Highlights

- Quantitative proteomics analysis was carried out on goat-kid omental adipose tissue after supplementing lactating mothers with different fatty acid diet integration
- Different diets modified the omental adipose tissue proteome
- A number 20 proteins were found to be differentially expressed, of which only NUCKS1, a physiological regulator of glucose metabolism, was found to be overexpressed
- The downregulation of ECI1 and Ceruloplasmin was also confirmed at gene expression level
- The results demonstrated that supplementing other diet with different PUFA may influence omental adipose tissue proteome.

Saturated or unsaturated fat supplemented maternal diets influence omental adipose tissue proteome of suckling goat-kids.

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Abstract

The aim of the present study was to investigate how maternal diet can influence the adipose tissue of goat kids. Omental adipose tissue proteomes of goat-kids from mothers fed with diet enriched with stearic acid (ST-kids), fish oil (FO-kids) and standard diets (CTRL) were determined by quantitative iTRAQ 2D-LC-MS/MS analysis. Twenty proteins were found to be differentially expressed in suckling kids' omental adipose tissue. Stearic acid induces changes in a higher number of proteins when compared to fish oil. Eleven proteins, namely AARS, ECl1, PMSC2, CP, HSPA8, GPD1, RPL7, OGDH, RPL24, FGA and RPL5 were decreased in ST-kids only. Four proteins, namely DLST, EEF1G, BCAP31 and RALA were decreased in FO-kids only, and one, NUCKS1, was increased. Four proteins, namely PMSC1, PPIB, TUB5X2 and EIF5A1, were be less abundant in both ST- and FO- kids. Most of the protein whose abundance was decreased in ST kids (10 out of 15) are involved in protein metabolism and catabolism pathways. Qualitative gene expression analysis confirmed that all the proteins identified by mass spectrometry, with the exception of FGA, were produced by adipose tissue. Quantitative gene expression analysis demonstrated that two proteins, namely CP, a minor acute phase protein, and ECl1, involved in fatty acid beta oxidation, were downregulated at mRNA level as well. ECl1 gene expression was downregulated in ST-kids AT as compared to Ctrl-kids and CP was downregulated in both ST- and FO-kids. The present results demonstrate that it is possible to influence adipose goat-kid proteome by modifying the maternal diet.

Introduction

The involvement of adipose tissue (AT) in several physiological and pathological processes, such as appetite regulation, reproduction, as well as inflammatory and immune response, has been thoughtfully acknowledged. In humans, AT has a key role in obesity and the development of metabolic diseases (Després and Lemieux, 2006). In farm animals, where obesity is not an issue due to the controlled environment in which they live, focus is on AT influence on animal health and meat quality (Sauerwein et al., 2014). In dairy animals, AT metabolism gained particular interest for its essential role in the transition period, when a hormonally-controlled lipid mobilization is established in order to support milk synthesis (Contreras and Sordillo, 2011; Wathes et al., 2012). The active role of AT in regulating a wide range of body functions is related to its capability to produce and secrete adipokines. Adipokines are signalling molecules with endocrine, autocrine or paracrine functions, secreted in response to neuroendocrine signals (Harwood, 2012). In goat, the species that is the object of the present study, fat depots can be influenced by diets. For example, linseed oil supplementation to Boer goats' diet leads to changes in fatty acid (FA) profile of subcutaneous adipose tissue and expression of genes related to fat metabolism such as PPARα, PPARγ and stearoyl-CoA desaturase (Ebrahimi et al., 2013). The transcriptomic profile of AT is modified by diets or feed deprivation (Faulconnier et al., 2011). Moreover, different fat sources have distinct impacts on AT, as shown by a study that investigated the effect of diets enriched in fish and soybean oils or saturated lipids on lipogenic and adipogenic gene expression in bovine subcutaneous AT (Thering et al., 2009). Finally, fish oil can delay fat mobilization in the adipose tissue after kidding (Invernizzi et al., 2016).

Fish oil is particularly rich in eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3) that can positively influence animal health due to their involvement in innate immune pathways (Lecchi et al., 2013, 2011; Pisani et al., 2009; Thanasak et al., 2004). On the other hand, Bueno and co-workers (Bueno et al., 2010) demonstrated that diets enriched with coconut oil

or lard, both rich in saturated fatty acids, can modify the pro-inflammatory environment of white AT by upregulating haptoglobin expression in rats.

Nutrition is the major determinant of milk fat synthesis and FA composition in goats, regulating in turn the quality of milk in cows (Toral et al., 2013a, 2013b). Diets enriched in fish oil increase the amount of n-3 PUFAs in colostrum and mature milk in pregnant dairy goats (Cattaneo et al., 2006). In addition, diets enriched in extruded linseed alone or in combination with fish oil have influence on the milk fatty acid composition in lactating goats (L Bernard et al., 2009).

A diet based on milk or milk replacer can influence meat quality and fat composition of suckling kids (Bañón et al., 2006). UCP1 expression and thermogenesis can be modulated by high fat diets in perirenal adipose tissue of newborn lambs (Chen et al., 2007), while overfeeding sheep during late gestation enhances adipogenesis in lamb's foetal muscles (Tong et al., 2008). It has been poorly investigated whether is possible to influence adipose tissue proteomics by modifying lactating mother diets. Maternal diets have effects on adipose tissue proteome in newborn pigs (Sarr et al., 2010). In goats, increasing the percentage of saturated or unsaturated fatty in maternal diets did not influence the expression of AT genes involved in thermogenesis, namely UCP1 and UCP2 (Restelli et al., 2015). No information about how maternal diets influence suckling kid adipose tissue proteome is available.

The present study aims to cover this gap by investigating the influence that the maternal diet has on kid AT. We performed a comparative investigation of visceral adipose tissue proteomes of suckling goat-kids, whose mothers were fed different high-fat diets. A quantitative 2D-LC-MS/MS analysis was carried out, using iTRAQ labelling, in order to evaluate the possible influence of fish oil (FO) or stearic acid (ST) mother's enriched diets on kids' omental protein expression. mRNA expression of significant proteins was also evaluated by quantitative PCR.

Materials and methods

The experimental protocol used in this study was approved by the ethic committee of the University of Milan (Protocol No. 5/11, 18 January 2011).

2.1 Animals, diets and tissue sampling

Samples of AT were obtained from twelve 29.8±2.8 day-old healthy suckling kids, which were part of a larger experiment aimed to evaluate the influence of the maternal diet on peripartum and goat kids' performances. A group of 23 multiparous Alpine goats, homogeneous for parity and milk production during the previous lactation, were fed with maternal lactating diets enriched with different fatty acids, either saturated (69:26 percentages ratio of ST (C18:0) and palmitic acid (C16:0)) or unsaturated (FO containing 10.22% of EPA=20:5 and 7.65 % of DHA=22:6), starting from a week before kidding until slaughtering of the kids at 30 days from birth. A third group of animals fed with a control diet without any specific diet supplementation was also used as control (CTRL). FO and ST enriched diets were adapted for the dry period (supplemented with 30 g of fatty acids) and lactation period (supplemented with 50 g of fatty acids). The diet ingredients and chemical composition are detailed in Table 1. After kidding, each goat shared the box with their relative suckling kids. From this larger group, twelve male kids, equally distributed among maternal control diet (CTRL-Kid=4), stearic acid (ST-Kid=4) and fish oil (FO-Kid=4), were randomly selected in order to be included in the present experiment. Samples were obtained from omental region. Tissue samples for both molecular biology and proteomic analysis were snap frozen in liquid nitrogen and stored at –80°C for further analysis.

2.2 Sample preparation for iTRAQ analysis and protein digestion

In addition to the 12 omental samples, a reference sample was created by pooling equal amounts of the four controls. The reference sample was divided into four identical aliquots, one for

each iTRAQ run and the 16 samples (12 experimental samples + 4 reference samples) were processed together throughout all the processes allowing the comparison of multiple iTRAQ runs.

Protein extraction procedures were carried out on ice or at 4°C as described previously (Danielsen et al., 2011). 200 mg of each adipose tissue were homogenized in 5 µl/mg TES buffer (10 mMTris-HCl, pH 7.6; 1 mM EDTA, 0.25 M sucrose) and centrifuged at 10000 x g, for 30 min, at 4°C. Protein concentration values of the tissue supernatants were determined by the Pierce BCA Protein Kit (VWR), using BSA as a protein standard, according to the manufacturer's manual. Proteins were precipitated by adding 6 volume of ice-cold acetone to a total of 120 µg of proteins from each tissue homogenate. The precipitated proteins were re-suspended in 20 µl of digestion buffer (0.5 M triethylammonium bicarbonate, 0.1% SDS); cysteine residues were reduced with 2.5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) at 60°C for 1 h, and then blocked with 10 mMmethylmethanethiosulfate at room temperature, for 1 h. Samples were digested with trypsin (1:10 w/w) (AB SCIEX) at 37°C, overnight.

2.3 iTRAQ(Isobaric Tag for Relative and Absolute Quantitation) labelling

iTRAQ labelling was performed according to the manufacturer's instructions (Applied Biosystems). Four independent iTRAQ runs were performed. Reference samples were labelled with reagent 114, control samples were labelled with reagent 115, fish oil treated samples were labelled with reagent 116, stearic acid treated samples were labelled with reagent 117 (as shown in Table 2). Each isobaric tagging reagent was added directly to the peptide mixture and incubated at room temperature for one hour. The 16 samples were then combined in 1:1:1:1 ratios into four tubes, each containing a common reference sample, a control and two treated samples (one stearic acid and one fish oil). In order to remove all the particulate matter that can interfere with later HPLC separation, all samples were passed through a 0.2 µm centrifuge filter (National Scientific Company) for 10 min at 10000 x *g*, vacuum-dried and eventually stored at -80°C until further analysis.

2.4 2D-LC-MS/MS analysis

2.4.1 Strong Cation Exchange (SCX) liquid chromatography

The peptides were re-dissolved in 0.03% formic acid and 5% acetonitrile in water. Peptides mixture generated from the digestion of 50 µg of protein were injected into an Agilent 1100 Series capillary HPLC equipped with a Zorbax Bio-SCX Series II, 0.8×50 mm column (Agilent Technologies) that provides peptide separation by strong cation exchange liquid chromatography.

Peptides were eluted with a gradient of increasing NaCl (0 min 0% B; 5 min 0% B; 10 min 1.5% B; 11 min 4% B; 25.5 min 15% B; 35.5 min 50% B; 45 min 100% B; 55 min 100% B). Buffer A contained 0.03% formic acid and 5% acetonitrile in water, buffer B contained 0.03% formic acid, 5% acetonitrile and 1 M NaCl in water. The flow rate was 15 µl/min and fractions were collected every minute for 65 minutes and then combined according to their peptide loads into 10 pooled samples to achieve approximately equal peptide loads for further LC-MS/MS analyses.

2.4.2 LC-MS/MS

The pooled samples were de-salted and concentrated prior to be further separated by reverse phase liquid chromatography on Agilent 1100 Series nano-flow HPLC system (Agilent Technologies). De-salting and concentration of the samples were carried out on an enrichment column (EASY Column, 2cm, ID 100µm, 5µm, C18 -Thermo Scientific) using an isocratic pump working at 20 µl/min (0.1% formic acid and 3% acetonitrile in water). Peptides were then eluted and further separated on an analytical column (EASY Column, 10cm, ID 75µm, 3µm, C18 -Thermo Scientific) with a nanoflow of 300 nl/min, using a gradient of increased organic solvent (0 min 5% B; 7 min 5% B; 70 min 40% B; 73 min 95% B; 78 min 95% B; 83 min 5% B; 100 min 5% B). Buffer A containing 0.1% formic acid in water and buffer B containing 5% water and 0.1% FA in acetonitrile. The eluted peptides were sprayed through nanospray needle (PicoTip®, silica, no coating,

OD 360µm, ID 20µm - New Objective) directly into the Q-star Elite mass spectrometer (Applied Biosystems).

2.5 Database searches and statistical analysis

The raw spectrum files from 16 individual shotgun LC-MS/MS runs were searched separately with Protein Pilot 1.0 software (Ab Sciex) using the ProGroup and Paragon algorithms for protein grouping and confidence scoring. The target database used for searching was constructed as a non-redundant union of UniProtKB Bovidae sequences (www.uniprot.org/uniprot/?query=taxonomy:9895) and NCBI Capridae sequences (www.ncbi.nlm.nih.gov/taxonomy/?term=9963). The False Discovery Rate (FDR) was estimated as the ratio of (2 x reversed sequence)/(reversed + forward sequence) in percentage (Elias and Gygi, 2007). Search parameters were set with an MS tolerance of 0.15 Da and a MS/MS tolerance of 0.1 Da, and using generic modifications including deamidation of glutamine and asparagines side chains, methionine oxidation as well as methyl methanethiosulfonate modification of cysteines. Samples were SCX fractionated and analyzed twice (technical replication) in order to gain higher reproducibility and proteome coverage as suggested by Chong and coworkers (Chong et al., 2006). The two data sets from each sample were searched together in ProteinPilot (Applied Biosystems). The confidence for protein identification was selected in Protein Pilot to a protein score of 1.3, equivalent to 95% confidence and a minimum of two peptides matching with MS/MS spectra per protein.

Data handling and analysis was performed using the statistical software package R (R Development Core Team). All data are presented as mean values and were analyzed by one-way ANOVA (Tukey's method; MATLAB R2016a (Mathworks, USA)). *P* values <0.05 were considered as significant.

2.5.1 Functional annotation and grouping

The open source online tool Blast2GO (http://www.blast2go.com) was used for the functional annotation of the identified proteins (Conesa et al., 2005). The default parameters were used and for the basic local alignment search tool (BLAST) protein sequences were mapped against the NCBI's non-redundant (nr) protein database (http://www.ncbi.nlm.nih.gov). We further narrowed the functional analysis by PANTHER classification into protein families and functional pathways in order to increase the confidence (Protein Analysis Through Evolutionary Relationship) system available at http://www.pantherdb.org (Thomas et al., 2003).

2.6 Qualitative and quantitative gene expression analysis.

Total RNA was extracted from the same omental adipose tissues used for the proteomic analysis from all the animals included in this study, stored at -80°C, by means of a commercial kit specific for all kind of tissues (RNeasy Plus Universal Mini Kit - Qiagen). A DNAse treatment was also carried out (RNase-Free DNase Set - Qiagen). The RNA concentration in each sample was quantified by NanoDrop ND-1000 UV-spectrophotometer. 1 µg RNA was retrotranscribed using the iScript cDNA Synthesis kit (Biorad). The resulting cDNA was used as template for qualitative and quantitative PCR reactions. In case of absence of goat sequences primers were designed on bovine sequences. The same primers were used in qualitative and quantitative PCR (primers sequences, accession numbers and length of the amplified fragments are listed in Table 3. A pool of cDNA from liver of all the 12 animals was created in order to use it as positive control in the qualitative PCRs and a pool of cDNA from AT of 4 CTRL animals was used as reference sample for the Real Time PCRs. Qualitative PCRs were performed in 10 µl final volume, containing 1 µl buffer (Vivantis), 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 1 µM each primer and 0.025 U Taq polymerase (Vivantis). No-template reactions were performed as negative control for each target.

PCRs were carried out on all the samples at the same conditions: 34 cycles at 96°C for 30s, 60°C for 30s, 72°C for 45s. Results were visualized on 1.6% agarose gel stained with ethidium bromide.

Real Time PCRs were performed in 12 µl Eva Green mixand primers' concentration as follows: 400 nM for GAPDH, 450 nM for LRP10, 250 nM for DLST, 200 nM for OGDH and RALA, and 300 nM for all the other targets, using the ECO™ Real Time PCR system (Illumina). Samples were tested in duplicate and no-template reactions were performed as negative control for each target. The PCR efficiency was evaluated by creating a standard curve with 1:3 serial dilutions of the liver pool. The thermal profile for each gene was 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 10 s and 60°C for 30 s; the melting curve was created running the samples at 55°C for 5 s and 80 cycles starting at 55°C up to 95°C, increasing 0.5°C each 5 s. Relative quantification was calculated using the comparative delta-delta-Ct method (Giulietti et al., 2001) and GAPDH, HPCAL1 and LRP10 as the most stable reference genes (Hosseini et al., 2010).

All data from the quantitative PCR evaluation were elaborated with an analysis of variance using the statistical software SAS (SAS Inst. Inc., Cary, NC). All data were evaluated for normal distribution using the Kolmogorov–Smirnov test. Post-hoc tests were carried out on parametric data using the Tukey-Kramer method.

Results and Discussion

In this study we presented for the first time the effects of FA introduced in the mother's diet on visceral adipose tissues of one month suckling kids and demonstrated how these effects can impact proteome AT via milk.

We used a quantitative iTRAQ 2-D LC-MS/MS based approach to compare omental adipose tissue's proteomes and identify differentially expressed proteins. Qualitative gene expression analysis confirmed that the differentially expressed proteins were effectively produced by AT.

3.1 Protein identification and abundance differential quantification by iTRAQ analysis

Four iTRAQ runs were performed, in which the identified proteins were 837, 749, 724, 802, respectively, with high confidence and coverage despite the high amount of lipids in the samples that can interfere with LC-MS/MS analysis. Indeed, in all experiments, the average unused ProtScore was 8.41 and the average protein sequence coverage was 32.8%. Three proteins were matched to decoy (reversed) protein sequences, which gives a fraction of incorrect assignments of (2 x reversed sequence)/(reversed + forward sequence)=3/635=0.0047%. In total, 635 unique proteins with at least two unique peptides matching with MS/MS spectra were identified and quantified.

In order to identify the differentially expressed proteins in animals whose mothers were fed with different fat-enriched diets, the proteomes of control animals (CTRL-Kid) was compared to that of animals whose mothers were fed with diets enriched with stearic acid (ST-Kid) or fish oil (FO-Kid). A protein was considered differentially expressed when the *p*-value was below 0.05. In total, 20 proteins were found differentially expressed in a statistically significant way. Results are presented in Fig. 1 and Table 4.

Quantitative proteomics results indicated that out of 20 proteins found to be differentially abundant, 19 were decreased after supplementation of diet with different fatty acids, and only one was found to be increased. Of them, 11 proteins were differentially expressed in ST-Kid samples compared to CTRL-Kid samples (ST vs CTRL), while 5 proteins were differentially expressed in FO-Kid samples compared to CTRL-Kid samples (FO vs CTRL). Four proteins were differentially expressed in both ST and FO samples (Fig. 2a). The functional grouping of the differentially expressed proteins, according to Biological Process and Molecular Function, was performed using Blast2GO. The generic Blast2GO annotation was subsequently reduced to PANTHER functional terms for a segregation of the proteins in four major categories, three of which were related to metabolism or catabolism, namely proteins involved in protein, carbohydrate and lipid, nucleic acid metabolic/catabolic processes and a fourth one containing proteins which were not found to be

involved in metabolic processes (Fig. 2b). Most of the proteins found during the present investigation in omental adipose tissue were also found in previous studies on the proteome of omental adipose tissues in goats (Restelli et al., 2014). Out of the twenty proteins found to be differentially expressed, fibrinogen alpha chain (FGA), tubulin beta-5 chain-like transcript variant X2, Peptidyl-prolyl isomerase B, Enoyl-CoA delta isomerase 1 (ECl1), Nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1) and V-ral simian leukemia viral oncogene homolog A (ras related) (RALA) were not found in the previous study, and are now reported for the first time in goat AT.

3.2 Proteins found to be differentially abundant in ST-kids

The abundance of 11 proteins, namely ceruloplasmin (CP), ECl1, Glycerol-3-phosphate dehydrogenase 1 (GPD1), alanine--tRNA ligase regulatory subunit 7 (AARS), Proteasome 26S subunit ATPase 2 (PMSC2), heat shock cognate 71 kDa protein (HSPA8), 60S ribosomal protein L5 (RPL5), 60S ribosomal protein L7 (RPL7), 60S ribosomal protein L24 (RPL24), oxoglutarate dehydrogenase (OGDH) and FGA, was found to be decreased in AT of ST-kids only.

Ceruloplasmin is a minor acute phase protein in wildlife ruminants and humans (Ceciliani et al., 2012; Rahman et al., 2010). Little information about its involvement in inflammatory reaction is available in goats. The presence of ceruloplasmin in several adipose tissue depots, including base tail, sternal, perirenal and omental, was recently demonstrated, presenting the evidence that ceruloplasmin is an adipokines, at least in human species, where it was found to be overexpressed in obese adipose tissue (Arner et al., 2014). The expression of ceruloplasmin by adipose tissue was also confirmed in goats (Restelli et al., 2014). Ceruloplasmin is the principal copper carrier and in as such is involved in its distribution, storage and reduction of potential toxicity. Ceruloplasmin is also involved in angiogenesis (Linder, 2016).

Of the proteins found to be decreased, ECl1, GPD1 and ODGH are involved in carbohydrate and lipid metabolism. A decrease in enzymatic activity of ECl1 in the livers of rats fed with saturated

fatty acids (palm oil) versus livers of rats fed with PUFA has been demonstrated (Kabir and Ide, 1996). Previous findings reported that specific fatty acids may influence ECl1 activity: for example, FO increases ECl1 activity in rat heart, thus stimulating beta oxidation (Kvannes et al., 1995). Moreover, it is known that diets enriched with specific fatty acids, such as EPA and DHA, may have different impact on different adipose tissues (Todorčević and Hodson, 2015), but no reports have been provided so far about the relationship between dietary specific fatty acids and the down-regulation of specific proteins in AT. On the background that ECl1 is directly involved in fatty acid beta oxidation, the present findings therefore support the hypothesis that saturated FA, such as stearic acid, introduced in the diet may increase the lipid biosynthesis.

GPD1 catalyses the reversible conversion of dihydroxyacetone phosphate (DHAP) and reduced nicotine adenine dinucleotide (NADH) to glycerol-3-phosphate (G3P) and NAD+. Being involve din lipid biosynthesis, GPD1 activity was found to be related to obesity in humans ((Swierczynski et al., 2003). In goats, the effects of integrating diets with sunflower-seed and linseed oils on GPD1 expression in visceral AT (perirenal), among other tissues, was also investigated (L Bernard et al., 2009; Laurence Bernard et al., 2009), and no effects were found on GPD1 mRNA gene expression. In the present study GPD1 was reported to be less abundant as compared to controls at quantitative proteomic level in ST-kids. This results is apparently contradictory to the lipogenesis-enhancing effect of ECl1 decrease.

A modification of pathways related to protein metabolism and catabolism is suggested by a decrease in the abundance of ribosomal proteins, such as RPL5, RPL7 and RPL24 proteins, and of proteins involved in protein biosynthesis, such as AARS, which catalyzes the attachment of alanine to tRNA(Ala). The abundance of proteins involved as chaperons in protein biosynthesis, such as PMSC2 and HSPA8, was also decreased in ST-kids. The finding that the abundance of HSPA8 is modulated by different diets is interesting, and corresponds to what had been previously reported in liver of rats fed with a short-term high-fat sucrose diet (Bondia-Pons et al., 2011). Consistently with

the present findings, the authors found that feeding rats with fat enriched diets induce a decrease in the abundance of liver HSPA8, indirectly linking this effect to the initiation of hepatic steatosis.

3.3 Proteins found to be differentially abundant in AT from FO-kids.

A total of 5 proteins were found to be differentially expressed in FO-kids as compared with controls, of which one of them (NUCKS1) was increased, and the others (dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex (DLST), eukaryotic translation and initiation factors (EEF1G), B-cell receptor-associated protein 31 isoform X1 (BCAP31) and ras-related protein Ral-A (RALA) were decreased.

NUCKS1 is the only protein that was found to be overexpressed in FO-kids as compared to CTRL-kids. NUCKS1 is a transcriptional regulator of insulin signalling (Qiu et al., 2014) as well as a physiological regulator of energy and glucose homeostasis. The present findings confirm what has been previously reported in goats, e.g. that NUCKS1 is overexpressed during a physiological phase where adipose tissue mass is growing (Liméa et al., 2009), but are somehow contradictory with reports in other species. In mice for example, whole body depletion of NUCKS1 leads to body fat accumulation (Qiu et al., 2014). It cannot be ruled out that an overexpression of NUCKS1 may also be related to the regulation of a possible excessive growth of adipose tissue (Qiu et al., 2015).

Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex (DLST) abundance is decreased in AT from FO kids. DLST is one of the catalytic unit of 2-oxoglutarate dehydrogenase and is involved in carbohydrate and lipid metabolism. The complex catalizes the conversion of 2-oxolutarate to succinyl CoA and CO_2, and contains multiple copies of three enzymatic components, namely 2-oxolutarate dehydrogenase (OGDH), DLST and lipoamide dehydrogenase. DLST has been shown to be induced during the differentiation of 3T3-L1 adipocytes (Carothers et al., 1988). DLST was also shown to be downregulated in human adipose tissue of high insulin resistance g index (HOMA-IR) group. The other two proteins whose abundance was reduced

in ST-kids are B-cell receptor-associated protein 31 isoform X1 (BCAP31) and ras-related protein Ral-A (RALA). Both BCAP31 and RALA are involved in proliferation and apoptosis (Ruchusatsawat et al., 2017) and control of cell cycle progression and survival (O Santos et al., 2016), thus suggesting the hypothesis that FO may interact with adipose tissue re-organisation. To the best of the knowledge of the authors, their presence in AT has not been reported so far.

3.4 Proteins found to be decreased in both ST- and FO-kids

A total of 4 proteins, namely 26S protease regulatory subunit 7 (PSMC1), peptidyl-prolyl cis-trans isomerase B (PPIB), eukaryotic translation initiation factor 5A-1 (EIF5A), tubulin beta-5 chain-like transcript variant X2 (TUB5X2) were downregulated in both ST- and FO-kids. The PMSC1, PPIB and EF5A are all involved in protein metabolism and catabolism, whereas TUB5X2 belongs to the cytoskeleton. The finding that TUB5X2 abundance is decrease after diet integration with ST and FO in adipose tissue is interesting. The exact function of the beta-5 isoform of tubulin is mostly unknown. Beta-tubulin dimerises with alpha-tubulin that has been suggested to take part, together with other proteins, to the intracellular scaffolding of the Glucose receptor GLUT4 (Bouwman et al., 2009). We might therefore speculate that a decrease of TUB5X2 may interact with insulin signalling of adipocytes.

3.4 Qualitative and quantitative mRNA expression

The intact adipose tissue is crossed by a wide capillary network. The finding of a protein by proteomic techniques does not confirm per se its expression by adipose tissue, given the background that several proteins can be expressed by liver or by other tissues and then delivered to AT through blood. Therefore, in order to assess that the proteins found by proteomic were effectively produced by adipose tissue, a qualitative PCR analysis was carried out to investigate the mRNA expression of the 20 proteins found to be differentially expressed due to different maternal

diets. The effective presence of all their respective mRNA coding genes in omental kid adipose tissue was demonstrated for all of them (Fig. 1 supplemental), with the exception of fibrinogen alpha chain, which was undetectable, suggesting that this protein is delivered to adipose tissues via blood capillaries, and confirming that the other proteins found were effectively expressed within omental adipose tissue depots. In a following step, the effects of different mother' diet on gene expression of kids' omental AT were studied by means of quantitative (Real Time) PCR, aiming to explore the quantitative correspondence between gene and protein expression. The mRNA quantitative gene expression analysis results are presented in Fig. 3. Most of the mRNA were found to be apparently downregulated, albeit not in a statistically significant way. Only ECl1 gene expression was downregulated in ST-kids AT as compared to CTRL-kids and CP was downregulated in both ST- and FO-kids as compared to CTRL-kids, in a statistically significant way ($p < 0.05$). The downregulation of ECl1 and CP in ST-kids also at mRNA expression level, are consistent with quantitative proteomics results.

Conclusions

In the present study we report the first proteomic analysis of goat visceral adipose tissue after maternal diet enrichment with different fatty acids. The analysis was carried out by 2D-LC-MS/MS and iTRAQ labelling on omental samples of goat kids. We demonstrated that kids' omental proteome can be modified by maternal diet enrichments with either saturated or unsaturated fatty acids. Stearic acid induces changes in a higher number of proteins when compared to fish oil. Although there was a general corresponding between a trend in downregulation of gene expression and protein under-expression, only two proteins were found to be downregulated at mRNA in a statistically significant way. The influence of maternal diet on kids' proteome, even if not confirmed by statistically significant gene expression changes, is noteworthy and suggests that further insights are worth exploring.

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Figure legends

Fig.1. The influence of maternal diets in suckling kids' omental adipose tissue proteome.

The figure presents the different abundance of proteins extracted from omental lipid depot from suckling kids whose mother were fed with stearic acid (ST-kids – black) and fish oils (FO-kids – grey). Values are expressed as fold changes between ST-kids or FO-kids as compared to controls.

Fig. 2. The influence of maternal diets in suckling kids' omental adipose tissue proteome: functional analysis.

Proteins were sorted four groups, namely those involved in nucleic acid metabolism, protein metabolism, carbohydrate and lipid metabolism and others, following PANTHER classification (Fig. 1a). Fig 1b presents the Venn diagram, with the distribution and overlap of proteins differentially expressed in suckling kids from mothers whose diets was integrated with Stearic Acid (ST-kids) or Fish Oil (FO-kids).

Fig.3. Real Time PCR analysis results. Graphs show the expression profiles of seven selected genes in omental adipose tissue as compared to liver samples (=1) in CTRL-Kid, ST-Kid and FO-Kid. Comparison of the mRNA expression profiles of the three experimental groups show no statistically significant differences. Statistical significance was accepted at $p< 0.05$ (*).

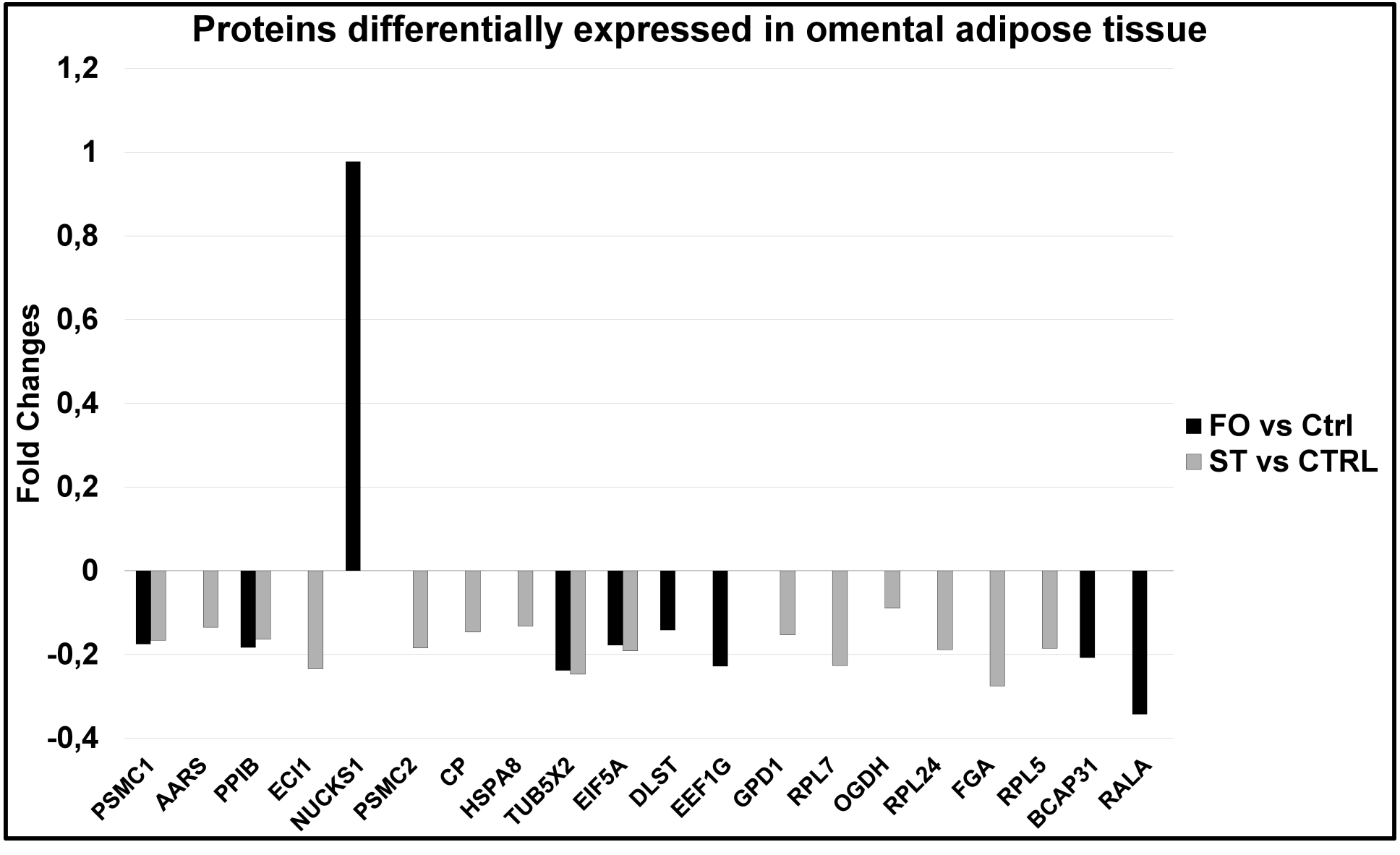
Table 1. Ingredients and chemical compositions of the experimental diets of the dairy goats fed either a basal diet (C) or a diet complemented with fish oil (FO) and stearate (ST).

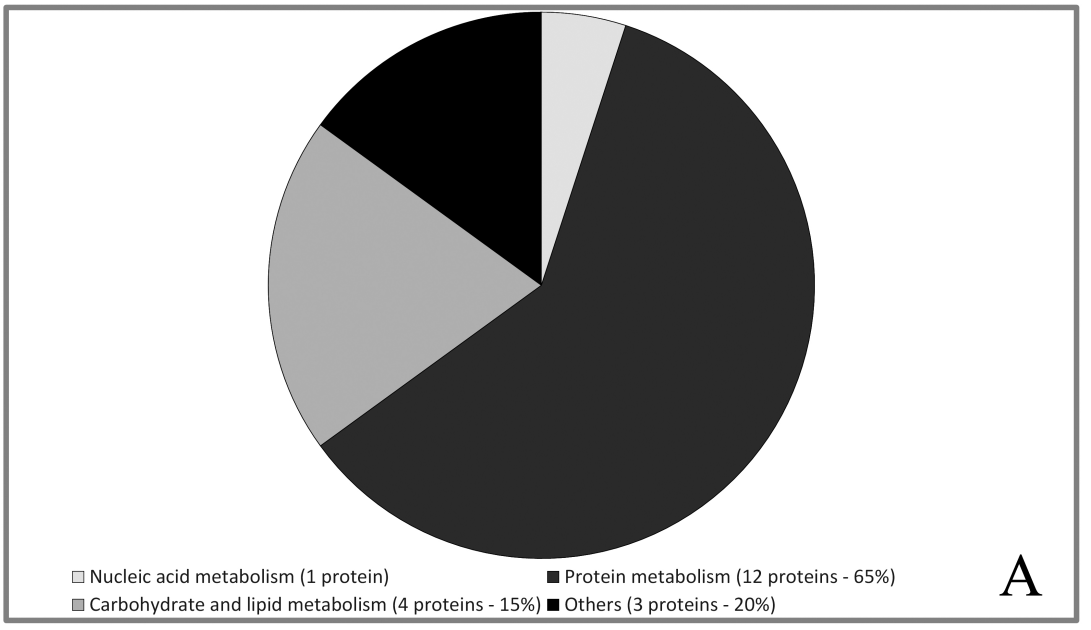
Table 2. iTRAQ labelling scheme

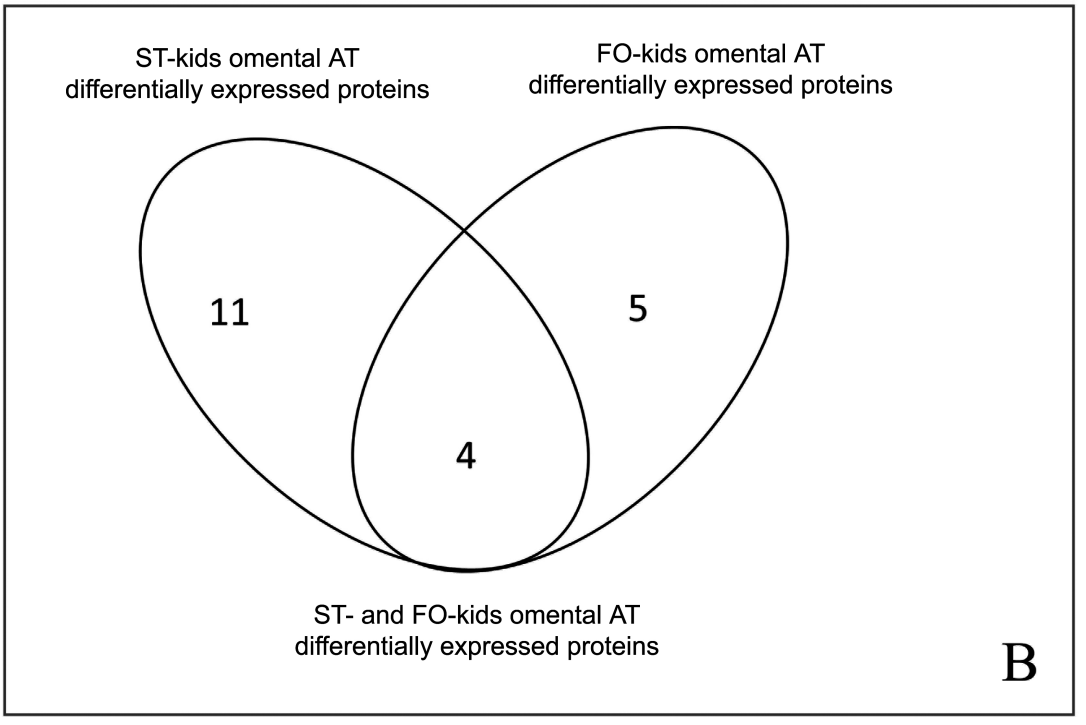
Table 3. Selected primers for mRNA expression analysis and reference genes.

Table 4. List of proteins differentially expressed in omental AT of CTRL-kids, FO-kids and ST-kids. Values are expressed as fold change between adipose tissue from kids whose mothers were fed with Fish Oil (FO vs CTRL) or Stearic Acid (ST vs CTRL) as compared to control. Functional classes are as follows: A: proteins involved in protein metabolism and catabolism pathways; B: proteins involved in lipid metabolism pathways; C: proteins involved in nucleic acid metabolism pathways; D: others.

Fig. 1 Supplemental: Qualitative PCR analysis of genes coding for proteins found as differentially expressed according to different maternal diets. Lane 1: CTRL-kids. Lane 2: FO-kids. Lane 3: St-kids. Lane 4: Positive control (Liver). Lane 5: negative control .


Proteins differentially expressed in omental adipose tissue
Fold Changes
1,2
1
0,8
0,6
0,4
0,2
0
-0,2
-0,4
FO vs Ctrl
ST vs CTRL
PSMC1
AARS
PPIB
ECI1
NUCKS1
PSMC2
CP
HSPA8
TUB5X2
EIF5A
DLST
EEF1G
GPD1
RPL7
OGDH
RPL24
FGA
RPL5
BCAP31
RALA


Nucleic acid metabolism (1 protein)
Protein metabolism (12 proteins - 65%)
Carbohydrate and lipid metabolism (4 proteins - 15%)
Others (3 proteins - 20%)
A


ST-kids omental AT
differentially expressed proteins
FO-kids omental AT
differentially expressed proteins
11
5
4
ST- and FO-kids omental AT
differentially expressed proteins
B

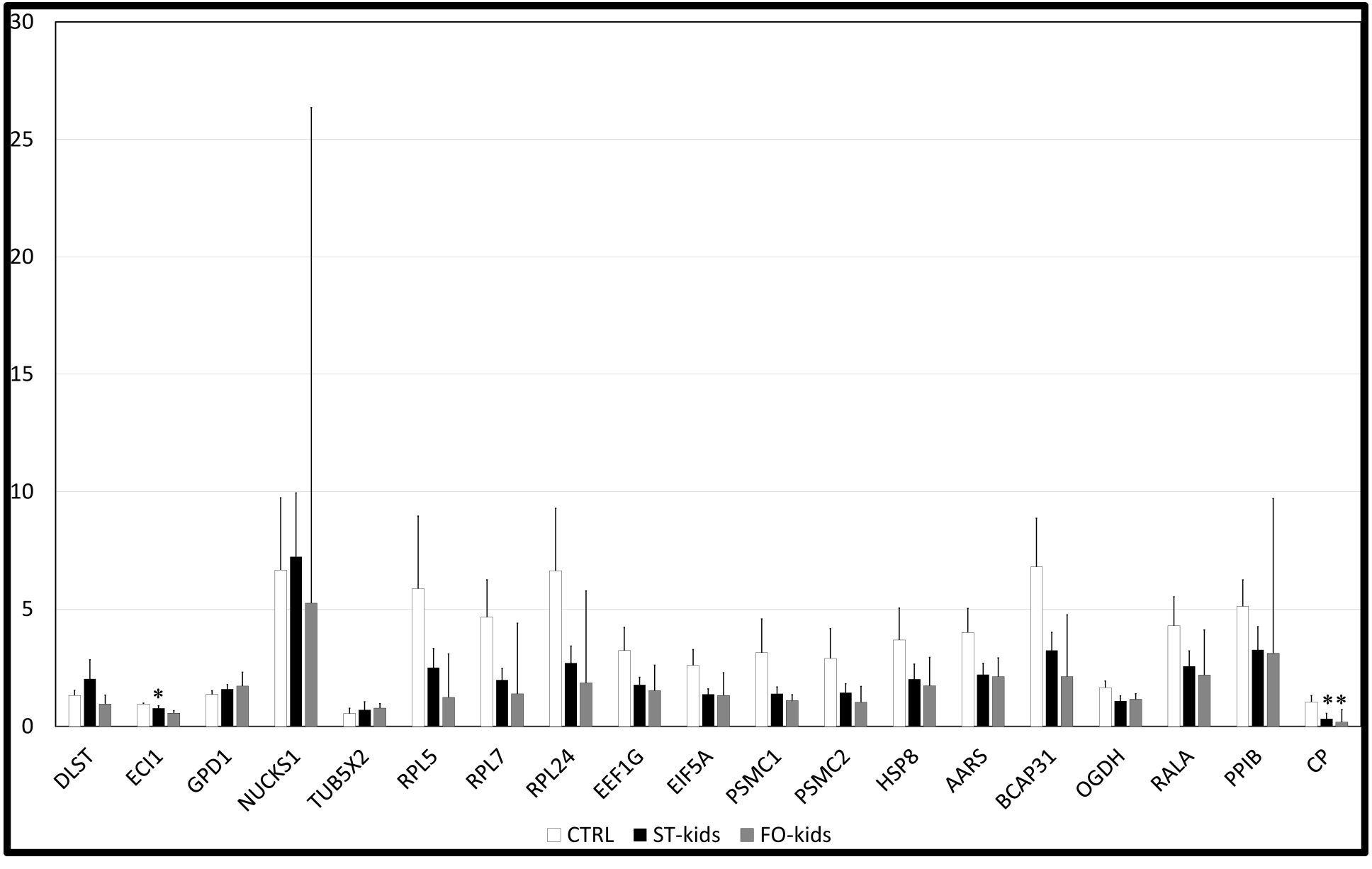

30
25
20
15
10
5
0
DLST
ECI1
GPD1
NUCKS1
TUB5X2
RPL5
RPL7
RPL24
EEF1G
EIF5A
PSMC1
PSMC2
HSP8
AARS
BCAP31
OGDH
RALA
PPIB
CP
*
**
CTRL
ST-kids
FO-kids

Table 1

Ingredients and chemical composition of the experimental diets of the dairy goats fed either a basal diet (CTRL) or a diet supplemented with fish oil (FO) or stearic acid (ST).

	Experimental diets					
	Pre-kidding			Post-kidding		
	CTRL	FO	ST	CTRL	FO	ST
Ingredient (%)						
Alfalfa hay	0.0	0.0	0.0	31.2	29.8	30.7
Mixture hay[2]	62.3	59.6	61.4	15.3	14.6	15.1
Concentrate mixture[1]	31.9	30.5	31.4	46.8	44.8	46.2
Corn meal	5.3	5.0	5.2	6.2	5.9	6.2
Fish oil	0.0	4.4	0.0	0.0	4.3	0.0
Calcium Stearate	0.0	0.0	2.0	0.0	0.0	1.9
$CaCO_3$	0.5	0.5	0.0	0.5	0.5	0.0
Chemical Composition (% of dry matter)						
Dry Matter (%)	88.4	88.7	88.6	89.3	89.5	89.4
Crude Protein	12.3	11.9	12.2	17.8	17.2	17.5
Ether Extract	2.9	4.9	4.5	3.2	5.2	4.8
NDF	43.9	43.8	43.3	33.7	34.0	33.2
Ashes	6.3	6.5	6.0	7.2	7.3	6.8
Ca	0.8	0.8	0.9	1.1	1.1	1.2
P	0.4	0.4	0.4	0.8	0.8	0.8
NE_L (Mcal/kg DM)[3]	1.61	1.66	1.67	1.67	1.72	1.72

[1] The concentrate mixture was a commercial dairy goat mixed feed, chemical composition: 22.25% crude protein, 5.00% ether extract, 22.98% neutral detergent fiber, 6.51% ashes, 1.28% Ca and 0.76% P (on dry matter basis).
[2]The mixture hay was a grass hay, chemical composition: 7.6% crude protein, 1.8% ether extract, 57.5% neutral detergent fiber, 5.9% ashes, 0.6% Ca and 0.2% P (on dry matter basis).
[3]Net energy of lactation concentration of the diets were determined using the Small Ruminant Nutrition System (SRNS) software (Tedeschi et al., 2010)

Table 2.

iTRAQ labelling scheme

	iTRAQ labelling and reporter ions			
iTRAQ runs	**114**	**115**	**116**	**117**
1	Ref[1]	C_1	FO_1	ST_1
2	Ref[1]	C_2	FO_2	ST_2
3	Ref[1]	C_3	FO_3	ST_3
4	Ref[1]	C_4	FO_4	ST_4

[1]reference sample was created by pooling equal amounts of the four control samples

Table 3. Selected proteins for mRNA expression analysis and housekeeping genes, accession numbers, primers sequences and length of the amplified fragments. In the last column Real Time PCR efficiency and R^2 are shown.

Sequence name	**Symbol**	**Accession number**	**Primer Forward (5'-3')**	**Primer Reverse (5'-3')**	**Lenght (bp)**	**PCR efficiency and R^2**
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_001034034	GGCGTGAACCACGAGAAGTATAA	CCCTCCACGATGCCAAAGT	119	104.53 0.993
Hippocalcin-like 1	HPCAL1	Hosseini et al., 2010	CCATCGACTTCAGGGAGTTC	CGTCGAGGTCATACATGCTG	99	105.72 0.995
Low density lipoprotein receptor-related protein 10	LRP10	Hosseini et al., 2010	CCAGAGGATGAGGACGATGT	ATAGGGTTGCTGTCCCTGTG	139	93.22 0.990
Ceruloplasmin	CP	NM_001256556.1	GAGCATGAAGGGGCCATTTATC	GCTGTCTTCCTCACCAGG	130	94.18 0.995
Fibrinogen Alpha chain	FGA	NM_001033626.1	TGAGATCCTGAGGCGCAAAG	TGTCCACCTCCAATCGTTTCAT	104	- -
Ribosomal protein L5	RPL5	XM_005678097.1	AGACGAGAGGGCAAAACTGA	ACGGGCATAAGCAATCTGAC	138	104.68 0.995
Ribosomal protein L7	RPL7	XM_005689063.1	TGCATTGATTGCTCGATCTC	TTCCACCTCGTGGAGAAGAC	143	101.32 0.995
Ribosomal protein L24	RPL24	XM_005674869.1	AAGAAAAGAACTCGCCGTGC	TTCCTTGGCAGCCCTGATAG	138	105.16 0.997
Alanyl-tRNA synthetase	AARS	XM_005691789.1	CTCCAGTGGGACCTACGTGT	TTCACCAGGTACCCTTCGTC	174	102.64 0.995
Tubulin beta-5 chain-like transcript variant X2	TUB5X2	XM_005696629.1	ACAATGAAGCCACAGGTGGC	CATCCAGGACCGAGTCAACC	204	108.59 0.991
Dihydrolipoamide S-succinyltransferase	DLST	XM_005686086.1	CTTCAGCCTTTGCCTTGCAG	TGGTTCGCTCAATATCGGCA	180	99.73 0.990
Glycerol-3-phosphate dehydrogenase 1	GPD1	XM_005679977.1	GCCGACATCCTGATCTTTGT	GCTCCCCAATCACTTCAGAG	160	91.94 0.990

Eukaryotic translation elongation factor 1 gamma	EEF1G	XM_005699776.1	CTGAGGAAGAATGCCTTTGC	CGTAGTCCACCTGCCAATCT	130	108.83 0.994
Eukaryotic translation initiation factor 5A	EIF5A	XM_005693488.1	ATCACTGCTCCAAGACAGCG	CCGTGATCAGGATCTCTTCTCC	113	107.57 0.997
Oxoglutarate dehydrogenase	OGDH	XM_005679326.1	TTCCATGTGAACTCGGATGA	GCTTCTGTTTTCGGATCTGC	187	106.97 0.993
Heat shock 70kDa protein 8	HSPA8	XM_005689565.1	AACCAAGTCGCAATGAATCC	AGCATCATTCACCACCATGA	126	102.12 0.992
Peptidyl-prolyl isomerase B (cyclophilin B)	PPIB	XM_005685667.1	AGGGCATGGATGTAGTACGG	GCTTCTCCACCTCGATCTTG	108	106.21 0.997
Enoyl-CoA delta isomerase 1	ECI1	XM_005697442.1	CTGGCTGACAACCCCAAGTA	TGCCCAATGGTGTTCACGTA	101	109.55 0.991
Nuclear casein kinase and cyclin-dependent kinase substrate 1	NUCKS1	XM_005690441.1	CACAGCTTCAAAGGCATCAA	ACCCTTCATCCCCAGATTTC	125	104.52 0.991
Proteasome (prosome, macropain) 26S subunit ATPase 1	PSMC1	XM_005686218.1	CTCACACTCAGTGCCGGTTA	AAGGCTTCATTTGCTCCTGA	102	109.44 0.996
Proteasome (prosome, macropain) 26S subunit ATPase 2	PSMC2	XM_005679109.1	CTGACTCAGAGGACCCGAAG	TCTTAGGAGGCAATGGGATG	159	92.54 0.993
B-cell receptor-associated protein 31	BCAP31	XM_005700513.1	ACCTGCTCAAGAAGGAAGCTG	CTTCAGGCTCCTGTTCTCTTCC	89	109.96 0.998
V-ral simian leukemia viral oncogene homolog A (ras related).	RALA	XM_005679348.1	TGGGCAAGAAGACTACGCTG	AAATCTGCTCCCTGAAGTCGG	125	108.64 0.992

Table 4 – List of proteins differentially expressed in omental adipose tissue					
Gene name	Protein name	NCBI accession code	FO vs Ctrl*	ST vs CTRL*	Functional class
PSMC1	26S protease regulatory subunit 7 [Capra hircus]	gi|548462854	-0,175584992	-0,166148404	A
AARS	alanine--tRNA ligase, cytoplasmic [Capra hircus]	gi|548502423		-0,13519305	A
PPIB	peptidyl-prolyl cis-trans isomerase B [Capra hircus]	gi|548482820	-0,183111474	-0,163187057	A
ECI1	enoyl-CoA delta isomerase 1, mitochondrial, partial [Capra hircus]	gi|548519773		-0,233717779	B
NUCKS1	nuclear ubiquitous casein and cyclin-dependent kinase substrate 1 [Capra hircus]	gi|548497778	0,977696419		C
PSMC2	LOW QUALITY PROTEIN: 26S protease regulatory subunit 4 [Capra hircus]	gi|548484734		-0,184552352	A
CP	ceruloplasmin-like [Capra hircus]	gi|548451476		-0,146306574	A
HSPA8	heat shock cognate 71 kDa protein [Capra hircus]	gi|548494982		-0,131878406	A
TUB5X2	tubulin beta-5 chain-like isoform X2 [Capra hircus]	gi|548518047	-0,238568455	-0,247178301	D
EIF5A	eukaryotic translation initiation factor 5A-1 [Capra hircus]	gi|548507861	-0,178151488	-0,190938696	A
DLST	dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial, partial [Capra hircus]	gi|548484104	-0,141939223		B
EEF1G	elongation factor 1-gamma [Capra hircus]	gi|548527699	-0,228011027		A
GPD1	glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic [Capra hircus]	gi|548465403		-0,152948648	B
RPL7	60S ribosomal protein L7 [Capra hircus]	gi|548493434		-0,226428777	A
OGDH	2-oxoglutarate dehydrogenase, mitochondrial isoform X4 [Capra hircus]	gi|548463635		-0,089515448	B
RPL24	60S ribosomal protein L24 [Capra hircus]	gi|548449536		-0,188844249	A
FGA	fibrinogen alpha chain [Capra hircus]	gi|548499843		-0,275586635	D
RPL5	60S ribosomal protein L5 [Capra hircus]	gi|548459269		-0,185251758	A
BCAP31	B-cell receptor-associated protein 31 isoform X1 [Capra hircus]	gi|548530270	-0,20763582		A
RALA	ras-related protein Ral-A [Capra hircus]	gi|548463681	-0,342846255		D

* Values are expressed as fold change between adipose tissue from kids whose mothers were fed with Fish Oil (FO vs CTRL) or Stearic Acid (ST vs CTRL) as compared to control. Functional classes are as follows: A: proteins involved in protein metabolism and catabolism pathways; B: proteins involved in lipid metabolism pathways; C: proteins involved in nucleic acid metabolism pathways; D: others.