Liver transcriptomic and proteomic profiles of preweaning lambs are modified by milk replacer restriction

A. Santos, F. J. Giráldez, J. Frutos, and S. Andrés*

Instituto de Ganadería de Montaña, CSIC-Universidad de León, Finca Marzanas, E-24346 Grulleros, León, Spain

ABSTRACT

Prenatal and early postnatal nutrition may promote long-term effects on both feed efficiency and health of animals. Therefore, moderated milk replacer restriction during the artificial rearing period might negatively affect feed efficiency during the postweaning phases in dairy ewes. The aim of this work was to identify differentially expressed (DE) genes by RNA sequencing and differentially accumulated proteins using MALDI-TOF mass spectrometry in the liver of artificially reared Assaf lambs to identify modified metabolic pathways as a consequence of milk replacer restriction in order to find possible solutions to correct any detrimental effect caused by this factor. Forty female Assaf lambs were used in this experiment. The animals were penned individually and assigned randomly to 1 of 2 treatments groups ($n = 20$ per treatment). The first group of lambs was fed *ad libitum* (AL), whereas the second (restricted, RES) only received approximately 62.5% of the level of intake measured in the AL group. Eight 35-d-old lambs from each group (16 lambs in total) were harvested and a piece of liver of 8 lambs (4 AL and 4 RES lambs) was excised for transcriptomic and proteomic analysis. Differential gene expression analysis identified 386 DE genes [198 of them being annotated genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway], with 176 downregulated and 210 upregulated in the early feed-restricted group relative to the AL group. A total of 26 spots were also differentially accumulated proteins in the liver of the 2 groups of lambs, 10 of them being down-accumulated whereas the other 16 evolved in the opposite way in the liver of RES relative to AL lambs. Collectively, both the transcriptomic and proteomic approaches revealed an upregulation of genes participating in oxidation of fatty acids in the early feed-restricted lambs. The expression of many genes involved in the degradation of several AA (e.g. alanine, valine, isoleucine, tyrosine) was also modified

in the RES lambs, probably to render substrates for gluconeogenesis. Moreover, other genes involved in apoptosis, inflammation, or hepatic oxidative pathways were upregulated in these lambs. Finally, the expression of genes implicated in oxidative phosphorylation was modified in RES animals. Altogether all these modifications suggest that the partitioning and utilization of nutrients in the early feed-restricted lambs might have been modified, being partially responsible for changes during the replacement phase later in life.

Key words: feed efficiency, feed restriction, developmental origins of health and disease, metabolic programming

INTRODUCTION

Early feed restriction (pre- and perinatal life) modifies gene expression in several organs and tissues of the offspring, affecting not only the digestive and metabolic utilization of nutrients in different biological functions (Lillicrop et al., 2007; Altmann et al., 2012; Lan et al., 2013; Geraghty et al., 2016; Khanal and Nielsen, 2017) but also promoting metabolic disorders later in life due to nutritional programming events, as described previously in humans (González-Barranco and Ríos-Torres, 2004; Roseboom et al., 2011; Ruchat et al., 2014) and rats (Kang et al., 2011; Ellis et al., 2014). Regarding lambs, several studies have shown that metabolic programming events are caused by early feed restriction (Davies and Owen, 1967; Greenwood et al., 2004; Galvani et al., 2014), with negative long-term effects after the period of feed restriction. In this sense, previous studies have demonstrated negative consequences not only on the health status (Frutos et al., 2018) and feed efficiency traits (Santos et al., 2018c,d), but also on the onset of puberty in replacement ewes lambs (Santos et al., 2018c). However, even though we have found several studies in children (Ruchat et al., 2014), cattle (Soberon et al., 2012; Soberon and Van Amburgh, 2013; Alexandre et al., 2015), and pigs (Vincent et al., 2015), to our knowledge no studies have described the effects of early feed restriction at the gene expression level in artificially reared female Assaf lambs. There-

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*Corresponding author: sonia.andres@eae.csic.es

fore, progress in this field may help us understand the mechanisms underlying nutritional programming and the adverse effects promoted by early undernutrition in replacement ewe lambs. Implementing early corrective strategies is crucial, as early feed restriction (which may happen in the farm as a consequence of environmental, health, or management conditions) might negatively affect future profitability of a dairy sheep farm. The present study aimed to identify both differentially expressed (**DE**) genes and differentially accumulated proteins (proteomics) in the liver of early feed-restricted lambs in order to understand both the mechanisms responsible for the alterations in the partitioning and utilization of nutrients of early feed-restricted lambs and those causing detrimental effects on reproductive and feed efficiency later in life in dairy sheep farms.

MATERIAL AND METHODS

Care and Use of Animals

All the recommendations of the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and the IGM-CSIC Animal Experimentation Committee (protocol number 2015-04) were carefully followed in all the handling practices involving animals.

Animal Diets and Sampling

Forty female Assaf lambs, individually penned during the artificial rearing period, were used in this experiment. The lambs were stratified based on live BW at birth (average = 4.73 ± 0.116 kg) and randomly allocated to 1 of 2 experimental treatments ($n = 20$ per dietary treatment) during the artificial rearing period. All the lambs were fed milk replacer (**MR**) 3 times daily (0830, 1400, and 2030 h) during the first week, and only twice daily (0830 and 1930 h) during the rest of the suckling period. However, whereas the first group of lambs was fed MR ad libitum (**AL**; expected intake of 1.5 MJ of gross energy/kg of metabolic BW, approximately 2.5 times maintenance energy requirements to gain a maximum of 250 g/d), the second group (restricted; **RES**) only received approximately 62.5% of MR intake as the AL group (approximately 1.5 times maintenance energy requirements to gain 100–120 g/d; NRC, 1985; Giráldez et al., 1999). The level of restriction was set according to previous studies carried out with milk-fed Assaf lambs (Manso et al., 1998; Castañares, 2008; Rodríguez et al., 2008). Ingredients and chemical composition of the MR administered are summarized in Table 1.

All the lambs were weighed twice a week throughout the artificial rearing period until they were 35 d old, when 8 representative animals from each group (16 lambs in total) were euthanized. Several pieces of liver were excised immediately, stabilized with RNAlater (Ambion Inc., Austin, TX) overnight at 4°C, and then stored at -80°C in order to study the liver transcriptomic and proteomic profiles. For this purpose, 4 liver samples from each group (8 lambs in total) were selected to cover the complete range of values of live BW at slaughter in each group and to avoid differences in birth weight between groups. Accumulated MR intake and animal performance of these preweaning lambs selected for omics (8 preweaning lambs in total) are summarized in Table 2 and Figure 1, whereas the rest of parameters measured at slaughter (16 preweaning lambs in total) are described in Santos et al. (2018c). The nonslaughtered animals (24 lambs in total) were weaned and fed ad libitum a TMR to study the effects of metabolic programming provoked by early feed restriction during the replacement phase (Frutos et al., 2018; Santos et al., 2018c). In the present study, only those data regarding liver transcriptomic and proteomic profiles of preweaning lamb (those slaughtered) are presented.

Transcriptomics Analysis of Liver Samples Using RNA Sequencing

A comparative liver transcriptome analysis was carried out using next-generation sequencing (RNA sequencing) as described in Santos et al. (2018c). Briefly, RNA extraction was performed in 4 liver samples per

Table 1. Ingredients and chemical composition (g/kg of DM, unless otherwise stated) of the commercial lamb milk replacer

Item	Value
Ingredient	
Skim milk powder	500
Milk whey powder	160
Vegetable oil (cocoa)	140
Animal fat (pork land)	120
Whey protein powder	40
Hydrolyzed wheat protein	15
Yeast/sodium bicarbonate/dextrose	15
PreVIT mineral ¹	10
Chemical composition	
DM, g/kg	960
CP	235
Ether extract	260
Ash	70
Metabolizable energy, kcal/kg of DM	5,089

¹Provided per kg of milk replacer: vitamin A, 80,000 IU; vitamin D₃, 4,250 IU; iron, 40 mg; cobalt, 0.2 mg; copper, 5 mg; manganese, 25 mg; zinc, 30 mg; selenium, 0.2 mg [Leches Maternizadas S.A. (LEMASA), León, Spain].

Figure 1. Accumulated live body weight (LBW) gain (kg) during the suckling period of lambs that were fed milk replacer (MR) ad libitum (AL) or restricted (RES).

group (8 samples in total), all of them presenting values for RNA integrity number larger than 8 (8.5 ± 0.04). The RNA sequencing (transcriptome sequencing) was then carried out on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA). The sequence and annotation of the most recent ovine genome assembly was obtained from the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>; accession number GCF_000298735.2, assembly Oar_v4.0).

A differential expression analysis was completed with the Bioconductor package DESeq2 (version 1.12.4; Love et al., 2014), whereas statistical analyses of differentially expressed genes were carried out using R (version 3.3.1; R Core Team, 2016). The data were deposited in the Gene Expression Omnibus database (GEO; accession number GSE110192; <https://www.ncbi.nlm.nih.gov/geo/>). Genes were considered to be differentially expressed if their *P*-value, adjusted for multiple testing using the Benjamini and Hochberg method (false

discovery rate), was less than 0.05 (Benjamini and Hochberg, 1995). The DE gene list was interpreted through enrichment analysis [Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8; <https://david.ncifcrf.gov/>) and kegg function (Kyoto Encyclopedia of Genes and Genomes, KEGG) of the edgeR package (Robinson et al., 2010)].

Proteomic Analysis of Liver Samples

The procedure applied to study the liver proteome has been published previously in Santos et al. (2018d). Briefly, liver samples (4 animals per group, 8 samples in total) were homogenized, quantified for protein concentration with the Bradford assay (Bio-Rad Protein Assay, BioRad Laboratories, Hercules, CA), and stored at -80°C or applied directly to 18-cm immobilized pH gradient (IPG) strips (nonlinear gradient) before proteome resolution. Proteins were resolved in the first and second dimensions and then high-sensitivity colloidal Coomassie Blue G-250 Staining (blue silver, Sigma-Aldrich, Steinheim, Germany) of all the two-dimensional gel electrophoresis was used for protein visualization (2 gels per sample, 8 gels per condition). Relative volume was calculated according to the volume of each spot divided by the total volume of all the spots in the gel. Differentially expressed proteins were considered when the ratio of the relative volume average between conditions was higher than 1.5 (fold change) and the *P*-value determined by Student's *t*-test was <0.05 . These proteins were selected for identification (4800 MALDI TOF/TOF Analyzer, Applied Biosystems, Foster City, CA). Names of the proteins differentially accumulated in the liver were assigned according to GCF_000298735.2_Oar_v4.0. Identification of a certain protein was considered correct if the score was higher than 93 (protein score >93 ; $P < 0.05$).

Table 2. Effect of milk replacer restriction on animal performance of preweaning lambs [4 ad libitum (AL) vs. 4 restricted (RES) lambs]

Item	AL	RES	RSD ¹	<i>P</i> -value
Birth weight, kg	3.99	4.12	0.192	0.409
Suckling period, ² d	35.0	34.3	1.50	0.494
Accumulated milk replacer intake, kg	41.8	25.1	4.77	0.002
ADG, ³ g/d	199	99.8	14.59	<0.001
Slaughter weight, kg	11.6	7.90	0.739	<0.001
Hot carcass weight, kg	5.65	3.57	0.407	<0.001
Cold carcass weight, kg	5.44	3.40	0.396	<0.001
Omental fat, g	101	19.4	11.57	<0.001
Mesenteric fat, g	102	53.3	14.11	0.003
Perirenal fat, g	104	34.6	19.99	0.002

¹Residual standard deviation.

²Artificial rearing phase, from birth to slaughter.

³ADG during the suckling period.

RESULTS AND DISCUSSION

Several studies have demonstrated that postnatal feed restriction of lambs may induce early nutritional programming, thus affecting the metabolism, growth, development, feed efficiency, and health throughout life (Davies and Owen, 1967; Greenwood et al., 2004; Galvani et al., 2014; Santos et al., 2018a,b,c,d). However, in-depth understanding of the early physiological processes promoted by this postnatal feed restriction on preweaning lambs is lacking. We present a complete differential transcriptome and proteome analysis of liver samples corresponding to artificially reared Assaf lambs fed *MR ad libitum* or restricted during this phase of life.

Differential gene expression analysis in the hepatic tissue of suckling Assaf lambs identified 386 DE genes, 198 of them being annotated genes in the KEGG pathway. Positive values of $\log_2$ -fold change showed that 210 genes were upregulated in the RES relative to the AL group, whereas negative $\log_2$ -fold change values denoted the downregulation of 176 genes (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15110>). The DAVID software and KEGG functional enrichment analysis identified functional gene clusters and pathways modified by milk replacer restriction, mainly related to the cytochrome P450, steroid hormone biosynthesis, AA metabolism, glycolysis/gluconeogenesis, and drug and xenobiotic metabolism. The most important terms and pathways are summarized in Tables 3 and 4.

The proteomic profile of the liver identified 26 differentially accumulated ($P < 0.05$) spots/proteins (Figure 2 and Table 5), 10 of them being down-accumulated whereas the other 16 evolved in the opposite way in the liver of RES lambs.

Carbohydrate Metabolic Pathways

After birth, animals have to be adapted to a new situation and emerging new metabolic pathways (Baldwin et al., 2004; Davis and Fiorotto, 2005; van den Borne et al., 2007). In this context, nutrient supply could modulate these adaptive changes, meaning suboptimal nutrition in the early life could promote long-term effects on health and feed efficiency with important economic implications during the postweaning phases (Santos et al., 2018a,b,c,d). For example, during the suckling period, gluconeogenesis is increased to maintain normoglycemia (Ferré et al., 1986; Girard, 1990) to the point that this metabolic route provides ~75% of the total glucose needs of neonatal ruminants (Nafikov and Beitz, 2007). However, in the present study, RES lambs were feed restricted, so a higher gluconeogenesis rate was expected in these animals to cover the glucose

requirements of the tissues. Accordingly, in the present study, the expression of some genes related to the glycolysis and gluconeogenesis (e.g., *GAPDH*, *PFKL*, *GPI*, and *ADH1C*) were upregulated, whereas that encoding glycogen synthase (e.g., *GYS2*, which is one of the main enzymes involved in the synthesis of glycogen) was downregulated in the RES lambs. These functional changes were corroborated by the proteomic profile of artificially reared lambs, where several proteins involved in glycolytic/gluconeogenic pathways (e.g., fructose-bisphosphate aldolase B and glyceraldehyde-3-phosphate DH) were differentially expressed in RES lambs (Table 5).

Likewise, it is interesting to note that *PDHA1* [pyruvate dehydrogenase (lipoamide) alpha 1], which catalyzes the irreversible conversion of pyruvate into acetyl-CoA (Fu et al., 2017), was down-expressed in the RES lambs. This fact might have directed at least part of the intermediates of glycolysis toward biosynthetic pathways (e.g., pentose phosphate pathway) instead of tricarboxylic acid cycle (TAC; Salway, 2004). Accordingly, the expression of genes involved in TAC (e.g., *IDH2* and *OGDH*), oxidative phosphorylation (e.g., *ATP6V0E2* and *LOC101119721*), and energy metabolism (e.g., *CKMT2*) was affected in RES lambs, whereas the upregulation of cytoplasmic aconitate hydratase (an enzyme involved in TAC) might be explained by its moonlighting functions, as it is involved in iron homeostasis when cellular iron levels are low (such as occurs under feed restriction). All these changes are in accordance with the results obtained by Akbar et al. (2013) in feed-restricted dairy cows, who observed an increased expression of genes associated with gluconeogenesis and the inhibition of mitochondrial oxidative phosphorylation during mid-lactation.

Conversely, as mentioned previously, the pentose phosphate pathway seemed to be activated in the RES lambs (e.g., upregulation of *PRPS2*), thus producing NADPH to be used in the biosynthetic pathways prevalent in the growing lambs (Wood, 1985). Together with the increased expression of genes related to nucleotides (e.g., *XDH*, *LOC101109035*, *NT5E*, *AK7*, *LOC101106419*, *CMAS*, and *PRPS2*), components of the cell membranes such as glycerophospholipids (e.g., *PEMT*; Gimenez et al., 2011), nucleoporins involved in RNA transport (e.g., *NUP210*, *NUP62*, and *NDC1*), ribosome biogenesis (e.g., *POP7*, *LOC101102787*, *LOC101104725*, and *RPL22L1*), and the production of vitamins and cofactors (e.g., *TKFC*, *PNPO*, *NNT*, *BTD*, *SPR*, *LOC101103439*, *LOC101104703*, *DHRS3*, *LOC101119050*, and *BCO1*), these modifications seem to be indicative of an attempt of the growing organism for maintaining natural physiological functions for

Table 3. Annotation clusters identified by the functional annotation tool of DAVID (<https://david.ncifcrf.gov/>) from the list of 386 differentially expressed genes [DESeq2; Love et al., 2014 (P . BH < 0.05¹)] in the livers of preweaning lambs fed milk replacer ad libitum (AL) or restricted (RES)

Term	No. ²	P-value
Annotation cluster 1 (enrichment score: 11.666)		
Steroid hormone biosynthesis	22	9.62E-18
Retinol metabolism	21	3.52E-17
Drug metabolism - cytochrome P450	19	1.86E-15
Metabolism of xenobiotics by cytochrome P450	19	1.61E-14
Ascorbate and aldarate metabolism	12	2.84E-12
UDP-glucuronosyl/UDP-glucosyltransferase	10	4.36E-12
Pentose and glucuronate interconversions	13	7.88E-12
Glucuronosyltransferase activity	10	4.99E-11
Drug metabolism - other enzymes	13	1.48E-10
Porphyrin and chlorophyll metabolism	13	1.48E-10
Metabolic process	14	1.76E-07
Glycosyltransferase	13	6.55E-07
Annotation cluster 2 (enrichment score: 6.521)		
Biosynthesis of amino acids	12	1.62E-06
Carbon metabolism	14	4.82E-06
Annotation cluster 3 (enrichment score: 3.605)		
NADP-dependent oxidoreductase domain	7	1.28E-06
Aldo/keto reductase subgroup	6	5.18E-06
Aldo/keto reductase	6	2.08E-05
Aldo/keto reductase, conserved site	5	9.49E-05
Aldo-keto reductase	6	1.18E-04
Oxidoreductase activity	10	1.78E-04
Ovarian steroidogenesis	4	1.53E-01
Arachidonic acid metabolism	4	3.42E-01
Annotation cluster 4 (enrichment score: 2.126)		
Iron	10	4.77E-04
Iron ion binding	10	1.75E-03
Heme binding	9	2.58E-03
Heme	7	3.44E-03
Cytochrome P450	5	1.61E-02
Monooxygenase	4	6.61E-02
Cytochrome P450, conserved site	3	1.67E-01
Annotation cluster 5 (enrichment score: 1.918)		
Pyridoxal phosphate-dependent transferase, major region, subdomain 2	5	6.63E-3
Pyridoxal phosphate-dependent transferase, major region, subdomain 1	5	9.45E-3
Pyridoxal phosphate-dependent transferase	5	9.45E-3
Pyridoxal phosphate binding	5	1.92E-2
Pyridoxal phosphate	4	2.25E-2
Annotation cluster 6 (enrichment score: 1.829)		
CO dehydrogenase flavoprotein-like, FAD-binding, subdomain 2	3	1.02E-2
Oxidoreductase activity, acting on CH-OH group of donors	3	1.28E-2
FAD-binding, type 2	3	1.29E-2
Flavin adenine dinucleotide binding	5	2.88E-2
Annotation cluster 7 (enrichment score: 1.679)		
Carbohydrate kinase, FGGY, C-terminal	3	5.60E-3
Carbohydrate kinase, FGGY, N-terminal	3	7.74E-3
Keto-sugar kinase	3	1.40E-2
Carbohydrate metabolic process	4	3.17E-1

¹ P . BH < 0.05 = P -value, adjusted for multiple testing using the Benjamini and Hochberg method (false discovery rate), was less than 0.05 (Benjamini and Hochberg, 1995).

²Number of genes involved in each term.

growth, such as cell proliferation, differentiation, and gene expression under feed restriction conditions.

Protein Metabolic Pathways

Keogh et al. (2016) demonstrated an increment of the expression of genes related to ribosomal functions (*RPL22*, *RPL23*, *RPL26*, *RP6S*, or *POP1*) that might

point towards enhanced protein biosynthesis in restricted-fed animals. This seems to be in agreement with the overaccumulation of glycine amidinotransferase (an intermediate of creatine) in the liver proteome of RES lambs as an attempt to increase muscle development under feed restriction conditions.

However, under feed restriction, protein accretion might be reduced by both a lack of nutrients and an

Table 4. Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) terms from the list of 386 differentially expressed genes [DESeq2 (Love et al., 2014; P BH < 0.05¹)] in the livers of preweaning lambs fed milk replacer ad libitum (AL) or restricted (RES)

Enriched KEGG pathways	No. ²	P -value
Metabolic pathways	97	<0.001
Steroid hormone biosynthesis	24	<0.001
Retinol metabolism	22	<0.001
Drug metabolism, cytochrome P450	19	<0.001
Metabolism of xenobiotics by cytochrome P450	19	<0.001
Ascorbate and aldarate metabolism	12	<0.001
Pentose and glucuronate interconversions	13	<0.001
Porphyry and chlorophyll metabolism	13	<0.001
Drug metabolism, other enzymes	13	<0.001
Biosynthesis of AA	12	<0.001
Carbon metabolism	14	<0.001
Glutathione metabolism	8	<0.001
Glycine, serine and threonine metabolism	7	<0.001
Cysteine and methionine metabolism	7	<0.001
Arginine and proline metabolism	7	<0.001
Biosynthesis of UFA	5	<0.001
Fatty acid elongation	5	<0.001
Vitamin B ₆ metabolism	3	<0.001
2-Oxocarboxylic acid metabolism	4	<0.001
Fatty acid metabolism	6	0.001
Arginine biosynthesis	4	0.002
Alanine, aspartate, and glutamate metabolism	5	0.003
Tyrosine metabolism	5	0.003
Sulfur metabolism	3	0.003
Valine, leucine, and isoleucine biosynthesis	2	0.003
Peroxisome	7	0.004
Glycolysis/gluconeogenesis	6	0.005
Tryptophan metabolism	5	0.007
Fructose and mannose metabolism	4	0.008
Nitrogen metabolism	3	0.009
PPAR signaling pathway	6	0.009
Selenocompound metabolism	3	0.013
Ovarian steroidogenesis	5	0.017
Fatty acid degradation	4	0.020
Glycosaminoglycan biosynthesis, heparan sulfate/heparin	3	0.022
Phenylalanine metabolism	3	0.024
Arachidonic acid metabolism	6	0.027
Amino sugar and nucleotide sugar metabolism	4	0.034
Nicotinate and nicotinamide metabolism	3	0.035
Pentose phosphate pathway	3	0.038
Valine, leucine and isoleucine degradation	4	0.039
Starch and sucrose metabolism	3	0.045
Citrate cycle (tricarboxylic acid cycle)	3	0.052
Biotin metabolism	1	0.071
Purine metabolism	8	0.072
Lysine degradation	4	0.078
Cell cycle	6	0.094

¹ P BH < 0.05 = P -value, adjusted for multiple testing using the Benjamini and Hochberg method (false discovery rate), was less than 0.05 (Benjamini and Hochberg, 1995).

²Number of differentially expressed genes involved in the KEGG term.

increased catabolism of some AA to produce gluconeogenic precursors (James, 1972; Goodman et al., 1980; Dhahbi et al., 2001; Velez and Donkin, 2005). Accordingly, in the present study, the expression of genes involved in the catabolism of several AA (e.g., Ala, Cys, Met, Val, Leu, Ile, Tyr, and so on) was upregulated in the RES lambs. Nevertheless, those

involved in lysine degradation (e.g., *PLOD2*, *AASS*) were downregulated. Moreover, the down-expression of genes related to protein processing in endoplasmic reticulum (e.g., *TXNDC12*) might have increased terminally misfolded proteins directed toward degradation through the proteasome (Galligan and Petersen, 2012; Lee and Lee, 2017), which also presented genes modified by feed restriction (e.g., *PSMB9* and *PSMD6*). Consequently, the accumulation of misfolded proteins in the endoplasmic reticulum might have increased protein catabolism, thus causing stress and, hence, the apoptosis of cells (e.g., *PERP* was upregulated in RES lambs), as further explained below. All these results at transcriptome level match those observed in the liver proteome profile, where peptidyl-prolyl *cis-trans* isomerase A-like isoform X2 (involved in both protein folding and targeting for proteasomal degradation) and protein-disulfide isomerase (involved in protein folding) were up- and downregulated, respectively, in the liver of RES lambs. Moreover, several enzymes involved in AA degradation (e.g., 3-hydroxyisobutyryl-CoA hydrolase, mitochondrial isoform X1, aminomethyltransferase, mitochondrial isoform X2) and protein catabolism (e.g., selenium-binding protein 1 isoform X1) were upregulated, whereas 2-iminobutanoate/2-iminopropanoate deaminase (involved in the isoleucine biosynthesis) was downregulated in the liver of RES lambs. It must be stressed that the expression of genes encoding enzymes involved in urea cycle (e.g., *CPS1*, *OAT*, and *ARG1*) was downregulated in the RES lambs, probably as a compensatory mechanism to favor nitrogen retention (James, 1972; Velez and Donkin, 2005). Therefore, the RES lambs might have increased the degradation of some AA to produce gluconeogenic precursors, but attempted to reduce time nitrogen losses at the same to maintain physiological normal growth under feed restriction conditions.

Lipid Metabolic Pathways

Feed restriction decreased the expression of genes related to the differentiation of adipocytes (e.g., *PLIN2*), synthesis of glycerolipids (e.g., *MOGAT1*), and long-chain fatty acids (e.g., *ELOVL4*, *ELOVL7*). This is in agreement with the down-expression of thiosulfate sulfurtransferase in the liver proteome of RES lambs, which is expressed selectively in adipocytes (Morton et al., 2016). Moreover, the expression of genes involved in the oxidation of a variety of organic substrates (e.g., *SORD*, *ADH1C*) and β -oxidation of fatty acids (e.g., *ECI1* and *ECHS1*) was increased in RES lambs. All these modifications at the transcriptome level might be related to functional changes [3-ketoacyl-CoA thiolase, peroxisomal isoform X2 (involved in β -oxidation

Figure 2. Representative colloidal Coomassie Blue (Sigma-Aldrich, Steinheim, Germany) stained 2-dimensional electrophoresis gel of a liver from early milk restricted lambs. A total of 450 μ g of protein was loaded, and 2-dimensional gel electrophoresis was performed using a pH range of 3 to 10 in the first dimension and SDS-PAGE (12.5–15% total acrylamide) in the second dimension. Marked spot numbers refer to spot identification in Table 5. Twenty-four spots/proteins were identified using MALDI-TOF/TOF tandem mass spectrometry analysis.

at peroxisomal level) was upregulated in the liver of RES lambs], which likely occur to render acetyl-CoA and glycerol (gluconeogenic precursor) through lipolysis and β -oxidation. In fact, Girard (1986) suggested that active hepatic fatty acid oxidation is necessary in the newborn to support hepatic gluconeogenesis by providing essential cofactors, such as acetyl CoA and NADPH.

Moreover, the hepatic fatty acid oxidation (coming either from the diet or fat depots) is fundamental to sustain protein accretion in growing lambs under feed restriction (Giráldez et al., 1999). However, fat mobilized from adipose tissue to liver for acetyl CoA production during feed restriction may cause hepatic steatosis (Xu et al., 2012; Santos et al., 2018b) and a concomitant increased expression of glucuronosyltransferases

(Yu et al., 2004; Xu et al., 2012). All these observations are in accordance with the results observed in our RES lambs (e.g., upregulation of *UGT1A6*, *UGT1A9*, *UGT2A1*, and *UGT2B7*) and may have implications in the conjugation reaction (via glucuronide) in phase II metabolism of drugs and xenobiotics. In addition, apart from the upregulation of *UGT* in the RES group, other genes related to the metabolism of drugs and xenobiotics were altered by early feed restriction. Thus, genes (e.g., *LOC101105651*, *LOC101112384*, and *EPHX1*) encoding phase I enzymes (i.e., oxidizing), belonging predominantly to the cytochrome P450 family (e.g., *CYP2C19*, *CYP2D14*), and others related to phase II (i.e., conjugation), such as glutathione transferases (e.g., *GSTA1-1*, and *GSTT2B*) and glycine N-acyltransferase (e.g., *LOC101108419* and *LOC101108685*), were over-

expressed in the RES lambs, which is in agreement with the results observed by Grubbs et al. (2013) in low-efficient pigs. However, protein synthesis could be repressed as a consequence of the lack of nutrients in the RES lambs (Yu et al., 2004; Xu et al., 2012), as previously stated.

Oxidative Stress and Cellular Damage Metabolic Pathways

Malnutrition provokes disorders in the peroxisomes, which are the organelles preventing the accumulation of steroid acids (van Zutphen et al., 2016). Accordingly, our results in the early feed-restricted group indicate the upregulation of many genes involved in metabolic pathways (e.g., steroid and steroid hormone biosynthesis, primary bile acid biosynthesis, and bile secretion). All these modifications might increase plasma levels of several cholesterol metabolites, such as oxysterols, bile alcohols, or bile acids, contributing to hepatic toxicity and damage (van Zutphen et al., 2016; Zhang et al.,

2016), thus inducing the production of reactive oxygen/nitrogen species and the initiation of apoptotic pathways (e.g., *PERP* is overexpressed in RES; Zhang et al., 2016). Therefore, in accordance with Grubbs et al. (2013), the increased expression of superoxide dismutase (e.g., *SOD1* and *LOC101115136*) and glutathione-S-transferase in the RES lambs (e.g., *MGST1*, which is involved in the production of inflammation mediators, such as leukotrienes or prostaglandin E) may indicate liver injury. Accordingly, recent studies have revealed that peptidyl-prolyl *cis-trans* isomerase A (which was overaccumulated in the liver of RES lambs) can be secreted by cells in response to inflammatory stimuli and oxidative stress (Jin et al., 2000, 2004; Nigro et al., 2013). In fact, these results corroborate the proinflammatory profile observed by metabolomics in the weaned lambs of the present experiment (nonslaughtered animals) during the replacement phase (Santos et al., 2018c). However, the apparently contradictory down-accumulation of mitochondrial superoxide dismutase in the liver proteome of RES lambs might have been

Table 5. List of spots/proteins identified by MALDI-TOF/TOF tandem mass spectrometry analysis on the lamb liver 2-dimensional gel electrophoresis gel and differentially accumulated in the livers of preweaning lambs fed milk replacer ad libitum (AL) or restricted (RES)

Spot ¹	log ₂ FC ²	Protein name	Accession number ³	Theor. MW ⁴	Exp. MW ⁴	Theor. pI ⁵	Exp. pI ⁵
B15	-0.93	Superoxide dismutase [Mn], mitochondrial	NP_001267632.1	24,580	23,658	8.89	7.89
A31	2.45	Selenium-binding protein 1 isoform X1	XP_012025411.1	52,516	52,466	6.33	6.38
B06	-2.17	2-Iminobutanoate/2-iminopropanoate deaminase	XP_004022295.1	14,327	14,000	7.82	5.29
B05	-2.05	2-Iminobutanoate/2-iminopropanoate deaminase	XP_004022295.1	14,327	15,426	7.82	7.02
A20	0.66	3-Hydroxyisobutyryl-CoA hydrolase, mitochondrial isoform X1	XP_011986429.1	40,110	37,845	5.83	5.54
A11	1.55	3-Hydroxyisobutyryl-CoA hydrolase, mitochondrial isoform X1	XP_011986429.1	40,110	37,437	5.83	5.80
A15	1.63	Aminomethyltransferase, mitochondrial isoform X2	XP_011977499.1	43,520	41,538	8.58	7.07
A29	4.99	Peptidyl-prolyl <i>cis-trans</i> isomerase A-like isoform X2	XP_012010030.1	17,869	17,079	7.68	7.93
B08	-1.34	Protein-disulfide isomerase	XP_011964799.1	57,266	53,722	4.80	5.02
B02	-2.64	Fructose-bisphosphate aldolase B	XP_011966534.1	39,571	34,834	8.54	7.35
A32	1.83	Fructose-bisphosphate aldolase B	XP_011966534.1	39,571	34,657	8.54	7.95
A39	1.33	Glyceraldehyde-3-phosphate DH	NP_001177319.1	35,869	33,725	8.51	6.96
A35	2.31	Glyceraldehyde-3-phosphate DH	NP_001177319.1	35,868	33,852	8.51	7.95
B03	-1.71	Acyl-coenzyme A synthetase ACSM1, mitochondrial isoform X1	XP_004020863.1	65,057	61,091	8.12	6.70
A07	2.81	3-Ketoacyl-CoA thiolase, peroxisomal isoform X2	XP_011995559.1	44,179	36,922	8.68	7.93
B01	-2.35	Thiosulfate sulfurtransferase	XP_012004218.1	33,277	32,757	7.06	6.71
A36	1.79	Glycine amidinotransferase, mitochondrial	XP_004010698.1	48,494	41,713	8.00	5.77
A33	1.69	Glycine amidinotransferase, mitochondrial	XP_004010698.1	48,494	40,995	8.00	6.01
A30	2.33	Glycine amidinotransferase, mitochondrial	XP_004010698.1	48,494	41,527	8.00	5.93
A04	1.14	Glycine amidinotransferase, mitochondrial	XP_012013381.1	48,357	34,904	8.00	7.00
A27	1.10	Cytoplasmic aconitate hydratase	XP_012005122.1	98,129	74,929	8.09	7.93
B16	-1.26	Plasminogen (fragment)	XP_011982598.1	9,397	25,657	7.10	5.39
A44	1.20	Uroporphyrinogen decarboxylase	NP_001012341.1	40,812	38,717	5.83	5.68
A05	1.48	LIM and SH3 domain protein 1	XP_004012879.1	29,677	27,980	6.61	5.20

¹Spots number labelled in Figure 2. Spots A09 and B07 were not included in Table 4 because they were not identified as *Ovis aries* proteins.

²Log₂ fold change of the relative volume average (positive value if RES/ADL >0.58; negative value if AL/RES >0.58).

³Accession number from NCBI database (<https://www.ncbi.nlm.nih.gov/>).

⁴MW = molecular weight (Dalton), theoretical (Theor.) and experimental (Exp.).

⁵pI = isoelectric point, theoretical (Theor.) and experimental (Exp.).

promoted by a reduced propagation of free radicals generated through oxidative phosphorylation because of the lower nutrient intake level (Sohal et al., 1994).

Other Metabolic Functions

Several other functions might have been affected by early feed restriction. It is noteworthy that *STS* was the only gene involved in the steroid hormone synthesis pathway that was down-expressed in the RES lambs. This gene has a crucial role in regulating the formation of biologically active sex steroids, such as estrogens (Reed et al., 2005); in fact, physiologically upregulated *STS* activity before puberty has been shown in humans (Idkowiak et al., 2016). In addition, it is interesting to note the downregulation of one of the genes (e.g., *SPR*) involved in the metabolic route related to folate biosynthesis; folate has been involved in epigenetic mechanisms (Crider et al., 2012), such as DNA methylation in the preovulatory oocyte or preimplantation embryo (Sinclair et al., 2007; Dupont et al., 2012). Consequently, the downregulation of both genes might be involved in the mechanisms that explain delays the onset of puberty after early feed restriction and impairment of reproductive functions in dairy ewes (Robinson et al., 2002, 2005). Accordingly, a delay in the onset of the first ovulation was observed in the replacement ewe lambs of the present study, as described previously in a different paper (Santos et al., 2018c).

Furthermore, Amadi et al. (2009) also described a reduced expression of heparan sulfate proteoglycan (*HSPG*) in enterocytes of children with Kwashiorkor that finally provoked intestinal protein loss and edema; in our study, genes involved in glycosaminoglycan biosynthesis and heparan sulfate (e.g., *GLCE* and *NDST3*) were down-expressed in RES lambs. Notwithstanding, the existence of differences in a specific tissue (e.g., liver) does not entail the same effect at other locations (Tizioto et al., 2015).

CONCLUSIONS

According to the results obtained in the present study, early feed restriction during the artificial rearing period of Assaf lambs increases the oxidation of fatty acids and the hepatic oxidative stress, whereas protein synthesis and degradation were also modified to sustain gluconeogenic pathways. All these changes, together with the differential expression of genes involved in oxidative phosphorylation, may modify the partitioning and utilization of nutrients in early feed-restricted lambs.

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