Journal of Dairy Research (2017) **84** 119–123. © *Proprietors of Journal of Dairy Research 2017*
doi:10.1017/S002202991700022X

Altered gene expression of epigenetic modifying enzymes in response to dietary supplementation with linseed oil

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Received 4 November 2016; accepted for publication 29 March 2017

Recently we showed that 5% linseed oil (LSO) and 5% safflower oil (SFO) supplementation of cow's diets reduced milk fat yield by 30·38 and 32·42% respectively, accompanied by differential expression of genes and regulation by microRNAs (miRNA). This research communication addresses the hypothesis that epigenetic regulation could be involved in the observed milk fat reduction. Thus, this study investigated the gene expression pattern of major epigenetic modifying enzymes in response to dietary supplementation with LSO or SFO. Twenty-six Canadian Holstein cows in mid lactation were randomly assigned to two groups (13/group) and fed a control diet for 28 d (day −28 to −1) (control period- CP) followed by a treatment period (TP) (control diet supplemented with 5% LSO (LSO treatment) or 5% SFO (SFO treatment) of 28 d (day +1 to +28). After treatment, cows in the two groups were returned to the control diet for another 28 d (day +29 to +56) (post treatment period-PTP). Milk samples were collected on day −1 (CP), +7, +28 (TP) and +56 (PTP) for RNA isolation and measurement of the expression of thirteen epigenetic modifying genes including two DNA methytrasferases (*DNMT1*, *DNMT3A*), four histone acetylases (*HAT1*, *KAT2A*, *KAT5* and *CREBBP*), five histone deacetylases (*HDAC1*, *HDAC2*, *HDAC3*, *SIRT1* and *SIRT2*) and two histone methyltransferases (*EHMT2* and *PRMT1*) by qPCR. Linseed oil supplementation significantly repressed the expression of *EHMT2*, *HDAC2 and HDAC3* on day +7 ($P < 0·05$) and *KAT2A* and *SIRT2* on day +28 ($P < 0·05$) as compared with the control period (day −1) while SFO had no effect. When LSO was withdrawn, the expression of some of the genes increased slightly but did not reach control (day −1) levels at the end of the PTP. Our study demonstrated a significant role of LSO in the epigenetic regulation of fatty acid synthesis as compared to SFO. The effect of LSO may be related to its higher degree of unsaturation and might represent a different regulatory mechanism which needs further investigation.

Keywords: DNA methytrasferases, histone acetylases, histone deacetylases, histone methyltransferases, milk fat, linseed oil, safflower oil.

Fat is the major energy component in bovine milk and its concentration and composition can be remarkably affected by dietary factors. There is growing evidence that nutrition may modify epigenetic marks which in turn may impact gene expression and the resultant phenotype (Mathers et al. 2010; McKay & Mathers, 2011). Supplementing dairy cow diets with unsaturated fatty acids (USFAs) has resulted in decreased milk fat yield and altered milk fatty acid (FA) composition coupled with reduced mRNA abundance of well-established lipogenic genes in mammary gland tissues (Harvatine et al. 2009; Jacobs et al. 2011; Mach et al. 2011; Ibeagha-Awemu et al. 2016), indicating massive transcriptional adaptions in mammary gland in response to dietary supplementary USFAs.

Epigenetics refer to changes in the phenotype or heritable states of gene expression caused by mechanisms (DNA methylation, histone tail modifications, chromatin remodelling and non-coding RNA regulation) other than changes in the DNA sequence (Rakyan et al. 2011; Allis & Jenuwein, 2016). Epigenetic mechanisms including DNA methylation, histone modifications and non-coding RNA regulation have been shown to be responsible for regulating gene expression not only during cellular differentiation in embryonic development but also throughout life (Choi & Friso, 2010). In mammals, DNA methylation patterns are established by the de novo DNA methyltransferases, *DNMT3A* and

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https://doi.org/10.1017/S002202991700022X Published online by Cambridge University Press

DNMT3B and maintained by the maintenance methyltransferase *DNMT1* (Law & Jacobsen, 2010). Besides covalent modifications of DNA, histone posttranslational modifications (acetylation, methylation, phosphorylation, ubiquitination, sumoylation, etc.) have also been implicated in the organisation of chromatin structure and regulation of gene transcription. Increasing evidence in recent years supports the notion that fatty acids, in particular polyunsaturated fatty acids, can modify the epigenome (Burdge & Lillycrop, 2014).

Previous studies have shown that dietary factors, such as feeding high fat, low protein or energy restricted diets in humans, rodents and farm animals can influence metabolic pathways through altered epigenetic marks and gene expression (Bouchard et al. 2010; McKay & Mathers, 2011; Ibeagha-Awemu & Zhao, 2015). For example, maternal dietary protein restriction and excess were reported to affect offspring epigenetic marks as well as influence on gene expression in pigs (Altmann et al. 2012). Recently we showed that 5% linseed oil (LSO) and 5% safflower oil (SFO) supplementation of cow's diets reduced milk fat yield by 30·38 and 32·42% respectively, accompanied by differential expression of genes and a greater impact of LSO on gene expression and metabolic pathways as compared to SFO (Ibeagha-Awemu et al. 2016). In the same light, SFO/LSO supplementation affected the expression of a number of miRNAs, which potentially targeted genes involved in de novo milk fatty acid synthesis (Li et al. 2015; Ibeagha-Awemu et al. 2016). However, whether epigenetic regulation is involved remains unclear. Therefore, this study aimed to investigate the gene expression pattern of epigenetic modifying enzymes in response to dietary supplementation with USFAs (LSO and SFO) and thus infer their possible roles in the regulation of bovine milk fat synthesis.

Materials and methods

Animals and diets

Animal selection and diet composition have been described in our previous study (Li et al. 2015) except that, a higher number of samples were included in the present study. Briefly, 26 Canadian Holstein cows in mid-lactation were randomly separated into two groups of 13 each. Animals in both groups were placed on a control diet (total mixed ration of corn and grass silages and concentrates) for 28 d (day −28 to −1) (control period, CP) followed by supplementation with 5% SFO (SFO treatment) (rich in linoleic acid, about 65% of total fat) on dry matter (DM) basis or 5% LSO (LSO treatment) (rich in linoleic acid: about 60–71% of total fat) on DM basis for 28 d (day +1 to +28) (treatment period, TP). After treatment, cows were returned to the control diet for another 28 d (day +29 to +56) (post treatment period, PTP).The oil supplements were added to the mixed ration on a daily bases and offered once daily in sufficient amounts to secure ad libitum intake. Water was freely available at all times.

Procedures for animal care and use were according to the Canadian national codes of practice for the care and handling of farm animals and approved by the animal care and ethics committee of Agriculture and Agri-Food Canada.

Sampling, RNA isolation and cDNA synthesis

Milk samples for isolation of RNA for gene expression analysis were collected 2 h after the morning milking on experiment day −1 (CP), +7, +28 (TP) and +56 (PTP). Samples were immediately centrifuged at 1900 ***g*** for 15 min at 4 °C and the fat layer (upper phase) transferred into sterile 50 ml RNase free falcon tubes. About 7·5 ml Qiazol lysis reagent (Qiagen Inc., Mississauga, Canada) was added to the fat, vigorously mixed by vortexing until the fat was well dispersed followed by total RNA isolation using RNeasy kit (Qiagen Inc.) according to manufacturer's instructions. RNA was treated with Turbo DNase I (Ambion, Inc., Texas, USA) according to manufacturer's instructions to reduce genomic DNA contamination. The quantity and quality (integrity) of RNA were determined by NanoDrop 1000 (NanoDrop Technologies, Wilmington, USA) and Bioanalyzer Nano chip (Agilent Technologies, Mississauga, Canada), respectively. The RNA integrity number of the samples ranged from 4·8 to 8·5. For synthesis of cDNA, 1 μg of total RNA was reverse transcribed in a 20 μl reaction containing 20 U Superscript II reverse transcriptase (Invitrogen, Carlsbad, USA), 0·5 mM of each dNTPs (Qiagen), and 128 ng random hexamer primers (Invitrogen).

Real-time quantitative PCR

Real-time qPCR primers for thirteen genes encoding epigenetic enzymes were designed using their respective reference sequences (online Supplementary Table S1) and PrimerQuest tool (Integrated DNA Technologies Inc., Coralville, USA, http://www.idtdna.com/Primerquest/Home/Index). These genes code for the key epigenetic modifying enzymes including two DNA methytrasferases (DNMT1, DNMT3A), four histone acetylases (HAT1, KAT2A, KAT5 and CREBBP), five histone deacetylases (HDAC1, HDAC2, HDAC3, SIRT1 and SIRT2) and two histone methytransferases (EHMT2 and PRMT1). Each primer pair was designed to span at least one exon boundary.

The qPCR reactions were performed in a volume of 10 μl containing 3 μl of cDNA, 0·3 to 0·6 μM of forward (F) and reverse (R) primers (online Table S1) and 5 μl Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA). Templates were amplified after a preincubation for 10 min at 95 °C, followed by 40 cycles of amplification (10 s at 95, 1 min at 60 °C). At the end of each qPCR, melt curve analysis was performed for all genes to check the specificity of the products. The melt curve analysis consisted of a 15 s denaturation at 95 °C, a 1 min annealing at 65 °C, and a temperature increase of 0·5 °C/s up to 90 °C, and a final cooling cycle at 4 °C for 30 s. In addition, no-template controls were added to each plate to ensure that qPCR mixes were not

https://doi.org/10.1017/S002202991700022X Published online by Cambridge University Press

contaminated with DNA. Furthermore, each sample was replicated three times during each qPCR run. All target genes showed acceptable amplification efficiencies (80–110%) and correlation coefficients (0·95–1·0). The expression profiles of genes were analysed by standard curve method using ABI StepOne Detection System (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems). The standard curve was generated on each plate by running a 6 serial four-fold dilution of a pool of all the samples. The geometric mean of *UXT* and *PPIA* genes was used to normalise the expression of target genes. *UXT* and *PPIA* were found to be the most stable out of four genes (*PPIA*, *GAPDH*, *UXT*, and *RPS9*) tested by Normfinder (Andersen et al. 2004).

Statistical analysis

The gene expression differences between experimental periods for each treatment and between treatments were determined using SAS version 9.1 (SAS Institute Inc., Cary, USA) and a regular ANOVA model with repeated measures. Multiple comparisons between means were conducted with Tukey's adjustment and declared significant at $P < 0{\cdot}05$. The following model was used:

$$Y_{ijk} = \mu + \text{treatment}_i + \text{cow}_{j(i)} + \text{time}_k + (\text{treatment} \times \text{time})_{ik} + e_{ijk}$$

where: μ = general mean; treatment_i = treatment effect (i = LSO, SFO); $\text{cow}_{j(i)}$ = random effect of cow j (experimental error for the global treatment effect) in treatment i; time_k = time effect (k: −1, +7, +28 and +56); $(\text{treatment} \times \text{time})_{ik}$ = treatment by time interaction; e_{ijk} = residual error.

Results and discussion

Out of four histone acetylases measured, linseed oil supplementation significantly repressed the expression of *KAT2A* on day +28 ($P = 0{\cdot}019$) as compared to the CP (day −1) (Fig. 1a). Apart from the expression of *HAT1* which was constant throughout, the expression of the histone acetylases decreased slightly (significant for KAT2A) in response to LSO, and remained low even after 28 d of withdrawal of treatment (Fig. 1a).

The expression of *HDAC2 and HDAC3* were repressed at day +7 ($P < 0{\cdot}05$) while *SIRT2* was repressed at day +28 ($P = 0{\cdot}018$) of LSO supplementation (Fig. 1b). When treatment was withdrawn, the expression of the genes remained low even after 28 d as compared to control values (Fig. 1b). The expression of one histone methytransferase (PRMT1) was not affected by LSO while that of *EHMT2* was significantly decreased at day +7 ($P = 0{\cdot}030$) (Fig. 1c). Following withdrawal of LSO supplementation, the expression of *EHMT2* and *PRMT1* still remained low.

The expression of de novo methyltransferase, *DNMT3A*, tended ($P = 0{\cdot}053$) to decrease with linseed oil supplementation at day +28 while DNMT1 was not affected (Fig. 1d, online Supplementary Table S2)

Generally, LSO had a decreasing effect on the expression of some of the genes, which was significant in some cases. The expression of the genes (except *HAT1*) were still below control (day −1) levels at the end of the PTP (Fig. 1 and online Supplementary Table S2).

Overall, the expression levels of all the investigated genes during the different experimental periods were not affected by SFO supplementation (online Supplementary Table S2). Slight decreases observed in the expression of *EHMT2* and *SIRT2* after 28 d of SFO treatment were not significant. Also, there were no significant differences between treatments in the expression of genes.

These results demonstrate that the mRNA abundance of some of the studied epigenetic modifying enzymes (*EHMT2*, *KAT2A*, *HDAC2*, *HDAC3* and *SIRT2*) were reduced on day +7 or day +28 under LSO supplementation with minimal effect of SFO supplementation. Following withdrawal of LSO treatment, the expression of the affected genes were still low four weeks after end of treatment indicating that the residual effects of LSO were still regulating gene expression. This demonstrates an effect of LSO on the expression of the studied epigenetic genes as compared to SFO, and potential involvement of genes in the epigenetic regulation of milk fat synthesis. This data supports observations on gene (mRNA) and miRNA expression on a subset of the same animals which showed a differential regulation by LSO as compared to SFO (Li et al. 2015; Ibeagha-Awemu et al. 2016). Although both supplements decreased milk fat yield similarly, 30·38% by LSO and 32·42% by SFO, RNA-Seq (mRNA expression) showed a greater impact of LSO on gene expression changes and metabolic pathways as compared to SFO (Ibeagha-Awemu et al. 2016). The gene expression changes included down regulation of key lipogenic genes involved in lipid synthesis. Similarly, miRNA expression showed that LSO and SFO affected significantly ($P < 0{\cdot}05$) the expression of 14 and 22 microRNAs, respectively (Li et al. 2015).

Histone modifications are much more diverse (Kouzarides, 2007; Tan et al. 2011) than DNA methylation thus potentially offering more opportunities for dietary factors to influence histone marks. Repressed expression of five genes (*EHMT2*, *KAT2A*, *HDAC2*, *HDAC3* and *SIRT2*) responsible for three types of histone modifications suggests that a wide range of histone marks responded coordinately to LSO supplementation. These results are not surprising as many studies have already described the involvement of histone methylation and acetylation in adipogenesis (Yoo et al. 2006; Haberland et al. 2010; Musri et al. 2010; Sato-Kusubata et al. 2011). Specifically, histone deacetylases and sirtuins have been shown to play central roles in lipid metabolism (Lomb et al. 2010; Feng et al. 2011).

The observed differential response of the gene expression of the epigenetic modifying enzymes to LSO and SFO could be due to the different degrees of unsaturation of the oils. SFO is rich in linoleic acid (C18 : 2*n*6) with two double bonds while linseed oil is rich in α-linolenic acid (C18 : 3*n*3) with three double bonds suggesting that the degree

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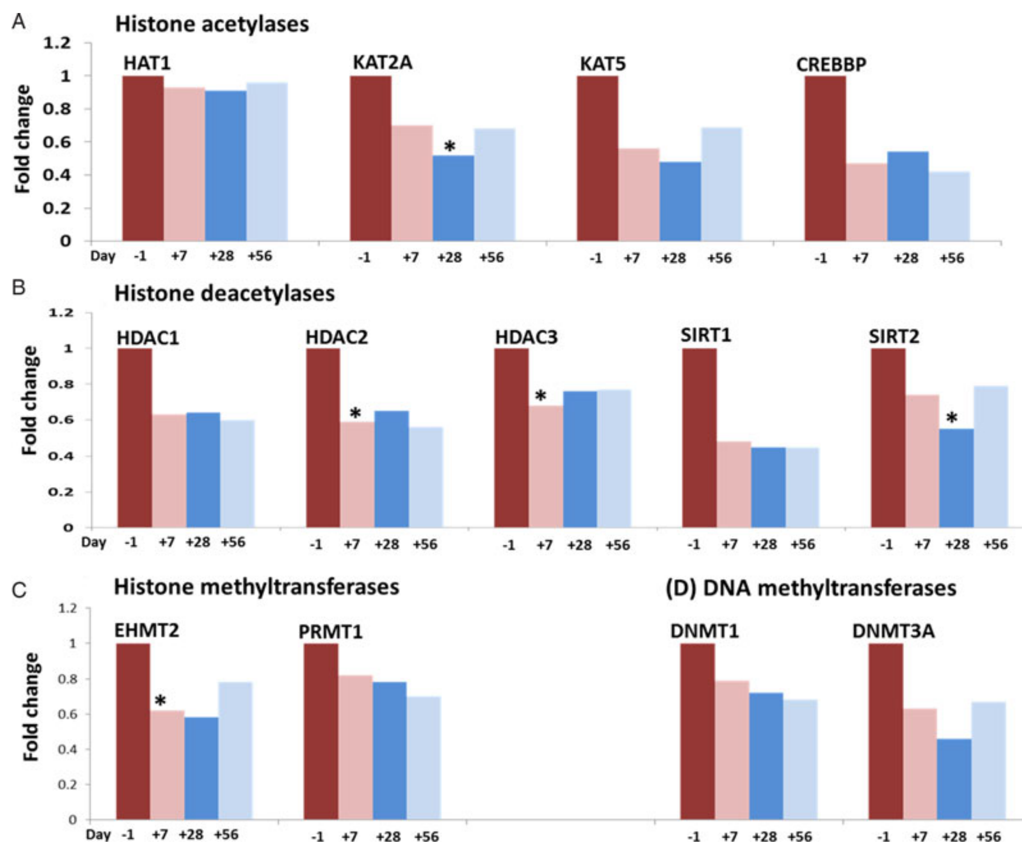

Fig. 1. Gene expression changes in (a) histone acetylases, (b) histone deacetylases, (c) histone methyltransferases and (d) DNA methyltransferases in response to dietary supplementation of cows' diet with 5% linseed oil. $n = 13$ biological replicates; * level of significance at $P < 0·05$.

of unsaturation of oil could impact the pathways involved in DNA methylation and histone modification differently, as observed with gene (mRNA) and miRNA expression data on a subset of the same animals (Li et al. 2015; Ibeagha-Awemu et al. 2016). Further studies are, however, necessary to understand the mechanisms underlying the differential dietary regulation of the expression of epigenetic modifying enzymes by LSO and SFO.

Our study has demonstrated the alteration of the expression of epigenetic modifying enzymes (*EHMT2*, *KAT2A*, *HDAC2*, *HDAC3* and *SIRT2*) by dietary supplementation with LSO which suggests involvement of histone marks in the regulation of fatty acid synthesis. The effect of SFO supplementation was less evident on the gene expression of the epigenetic modifying enzymes but still caused milk fat reduction, suggesting that oil supplements could affect the milk fat synthesis by different pathways.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S002202991700022X

Funding for this study was provided by Agriculture and Agri-Food Canada.

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