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Review article

Application of nutrigenomics in small ruminants: Lactation, growth, and beyond

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ABSTRACT

Ruminants have a very special niche in the animal kingdom, and are the most important livestock species providing milk, meat, and wool for humans from consumption of highly-fibrous feedstuffs. Cattle, goat and sheep have been widely-used for years as models to study ruminal fermentation and the mechanisms whereby tissues utilize nutrients for milk synthesis, growth, wool accretion, and reproduction. The advent of high-throughput technologies to study an animal's genome, proteome, and metabolome (i.e., "omics" tools) offered ruminant scientists the opportunity to study multiple levels of biological information to better understand the whole animal response to nutrition, environment, physiological state, and their interactions. The omics revolution gave rise to the field of nutrigenomics, i.e. the study of the genome-wide influences of nutrition through alteration in mRNA, protein, and metabolite expression or abundance. This field of research is relatively new in ruminants, and particularly sheep and goats. Dietary compounds affect gene expression directly or indirectly via interactions with transcription factors including ligand-dependent nuclear receptors. New knowledge generated through the application of functional analyses of transcriptomic, proteomic, and metabolomic data sets in goat and sheep is discussed.

1. Introduction

Phenotypic variables have been the foundation of traditional nutritional science, where scientists have based their hypothesis and findings. However, as discussed in the "nutrigenomics technology" section, the recent advancements in molecular biology (and bioinformatics) have provided new tools to evaluate fundamental effects of nutrients on physiologic outcomes. The latter has allowed new fields of research such as nutrigenomics to bloom. Nutrigenomics has been described as "the study of genome-wide influences of nutrition" (Muller and Kersten, 2003), or how dietary nutrients can affect gene expression and consequently affect protein expression and metabolism of the entire organism.

2. Nutrigenomics technologies

2.1. Transcriptomics

Technological advances, such as microarray platforms and RNA-sequencing, allowed scientists to quantify the expression of almost the entire set of transcribed genes in a sample, hence, the term transcriptome. The transcriptome is the total transcribed RNA (i.e., mRNA, noncoding RNA, rRNA, and tRNA) in a cell or tissue, and reflects the cellular metabolic and non-metabolic response to a particular environment (e.g., diet, management, treatment) allowing a "still-frame" of cellular activity. Bovine microarrays were first used in small ruminants in 2007 during a nutrigenomic study of goat mammary transcriptome responses to feed deprivation (Ollier et al., 2007). This study highlighted genes responsible for the drop in milk protein, lactose, and fat secretion, and genes responsible for a slowdown in mammary cell proliferation and differentiation and/or an increase in programmed cell death leading to activation of early mammary involution.

Recently, RNA sequencing has replaced microarray platforms, and this technology has been used in omics studies since at least 2011. Despite the fact that none of those experiments had a nutrigenomics focus, this technology has been applied to study not only the transcriptome (Shi et al., 2015) but also the microRNAome (Li et al., 2012) in small ruminants such as goats and sheep (Jager et al., 2011; Cox et al., 2012).

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2.2. Gene reporter technology

Gene reporter technologies have been extensively used in the study of transcription factors and their involvement in cellular signaling cascades. The reporter used is often a gene that transcribes a protein with a readily-measurable phenotype that can be distinguished easily from a background of endogenous proteins (Alam and Cook, 1990). Normally, the reporter gene is linked to a promoter sequence through an expression vector (e.g. chimera plasmid) that is further transferred into cells, both *in vivo* or *in vitro*. When activated, the interaction between transcription factor and its *cis*-regulatory sequences (promoters, or response elements) included in the sequence of the reporter gene will elicit the expression of the reporter that can then be easily monitored.

In ruminant physiology these technologies have been used to address involvement of transcription factors in mammary metabolism and the response to nutrients and nutritional stages (discussed in Section 4). Goats have been recently used as models, for example, to characterize the activity and involvement of peroxisome proliferator-activated receptors (PPAR) and sterol regulatory element binding protein 1 (SREBP1) through the use of gene reporters (Xu et al., 2016a,b; Shi et al., 2017).

2.3. Proteomics

When considering the mRNA portion of the transcriptome, a direct hypothesis can be formulated on the protein expression of a cell, which due to the complexity of post-transcriptional regulations (e.g., alternate splicing, and phosphorylation or dephosphorylation), has a greater degree of complexity compared with the genome. Thus, proteomics has been developed to identify and differentially quantify protein species in complex biological samples.

The core technology is mass spectrometry (MS), which has been adopted by livestock scientists (Lippolis and Reinhardt, 2008; Sauerwein et al., 2014). In the past decade, various proteomics studies in sheep have addressed the proteome response during pathogen-related reproductive disease (Wu et al., 2014b; Du et al., 2016; Miao et al., 2016), digestive diseases (Athanasiadou et al., 2008; Nagaraj et al., 2012; Pisanu et al., 2017), or immunological disease (Marsh et al., 2006; Chiaradia et al., 2012) namely as a way to identify novel biomarkers. In addition, Al-Gubory et al. (2014) used this technique to understand basic physiological mechanism during the establishment of pregnancy in sheep. Restelli et al. (2014) used it to identify differences in the visceral and subcutaneous fat proteome in goats, also uncovering novel adipokynes in this species. Unlike large ruminants, proteomics use for nutrigenomics in small ruminants remains limited. Recently, Ren et al. (2016) investigated the effect of overgrazing on the sheep liver proteome, identifying a shift in energy resources from carbohydrates to proteins, causing an impairment of nutrient metabolism (protein and lipid) and immunity, which may be reasons for the reduced growth observed under this nutritional condition.

2.4. Metabolomics

Following the physiological flow of biological information processing and synthesis from gene expression to protein synthesis and metabolite changes, the analysis of the metabolome naturally completes and complements the transcriptome and proteome. The metabolome consists of the global profiling of metabolites in a sample, using high-resolution analysis together with statistical tools such as principal component analysis and partial least squares (Zhang et al., 2012). The small molecules detectable by this approach include peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, alkaloids, and inorganic species.

Metabolomics studies may be conducted on a variety of biological fluids and tissue types with a number of different technology platforms, like nuclear magnetic resonance (NMR) and MS, with the latter becoming the technique of choice. Metabolomics applications, however, are almost inexistent in small ruminants, and only few recent examples exist in the published literature. Comparative studies of the metabolic profile of milk have been conducted in goat to address differences due to genotype or with other species (e.g. cow) (Scano et al., 2014; Caboni et al., 2016). Nutrigenomics applications have also been attempted in sheep, specifically in relation to maternal nutrient restriction during pregnancy, focusing on the global composition of umbilical venous blood (Sun et al., 2017) or on breed adaptability to harsh environmental conditions (Palma et al., 2016). The first study revealed a beneficial effect of dietary rumen-protected arginine or N-carbamylglutamate supplementation on mammalian reproduction to avoid detrimental effects of undernutrition during pregnancy. Their effect was associated with complex metabolic networks and signal transduction involving amino acids, protein, carbohydrate, energy, and lipid metabolism, as well as oxidative stress. In the latter case, both the liver and muscle metabolome were evaluated to investigate the response of different sheep breeds to seasonal weight loss due to pasture scarcity in the dry summer period. The data suggested that Dorper and Damara breeds are more tolerant to these conditions and, thus, more suitable than Merino for harsh environmental conditions.

3. Transcription factors in small ruminants

Dietary nutrients can alter gene expression in the short-to-medium term; however, these alterations or effects are carried out by specialized proteins within the cell, i.e. transcription factors (TF). TF are fundamental to the study of nutrigenomics; they can act as intermediaries between dietary nutrients and the ultimate alteration in gene expression. The TF can be activated directly or indirectly by dietary nutrients, and upon activation they translocate from the cytoplasm to the nucleus where they alter the transcription of specific target genes. The ability of TF to bind specific regulatory sites on DNA (i.e., response elements) to regulate gene expression confers these proteins a central stage in the field of nutrigenomics. It is because of this important role of TF in nutrigenomics that the accurate identification and characterization of TF that respond to specific dietary nutrients and to what extent these can be manipulated through dietary effects should be a focus of researchers in the near future.

3.1. General aspects

The normal cellular functions, as well as adaptations to external stimuli, are governed by a precise pattern of gene expression. In turn, the gene expression patterns are highly-regulated by the coordinated action of regulatory elements known as enhancers or *cis*-regulatory modules (Shlyueva et al., 2014). These contain short DNA motif sequences (i.e., 6–10 nucleotides) also known as response elements, that act as binding sites for TF. Once the TF bind a response element, they will recruit coactivators, chromatin remodeling proteins, and the RNA polymerase components.

Initial estimates indicated a range of 2000 to 3000 sequence-specific DNA-binding TF in the human genome (Vaquerizas et al., 2009). This discovery was followed by a comprehensive analysis of nearly 1500 manually curated TF, from which ~100 have been experimentally verified for their DNA-binding and regulatory functions (Vaquerizas et al., 2009). The combination of chromatin immunoprecipitation and DNA sequencing (ChIP-seq) has allowed the construction of databases such as ENCODE (Yip et al., 2012) and AnimalTFDB (Zhang et al., 2015) with current up-to-date information on verified TF. While ENCODE provides mainly information on human and rodent TF, AnimalTFDB offers an array of 50 animal species including *Bos taurus,* but no information specific to small ruminants is available. However, other web-based software such as LASAGNA (Length-Aware Site Alignment Guided by Nucleotide Association), which allows an automatic retrieval and analysis of TF binding sites (i.e., response elements) and related TF

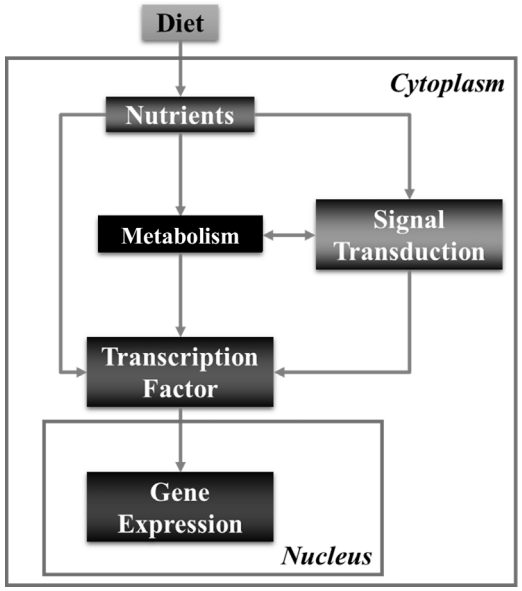

Fig. 1. Proposed model for transcription factor activation by nutrients in the cell.

in the promoter region of genes, contains information for sheep but not goat. Because of the plethora of response elements present in promoter regions, enhancer, and silencer sequences where TF can bind to the DNA, these (i.e., TF) do not work in isolation but rather as a network of TF, and as such they coordinate the response to external stimuli and translate this into changes in gene expression.

At the cellular level, nutrients and bioactive compounds can: i) act as ligands for TF receptors [i.e., ligand-dependent nuclear receptors (LdNR)] (Dauncey et al., 2001; Jacobs and Lewis, 2002); ii) be metabolized by primary or secondary metabolic pathways, thereby altering concentrations of substrates or intermediates (Nobel et al., 2001); and iii) positively or negatively affect signaling pathways (Clarke, 1999; Eastwood, 2001). It is noteworthy to emphasize that these coordinated effects, which are product of alterations in the ordinary supply of specific nutrients may affect the normal function of TF (Figure 1).

3.2. Transcription factors with nutrigenomic potential in small ruminants

The nuclear receptor superfamily of TF, with 48 members in the human genome, is the most important group of nutrient sensors (Chawla et al., 2001). From this superfamily, a short list of TF have been identified as LdNR, encompassing PPAR, liver X receptors (LXR), and hepatic nuclear factor 4 (HNF4) (Muller and Kersten, 2003). The importance of these TF resides in their ability to bind and be activated by macronutrients, including fatty acids (Khan and Vanden Heuvel, 2003) and metabolites of cholesterol for LXR (Zhao and Dahlman-Wright, 2010). Other LdNR, e.g. vitamin-specific, also are among the micronutrient responders including the retinoid X receptors (RXR) and retinoic acid receptors (RAR) activated by retinoic acids [metabolites of the vitamin A; (Minucci et al., 1997), as well as vitamin D receptor (VDR), and pregnane X receptor activated by vitamin E (Landes et al., 2003)].

There are non-LdNR TF, through which nutrients can control gene expression. However, this activation is indirect and mediated by other factors. Among these, the non-LdNR TF encompass SREBP1, which can be either inhibited by polyunsaturated fatty acids (PUFA) (Georgiadi and Kersten, 2012) or activated by glucose (Uttarwar et al., 2012); Spot14 or thyroid hormone responsive protein (THRSP), which is affected by PUFA (Cunningham et al., 1998); carbohydrate responsive element binding protein (ChREBP), which can be activated by glucose 6-phosphate and xylulose-5-phosphate (Li et al., 2006; Oosterveer and Schoonjans, 2014); and amino acid deprivation has been associated with alterations of CCAAT/enhancer-binding protein (C/EBP), activating transcription factor 4 (ATF4) (Kilberg et al., 2012). The nutrigenomics potential of some of these TF has been studied previously in small ruminants or in other ruminants (e.g., dairy cow). In the following sections, we provide an overview of the relatively few investigated LdNR and non-LdNR TF and their nutrigenomics roles in small ruminants.

4. Ligand-dependent nuclear receptors with nutrigenomics potential in small ruminants

Recently, Bionaz et al. (2015) reviewed the main LdNR with a potential role in nutrigenomics with an emphasis in ruminants, providing a short list of 13 LdNR from the nuclear hormone receptor subfamily I and II. These were selected primarily for their ability to bind and be activated by molecules or compounds present in regular ruminant diets and include mostly fatty acids and vitamins. Upon activation, some LdNR will undergo a translocation from the cytoplasm to the nucleus where they form hetero or homodimers before binding the DNA (Burris et al., 2013). However, there are some other LdNR that can be found exclusively in the nucleus or both fractions (i.e., cytoplasm and nucleus) (Hager et al., 2000). Although some LdNR do not avidly interact with heat shock proteins, the inactive forms of some LdNR are complexed with heat shock protein 90, heat shock protein 70, and other proteins that sequester the LdNR until its activation in a ligand-depend fashion (Khan and Vanden Heuvel, 2003). For instance, the PPAR, RXR, VDR, and RAR, members of the type II nuclear hormone receptor, do not interact with heat shock proteins and reside primarily in the nucleus but can also be detected in the cytoplasm (Khan and Vanden Heuvel, 2003; Patel et al., 2005). In the specific case of PPAR, they shuttle between the cytoplasm and the nucleus by export receptors that recognize two different domains on the PPAR protein (Umemoto and Fujiki, 2012). While specific criteria can be met to produce a nutrigenomic effect such as the presence of exogenous agonist, LdNR, and its coactivators, the shuttling of LdNR between cytoplasm and nucleus seems to play a role in the final activity of the LdNR.

4.1. Peroxisome proliferator-activated receptors

Among the currently known LdNR with a nutrigenomic potential, the PPAR have received the greatest attention by the scientific community both, in monogastrics and ruminants (Afman and Muller, 2012; Bionaz et al., 2015). Such interest resides on the fact that PPAR consistently respond, at least *in vitro*, to long-chain fatty acids (LCFA) in a ligand-dependent manner (Bocos et al., 1995; Desvergne and Wahli, 1999; Duplus and Forest, 2002). Recently, a comprehensive review on PPAR and their potential nutrigenomics role in ruminants was published (Bionaz et al., 2013), and some of the authors of the present review published a subsequent review on dairy cattle nutrigenomics (Bionaz et al., 2015), where up-to-date information on PPAR and other potential TF was compiled. Therefore, in the present paper, we will focus and discuss mainly literature directly related to nutrigenomics effects of PPAR in small ruminants. For instance, an RNA-seq study in ovine mammary gland between late pregnancy and lactation revealed the importance of genes related to milk fat synthesis including the activation of PPAR pathways (Paten et al., 2015). As in cow mammary gland (Bionaz et al., 2012ab), the latter indicates a potentially key role of PPAR in mediating the adaptive transcriptional changes the ovine mammary gland undergoes in support of lactation.

The PPAR belong to the subfamily I within the nuclear receptor superfamily and during activation form heterodimers with RXR, which belong to the subfamily II (Burris et al., 2013). There are three PPAR isotypes α, β/δ, and γ which operate by controlling the expression of genes involved in lipid metabolism and inflammation. A primary role in hepatic LCFA catabolism in mitochondria, peroxisome, and microsomes has been established for PPARα (gene symbol *PPARA*) (Desvergne and

Wahli, 1999), while PPARγ (gene symbol *PPARG*) has been associated with a pivotal role controlling the switch between adipogenesis and osteogenesis (Escher and Wahli, 2000; Takada et al., 2009) as well as insulin sensitivity (Olefsky and Saltiel, 2000). In contrast to PPARα and γ, PPARβ/δ (gene symbol *PPARD*) has been perhaps the least studied of the isotypes. However, this isotype can (at least in non-ruminants) control fatty acid catabolism in skeletal muscle and heart (Desvergne et al., 2006). An additional function for PPARβ/δ was recently proposed (Osorio et al., 2016b), and relates to its role in milk synthesis via regulation of glucose uptake in the mammary gland.

The relative amount of PPAR isotypes, in either mRNA or protein basis, across tissues in small ruminants has not been investigated as in dairy cows (Bionaz et al., 2013). However, Shi et al. (2014) observed that *PPARG* mRNA abundance was predominant in adipose tissue, similar to what was observed by Bionaz et al. (2013). It is well-accepted that *PPARA* mRNA is highly expressed in liver, kidney, heart, skeletal muscle, and any other tissue that requires fatty acid oxidation; *PPARG* is predominantly expressed in adipose tissue; while *PPARD* is ubiquitously-expressed in almost all tissues (Bionaz et al., 2013). Differences in *PPAR* isotype mRNA abundance across ruminant species have been observed, where expression of *PPARG* was lower in mammary tissue from lactating dairy goats than cows (Bernard et al., 2013). Based on the previous differences across ruminants species, it will be important to elucidate if a similar pattern of *PPARA* and *PPARD* isotype mRNA abundance across tissues observed in dairy cows (Bionaz et al., 2013) is conserved in small ruminants.

4.1.1. Synthetic PPAR agonists and antagonists

The pharmacological use of synthetic compounds such as those derived from clofibric acid [e.g., thiazolidinedione (TZD) and rosiglitazone] that can bind and activate PPAR has been tested *in vitro* and *in vivo*, and has provided the basis for the claimed regulatory effects of this TF (Desvergne et al., 2006). The latter, primarily *in vitro*, has been associated with an up-regulation of genes related to lipid metabolism after treatment with a synthetic agonist. However, such effects have been more predominant in monogastrics and to a lesser extent in ruminants (Bionaz et al., 2013). Cappon et al. (2002) published one of the first *in vivo* experiments in small ruminants (i.e., lactating goats) using the compound Wy-14643, a synthetic agonist for PPARα, where the authors reported increased hepatic β-oxidation and decreased blood cholesterol. Then, a year later French researchers confirmed the role of PPARγ in controlling follicular differentiation by incubating sheep granulosa cells with rosiglitazone (Froment et al., 2003). The activation of PPARγ by rosiglitazone was recently confirmed in goat mammary epithelial cells, and consequently such effect has been accompanied by an up-regulation of several genes associated with triacylglycerol synthesis and secretion as well as genes related to lipid accumulation such as adipose differentiation-related protein (currently known as perilipin 2 or *PLIN2*) (Shi et al., 2013; Kang et al., 2015).

Recent work has utilized other synthetic agonist such as TZD for the activation of PPARγ in lactating goats (da Rosa et al., 2015). The lack of overall TZD effect on mRNA expression of putative PPARγ target genes measured in adipose or mammary tissues provided preliminary data indicating that TZD is not a potent PPARγ agonist. Similar results have been observed in dairy cow adipose tissue (Schoenberg and Overton, 2011). Based on this lack of effect of TZD, a follow up *in vitro* study was conducted to determine if such effect could be associated with a low basal activation of RXR. The latter forms a heterodimer with RXR prior to binding the DNA (Burris et al., 2013). The marked increase in activity of PPARγ only when immortalized bovine cells (i.e., MACT and MDBK) were incubated with both TZD and 9-*cis*-retinoic acid [specific agonist for RXR (Wang et al., 2010)] confirms that *in vivo* a low activation of RXR precluded the full effect of TZD (Bionaz et al., 2015). Therefore, those data suggest that adequate levels of vitamin A supplementation might be required in order to have a functional activation of PPARγ *in vivo*.

In contrast to activation of PPAR, decreasing the activity of PPAR isotypes could also provide fundamental knowledge on how these TF work at the molecular level and which are the main gene networks affected by such effect. For this, several synthetic antagonists, which decrease PPAR activation, such as GW6471 (Xu et al., 2002), GW9662 (Wright et al., 2000), and GSK0660 (Shearer et al., 2008) have been tested for PPARα, PPARγ, and PPARβ/δ, respectively. Currently, there is a lack of data showing the effects of these synthetic antagonists in small ruminants. However, an *in vivo* experiment conducted with neonatal lambs receiving intravenous injections of GW9662 led to a significant decrease in PPARγ protein concentration and activity (Sharma et al., 2013). The results also demonstrated that GW9662 induced mitochondrial dysfunction mainly associated with a disruption in carnitine metabolism. Such effect is of great importance in ruminants, especially in dairy cattle transitioning from pregnancy into lactation (Carlson et al., 2007) when energy balance becomes negative and the liver is flooded with LCFA arising from adipose tissue lipolysis.

Induction of negative energy balance affects lactating ewes in a similar way as dairy cows (Bouvier-Muller et al., 2016), i.e., LCFA concentration in plasma increases and are transported to the liver where carnitine is needed for translocation into the mitochondria for β-oxidation. If LCFA are not processed at an adequate rate through β-oxidation or other hepatic mechanisms, they can accumulate leading to fatty liver and predispose the animal to other health issues. Within this context, the activation of PPARγ in sheep seems of utmost importance because the results with GW9662 (Sharma et al., 2013) showed a dependency of carnitine metabolism on PPARγ. It remains to be determined if the activation of PPARγ can enhance carnitine metabolism or if activation of this TF is only required to maintain a basal level of carnitine metabolism.

4.1.2. Natural PPAR agonists

The ability of PPAR isotypes to bind and be activated by LCFA, which are commonly present in small ruminant diets, underscores the importance of these TF from an applied nutrigenomics standpoint (Bionaz et al., 2013; Bionaz et al., 2015). About a decade ago Muhlhausler et al. (2007) reported that a nutrient-dense diet fed to pregnant ewes during late-gestation increased mRNA expression of *PPARG* in fetal perirenal fat. These data were the first evidence *in vivo* of the potential for nutrigenomics effects in small ruminants. Subsequently, Ebrahimi et al. (2013) reported similar results in growing goats that were fed a high linolenic acid diet, which resulted in greater mRNA expression of *PPARG* in adipose tissue. In a follow-up study using male goats, researchers fed diets that increased flaxseed oil as a source of α-linolenic acid (18:3n-3) at 0, 0.4, and 1.3% of diet dry matter (Ebrahimi et al., 2014). The results showed a linear up-regulation of *PPARA* and *PPARG* in muscle, consistent with their previous observation in adipose tissue.

In contrast to adipose and muscle tissues, the activation of PPAR isotypes through dietary effects in mammary tissue of small ruminants has been less consistent. For instance, the lipogenic activity was increased in goat mammary slices incubated with α-linolenic acid, while the mRNA expression of *PPARG1* (i.e., an isoform of *PPARG*) was not affected by this LCFA (Bernard et al., 2013). Similarly, an *in vivo* study under- (70%) or overfeeding (130%) lactating goats according to energy and protein requirements did not detect an effect on *PPARG2* expression in mammary tissue (Tsiplakou et al., 2015b). In a similar experiment, Tsiplakou et al. (2015a) observed an increase in *PPARG2* mRNA expression between under and overfeeding in sheep mammary tissue indicating a greater degree of responsiveness. However, *PPARG2* expression in both conditions was similar to the control. The difference in isotype responsiveness is noteworthy because *PPARG1* has been reported to be the predominant *PPARG* isoform in mammary and other tissues in goats (Li et al., 2013; Shi et al., 2014).

Increasing the nutrient density in diets might not be an accurate method to target PPAR activation as observed by Tsiplakou et al.

(2015a; 2015b). Perhaps using specific dietary nutrients such as LCFA to target PPAR might be a more reliable way to accomplish such activation. For instance, Bionaz et al. (2015) reported an increase in PPAR activation using a luciferase gene reporter method when goat mammary epithelial cells were incubated with media containing palmitic acid (C16:0) at 100 m*M* or fetal bovine serum (FBS) at 10%. The latter has been reported to contain significant amounts of oleic acid (C18:1n-9), C16:0, and stearic acid (C18:0) (i.e., 80.8, 67.6, and 32.7 m*M*, respectively) (Lagarde et al., 1984). Interestingly, the greater PPAR activation of FBS over C16:0 could only be explained by an additive effect of oleic and stearic acid in FBS (Bionaz et al., 2015). Oleic acid has received less attention as a PPAR activator in ruminants since this LCFA does not appear to have an effect on milk fat synthesis in bovine mammary cells (Bionaz et al., 2015). In contrast to C18:1n-9, C18:0 upregulates *PPARG* mRNA expression and consequently target genes in goat mammary epithelial cells under lactogenic activation (Zhao et al., 2014). Therefore, most of that increment in PPAR activation observed by Bionaz et al. (2015) could be associated to the C18:0 acid in FBS. It is noteworthy, however, that C18:1n-9 in a previous *in vivo* experiment increased milk fat synthesis in lactating goats (Bernard et al., 2005). To shed light on alternative nutrigenomic applications, the degree of PPAR activation by C18:1n-9 and C18:0 in the mammary gland of small ruminants should be further investigated in the future.

In dairy cattle, the liver is responsible for orchestrating major metabolic and inflammatory adaptations during the transition period from pregnancy into lactation, and such adaptations will likely determine the health status during early lactation and consequently affect performance in terms of dry matter intake and milk yield (Loor, 2010). Within this context the activation of PPARα by LCFA in the liver is a central element of the proposed model by Bionaz et al. (2013), where simultaneous activation of PPAR isotypes in various tissues and cells in the periparturient ruminal would promote a healthier transition period by enhancing lipid metabolism in the liver and improving insulin sensitivity in adipose tissue among others effects.

Those effects have been partly observed in sheep when hydrogenated palm oil containing mainly C16:0 and C18:0 was offered to peripartal ewes at 47 g/day from 2 to 3 weeks relative to parturition (Agazzi et al., 2010). In that study, the palm oil diet up-regulated the mRNA expression of *PPARA* and target genes for this TF such as carnitine palmitoyl-transferase 1A (*CPT1A*) by 21 days postpartum. Compared with a control diet, these effects were reflected in a greater (3.65 versus 2.42 kg/d) milk yield. Others have not observed an upregulation of *PPARA* mRNA expression in liver by feeding LCFA, specifically diets containing marine algae to lactating ewes (Bichi et al., 2013a). However, Bichi et al. (2013a) observed that the marine algae diet upregulated hepatic mRNA expression of 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*HMGCS2*), a target gene of PPAαR that is directly involved in lipid metabolism via ketogenesis. Such effect suggested PPARα activation at the protein level.

4.2. Liver x receptor

The nutrigenomics effects of LXR have been recently studied in ruminants (McFadden and Corl, 2010), and in monogastrics this LdNR is associated with cholesterol synthesis (Desvergne et al., 2006). To date, two isoforms of LXR have been reported: LXRα (gene symbol *NR1H3*) and LXRβ (gene symbol *NR1H2*). Oxysterols and derivatives of cholesterol are the primary ligands for LXR, and to a lesser extent, fatty acids (Burris et al., 2013). Both isoforms of LXR can form heterodimers with RXR, and subsequently, bind to LXR response elements in the promoter of target genes (Fievet and Staels, 2009). In dairy cows, the expression of *NR1H3* (LXR) is most abundant in the liver (Harvatine et al., 2014), while in lactating goats it is most ubiquitously expressed but at different levels, with small intestine and liver being higher (Wang et al., 2012). In contrast to dairy cows, the goat mammary gland has comparable mRNA abundance of *NR1H3* as in liver (Wang et al., 2012).

There is less information available on LXR for sheep, but *NR1H3* mRNA expression has been assessed in the perirenal adipose tissue of neonatal lambs (Basse et al., 2015), while Chromatin Immunoprecipitation technology (ChIP) has been used to determine the activity of both LXR isoforms in corpora lutea of ewes (Seto and Bogan, 2015). The ability to control SREBP1 is one of the primary mechanism by which LXR regulate lipid metabolism. This mechanism has been confirmed by the presence of an LXR response element in the promoter region of sterol regulatory element binding transcription factor 1 (*SREBF1*; gene encoding SREBP1) in goat mammary epithelial cells (Wang et al., 2012). A recent elegant experiment conducted in goat mammary epithelial cells demonstrated the importance of LXR in the transcription of essential mammary enzymes such as stearoyl-coenzyme A desaturase 1 (encoded by *SCD1*) (Yao et al., 2016a). The *SCD1* is a vital mammary enzyme for the synthesis of monounsaturated fatty acids (MUFA) and specific conjugated linoleic acid (CLA) isomers, but it can be down-regulated by linoleic acid. Such effect is carried out through a decrease in *SCD1* gene promoter activity and also a decrease in *SREBF1* expression. However, Yao et al. (2016a) observed that the adverse effects of linoleic acid to some extent could be reverted via a synthetic agonist for LXR (i.e., T09) and, consequently, this could stimulate the *SCD1* promoter even in the presence of linoleic acid. Another vital enzyme for milk fat synthesis in the mammary gland is fatty acid synthase (encoded by *FASN*). Similar to *SCD1*, the importance of LXR for the transcription of *FASN* in goat mammary epithelial cells has been proven recently (Li et al., 2015c).

Although there is no doubt on the importance of LXRα for milk fat synthesis in small ruminants, future studies should focus on confirming the activation of this TF by LCFA, to provide a more solid case for the use of LXRα in nutrigenomics interventions. For instance, such activation of LXR by LCFA has been proven in monogastrics (Vanden Heuvel et al., 2006). At least in bovine mammary cells, LXRα does not respond to *trans*-10, *cis*-12 CLA (Harvatine et al., 2014; Ma et al., 2014), which suggests that this TF does not participate in the transcriptional inhibition induced during milk fat depression by this CLA. However, this remains to be determined in small ruminants.

Besides cholesterol metabolism, there are additional effects where LXR is involved in monogastrics; this TF can modulate glucose uptake and lipogenesis in adipose tissue, while in liver it can modify lipogenesis, bile acid metabolism, and glucose metabolism, and decrease inflammatory genes in immune cells (Calkin and Tontonoz, 2012). The exploration of these effects should be done from a nutrigenomics standpoint and among ruminants species since comparisons across literature suggest major differences in the LXR.

4.3. Vitamin D receptor

The VDR is a LdNR that responds to vitamin D and is capable of forming a heterodimer with RXR (Bionaz et al., 2015). The latter suggests an intertwined relationship between vitamin D and A in the final activation of VDR, and understanding this relationship could lead to uncovering the potential nutrigenomics effect of this TF. To the authors' knowledge, a characterization of *VDR* gene expression profiles across tissues has not been carried out in small ruminants. The main biological effect of VDR in ruminants species has been associated with Ca and P absorption in the small intestine primarily during early postpartum when there is an increased demand for Ca output into milk. Impaired Ca absorption coupled with the lower capacity to remove Ca from bones can often lead to developing "milk fever" (Horst et al., 1994; Liesegang et al., 2007).

Based on rodent data, the primary VDR target genes are cytochromes or mitochondrial proteins such as cytochrome P450 family 24 subfamily A member 1 (*Cyp24a1*) and cytochrome P450 family 24 subfamily B member 1 (*Cyp27b1*) (Carlberg and Campbell, 2013) as well as small intestine P (solute carrier family 34 member 2, *Slc34a2*) (Hattenhauer et al., 1999) and Ca transporters (ATPase plasma

membrane Ca2+ transporting 1, *Atp2b1*) (Lee et al., 2015). Calcitriol, a metabolite of vitamin D, can activate VDR, and in fact, this has been tested in sheep and goats (Herm et al., 2015; Wilkens et al., 2015). Interestingly, it has been reported that intestinal *VDR* mRNA expression in sheep is more responsive to supplementation of calcitriol than goats (Herm et al., 2015). A similar effect has been observed in growing goats, where a reduction in Ca and P in the diet did not affect the intestinal mRNA expression of *VDR* and *SLC34A2* (Muscher et al., 2012). However, this effect should be further researched and replicated to pinpoint the actual mechanisms by which Ca supplementation might differentially affect vitamin D metabolism between sheep and goats.

In addition to the small intestine, the mRNA expression of *VDR* and its target gene *SLC34A2* has been measured in the mammary gland of goats (Muscher et al., 2009). Results from this study demonstrated that both mRNA expression of VDR and *SLC34A2* could be modulated via dietary P (Muscher et al., 2009). Interestingly, in contrast to *in vivo* data, a recent *in vitro* study using goat mammary epithelial cells revealed a marked response of *VDR* and its target genes *ATP2B1*, *ATP2B2*, and S100 calcium binding protein G (*S100G*) in a dose-dependent manner to concentrations (i.e., 0, 0.1, 1, and 10 nmol/L) of 1,25-Dihydroxyvitamin D3, the active form of vitamin D (Sun et al., 2016). Sun et al. (2016) also confirmed the action of calcitriol not only on calcium absorption but also on glucose uptake via the upregulation of the glucose transporters *GLUT1* and *GLUT12*. Taken these data together, it suggests that *VDR* in small ruminants responds to dietary P concentrations and vitamin D metabolites, calcitriol, and energy supply. RXR activation by 9-*cis*-retinoic acid also could further increase the response of *VDR* in the small intestine. If the latter effects can be optimized in peripartal ruminants, it could lead to potential nutrigenomics interventions to minimize the occurrence of milk fever during early postpartum.

4.4. The pregnane x receptor

This LdNR (PXR; gene symbol NR1I2) has received little to no attention in ruminants, but certainly, has a great potential for nutrigenomic applications because it can be activated by xenobiotics, natural and synthetic glucocorticoids, steroids, vitamin E, and vitamin K2 as well as specific herbal extracts (Chang, 2009; Ihunnah et al., 2011). Although neither mRNA expression nor the activity of PXR has been measured in goats, in sheep it is expressed in the liver (Mate et al., 2012). Mate et al. (2012) reported that PXR was unresponsive to doses of dexamethasone, a glucocorticoid, and therefore contrasted with data from monogastrics. In sheep, PXR mRNA expression has been observed to respond to vitamin D metabolites, calcitriol and calcifediol (i.e., calcitriol precursor), in kidney and jejunum, respectively (Wilkens et al., 2015). The latter suggests a pivotal role of vitamin D in the activation of either VDR or PXR in small ruminants. The study of PXR from a nutrigenomic standpoint is warranted based on its role in gluconeogenesis, triacylglycerol synthesis, and bone mineral homeostasis reported in monogastrics (Ihunnah et al., 2011).

4.5. Hepatic nuclear factor 4

This LdNR is probably the only one discussed in this review which does not form a heterodimer with RXR (Bionaz et al., 2015). However, this does not minimize its importance in terms of lipid metabolism, glucose metabolism, cell junctions, differentiation and proliferation in the liver and intestinal epithelial cells (Babeu and Boudreau, 2014). Although HNF4 requires binding of an LCFA to be activated (Nakamura et al., 2014), it seems that the final activity is also regulated by a specific coactivator, the steroid receptor Coactivator-1 (SRC-1) (Duda et al., 2004). Despite the need for SRC-1, the acyl-CoA thioester of myristic acid (C14:0) and C16:0 can serve as agonists, whereas PUFA and C18:0 are antagonists (Hertz et al., 1998). Although the HNF4a has not been measured in small ruminants, in dairy cows the mRNA expression is upregulated in the liver during early postpartum, indicating that this TF might play important roles in lipid metabolism of peripartal ruminants (Loor et al., 2005). The activation of this TF by LCFA has not been tested in ruminants.

5. Other non-LdNR transcription factors in small ruminants

The LdNR discussed above exhibit, for the most part, a linear axis of regulation with their respective ligands; in contrast, the non-LdNR will require a more elaborate mechanism(s) to be activated by specific dietary nutrients and compounds (Fig. 1). Those mechanisms are likely to involve primary or secondary metabolites or original nutrients or signal transduction cascades that “sense” or respond to nutrient availability, as depicted in Fig. 1. Therefore, rendering this type of TF less desirable for nutrigenomics interventions in ruminants. In fact, the nutrigenomics manipulation of LdNR in ruminants remains a difficult task at best, with a proper manipulation of non-LdNR less likely to be accomplished in the near future; however, this does not diminish the importance and the value of understanding the biology of these TF in small ruminants.

5.1. Sterol regulatory element-binding protein 1

Efforts to understand the molecular mechanisms associated with the well-known phenomenon of milk fat depression (MFD) in dairy cows led to the recognition of the pivotal role of SREBP1 in milk fat synthesis (Bauman et al., 2011). Because of the practical importance of MFD in ruminants, there is a significant amount of review papers and data available addressing this topic (Bionaz et al., 2013; Osorio et al., 2016b). Therefore, we will primarily discuss the involvement of SREBP1 during MFD and milk fat synthesis as well as the main differences among ruminant species.

Ahnadi et al. (2002) working with a fish oil-induced MFD model were the first to propose *SREBF1* as the primary TF orchestrating the transcriptional alterations during MFD. However, at the time this study was published, the central role of *trans*-10 *cis*-12 CLA in the function of SREBF1 was yet to be confirmed. It was not until 2014, when Ma et al. (2014) confirmed, through a gene reporter assay, the inhibitory activity of *trans*-10 *cis*-12 CLA on SREBP1. The exact mechanisms for the inhibitory action of CLA on SREBP1 have been previously discussed (Bionaz et al., 2015; Osorio et al., 2016b), and it is well-established from non-ruminant data that these effects are carried out through both decreased transcription of *SREBF1* (both isoform 1a and 1c) as well as proteolytic processing (Hannah et al., 2001). The latter refers to an inhibition of the cleavage of the immature SREBP1 in the Golgi, and its subsequent maturation. Thus, if the SREPB1 does not mature, it will not migrate to the nucleus and exert its transcriptional activity (Hannah et al., 2001). Such transcriptional activity has been associated with upregulation of target genes associated with the de novo fatty acid synthesis and LCFA desaturation, encompassing as acetyl-CoA carboxylase alpha (*ACACA*), *FASN*, and *SCD*.

The study published by Barber et al. (2003) was the first in which SREBP1 was confirmed as a key regulator of the lipid synthesis in the mammary gland of small ruminants, specifically sheep. In goats, this was ascertained in 2012 by Wang et al. (2012), where *SREBF1* mRNA expression was up-regulated by a synthetic agonist of LXRα. Interestingly, by 2010 available data indicated that small ruminants compared with cows were more resistant to the MFD effect induced by CLA (Shingfield et al., 2010). This was associated with a lower susceptibility in small ruminants to alterations in diet-induced ruminal biohydrogenation pathways that produce more *trans*-10 18:1 than *trans*-11 18:1. However, the negative effects of *trans*-10 *cis*-12 CLA, including a decreased in *SREBF1* mRNA expression, were recently confirmed in lactating ewes (Hussein et al., 2013). In contrast to this CLA effect, a high-concentrate diet (130% of energy requirements) increased the expression of SREBF1, which could be associated with a lower

specificity of a high-concentrate diet vs. *trans*-10 *cis*-12 CLA (Tsiplakou et al., 2015a). In contrast to sheep, the expression of *SREBF1* in goat mammary gland seems to be less responsive to high-concentrate diets (Tao et al., 2015; Tsiplakou et al., 2015b), an effect confirmed when goat mammary slices were incubated with incremental concentrations of *tran*-10 *cis*-12 CLA (Bernard et al., 2013).

Although these data show an inconsistent response to *trans*-10 *cis*-12 CLA in sheep, for the most part, the lower susceptibility of small ruminants to MFD can be partly explained by reduced responsiveness of *SREBF1* in the goat mammary gland. In addition to *trans*-10 *cis*-12 CLA, the PUFA contained in fish oil and marine lipids have been consistent in producing MFD in sheep (Bichi et al., 2013b; Carreno et al., 2016; Toral et al., 2016a). These PUFA sources seem to exert their MFD effect through changes in the transcription of *SREBF1* (Carreno et al., 2016; Toral et al., 2016b).

Besides the mammary gland, the involvement of SREBP1 in skeletal muscle in small ruminants has been less investigated (Dervishi et al., 2010; Dervishi et al., 2012). Dervishi et al. (2010) reported that the effect of feeding systems (i.e., grazing vs confinement) did not affect the mRNA expression of *SREBF1*, *PPARA*, and *PPARG*, but they observed that confinement up-regulated *SCD* mRNA expression and consequently CLA concentration in semitendinosus muscle. This effect on *SCD* could be mainly associated with differences in dietary energy availability between feeding systems. In a subsequent experiment, researchers observed that *SREBF1* expression increased in muscle of female lambs suckling from mothers fed different types of forages (Dervishi et al., 2012).

Without a doubt, SREBP1 plays a significant role in lipid metabolism of small ruminants, and in all ruminant species in general. However, important differences exist in comparison with dairy cows, likely due to metabolic differences between ruminant species. Despite being an important LdNR, the usefulness of SREBP1 for nutrigenomics applications are limited at this point. In contrasts to PPAR, SREBP1 seems to be less responsive to activation by LCFA, in fact, until now only LCFA such as CLA-*trans*-10 *cis*-12 can down-regulate *SREBF1* expression and activity, hence, reducing the concentration of this CLA can only be applied as mean to maintain a functional lipid metabolism but not enhance it.

5.2. Other transcription factors with potential nutrigenomics effects in small ruminants

Although the nuclear factor Y (NF-Y) has not been evaluated in sheep, its response element or transcription factor binding sites (TBFS) have been associated with alterations in the transcription of essential milk fat synthesis-related genes such as *SCD* and *FASN* (Li et al., 2015c; Yao et al., 2016a). The *THRSP* or *SPOT14* protein has been linked to MFD in dairy cows (Harvatine and Bauman, 2006), and in goats its expression is greatest in adipose, followed by muscle and liver, and is lower in the mammary gland (Yao et al., 2016b). Yao et al. (2016b) tested the overexpression of *THRSP* in goat mammary epithelial cells, and detected an up-regulation of important genes related to fatty acid synthesis (i.e., *FASN* and *SCD*) and triacylglycerol synthesis (i.e., *DGAT1*, *GPAM*, and *PLIN2*). These data coupled with the fact that *THRSP* expression increased from pregnancy into lactation, underscored the nutrigenomics potential of this TF (Yao et al., 2016b).

As new data on these TF are reported in ruminants, a clearer application in the nutrigenomics context is likely to be elucidated. Important factors such as activation mechanisms *in vivo* and how to achieve them through diet formulation are essential pieces of information. Because of the multiple TF expressed in tissues for which we have some knowledge, it is likely that future integrative work will be needed to build networks of TF that can spam ligand-dependent to inflammation-responsive TF. Therefore, uncovering these links within this network of TF is an important goal as we aim to improve health and performance through dietary manipulations in small ruminants.

6. In silico analysis of transcription factor networks in small ruminants

Based on the limited data available in small ruminants few additional TF with nutrigenomic potential could be suggested at this point. However, the differences outlined above between ruminant species makes for an exciting and promising future regarding how much we can learn, and how this knowledge can be transferred into nutrigenomics applications. To uncover and suggest potential new TF involved in controlling milk fat synthesis, we have performed an in silico analysis of the promoter region of critical genes using LASAGNA (Lee and Huang, 2013). This is the only web-based software currently available for the analysis of TF in small ruminants (i.e., sheep). This software allows an automatic retrieval and analysis of transcription factor binding sites (TFBS) and related TF in the promoter region of genes in several species. It also allows the use of the TRANScription FACtor database (TRANSFAC), which is a manually curated database of over 250 eukaryotic TF and their respective genomic binding sites and DNA-binding profile. We compared the results to previous data from dairy cows (Osorio et al., 2016b).

Because of the lack of data in sheep, some of the important genes related to milk-fat synthesis such as *FASN*, *CD36*, *AGPAT6*, *GPAM*, *BTN1A1*, and *ACSL1* previously used in Osorio et al. (2016b) were not available for the current analysis. Considering all the TFBS for TF within TRANSFAC with significant ($P < 0.001$) hit, we obtained over 250 putative TF that can control the expression of milk fat synthesis-related genes (results not shown). When a more stringent *P*-value cut-off was applied ($P < 0.00001$), we found 51 putative TF for all the genes assessed (Fig. 2). This analysis showed that 8 of 51 TF can regulate two genes simultaneously (i.e., common upstream regulators), and the activating enhancer binding protein 4 (AP-4) was the only TF that could regulate the transcription of 3 genes (i.e., *LPIN1*, *FABP3*, and *LPL*).

Among the TF discussed above, SREBP1 and HNF4α (HNF-4alpha1) were the only present in this analysis. Interestingly, HNF4a (the only LdNR present in this analysis was associated with *SCD* while SREBP1 was associated with *FABP3*. The latter also was detected in a previous analysis in bovine (Osorio et al., 2016b). Another similarity between this and the previous analysis from Osorio et al. (2016b) is that PPAR were not present among the selected TF containing TBFS for *FABP3* between −950 to +50 nt relative to the transcription start site (TSS). This strongly suggests that functional TBFS for PPAR are present beyond the −950 nt relative to TSS, which has been observed in PPAR isotypes (Bionaz et al., 2012). This information, if confirmed in the future, will indicate that PPAR do not act at the promoter region (i.e., closer upstream DNA sequence from the TSS), but rather from a more distal site relative to the TSS. Because the previous analysis (Fig. 2) encompassed between −950 and +50 nt relative to the TSS, and the LASAGNA software can analyze up to −5000 to +1000 nt relative to TSS, there are TBFS that can be present up to 10,000 nt upstream or in the coding region, which has been observed for *SREBP-1c* (SREBP1 isoform) (Lengi and Corl, 2010). Unfortunately, the milk-fat synthesis related genes missing create a significant gap on the overall scope of this *in silico* analysis. Thus, it is possible that other TF might play a major role in this biological process.

In order to uncover major differences among ruminant species, a follow-up *in silico* analysis was performed with the LASAGNA software. This analysis included the LdNR discussed above (PPARα, HNF4α, LXRβ, RAR, and VDR) in addition to NF-Y and SREBP1. Unfortunately, the LASAGNA software does not contain a TFBS matrix for LXRα, therefore, we used LXRβ. This evaluation included the same target genes related to milk fat synthesis as in the previous analysis (Fig. 2) containing the most significant TFBS ($P < 0.001$) in the promoter region between −950 to +50 nt relative to the TSS in ovine and bovine (Fig. 3A and C, respectively). This analysis included the TRANSFAC and JASPAR databases. The results from this analysis indicated substantial

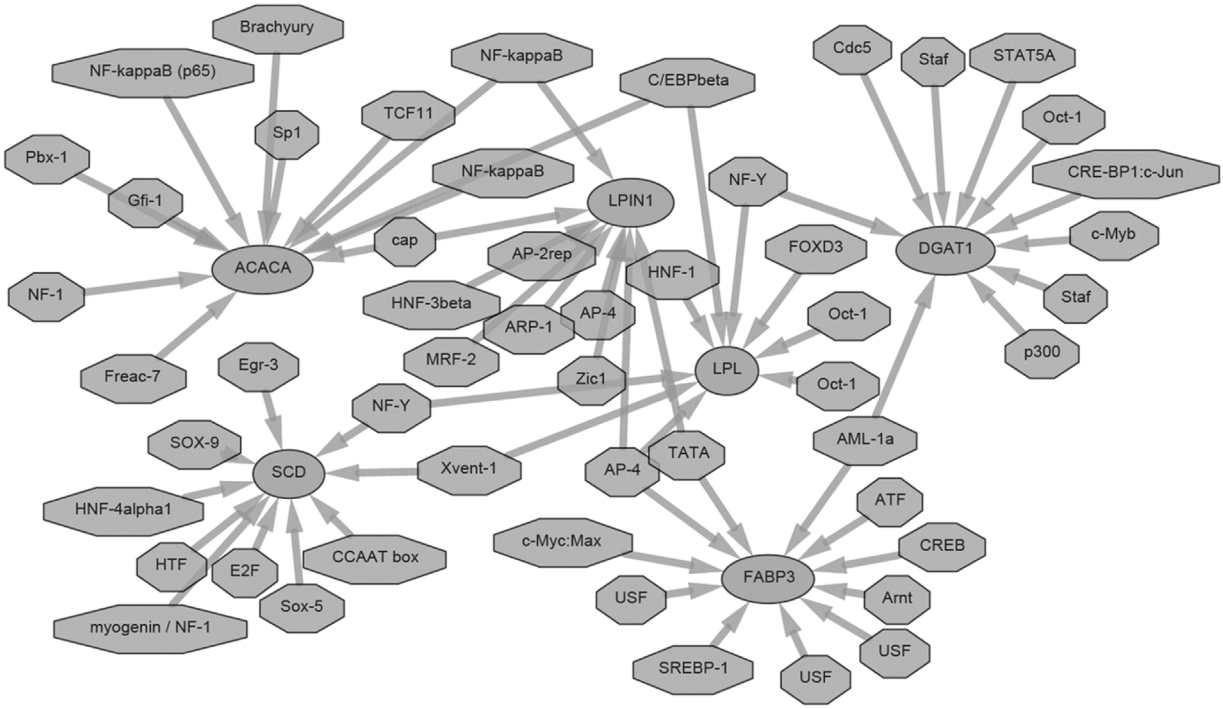

Fig. 2. In silico analysis of transcription factor binding sites (TFBS) in milk fat-related genes. Most significant ($P < 0.00001$) TFBS in the promoter region [-950 to +50 nt relative to the transcription start site (TSS)] of key ovine genes encoding known proteins involved in milk fat synthesis (oval). The TFBS are reported as octagons. The analysis was performed using the LASAGNA software with all TRANSFAC matrices. The *P* value indicates the probability of observing a score of the hit equal to or higher than the score by chance alone.

similarities between ovine and bovine. However, for the gene *FABP3,* we observed 4 possible TBFS hits in ovine in contrast with 7 in bovine. This difference suggests that a greater degree of regulation occurs in bovine. Interestingly, the 3 additional TBFS hits in *FABP3* in bovine were produced by the same PPARγ (Fig. 3A and C). This finding is in agreement with previous confirmation of *FABP3* being a PPAR target gene in bovine mammary epithelial cells (Liang et al., 2014).

To a lesser extent, *ACACA* has fewer TBFS hits in ovine in comparison with bovine (5 vs 7; Fig. 3A and C). Because this type of in silico analysis can be considered only as indicative of TBFS hits (i.e. this is highly determined by the epigenetic status through DNA methylation), the same type of analysis in Fig. 3A and C was performed but with a more stringent *P*-value cut-off ($P < 0.00001$). The latter was done with the aim to uncover the more reliable and resilient TBFS hits within this range in the promoter region between the ovine and bovine (Fig. 3B and 3D). In contrast to the previous analysis (Fig. 3A and C), there were more pronounced differences between ovine and bovine in terms of TBFS hits for the milk fat-related genes evaluated here (Fig. 3B and D). One of the most striking differences between the sheep and cow genetic makeup is the absence of PPARg TBFS hit for ovine in the proposed milk fat-related genes (Fig. 3B). In contrast, PPARγ still plays a significant role in the activation of *FABP3* and *SCD* genes in bovine. The lack of PPARγ TBFS hits in milk fat-related genes in ovine (Fig. 3B) is in agreement with previous data suggesting that to some extent small ruminants (i.e., primarily caprine) have lower PPARγ expression (Bernard et al., 2013) in the mammary gland, which consequently might compromise its activation (Bionaz et al., 2013; da Rosa et al., 2015). However, as mentioned above it is likely that the functional TBFS hits for PPAR might be located beyond −950 nt from the TSS. This has been confirmed by the upregulation of milk fat-related genes such as *FASN*, *ACACA*, and *SCD* in goat mammary epithelial cells after overexpressing *PPARG* (isoform 1) (Shi et al., 2014). In contrast, the downregulation of several genes related to triacylglycerol synthesis and secretion including *FABP3*, *SCD*, and *DGAT1* in goat mammary epithelial cells after PPARγ knockdown (Shi et al., 2013) confirmed the crucial role of this TF in milk fat synthesis in small ruminants.

Interestingly, neither *LPIN1* nor *ACACA* had significant TBFS hit in the promoter regions when a more stringent *P*-value cut-off ($P < 0.00001$) was applied in ovine and bovine, respectively (Fig. 3B and 3D). While SREBP1 was the only TF that had a TBFS hit for *FABP3* in ovine, the same gene (i.e., *FABP3*) had significant TBFS for PPARγ and SREBP1 in its promoter region in bovine. The *ACACA* had NF-Y as the only TF with a potential TBFS hit in the promoter region in ovine (Fig. 3B). Similar to *ACACA* and *FABP3* in ovine, *LPIN1* in bovine only had one TF with a TBFS, VDR (Fig. 3D). In fact, the vitamin ligand-dependent TF VDR and RAR seem to have important roles in controlling milk fat-related genes in either ovine and bovine. Interestingly, based on the total number of TBFS hits, the NF-Y was the TF with most hits in ovine and bovine, underscoring its importance. This high number of TBFS hits is in agreement with the ubiquitous presence of NF-Y among cell types (Oldfield et al., 2014). One of the primary roles of this TF is to promote chromatin accessibility to other TF by bending the DNA after binding at specific sequence (Oldfield et al., 2014). In recent years the importance of NF-Y in small ruminants has been reported, because the TBFS for this TF are in the vicinity of TBFS of important TF such as SREBP1 (Li et al., 2015b; Yao et al., 2017).

An additional LASAGNA analysis was performed with the same features as in Fig. 3B and 3C, with the only modification that instead of evaluating the promoter region between −950 to +50 nt relative to the TSS a wider region was covered from −5000 to 0 nt (Fig. 4A and 4B). Interestingly, the genes *LPIN1* and *ACACA* in ovine and bovine, respectively, now both had TBFS hits for PPARγ, HNF4α, RAR, and LXRβ for *LPIN1* as well as HNF4α and LXRβ for *ACACA* (Fig. 4). This suggests that these genes are regulated through TF on the far regions of the promoter sequence between −5000 to −950 nt.

Another interesting finding from this analysis was the appearance of PPAR in ovine genes. This suggests that PPARγ operates in the far upstream promoter region (i.e., −5000 to −950 nt from the TSS) of milk fat-related genes in ovine, and further confirmed our hypothesis above. Among the similarities across ruminant species observed was the presence of TBFS between −5000 to −950 nt relative to the TSS for LRXβ in both species (Fig. 4), since this TF was not present between −950 to

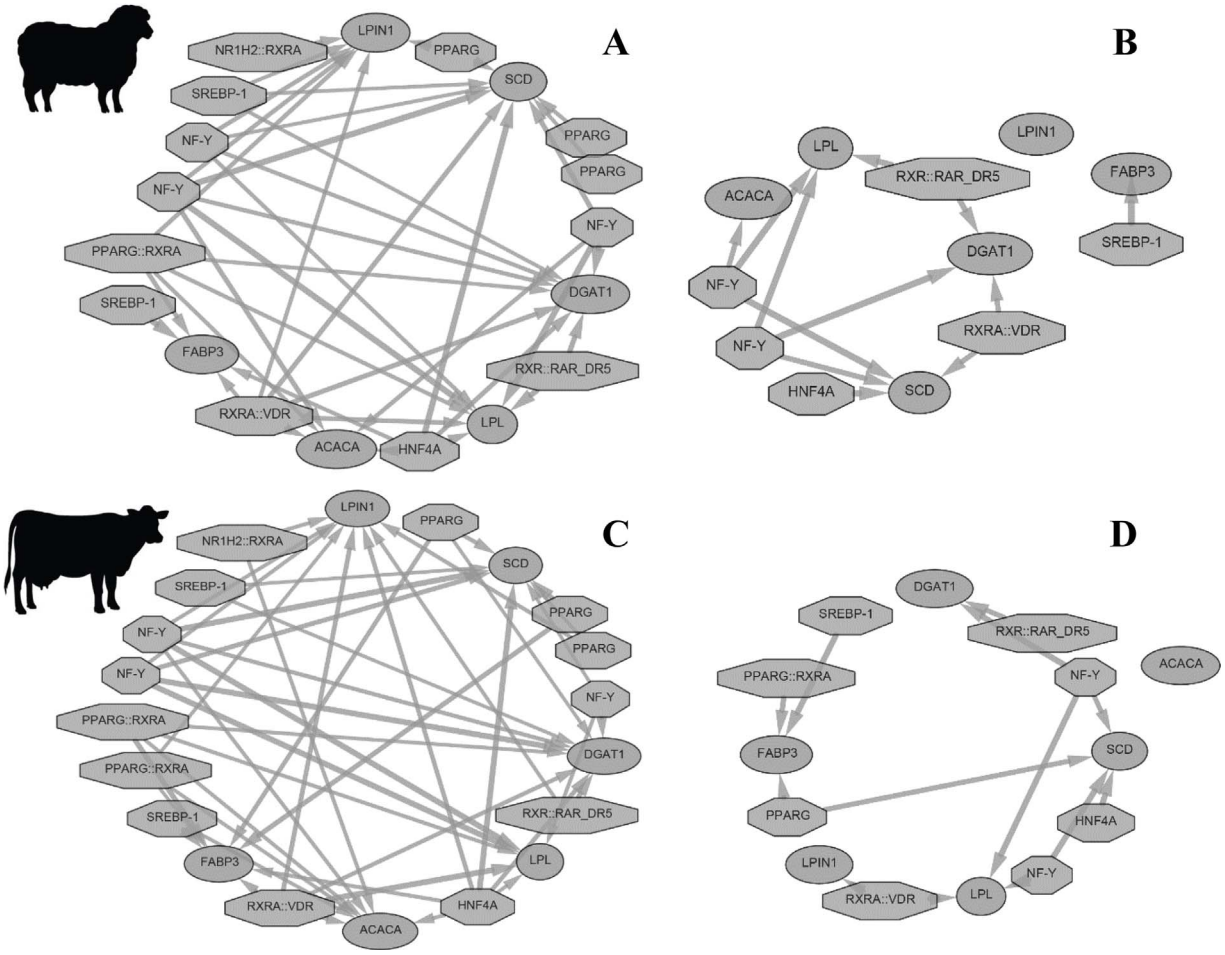

Fig. 3. In silico analysis of TFBS for PPARγ, SREBP1, HNF4α, LXRβ, RAR, VDR, and NF-Y in milk fat-related genes. Analysis of most significant ($P < 0.001$) TFBS for PPARγ (PPARG and PPARG:RXRA), SREBP1, HNF4α (HNF4A), LXRβ (NR1H2:RXRA), RAR (RXR:RAR_DR5), VDR (RXRA:VDR), and NF-Y in the promoter region between −950 to +50 nt relative to the TSS of ovine (A) and bovine (C) genes encoding proteins involved in milk fat synthesis (ovals). The TFBS for specific TF are reported as octagons. Arrow size thickness is proportional to the significance of the hit. The same analysis was performed with more stringent ($P < 0.0001$) parameters in ovine (B) and bovine (D). The *P* value indicates the probability of observing a score of the hit equal to or higher than the score by chance alone.

+50 nt (Fig. 3B and 3D). Additionally, the TBFS hits for HNF4, RAR, and VDR seem to be conserved across species and are present throughout the entire DNA sequence analyzed (i.e., −5000 to +50 nt from the TSS). Overall, these in silico analyses indicate major differences among ruminant species that could partially explain other physiological effects such as the lower susceptibility of small ruminants to MFD in comparison with bovine. As a consequence, future proposed nutrigenomics interventions might differ among cow, sheep, and goat.

7. Nutriepigenomics

7.1. General

In recent years it has been widely accepted that environmental factors such as diet and ambient conditions not only affect the short- and medium-term gene expression, but there is also a medium- to long-term regulation of genes. The latter is primarily carried out through changes in the availability of gene sequences to be transcribed into mRNA. This concept is referred to epigenetics, where “epi” is a Greek-derived term meaning “over,” i.e. epigenetics is commonly defined as “on-top-of genetics.” This means that there could be a set of inherited characteristics, phenotypes, and chemical entities that are superimposed on the DNA and do not follow basic Mendelian laws. Every individual will have a set of these features or epigenetic marks throughout the genome, and consequently, this is known as the epigenome.

The epigenome can be so unique that even individuals with the identical genetic code could have significantly different epigenomes as, for example, it could occur as a result of distinct nutritional history (Levesque et al., 2014). Within the context of ruminants, epigenomic changes could serve important physiological adaptations during the onset of lactation including increasing the availability of gene sequences (i.e., through decreased DNA methylation) for the transcription of essential milk proteins (i.e., caseins) in the mammary gland of dairy cows (Vanselow et al., 2006). Without a doubt, this relatively new spinoff of nutrigenomics (i.e., nutriepigenomics) will provide essential information to our understanding of how nutrients can affect the biology of ruminants at a molecular level. However, at the same time, nutriepigenomics will add another layer of complexity to our field, where such interactions have to be fully understood, and in time, manipulated through dietary interventions. Until now, the body of knowledge in ruminants on the specific roles of nutrients on main epigenetics effects that can result in alterations in the transcriptome such as DNA methylation, histone modifications, and noncoding RNA, remains limited.

7.2. Deoxyribonucleic acid methyltransferases

Among the epigenetic mechanisms mentioned above, DNA methylation is perhaps the one that has received the most attention in the research community in general (Jaenisch and Bird, 2003). Among all other nucleotides, cytosine is essential for DNA methylation, since it is

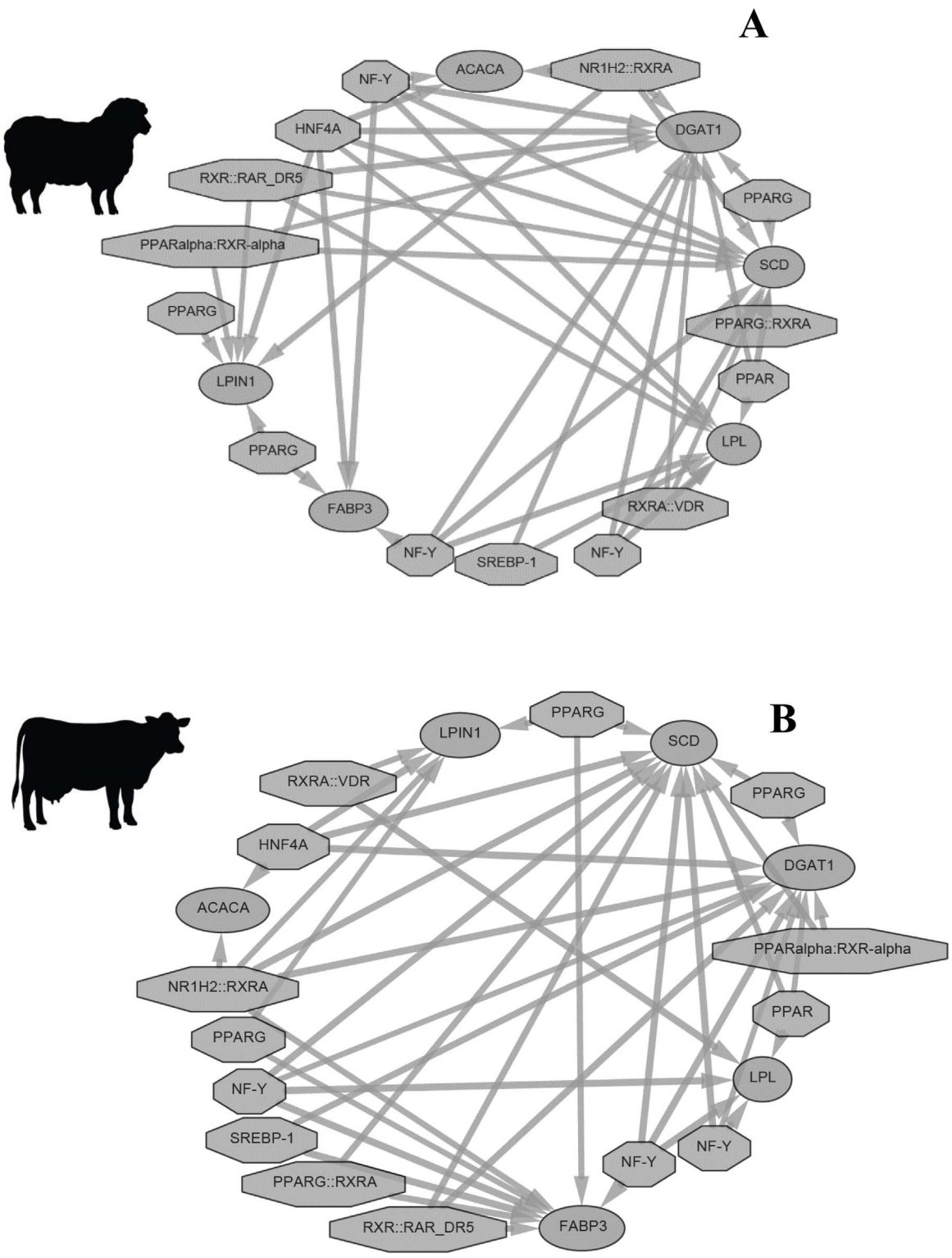

Fig. 4. In silico analysis of TBFS for PPARγ, SREBP1, HNF4α, LXRβ, RAR, VDR, and NF-Y in milk fat-related genes. Analysis of most significant ($P < 0.0001$) TFBS for PPARγ (PPARG and PPARG:RXRA), SREBP1, HNF4α (HNF4A), LXRβ (NR1H2:RXRA), RAR (RXR:RAR_DR5), VDR (RXRA:VDR), and NF-Y in the promoter region between −5000 to 0 nt relative to the TSS of ovine (A) and bovine (C) genes encoding proteins involved in milk fat synthesis (ovals). The TFBS for specific TF are reported as octagons. Arrow size thickness is proportional to the significance of the hit. The *P* value indicates the probability of observing a score of the hit equal to or higher than the score by chance alone.

the primary target of this mechanism, especially when it is adjacent to guanine. The dinucleotide cytosine-guanine in the DNA sequence is known as CpG and DNA regions with a high-frequency of this dinucleotide are often called "CpG islands." When a high degree of methylation (i.e., hypermethylation) occurs in the CpG islands across the promoter region of a gene, it decreases its level of expression; whereas, a low degree of methylation (i.e., hypomethylation) at CpG islands is indicative of an increase in gene expression.

Because of the importance of DNA methylation of the promoter region of genes, we conducted an exploratory analysis of genes related to milk fat synthesis in both ovine and bovine (Fig. 5). This analysis was performed using MethPrimer, a web-based software for designing PCR

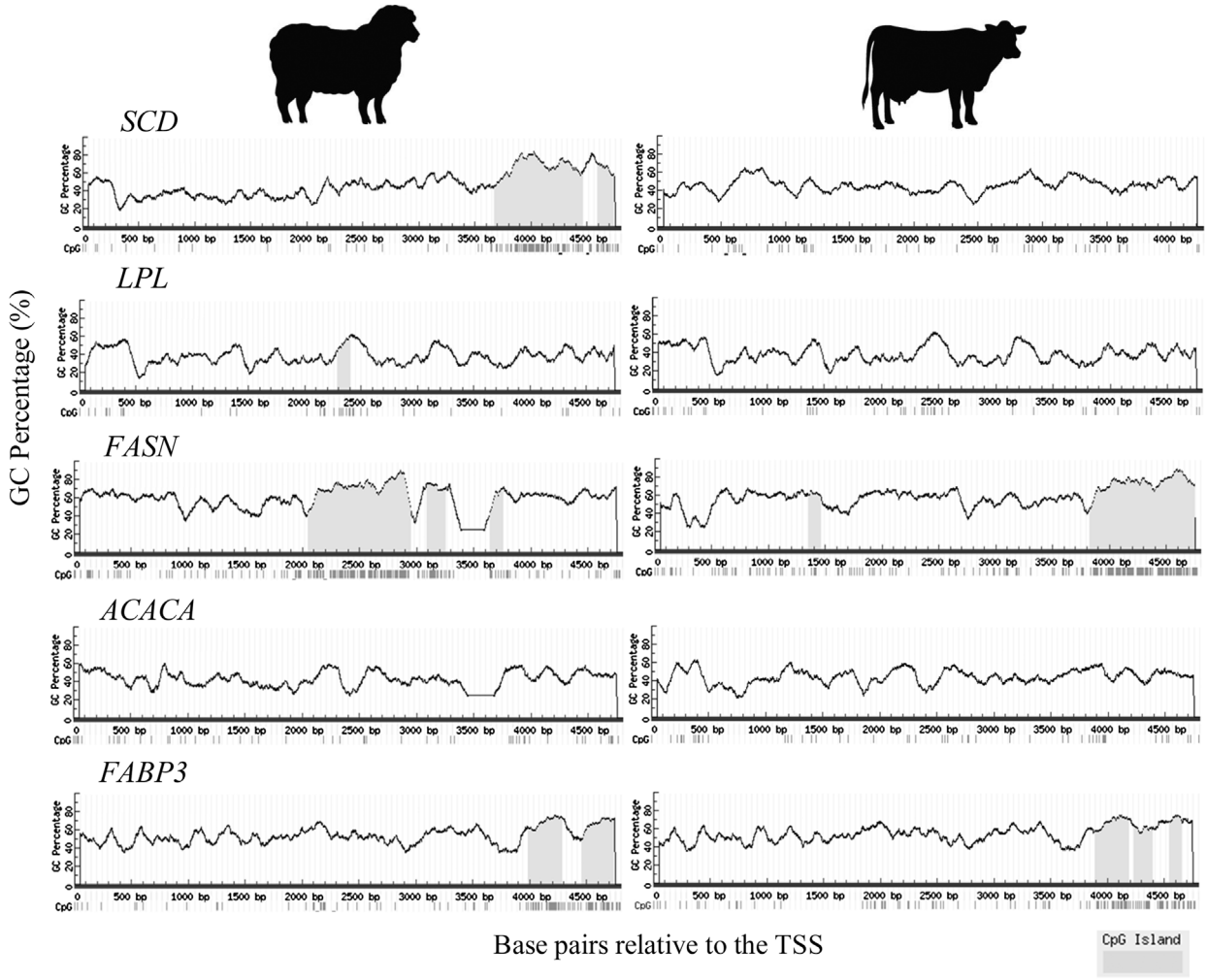

Fig. 5. Detection of CpG islands in the promoter region between −5000 to 0 bp (i.e., 0 and 5000, respectively in X-axis) relative to the TSS of ovine and bovine of genes encoding key proteins (*SCD*, *LPL*, *FASN*, *ACACA*, *FABP3*) involved in milk fat synthesis. This analysis was performed with the web-based software MethPrimer, a program for designing PCR primers for methylation mapping that also searches input DNA sequences to identify potential CpG islands (Li and Dahiya, 2002). MethPrimer output presents results as the DNA sequence is uploaded into the software; therefore, 0 and 5000 bp in the X-axis represent −5000 and 0 bp relative to TSS, respectively. Promoter region sequences were obtained from the UCSC Genome Browser (Speir et al., 2016). Light gray shading under the curve denotes a CpG island.

primers for methylation mapping that and also searches input DNA sequences for potential CpG islands (Li and Dahiya, 2002). The promoter regions between −5000 and 0 bp relative to the TSS of *SCD*, *LPL*, *FASN*, *ACACA*, and *FABP3* were evaluated. The DNA sequences for such promoter regions in each species were obtained from the UCSC Genome Browser (Speir et al., 2016). Interestingly, there were evident differences between ruminant species, primarily for *SCD* and *LPL*, where at least a CpG island was identified in ovine in contrast to the lack of CpG islands in bovine (Fig. 5). Such differences between species might have fundamental implications of how gene expression is regulated, for instance, in Fig. 4 the *SCD* gene had a TFBS for LXRβ and HNF4α for both ruminant species. However, the presence of CpG islands in the promoter region of *SCD* in ovine (Fig. 5) could render such TF (i.e., LXRβ and HNF4) unfit to induce gene expression, especially if the TBFS for such TF fall adjacent to the CpG island. A different pattern of CpG islands in the promoter region of *FASN* and *FABP3* between ovine and bovine was observed (Fig. 5), which could elicit major differences in gene expression depending on the location of TBFS for such genes. At least up to −5000 bp, for both species evaluated, the *ACACA* gene seems to lack CpG islands. This indicates that *ACACA* is less susceptible to epigenetic modifications, and such feature appears conserved across ruminant species.

The methylation of DNA is carried out by specialized proteins called DNA methyltransferases (DNMT), of which there are several isoforms: DNMT1b, DNMT2, and DNMT3b. These proteins are responsible for methylating cytosines and, consequently, create and maintain the methylated CpG patterns in the mammalian genome (Siedlecki and Zielenkiewicz, 2006). It has been observed that DNMT isoforms have specific tasks to create and maintain DNA methylation patterns. For instance, DNMT3a and DNMT3b are responsible for identifying unmethylated CpG regions and initiating de novo transfer of methyl groups; whereas, DNMT1 is in charge of methylation of remaining unmethylated cytosines within CpG regions (Hsieh, 2005; Siedlecki and Zielenkiewicz, 2006). To our knowledge, the specific manipulation of DNMT through diet has not been evaluated in small ruminants, and only few data for a role in ruminants has been generated from cow studies. For instance, Osorio et al. (2014) detected up-regulation of hepatic *DNMT3A* expression when cows were fed a rumen-protected methionine supplement during the last 3 weeks prior to parturition through the first 4 weeks postpartum. This suggested a greater availability of DNMT protein to initiate de novo DNA methylation.

Methionine metabolism serves as an essential methyl donor through the production of S-adenosylmethionine (SAM), that in turn is utilized by DNMT for DNA methylation, conferring this amino acid a potential role in nutriepigenomics. Osorio et al. (2016a) reported alterations in global DNA methylation and promoter region DNA methylation of *PPARA* when dairy cows were fed rumen-protected methionine (Osorio et al., 2016a). In sheep, although nutriepigenomics studies *per se* are

scarce or not available, there is a substantial amount of data on the effect of maternal methyl donor nutrition on the DNA methylation in the offspring (Sinclair et al., 2007; Lan et al., 2013). For instance, methionine and vitamin B deficient diets fed to pregnant ewes resulted in extensive alterations in DNA methylation in the offspring that were associated with an impaired health condition including insulin resistance, elevated blood pressure, and altered immune response among others (Sinclair et al., 2007).

In contrast to DNMT, the tet methylcytosine dioxygenase 1 (*TET1*; gene symbol) is a protein that catalyzes the initial step (Tahiliani et al., 2009) for demethylation of DNA cytosines (Liu et al., 2011; Maiti and Drohat, 2011). Clearly, this protein could have major epigenetic effects through its role in DNA demethylation; however, such implications in the context of ruminants remain to be verified. Furthermore, its manipulation through dietary interventions (nutriepigenomics) is unknown. A recent experiment evaluated the transcription of *TET1* in the mammary gland of ewes from pregnancy to lactation (Paten et al., 2014). The results from the study showed a down-regulation of *TET1* from pregnancy to lactation, which led authors to suggest that this protein exerts valuable epigenetic remodeling for maturation of the sheep mammary gland prior to lactation.

Although from a nutritional standpoint the currently-available data on epigenomics in small ruminants is scarce or inexistent, in this review we seek to provide fundamental information with the expectation that it will raise awarness of the potential effects of methyl donors such as methionine, which is an essential nutirent for rumimants. If nutrigenomics deals with nutrient-gene interactions with the ultimate goal of manipulating such interactions through fine-tunning dietary effects, nutriepigenomics investigates how dietary compounds can reveal or conceal genetic information to be utilized by the body. Therefore, it is only logical to envision an overlap between nutrigenomics and nutriepigenomics. Future gene manipulation through dietary effects will only be accomplished by a deep understanding of these disciplines.

7.3. MicroRNA

Since the early 21st century, among the various epigenetic mechanisms, microRNA (miRNA) a class of small noncoding RNA (18–25 nucleotides), have received the greatest notoriety. Such attention is well founded since miRNA play a major role in controlling post-transcriptional regulation by preventing translation of mRNA (Romao et al., 2011). One of the initial studies on miRNA in small ruminants was performed with the aim of identifying miRNA with key roles in hair growth (Wenguang et al., 2007). This study characterized the expression of 159 miRNA in body side skin and ear skin of goats and sheep using microarray analysis. There were 105 miRNA that were conserved between goat and sheep with significant roles in hair follicle differentiation. Subsequent high-throughput sequencing studies increased this list of miRNA in goat from 326 (Liu et al., 2012) to 399 miRNA (Yuan et al., 2013). Yuan et al. (2013) also observed that depending on the follicular cycling stage (i.e., anagen, catagen, and telogen) a reduced number (< 15) of miRNA were expressed at each stage, confirming their importance in hair follicle growth. Interestingly, using similar sequencing techniques, other researchers observed that miR-10b and miR-211 by affecting specific signaling pathways such as Notch and MAPK might play a major role in black and white follicle formation in goats (Wu et al., 2014a). There is no doubt that miRNA play an essential role in follicle formation, which is of great importance for the wool industry. Therefore, future research should focus on possible connections between dietary effects and the expression of miRNA in sheep.

While there are miRNA with ubiquitous expression, there are other exclusively or preferentially expressed miRNA in tissues such as muscle also known as myomiRs (McCarthy, 2008). As such, these miRNA play important roles in muscle development (Horak et al., 2016). A deep-sequencing study of muscle (i.e., *longissimus dorsi*) miRNA in sheep substantially extended the library of miRNA or miRNAome to 2914 miRNA (Zhang et al., 2013). A similar analysis identified and characterized 562 miRNA in the muscle of 6-moth-old Boer goats (Ling et al., 2013). The understanding of pathways and biological processes affected by miRNA is of great importance in muscle development. For instance, Miao et al. (2015) observed 157 differentially expressed miRNA between sheep breeds, i.e., the Dorset and small tail Han Chinese, where the former is known to have a greater growth rate. From those 157 differentially expressed miRNA, 16 were up-regulated and 141 down-regulated in the Dorset breed in comparison with the Han Chinese. Among the predicted target genes for those differentially expressed miRNA were the toll-like receptor 9 (*TLR9*), carbonic anhydrase 4 (*CA4*), and period circadian clock 1 (*PER1*) (Miao et al., 2015). These genes play key roles in biological processes such as pathogen recognition and inflammatory response (Boyd et al., 2006), lactic acid transport in skeletal muscle (Wetzel et al., 2001), and circadian clock metabolism (Harfmann et al., 2015), respectively, all of which play direct or indirect roles in muscle biology.

A comprehensive comparative profiling of caprine muscle miRNA was performed at two developmental stages, fetal and six-month of age (Wang et al., 2014). The results from this experiment identified > 500 miRNA of which 336 were differentially expressed between the two developmental stages, and suggested that miR-424-5p and miR-29a might have important roles in muscle development in goats. Additionally, *in vitro* data suggested a positive role of miR-101a in goat muscle development (Li et al., 2015a). More recently, the implications of specific miRNA such as miR-192 in muscle development have been reported in sheep, where this miRNA regulates the transcription of retinoblastoma (*RB1*), a known regulator of myogenesis (Zhao et al., 2016). Lie et al. (2015) observed that maternal undernutrition in periconceptional sheep, primarily during early embryogenesis, increased the transcription of miR-30a-5p in fetal skeletal muscle. In turn, this miRNA was negatively correlated with essential proteins related to muscle development such as mammalian target of rapamycin (mTOR) and myogenic factor 5 (MYF5). This study suggested a potential connection between dietary effects and the expression of specific miRNA in muscle.

In terms of milk production in small ruminants, the effects of miRNA in the mammary gland have been explored to a greater extent in goats than sheep. For instance, one of the first studies in goats evaluated the variability in the 3' untranslated region (UTR) of goat casein genes across 5 breeds (Zidi et al., 2010). The binding of miRNA to their target mRNA is done in the sequence motifs located in the 3¢ UTR of those target mRNA. The results showed that the 3' UTR of casein alpha s1 (*CSN1S1*), casein alpha s2 (*CSN1S2*), and casein beta (*CSN2*) genes are polymorphic, with a greater level of variation for *CSN1S2* across goat breeds. Those data suggested that the degree of polymorphism on the 3' UTR of casein genes could affect the ability of miRNA to target such genes, consequently altering the phenotype across goat breeds.

More recent studies have profiled the miRNAome in the goat mammary gland (Ji et al., 2012a,b; Li et al., 2012), resulting in a significant amount of information identifying and characterizing miRNA. Furthermore, the various miRNA profiles have been correlated with physiological changes during pregnancy, non-lactating, and lactating stages. For instance, Li et al. (2012) observed 169 miRNA differentially expressed between peak lactation and the dry period, where 165 were down-regulated, and 4 were up-regulated. This response suggested that a high number of miRNA decrease during peak lactation, and consequently it can be argued that miRNA exert a greater epigenetic control during the dry period. A similar analysis was done in sheep, where a mouse miRNA oligoarray was used to establish miRNA profiles of RNA extracted from mammary glands of sheep collected at different developmental stages: preconception, early, mid-, late pregnancy, and lactation (Galio et al., 2013). Results from this experiment showed that from a pool of 137 miRNA differentially expressed, the majority of (78 or 57%) decreased in expression as sheep progressed from preconception to lactation. Such results are, to some extent, in agreement with

those observed by Li et al. (2012) in goats.

After the identification and characterization of miRNA at a broad spectrum (i.e., high-throughput sequencing), specific miRNA have emerged as potential modifiers of critical biological processes for milk production in small ruminants. For instance, to date, fatty acid metabolism is by far the biological process with the most functional miRNA identified in the mammary gland. Other biologic processes such as prolactin sensitivity seem to be affected by miR-135a (Ji et al., 2015). Interestingly, while Li et al. (2012) observed via high-throughput sequencing that the majority of miRNA expressed in mammary gland decreased from pregnancy to lactation, to date, the majority of functionally-characterized miRNA related to fatty acid metabolism exhibit an increase in expression during lactation. For instance, the transcription of miR-27a (Lin et al., 2013c), miR-103 (Lin et al., 2013a), miR-24 (Wang et al., 2015), miR-26a/b (Wang et al., 2016a), and miR-145 (Wang et al., 2016b) increased between pregnancy to lactation, while miR-130b (Chen et al., 2015) and miR-181b (Chen et al., 2016) decreased.

Except for miR-27a (Lin et al., 2013c), the data seem to indicate that alterations in the expression of the various miRNA during lactation can elicit a regulatory effect that activates or inhibits fatty acid metabolism in general. The function of miR-27a is puzzling because, despite its increase during lactation, it also inhibits lipogenesis and triacylglycerol synthesis presumably via targeting *PPARG* or other TF (Lin et al., 2013c). Another possible explanation is that miRNA such as miR-27a do not work alone but interact with other miRNA (Lin et al., 2013b). In fact, Lin et al. (2013b) observed a high positive correlation ($P < 0.001, r > 0.57$) at the transcriptomic level between a miR-27b (i.e., an isoform of miR-27) and miR-23a and miR-200a, and contrary to miR-27b, miR-200a and miR-23a have positive effects on lipid metabolism. Future research needs to be done to explore such relationships among miRNA not only in the context of fatty acid metabolism but also on the potential for nutrition to alter these relationships.

One of the first experiments evaluating the miRNAome response in the goat mammary gland to a nutritional effect was published recently (Mobuchon et al., 2015). This experiment found 30 differentially expressed miRNA after goats were food deprived for 48-h, 16 were down-regulated, and 14 were up-regulated. Utilizing bioinformatic analyses including Ingenuity Pathway Analysis®, Mobuchon et al. (2015) analyzed the molecular and cellular functions affected by the target genes of the differentially expressed miRNA. Such analysis revealed that gene expression, cellular development, and cellular growth and proliferation were among the most affected pathways. These findings indicated that food deprivation could lead to transcriptomic alterations via the activation of miRNA.

Overall, data on miRNA in small ruminant continues to accumulate, underscoring the importance of these small noncoding RNA in key physiological processes. Moving forward, we believe that pioneering research evaluating high-throughput miRNA transcriptional changes to dietary effects will fill important gaps in knowledge on how miRNA can be manipulated through diet. A plausible route for dietary effects or compounds to exert their alterations in the miRNome is through the activation of TF, that in turn could alter the transcription of specific miRNA (Ruffalo and Bar-Joseph, 2016). Such link will emphasize the above menitoned interaction and overlap between nutrigenomics and nutriepigenomics, which will likely yield a more functional approach for dietary interventions to improve performance in small ruminants.

8. Conclusions

Omics and bioinformatics tools are poised to accelerate our understanding of the multiple levels of regulation induced in small ruminants by dietary nutrients during their utilization for milk, meat, wool, or reproduction. Initial data indicate that the nutrigenomics approach may eventually lead to more precise management of goats and sheep, hence, helping improve utilization of feed resources in a more optimal fashion. For instance, transcriptomics underscored the negative effect of undernutrition on genes regulating milk component synthesis and mammary cell proliferation while activating apoptosis and involution. This highlights the role of proper feed allocation or nutritional management to ensure optimal mammary gland function. Progress in understanding the control of tissue-specific transcription regulators in goats has provided new avenues for manipulating milk fat synthesis in vivo through the use of specific types of long-chain fatty acids. Similarly, the use of dietary lipid during the transition period in dairy goats could be beneficial for activating transcriptional programs in the liver of the animal as way to optimize a smooth transition into lactation, i.e. lower the risk of metabolic disorders and improve welfare. Application of proteomics, for example, has already allowed the discovery of a number of disease-related biomarkers. Moving forward, we believe that focus on the role of “nutritional programming” (epigenetics) of the offspring will yield practical ways of rearing small ruminants without compromising the ability of the animal to express its full biologic potential.

Conflict of interest

The authors declare no conflict of interest exist.

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