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P u b l i s h e r s

Modifications in liver transcriptomic profile of fattening lambs by early suckled milk intake level

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The increment of world population increases the need of improving feed efficiency traits of livestock. However, the molecular mechanisms behind different feed efficiency traits and their regulation by nutrition remain poorly understood. Nowadays, Next Generation Sequencing methods allow understanding differences in gene expression and identifying functional candidate genes and pathways that control target traits in order to design strategies to increase feed efficiency. The present study was designed to identify (RNA-seq) differentially expressed (DE) genes in the liver tissues of fattening merino lambs caused by milk restriction during the suckling period. Forty male lambs were assigned randomly to two intake levels (n=20 per group) during the suckling period, namely *ad libitum* (ADL) and restricted (RES) groups. When they reached 15 kg of live body weight (LBW), all the animals were offered the same complete pelleted diet at a restricted level (40 g/kg) to ensure no selection of ingredients and no differences in dry matter intake during the fattening period. All the lambs were slaughtered with 27 kg of LBW and four animals from each group were selected for RNA-seq. Thirty-eight DE annotated genes were identified, with 23 DE genes being down-regulated and 15 up-regulated in the RES relative to the ADL group. RES lambs showed over-expression of lipid and xenobiotic metabolism pathways. Moreover, those genes involved in protein synthesis or protease inhibitors were down-regulated in the RES group, whereas those related to proteolytic degradation were up-regulated, thus suggesting a higher catabolism of proteins in these lambs. In conclusion, a restricted milk intake level during the suckling period of merino lambs promoted long term effects on hepatic transcriptomic profile which might have modified fatty acids metabolism and increased catabolism of proteins and detoxification of xenobiotics during the fattening period.

MODIFICATIONS IN LIVER TRANSCRIPTOMIC PROFILE OF FATTENING LAMBS BY EARLY SUCKLED MILK INTAKE LEVEL

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1 Introduction

Increase in world population and demand for animal products

Limited resources: strategies to increase feed efficiency

Genetic and environmental factors: pre and postnatal nutrition

Underlying molecular mechanisms

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2 Objective

To identify **differentially expressed genes** in the liver tissues of fattening merino lambs with different levels of nutrition during the suckling period

"Next Generation Sequencing"

3 Material and Methods

Suckling period, early feed restriction

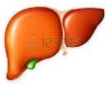
12 lambs *ad libitum* (ADL)

12 lambs early feed restricted (RES)

Fattening period, same level of DMI

3 Material and Methods

Slaughter target body weight, **27 kg**



RNA-seq ⟷ Gene Expression

4 ADL 4 RES

4 Material and Methods

RNA-seq Workflow

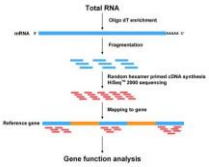
RNA extraction

Library preparation

Sequencing

Differential expression analysis → DESeq2

Functional annotation



5 Results and Discussion

38 DE genes

RES/ADL

23 down

15 up

5 Results and Discussion

RES/ADL ↑ CYP450

Steroid hormone biosynthesis

Lipid and xenobiotic metabolism

5 Results and Discussion

Fatty acid metabolism

RES/ADL

↓ *ACSM3* *ACOT13* → Fatty acid activation

↓ β-oxidation

ND3 → Oxidative phosphorylation and respiratory chain

5 Results and Discussion

Biosynthesis of fatty acids

RES/ADL

ACOX3
FADS6

→ Polyunsaturated FA → **Fatty acids profile**

5 Results and Discussion

Protein metabolism

RES/ADL

LOC101102122
RPLP1
RPL3
LOC101114033
→ **Ribosomal proteins**

CST3
SERPINA11
→ **Protease inhibitors**

Proteolytic degradation ← *DNAJA1*, *LOC101105132*, *LOC105605968*, *LOC101101922*

Higher catabolism of proteins

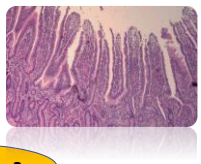
5 Results and Discussion

Cellular growth and proliferation

RES/ADL

ACP5
TF
RBMS2
F2
DPYS

Villous atrophy in small intestine



Malabsorption ?

5 Results and Discussion

RES/ADL

- Inflammatory response → *CYP2C19* ↑, *TF* ↓
- Susceptibility to infections → *GUCY2C* ↑
- Coagulation disorders → *APOH* ↓, *F2* ↓, *TF* ↓
- Oxidative stress → *C1H21orf33* ↓

6 Conclusions

A restricted milk intake level during the suckling period of merino lambs promoted long term effects on the **hepatic transcriptome profile** during the fattening period:

- ↓ β-oxidation of fatty acids
- ↑ Catabolism of proteins
- ↑ Detoxification of xenobiotics

Thank you for your attention

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Background

One of the main challenges for the world is feeding 9 billion people within the carrying capacity of planet Earth. For this reason, as livestock sector is concerned, it is important to look for strategies to increase the feed efficiency of animals. It should be noted that differences in feed efficiency may be determined not only by genetic but also environmental factors, such as pre and postnatal nutrition [1]. However, the molecular mechanisms behind different feed efficiency traits and their regulation by nutrition remain poorly understood. Consequently, a deeper knowledge in this field is needed in order to design strategies to obtain more efficient animals. In this sense, gene expression differences between individuals is a key source of variation that can be used to identify candidate genes and pathways that control target traits. The availability of high-throughput sequencing methods (referred to as Next Generation Sequencing), enables unraveling the genetic regulation of complex phenotype [2].

The present study is focused on long term effects promoted by early nutrition of lambs, trying to identify differentially expressed genes in the liver tissues of fattening merino lambs with different levels of nutrition during the suckling period. The study aims to identify molecular pathways activated by early feed restriction, reasoning that this may highlight potential strategies to improve feed efficiency through either selection or improved management practices.

Material and methods

Forty male merino lambs were used in this experiment. The lambs were stratified on the basis of live body weight at birth (average LBW 4.77 ± 0.213 kg), and then assigned randomly to each of two different groups (n=20 per dietary treatment) during the suckling period. The first group of lambs (ad libitum, ADL) was kept permanently with the dams whereas the other twenty lambs (restricted, RES) were separated from the dams from 9 to 18 h. Dams were milked at 17 h before the reintroduction of lambs. When they reached 13.5 kg of LBW, lambs were weaned progressively until they weighed 15 kg. Then, all the animals were penned individually and offered the same complete pelleted diet (CPD) at a restricted level (40 g/kg LBW) to ensure, on the one hand, no selection of ingredients and, on the other hand, no differences in dry matter intake during the fattening period. All the animals were slaughtered with a target body weight of 27 kg, and a piece of liver was excised,

stabilized with RNAlater (Ambion Inc., Austin, TX, USA) and stored at 80 ºC for transcriptomics analysis.

A transcriptome analysis was carried out using next-generation sequencing (RNA-seq). RNA extraction from liver samples (4 animals per group, 8 in total) was performed using RNeasy Mini kit and RNase-Free DNase Set kit (Qiagen, Hilden, Germany). RNA concentration, purity and integrity were tested by spectrometry using a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and by capillary electrophoresis with the RNA 6000 Nano Kit (Agilent, Santa Clara, CA, USA) on a Bioanalyzer 2100. Library preparation for RNA-seq (transcriptome sequencing) was performed on a HiSeq 2500 sequencer (Illumina, San Diego, CA, USA) to obtain readings of 100 bp in length from each sample and cDNA libraries were obtained according to manufacturer instructions. The pool of the libraries was sequenced by paired-end sequencing (2 x 100 bp) on an Illumina HiSeq 2500 sequencer.

The sequence and annotation of the most recent ovine genome assembly was obtained from the NCBI database (accession number GCF_000298735.2, assembly Oar_v4.0). The identification of new and annotated transcripts that presented differential transcription between the experimental groups was made by mapping with HISAT. A differential expression analysis was performed with the DESeq2 v 1.12.4. Statistical analyses were performed in R (version 3.3.1). In addition, the TPM (Transcripts Per Kilobase Million) values were calculated according to Conesa et al. [3].

Results and discussion

Differential gene expression analysis in liver tissue of merino lambs with different levels of milk intake during the pre-weaned period identified 38 differentially expressed (DE) annotated genes. The sign of the $\log_2$(fold change) allowed separating the DE genes into up- and down-regulated groups with 23 DE genes being down-regulated and 15 up-regulated in the RES relative to the ADL group respectively (Table 1).

Table 1. Differential expression of genes in the livers of fattening lambs allowed ad libitum (ADL) or restricted milk intake (RES) during the suckling period.

Gene ID and function	TPM Mean ADL	TPM Mean RES	$\log_2$ FC (*)	p-value	q-value
Steroid hormone biosynthesis, lipid and xenobiotic metabolism					
LOC101110202	5079	7494	0.433	8.45E-08	0.0005
CYP2C19	3475	8193	0.708	3.48E-05	0.0218
CYP3A24	11 500	19 564	0.548	6.48E-05	0.0304
Coagulation					
CDC37L1	153	217	0.381	2.35E-06	0.0026
APOH	54 235	44 837	-0.359	9.67E-06	0.0091
F2	12 697	10 378	-0.366	1.00E-04	0.0433
Inflammatory response					
TF	97 750	76 426	-0.420	1.75E-05	0.0123
GUCY2C	51.6	122	0.619	0.00043	0.0893
Fatty acid metabolism					
ACSM3	7546	6040	-0.382	0.00030	0.0877

ACOT13	1663	1283	-0.419	0.00042	0.0893
ACOX3	445	277	-0.561	0.00048	0.0893
FADS6	1863	1364	-0.459	0.00052	0.0893
Respiratory electron transport					
ND3	17 512	10 566	-0.686	3.21E-07	0.0005
Cholesterol metabolism					
APOM	1209	9613	-0.392	0.00023	0.0843
ABCA10	1181	959	-0.364	0.00065	0.0972
Protein catabolism					
CST3	3141	2217	-0.515	5.22E-05	0.0280
SERPINA11	2346	1986	-0.318	0.00047	0.0893
DNAJA1	137	207	0.422	0.00041	0.0893
LOC101105132	244	372	0.430	0.00042	0.0893
LOC105605968 (**)	360	561	0.468	5.47E-05	0.0280
LOC101101922 (**)	291	464	0.474	0.00029	0.0877
Protein synthesis					
LOC101102122 (**)	5958	4707	-0.420	3.84E-07	0.0005
RPLP1	8712	6923	-0.403	1.37E-05	0.0110
RPL3	5485	4944	-0.245	0.00029	0.0877
LOC101114033	2076	1792	-0.297	0.00065	0.0972
DPYS	3579	2332	-0.541	0.00042	0.0893
Cellular growth and proliferation					
ACP5	818	562	-0.571	3.55E-07	0.0005
RBMS2	76.6	57.6	-0.436	0.00045	0.0893
Glycine, serine and threonine metabolism					
SERINC2	129	285	0.633	0.00024	0.0843
GCAT	1900	1392	-0.454	0.00066	0.0972
Others					
NUFIP2	160	249	0.454	0.00024	0.0843
PLXNA2	41.5	25.7	-0.555	0.00057	0.0911
LOC101106024	468	920	0.592	0.00033	0.0893
ELK4	97	143	0.402	0.00054	0.0893
EPS8	98.2	140	0.363	0.00054	0.0893
C1H21orf33	1611	1264	-0.399	0.00050	0.0893
LOC101121401 (**)	2732	2266	-0.344	0.00048	0.0893
CCDC117	162	225	0.344	0.00053	0.0893

*Fold change estimates are relative to ADL group, so positive values indicate greater expression in RES lambs.
**Non-expressed pseudogenes.

The liver is the major metabolic organ in the body and regulates a wide variety of metabolic processes, including glycogen storage, protein synthesis, lipogenesis, detoxification and digestion [4]. To our knowledge, it is not known if all these processes might be altered by different levels of milk intake in pre-weaned lambs, thus promoting long term effects in hepatic metabolism during the fattening period.

This study revealed that low milk intake animals (RES) showed over-expression of several cytochrome P450 forms, such as *CYP3A24* and *CYP2C19*. The cytochrome P450 constitutes the mayor enzyme family capable of oxidizing a variety of structural compounds, including steroids, fatty acids and xenobiotics [5]. Therefore, there might have been alterations at the reproductive level, but also on the detoxification of xenobiotics in the RES animals. In fact, it is well known that oxidation of some inactive antithrombotic prodrugs by hepatic CYP450 (e.g. *CYP2C19* and *CYP3A*) is essential for the inhibition of the platelet aggregation [6]. Consequently, the higher expression of

genes involved in coagulation cascades (*CDC37L1*, *APOH*, *F2*) in RES lambs, might be the reaction against a exacerbated bio-activation by CYP450 of some compounds naturally present in the diet of fattening lambs. Additionally, *CYP2C19* also takes part of arachidonic acid metabolism and inflammation, so alterations at this level in the RES animals also can be expected. Indeed, the lower expression of transferrin (*TF*) in RES lambs might be related to an increase of inflammation in these animals, whereas a higher expression of *GUCY2C* can be related to a greater susceptibility to infections.

As far as fatty acid metabolism is concerned, several mitochondrial (*ACSM3*) and peroxisomal (*ACOX3*) enzymes involved in fatty acid degradation (β-oxidation) were down-regulated in the RES group. This, together with the lower expression of one of the genes involved in oxidative phosphorylation and respiratory electron transport chain (*ND3*) might indicate that β-oxidation of fatty acids (and hence ATP production through this pathway) was down-regulated in RES lambs. Moreover, genes involved in biosynthesis of long chain fatty acids (*ACOT13*) or polyunsaturated fatty acids (*ACOX3*, *FADS6*) were down regulated in the RES lambs. It would be interesting to test if these circumstances are related to a higher storage of lipids in RES fattening lambs and hence affect meat quality or feed efficiency traits. Moreover, other genes such as *SERINC2*, that are over-expressed in the RES group and that are related to sphingolipid biosynthesis, might have a role in inflammation processes.

On the other hand, genes involved in protein synthesis like the genes encoding ribosomal proteins (*LOC101102122*, *RPLP1*, *RPL3* and *LOC101114033*) or protease inhibitors (*CST3* and *SERPINA11*) were down-regulated in the RES group, whereas those related to proteolytic degradation (*DNAJA1*, *LOC101105132*, *LOC105605968* and *LOC101101922*) were up-regulated, suggesting a higher catabolism of proteins. The lower expression in RES lambs of genes related to cellular growth and proliferation (*ACP5*, *TF*, *RBMS2*, *F2* and *DPYS*) would be in consonance with the previous statement. Especially remarkable is the lower expression of *DPYS* in RES lambs, since this condition has been related to villous atrophy in the small intestine, thus leading to malabsorption problems and failure to gain weight at the expected rate.

A lower expression of *C1H21orf33*, which encodes a potential mitochondrial protein with protective effects against oxidative stress, was also noticed in RES lambs.

In conclusion, according to the data of the present study, a restricted milk intake level during the suckling period of merino lambs promoted long term effects on the hepatic transcriptome profile, which might have impaired β-oxidation of fatty acids and increased catabolism of proteins and detoxification of xenobiotics during the fattening period.

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