i>	0.004	0.004	0.005	0.006	0.003	0.005	0.003	0.004	0.0007	0.28	0.19	0.72
<i>GPAT1</i>	0.025	0.022	0.028	0.021	0.019	0.019	0.013	0.018	0.0327	0.05	0.89	0.16
<i>CPT1A</i>	0.134	0.190	0.198	0.193	0.107	0.204	0.114	0.130	0.0304	0.08	0.16	0.44
<i>SLC22A5</i>	0.005	0.005	0.005	0.002	0.002	0.007	0.004	0.003	0.0011	0.59	0.02	0.07
<i>FATP2</i>	0.064	0.081	0.067	0.027	0.035	0.048	0.031	0.028	0.0193	0.17	0.24	0.68
<i>FATP5</i>	0.275	0.215	0.251	0.239	0.224	0.230	0.196	0.260	0.0283	0.40	0.65	0.43
<i>FABP1</i>	1.137	1.007	1.167	1.234	0.458	0.594	0.836	0.868	0.1947	0.01	0.02	0.29
<i>GLUT2</i>	0.155	0.093	0.090	0.073	0.077	0.091	0.095	0.099	0.0092	0.05	0.15	0.02
TAG (mg/100 g tissue)	30.43	81.23	94.83	56.24	28.01	99.9	119.84	54.75	14.635	0.51	0.003	0.89
Adipose tissue												
<i>FASN</i>	1.972	0.056	0.033	0.075	1.387	0.031	0.026	0.042	0.2098	0.28	0.001	0.46
<i>LPL</i>	0.575	0.147	0.076	0.101	1.068	0.070	0.056	0.0092	0.2576	0.60	0.02	0.66
<i>PPAR<math>\gamma</math></i>	0.049	0.039	0.017	0.022	0.023	0.038	0.018	0.018	0.0653	0.34	0.23	0.42

T, treatment effect; W, effect of time; *MTTP*, microsomal triglyceride transfer protein; *PPAR $\alpha$* , peroxisome proliferator-activated receptor  $\alpha$ ; *PPAR $\delta$* , peroxisome proliferator-activated receptor  $\delta$ ; *GPAT1*, glycerol-3-phosphate acyltransferase; *CPT1A*, carnitine palmitoyltransferase I; *SLC22A5*, solute carrier family 22 member 5; *FATP2*, fatty acid transport protein 2; *FATP5*, fatty acid transport protein 5; *FABP1*, fatty acid-binding protein 1; *GLUT2*, glucose transporter 2; *FASN*, fatty acid synthase; *LPL*, lipoprotein lipase; *PPAR $\gamma$* , peroxisome proliferator-activated receptor  $\gamma$ .

\*Included  $n = 14$  cows.

2009). These findings are in contrast with experiments with cows in established lactation demonstrating a reduction in lipogenic activity of perirenal or subcutaneous adipose tissue in response to dietary fat (McNamara et al., 1995) or post-ruminal infusions of rapeseed oil (Chilliard et al., 1991). In addition, these

observations differed from previous findings with non-ruminants where increasing the intake of PUFAs, either n-6 or n-3 FA, increased lipid oxidation, decreased lipid storage and changed regulation of gluconeogenesis (Jump et al., 1999). The lack of an effect of linseed supplementation on key metabolic gene



**Fig. 4** Gene set enrichment analysis (GSEA) for gene expression in the mammary gland when comparing dairy cows supplemented with linseed relative to those fed control diet basal ration. The GSEA derives its power by focusing on gene sets, that is, groups of genes that share common biological functions (e.g. biochemical, metabolic or signal transduction routes), chromosomal location or regulation. The GSEA method first calculates for each group of genes an enrichment score (ES) that reflects the degree of association between the gene expression and the treatment. Then, it estimates the significance level of ES by using an empirical phenotype-based permutation test procedure that preserves the complex correlation structure of the gene expression data, and lastly, there is an adjustment for multiple hypothesis testing. Gene set size filter considered a minimum of 15 and a maximum of 500 genes, and the number of permutation was set to 1000. Gene sets were considered significantly enriched at a FDR < 0.05. Normalized enriched scores (NES) of significantly enriched pathways between treatments were calculated. A positive and negative NES indicate that the gene set is upregulated and downregulated in cows supplemented with linseed relative to those fed control diet basal ration respectively. In the figure, gene sets are grouped by main biological functions. FDR, false discovery rate.

expression and TAG concentrations could be due to the limited uptake of ALA by the intestine [on most diets, biohydrogenation of ALA in ruminants varies between 85% and 100% (Glasser et al., 2008)], which might be below a threshold needed to alter hepatic and adipose gene expression. Also, Agazzi et al. (2010) showed that gene expression in the liver of ruminants could be affected not to the n-3 FA itself, but to liver concentration of saturated FAs. Palmitic and stearic acid, which are the main FAs in lipid deposits, can be strongly mobilized around calving (Agazzi et al., 2010). Therefore, hepatic FA oxidation could be particularly responsive to supraphysiological concentrations of these FAs. Furthermore, liver metabolism seems to be highly variable among cows (Hammon et al., 2008). Nevertheless, the expression of *GLUT2* mRNA in liver tissue was influenced ( $p < 0.05$ ) by the interaction between dietary treatment and time, suggesting that the differences in the expression of *GLUT2* mRNA between dietary treatments were not constant across time. The expression

of *GLUT2* mRNA in liver tissue increased gradually from week -3 through week +1 post-partum in cows supplemented with linseed, whereas that of control cows was reduced from week -3 to week +6. In parallel, we observed a linseed-induced increase in lactose yield. In ruminants, *GLUT2*, located in the hepatic plasma membrane, plays a crucial role in the release of glucose in the liver (Zhao et al., 1996). Most of this glucose is required for the lactating mammary gland. Here, apart from its role in cellular respiration (to generate ATP and NADPH), glucose is a precursor of lactose (Zhao and Keating, 2007). Together with the observation that lactose in milk was increased in cows fed with linseed, these data suggest that dietary linseed supplementation stimulates the release of glucose by the liver, which in turn increases the bioavailability of glucose for lactose synthesis in the mammary gland of periparturient cows. Also, Hammon et al. (2008) reported that when long-chain FAs are supplemented to dairy cows, they are preferentially taken up by the mammary gland and directly incorporated into milk

fat. A lower demand on the mammary *de novo* FA synthesis requires less consumption of NADPH, a product of the pentose phosphate pathway. In this way, a larger portion of glucose could be utilized for lactose synthesis. Furthermore, the higher milk yield of cows supplemented with linseed can be associated with greater release of glucose, as DM intake was unaffected by diets.

#### Gene functional clustering in the mammary gland

The effect of linseed supplementation on the functional characterization of mammary gland of early lactating cows, including the underlying gene pathways, remains to be fully elucidated. In that end, we have attempted to provide molecular knowledge regarding the effect of linseed supplementation on mammary gland function of early lactation dairy cows using microarrays. The value of microarrays lies in its ability to provide a holistic view of the molecular events that occur when the mammary gland adapts to changes in the supply of dietary lipids and consequent changes in the milk yield and composition through feeding strategies.

After correcting for multiple hypotheses testing, no individual gene met the threshold for statistical significance, probably because differences in gene expression were modest relative to the technique noise, which is inherent to technical features of experiment (RNA isolation and handling, chip hybridization conditions, scanner characteristics, etc.), and may be of limited uptake of ALA by the intestine and individual variation (coefficient of variation in gene expression between cows). Therefore, to overcome these analytical challenges, we applied the method GSEA, which evaluates microarray data at the level of gene sets based on prior biological knowledge, instead of analysis at the level of individual genes (Su *et al.*, 2009).

Several pathways were differentially expressed in the mammary gland tissue when supplementing dairy cows with linseed compared with cows fed a control diet, suggesting transcriptional adaptation to the dietary linseed supplementation. The majority of pathways that could be classified as preferentially upregulated in cows fed with linseed in relation to those fed a control diet were associated with cell organization together with cell function, nutrient and energy metabolism, as well as immune system functions. Unfortunately, there are no other data evaluating the effects of supplemental linseed on the regulation of genes functioning in cell proliferation and remodelling of the mammary gland in early lactation dairy cows. However, Mach *et al.* (2011a) reported that changes occurring within the mammary

gland after UFA supplementation in mid-lactation dairy cows include a reduced mammary cell proliferation and differentiation (e.g. integrin-mediated cell adhesion, focal adhesion, and regulation of actin cytoskeleton pathways and the major histocompatibility complex, particularly the class I). Overall, the variation between these two studies most likely resulted from the difference in lactation stage. From a functional point of view, it can be suggested that the organizational and morphological changes in the mammary gland tissue are greater during the first weeks of lactation to cope with increased milk production, whereas during lactation, the tissue attempts to maintain a stable organization and transcriptional programme. In fact, when comparing mammary development during pregnancy, lactation and involution, it has been described that relative to parturition, most of the genes were downregulated by the time of mature lactation (Lemay *et al.*, 2007).

Furthermore, in the present study, some other significantly expressed pathways were associated with the development of immune system functions (e.g. the T-cell receptor signalling pathway, leucocyte transendothelial migration, induced chemokine gene expression in HMC-1 cells and the antigen-processing and presentation signalling pathway). Little is known about defence, inflammatory and immune-related genes in response to dietary linseed supplementation in early lactation dairy cows, although these results provide evidence that these immune-related pathways might be biologically relevant in the context of mammary gland when cows are supplemented linseed. Whereas we are not aware of similar transcriptomic characterization of mammary gland in early lactating dairy cows, Mach *et al.* (2011b) reported that UFA supplementation in mid-lactation cows downregulated genes associated with cell defence, as well as inflammatory and immune-related genes. Similarly, Lemay *et al.* (2007) reported that genes associated with antigen processing and presentation were also downregulated during lactation relative to late pregnancy. During the peripartum period, the incidence of metabolic and infectious disorders increases (Sordillo *et al.*, 2010). The increased metabolic demands of the periparturient cow may contribute to compromised host defence, particularly as related to the regulation of inappropriate inflammatory response (Sordillo *et al.*, 2010). This might be a possible explanation for the activation of some immune pathways during the peripartum period and its suppression through the onset of lactation. Experiments specifically designed to test these hypotheses are warranted to verify the roles of n-3 FA on genes involved in immune system

response pathways, together with cell cycle and proliferation, and to determine nutritional conditions that optimize the mammary gland function of the dairy cows.

Our microarray data also provide insight into the nutrient metabolism adaptations in the mammary gland as a result of linseed supplementation. Our finding suggested that through linseed supplementation, the mammary gland reduced overall fat and carbohydrate metabolic activity (e.g. propionate, pyruvate and butanoate metabolism). Among lipid metabolism, glycerolipid metabolism and linolenic acid metabolism were the most inhibited, which could be an explanation for the reduction of fat percentage in the milk. In agreement, Ahnadi *et al.* (2002), Harvatine and Bauman (2006) and Piperova *et al.* (2000) described a substantial downregulation of the mRNA expression of genes in the mammary gland involved in lipid metabolism when supplementing dairy cows with unprotected UFA.

To our knowledge, this is the first study that addresses the role of linseed supplementation on transcriptional adaptations in different tissues of periparturient dairy cows. With the present findings, we conclude that dietary supplementation of linseed, at a level not to influence DMI, affects the mammary gland metabolism. Key evidence for this hypothesis is the substantial milk FA profile modification, in addition to the expression patterns of mammary gland pathways. Although feeding periparturient dairy cows up to 1.0 kg of linseed/day did not affect key metabolism genes in liver and adipose tissues, it cannot be discarded whether linseed supplementation affected processes not related to lipid and glucose metabolism and the expression of less obvious genes in liver and adipose tissues. In the light of the above findings in mammary gland, determination of wide-genome expression in adipose and liver tissue needs to be tested to verify the hypothesized crosstalk between adipose tissue, the liver and the mammary gland. In addition, knowledge of the relationship between mRNA and protein is a complex one (Hack, 2004). While mRNA analysis provides incredible insight into cell operation, there are still limited conclusions that can be drawn from just measuring mRNA as post-translation, and the amount of active protein is dependent on many factors (Rogers *et al.*, 2008). Therefore, large-scale measurements of other molecular species, particularly proteins, should be combined with mRNA concentration to enable us to make inferences and predictions about how the network of regulatory control varies at the mRNA and protein levels. Such molecular information on the physiology of the different tissues might provide the

basis for functional studies for research and for the dairy industry as a whole, to improve strategies to enhance metabolism and health status of the dairy cow and to alter the milk composition to be more compatible with recommendations for improving human health.

## Conclusions

The results of our study suggest that supplementing dairy cows with up to 1.0 kg of linseed/day does not modify DMI, but increases milk yield, lactose yield and reduces milk fat percentage. Due to the linseed supplementation, the proportion of C16:0 reduces, whereas the omega-3 fatty acids and conjugated linolenic acid concentrations increase and, in turn, improve the milk quality aspects. Furthermore, supplementing dairy cows with linseed increased the expression of *GLUT2* in the liver, suggesting that more glucose was available for lactose synthesis compared with cows fed control diet. In the mammary gland, the functional analysis indicated that dietary supplementation of linseed not only reduces the expression of pathways associated with lipid and carbohydrate metabolism, but also increases pathways involved in cell proliferation, cytoskeleton organization and immune response. Such molecular knowledge might provide the basis for more detailed functional studies for research and for the dairy industry to improve milk quality and animal health.

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## Conflicts of interest

R.E. Weurding is employed by one of the financial sponsors of this study. He contributed to this study by formulating the experimental diets, designing the feeding strategy post-partum and reviewing earlier

versions of the manuscript. There were no circumstances that present a conflict of interest for the first and other co-authors.

### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Data S1** Supplementary material.

**Table S1** Genes included in the overrepresented lipid metabolism pathway when comparing dairy cows supplemented with linseed relative to the cows fed a control diet..

**Table S2** Sequences of the primers used for quantitative PCR.

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