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Metabolome and proteome changes in skeletal muscle and blood of pre-weaning calves fed leucine and threonine supplemented diets

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Highlights

- Leucine and threonine are essential amino acids for calves.
- Calves received milk replacer supplemented or not with 3% leucine and 3% threonine.
- Supplementation with Thr and Leu leads to higher citrate cycle activity and OXPHOS.
- Supplementation with Leu and Thr increased antioxidant defenses.

- Supplementation with Leu and Thr increases the muscle Phospho-S6/S6 ratio.

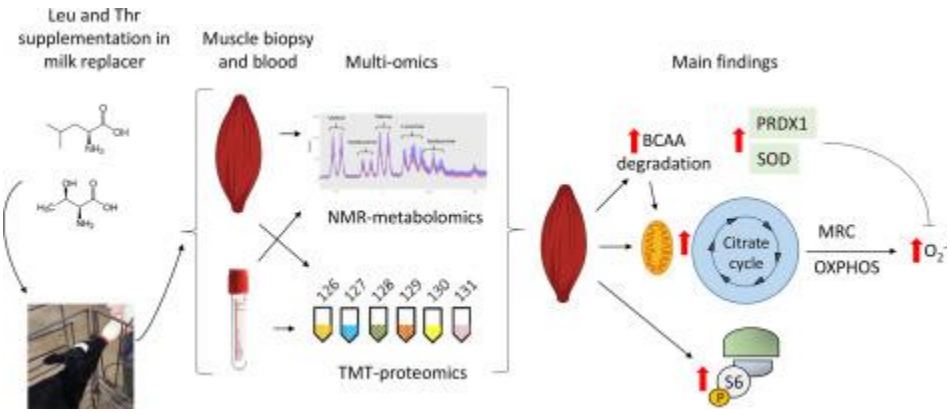
Abstract

In pre-weaning calves, both leucine and threonine play important roles in growth and muscle metabolism. In this study, metabolomics, proteomics and clinical chemistry were used to assess the effects of leucine and threonine supplementation added to milk replacer on 14 newborn Holstein male calves: 7 were fed a control diet (Ctrl) and 7 were fed the Ctrl diet supplemented with 0.3% leucine and 0.3% threonine (LT) from 5.6 days of age to 53.6 days. At this time, blood and semitendinosus muscle biopsies were collected for analysis. Integrated metabolomics and proteomics showed that branched-chain amino acids (BCAA) degradation and mitochondrial oxidative metabolism (citrate cycle and respiratory chain) were the main activated pathways in muscle because of the supplementation. BCAA derivatives and metabolites related to lipid mobilization showed the major changes. The deleterious effects of activated oxidative phosphorylation were balanced by the upregulation of antioxidant proteins. An increase in protein synthesis was indicated by elevated aminoacyl-tRNA biosynthesis and increased S6 ribosomal protein phosphorylation in skeletal muscle. In conclusion, LT group showed greater BCAA availability and mitochondrial oxidative activity; as the muscle cells undergo greater aerobic metabolism, antioxidant defenses were activated to compensate for possible cell damage. Data are available via ProteomeXchange (PXD016098).

Significance

Leucine and threonine are essential amino acids for the pre-weaning calf, being of high importance for growth. In this study, we found that leucine and threonine supplementation of milk replacer to feed pre-weaning calves led to differences in the proteome, metabolome and clinical chemistry analytes in skeletal muscle and plasma, albeit no differences in productive performance were recorded. This study extends our understanding on the metabolism in dairy calves and helps optimizing their nutritional status.

Graphical abstract



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Keywords

Amino acid
Calf
Muscle
Plasma
Metabolomics
Proteomics

Abbreviations

AA
amino acid(s)
BCAA
branched-chain amino acid(s)
BCKA
branched-chain keto acid(s)
BW

body weight
CT
computed tomography
DM
dry matter
MR
milk replacer
mTOR
mammalian target of rapamycin
PON1
paraoxonase-1
TSP
total protein staining

1. Introduction

Amino acids (AA) are important for the growth of pre-weaning calves being pivotal for tissue development, endocrine, and immune function [1,2]. Leucine is an essential AA and one of the more studied branched-chain AA (BCAA) in several species due to its function in promoting protein synthesis through the mammalian target of rapamycin (mTOR) pathway in skeletal muscle [3,4]. Leucine also enhances energy homeostasis through increasing fatty acid oxidation and provides skeletal muscles with an increased flux of lipids to supply energy and intermediary substrates to support protein synthesis [5]. Leucine from dietary protein can bypass metabolism in the liver, which results in a rise of plasma leucine levels and the activation of leucine signaling in peripheral tissues in response to a meal [6] and, among these tissues/organs, the skeletal muscle is the main site for BCAA oxidation [7]. Threonine is another essential AA that is often considered limiting in milk replacers (MR) for calves [8]. It participates in the synthesis of several other important AA such as glycine and serine which are involved in the production of collagen and muscle tissue, it also plays a central regulatory role in the immune system and is a major component of intestinal mucins and plasma γ-globulins [1,9].

To understand the role of nutritional compounds at the molecular level [10] and to analyze the coordination of multiple systems including for instance the immune, digestive and endocrine, the identification of large sets of biological molecules is required. Accordingly, “multi-omics” approaches have gained popularity in animal science. The combination of proteomics and metabolomics in food and nutrition science, more recently named as “foodomics”, has been shown to be a more suitable approach compared to other “-omics” such as genomics or transcriptomics [10].
The objective of this work is to use a foodomics-based approach in calves' skeletal muscle and plasma in order to fully assess the effects of leucine and threonine supplementation in the milk replacer (MR) during the pre-weaning period, under the hypothesis that AA supplementation will modify the energy metabolism, especially in the muscle tissue. Furthermore, the activation of the mTOR pathway was studied in skeletal muscle by determining the phosphorylation of S6 ribosomal protein, a key event in protein synthesis.

2. Materials and methods

2.1. Animal housing and diet management

Animal management followed the recommendations of the Animal Care Committee of *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA) under the approval research protocol FUE-2017-00587321, authorization code 9733.
Fourteen Holstein male calves (41.9 ± 0.67 kg of body weight (BW) at age of 5.6 ± 0.67 days) were obtained from *Granja Murucuc* (Gurb, Barcelona, Spain) and housed individually at IRTA *Torre Marimon* (*Caldes de Montbui*, Barcelona, Spain) and fed a milk protein-based MR twice a day. The MR contained 24% crude protein (based in skimmed milk protein and whey protein concentrate) and 20.3% fat, on a DM basis. Calves were fed 4 L/day at 15% dry matter (DM) concentration from days 1 to 7 of experiment. Then, MR volume was increased to 6 L/day from 8 to 49 days of age, and it was reduced to one single feeding of 3 L/day until animals were weaned. Calves were divided into control (Ctrl, $n = 7$) and Ctrl supplemented with 0.3% leucine and 0.3% threonine (LT, $n = 7$) groups according to the AA profile in the MR formula. Ingredient and chemical composition are shown in Supplementary material (Table S1). All AA were provided by Livzon Group (Fuxing Pharmaceutical, Ningxia,

China). All animals had free access to water, and restricted access to a pelleted concentrate starter feed to minimize the impact of concentrate feeding on AA supply. The starter feeding program is shown in Supplementary material (Table S2). Chopped barley straw was offered ad libitum during the study.

2.2. Sample collection

At 53.6 ± 0.67 days of age and 81.8 ± 3.49 kg of BW, blood was obtained from the jugular vein, 4 h after the morning MR feeding. Plasma and serum were obtained by centrifugation at 1500*g* for 10 min. Plasma and serum were aliquoted and stored at −80 °C until further analysis. Biopsies were obtained from the semitendinosus muscle as previously described [11] and kept at −80 °C until further analysis. Prior to Omics assays, frozen muscle tissues were ground into a fine powder with mortar and pestle in liquid nitrogen and at least two 0.5 g aliquots were made and kept in a 15 mL polypropylene vial at −80 °C.

2.3. Serum clinical chemistry and plasma AA

Serum clinical chemistry analytes were determined with an Olympus AU400 analyzer (Olympus corp., Tokyo, Japan). Methods, reagents and intra-assay CV (%) were previously reported [12]. Plasma AA were determined by HPLC as described [11] with some modifications. Briefly, an Elite LaChrom (Hitachi, Tokyo, Japan) equipped with an UV detector (Hitachi L-24200, Tokyo, Japan) with a Novapak C18 column (300 mm × 3.9 mm) from Waters (Milford, MA, USA) were used. The flow rate was 1.0 mL/min, and the column temperature was kept at 42 °C. The injection volume was 10 µL and the detection wavelength was set at 254 nm. The solvent system consisted of two eluents: (A) 140 mM sodium acetate trihydrate, 12 mM TEA and 1 mg/L EDTA at pH 5.02 and (B) 60% acetonitrile/40% water. The Agilent software EZChrom Elite system V3.1.7 (Santa Clara, CA, USA) was used for system control and data acquisition.

2.4. Proteomic analysis

2.4.1. Protein extraction and quantification

One aliquot of semitendinosus muscle fine powder in a 15 mL tube was sonicated in 3 mL extraction buffer (137 mM NaCl, 50 mM HEPES, 2% SDS, 1% NP-40, 10% glycerol, 2 mM Na_3VO_4, 100 mM NaF and 1% protease inhibitor cocktail (Sigma), pH 7.4) at 40% amplitude following a sequence of 10-s on and 5-s off, three times on ice. The homogenate was centrifuged at 12,000*g* for 15 min at 4 °C and the supernatant (muscle extract) retrieved. Protein concentration was determined using the Bradford method. Samples were kept at −80 °C until analyses.

2.4.2. Protein immunoblot for S6 and Phospho-S6 in muscle

All antibodies were purchased from Cell Signaling Technology (Denvers, MA, USA). 15 µg of muscle extract per sample were subjected to 12% SDS-PAGE and transferred to PVDF membranes. Total protein staining (TSP) by Ponceau S was performed for band intensity normalization on membrane after transfer. The membranes were then immunoblotted with primary antibodies (1:2000) against S6 ribosomal protein (#2217) and phospho-S6 ribosomal protein (#4858). The dilution for secondary antibodies were 1:5000 and 1:2000 for S6 and phospho-S6, respectively. Immunoblot bands were processed using Multi Gauge software (Fujifilm, Tokyo, Japan). Each sample was normalized by dividing the band density by its normalization factor. The latter was in turn calculated taking into account the mean TPS density of all gels and the mean TPS density of the gel where the sample was placed. Immunoblots were repeated three times with a random distribution of samples between gels.

2.4.3. Sample preparation for high resolution LC-MS/MS

Muscle samples were processed using Filter-Aided Sample Preparation (FASP) with some modifications [13]. Briefly, for each sample and internal standard (pooled sample), 35 µg of proteins were diluted in UA buffer (8 M urea in 0.1 M Tris/HCl, pH 8.5) to 200 µL. They were then added to a 10 kDa filter unit (Microcon YM-10, Merck Millipore, Burlington, MA, USA), centrifuged at 13,000*g* for 15 min at 20 °C and washed by adding 200 µL of UA buffer followed by centrifugation. Samples were alkylated with 100 µL iodoacetamide (0.05 M IAA in UA buffer) for 20 min in the dark at RT. Additional series of washes with 100 µL of UA buffer (twice) and 100 µl TEAB (0.1 M triethylammonium bicarbonate) (twice) with subsequent centrifugation

were conducted. Overnight digestion at 37 °C was performed with 50 µL of 0.1 M TEAB containing 2% trypsin (*v*/v). Tryptic peptides were eluted by centrifugation at 13,000 *g* for 10 min and washed with 50 µL of buffer (50% acetonitrile in 0.1 M TEAB, v/v). Collected flowthrough was dried and used for TMT labelling. Before labelling, pellets were dissolved in 50 µL of 0.1 M TEAB.
Plasma-EDTA samples were prepared as described [14] with some modifications. Briefly, 35 µg of proteins per sample or internal standard were diluted with 100 µL 0.1 M TEAB containing 1% SDS, reduced, alkylated and acetone precipitated overnight. Protein pellets were collected by centrifugation and dissolved in 50 µL TEAB containing 1 µg trypsin for overnight digestion at 37 °C.

2.4.4. Tandem Mass Tag (TMT) labelling for muscle and plasma proteins

TMTsixplex™ Isobaric Label Reagent Set (#90061) from Thermo Fisher Scientific (Waltham, MA, USA) was used for peptide labelling. All reagents were prepared as previously reported [14,15]. Briefly, for the labelling, 19 µL of reconstituted TMT reagent was added to each tryptic digested sample and incubated for 1 h at room temperature. Internal standard was labelled with the TMT *m/z* 126 while other tags were randomized. The reaction was quenched by adding 8 µL of 5% hydroxylamine to each sample and a 15 min incubation. For each sixplex, samples were combined at equal amounts, aliquoted, dried in a vacuum centrifuge and kept in −80 °C until further analysis.

2.4.5. High resolution LC-MS/MS analyses and bioinformatics

Mass spectrometric analysis was conducted on an Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) and Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as reported [14]. In short, after desalting on the trap column, TMT-labelled peptides were separated on the analytical column (PepMap™ RSLC C18, 50 cm × 75 µm) using a 120-min linear gradient of 5–45% mobile phase B (0.1% formic acid in 80% ACN) at the flow rate of 300 nL/min. Peptides were introduced into the mass spectrometer using a 10 µm-inner diameter SilicaTip emitter (New Objective, Woburn, MA, USA) in a nanospray Flex ion source. For DDA analysis, the Top-8 method was used.

Proteome Discoverer (version 2.0, Thermo Fisher Scientific, Waltham, MA, USA) was used for peptide identification and relative quantification. SEQUEST algorithm database search against *Bos taurus* FASTA files (NCBI database, 7/12/2017, 46,105 entries) was performed. Proteins with at least two unique peptides and 5% FDR were reported as validly identified. Furthermore, for reporter ion-based protein quantification, internal standards were used to compare the data between the TMT sixplexes. For bioinformatics, NCBI accession number (gi number) was converted to gene symbol by Biological Database Network (https://biodbnet-abcc.ncifcrf.gov/db/db2db.php) and protein function was explored with PANTHER (http://pantherdb.org/).

2.4.6. Validation

PON1 esterase activity was measured in plasma-heparin samples by analyzing the hydrolysis of phenyl acetate (arylesterase activity, ARE), as previously described [16].

2.5. Metabolomic analysis

2.5.1. Muscle and serum extractions

The extraction of the aqueous metabolites from muscle powder was performed as described [11]. For serum, 300 µL of each sample were extracted by methanol protein precipitation method as described [17], with the exception that dried samples were resuspended in 600 µl of NMR buffer.

2.5.2. Data acquisition, processing and analyses

For muscle, data acquisition method and parameters, data processing protocols and analyses were as described previously [11]. For serum samples, 128 scans and 8 test scans were applied. All raw data have been deposited to the EMBL-EBI MetaboLights database (https://www.ebi.ac.uk/metabolights/MTBLS1045).

2.5.3. Spectral profiling for chemometrics

All 1r data from either biological sample collected from Bruker were uploaded to NMRProcFlow 1.2 Online spectral processing tool interface [18]. Spectra were calibrated referring to the ppm and some peaks were aligned. H_2O peak was zeroed. For bin data, peaks were bucketed following previous knowledge of assignment. Peak intensities for each sample were normalized by its own weight for muscle, no normalization for serum, and centered-scaled for both.

2.6. Statistics

All statistical analyses were performed using R (version 3.5.2) under RStudio (version 1.2.1335), "dplyr" [19], "reshape2" [20], "ggpubr" [21], "ggplot2" [22], "corrplot" [23], "nortest" [24] and "openxlsx" [25] packages were used for data manipulation, statistics and graphic processes. Performance and intake data were analyzed using a mixed-effects model accounting for the fixed effects of treatment, time of measurement, and their 2-way interaction, and the random effect of calf. Time entered the model as a repeated measure using an autoregressive covariance matrix. Initial BW was used as a covariate in the model. For statistical difference (univariate analyses) in the serum clinical chemistry, plasma AA and metabolomic results, a one-way analysis of variance (ANOVA) was used and for western blot and proteomic data, a Wilcoxon test was used, p between 0.05 and 0.1 was inspected, while <0.05 was considered significant. For MS-proteomics, fold change between two groups was calculated using the function of log2(Mean(LT)/Mean(Ctrl)). Absolute value of fold change ≥0.20 and $p < .05$ were considered significant. Additionally, some proteins/metabolites with p between 0.05 and 0.1 or/and with fold changes between 0.10 and 0.20 showing significant correlations with representative metabolites, were also included in the analysis.
For univariate analyses in metabolomics, significant chemical shifts from chemometrics were inspected to assist the identification. For multivariate analyses, principle component analyses (PCA) with loadings of both quantified and chemometric data were plotted. The correlation plot in metabolomic results was made and Pearson r was used as distance measurement.

All concentrations are presented in form of mean ± SE.

2.7. Proteomic-metabolomic interactive analyses

For significant proteins and metabolites, their official gene symbols (proteins) and compound names (metabolites) were analyzed using Reactome (version 69 released on June 12, 2019) (https://reactome.org/) [26] and Joint Pathway Analysis provided by MetaboAnalyst. Human (*Homo sapiens*) was selected as species since *Bos taurus* was not available in both databases. The hypergeometric test, degree centrality and gene-metabolite pathways options were used for enrichment, topology and pathway analyses, respectively, in the MetaboAnalyst. For unannotated proteins, literature and Uniprot were referred.

3. Results

3.1. Performance results

The mean average daily gain (ADG) was 0.89 kg/d and 0.96 kg/d in Ctrl and LT calves respectively. No significant differences between groups in ADG, BW or food intake were recorded. Complete results are shown in Supplementary material (Table S3).

3.2. Blood clinical chemistry and AA profile

Analytes with significant differences between groups are shown in Table 1. Decreased GPx, increased cholesterol and a tendency for an increase in triglycerides were observed in the LT group. There was an increase in plasma isoleucine and histidine, and a tendency for an increase in leucine concentrations. Complete results are shown in Supplementary material (Table S4). All blood biochemistry parameters were within the reference interval for this species [12].

3.3. Proteomics in skeletal muscle and plasma

The differentially abundant proteins in muscle and plasma from both groups are shown in Table 2, Table 3, respectively, and the raw proteomic data are presented in Supplementary material (Table S5 for muscle and Table S6 for serum, respectively. The mass spectrometry proteomics data have been deposited to the ProteomeXchange

Consortium via the PRIDE [27] partner repository with the dataset identifier PXD016098.

In muscle, nine proteins were found with higher abundances (HADHA, PRDX1, SOD2, COX2, COX5, NDUFAB1, ACO2, SLC25A4 and VCP) and two with lower abundances (GYS1 and RTN2) in LT animals in comparison to Ctrl animals. For increased abundance proteins, GO analysis indicates a positive regulation of ATP biosynthetic process (GO:2001171) and the removal of superoxide radicals (GO:0019430) as the most relevant biological processes and the antioxidant activity (GO:0016209) as the first molecular function. Complete GO analysis is shown in Supplementary material (Table S7).

In plasma, five proteins were found with higher abundance (APOA2, APOC3, CRP, F2 and PON1) in treated animals. The GO analysis for biological process showed that three of them (APOA2, APOC3 and PON1) are related to regulation of cholesterol transport (GO:0032374) and chylomicron assembly (APOA2 and APOC3) (GO:0034378). CRP and F2 are associated to the acute phase response (GO:0006953). The GO analysis for molecular function showed three proteins related to lipoprotein receptor binding (APOA2, APOC3 and PON1) (GO:0070325). Proteins with lower abundances in the LT group included six isoforms of the SERPINA3 protein (SERPINA3–1, −2, −5, −6, −7 and − 8), ITIH4 and FN1. All eight proteins are involved in the GO peptidase regulator activity (GO:0061134) molecular function and all, with the exception of FN1 are also related to serine-type endopeptidase inhibitor activity (GO:0004867). Complete GO analysis is shown in Supplementary material (Table S8).

For validation of proteomic results, plasma PON1 was determined and found increased with a statistical tendency ($p = .06$) in LT (125.55 U/L ± 9.70) compared to Ctrl (88.16 U/L ± 11.58).

Table 1. Concentration of serum analytes and plasma AA which were found significantly different between Ctrl and LT groups (see Table S4 for complete results).

Analyte	Ctrl	LT	*p*-value
Serum clinical chemistry			
Cholesterol (mg/dL)	60.29 ± 4.8	84.56 ± 9.48	0.041
Glutathione Peroxidase (GPx) (U/L)	423.84 ± 38.22	321.89 ± 16.71	0.031
Triglyceride (mg/dL)	19.69 ± 3.26	40.26 ± 11.21	0.058

Analyte	Ctrl	LT	*p*-value
Plasma AA (μM)			
Histidine	71.45 ± 4.53	92.29 ± 9.06	0.048
Isoleucine	94.72 ± 3.52	127.28 ± 13.37	0.017
Leucine	164.74 ± 10.36	220.37 ± 28.84	0.056

Table 2. Proteins with significantly differential abundances in skeletal muscle identified in Ctrl and LT groups.

Gene name (*Bos taurus*)	Protein description	*p*-value	fold change (LT vs Ctrl)
HADHA	Trifunctional enzyme subunit alpha, mitochondrial	0.037	0.37
PRDX1	Peroxiredoxin-1	0.030	0.26
SOD2	Superoxide dismutase 2, mitochondrial	0.043	0.26
SLC25A4	ADP/ATP-carrier protein	0.041	0.21
VCP	Transitional endoplasmic reticulum ATPase	0.041	0.20
COX2	Cytochrome c oxidase	0.030	0.19
COX5A	Cytochrome c oxidase, subunit 5A, mitochondrial	0.030	0.19
NDUFAB1	Chain U, Acyl Carrier Protein, mitochondrial	0.041	0.19
ACO2	Aconitase 2, mitochondrial	0.071	0.11
GYS1	Glycogen synthase 1 protein	0.025	−0.29
RTN2	Reticulon-2	0.010	−0.25

Table 3. Proteins with significantly differential abundances in plasma identified in Ctrl and LT groups.

Gene name (*Bos taurus*)	Protein description	*p*-Value	fold change (LT vs Ctrl)
PON1	Paraoxonase 1	0.004	0.41
APOA2	apolipoprotein A-II	0.009	0.35
CRP	C-reactive protein	0.025	0.35
APOC3	Apolipoprotein C-III	0.015	0.33
F2	Prothrombin	0.041	0.20
SERPINA3–5	Serpin A3–5	0.018	−0.44
SERPINA3–6	Serpin A3–6	0.018	−0.44
ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4	0.015	−0.34
SERPINA3–8	Serpin A3–8	0.006	−0.31
FN1	Fibronectin	0.034	−0.24

Gene name (*Bos taurus*)	Protein description	*p*-Value	fold change (LT vs Ctrl)
SERPINA3–7	Serpin A3–7	0.041	−0.20
SERPINA3–2	Serpin A3–2	0.013	−0.19
SERPINA3–1	Serpin A3–1	0.018	−0.19

3.4. Phosphorylation status of S6 in skeletal muscle

The ratio of Phospho-S6/S6 in muscle was significantly increased in LT compared with Ctrl calves, as shown in Fig. 1.

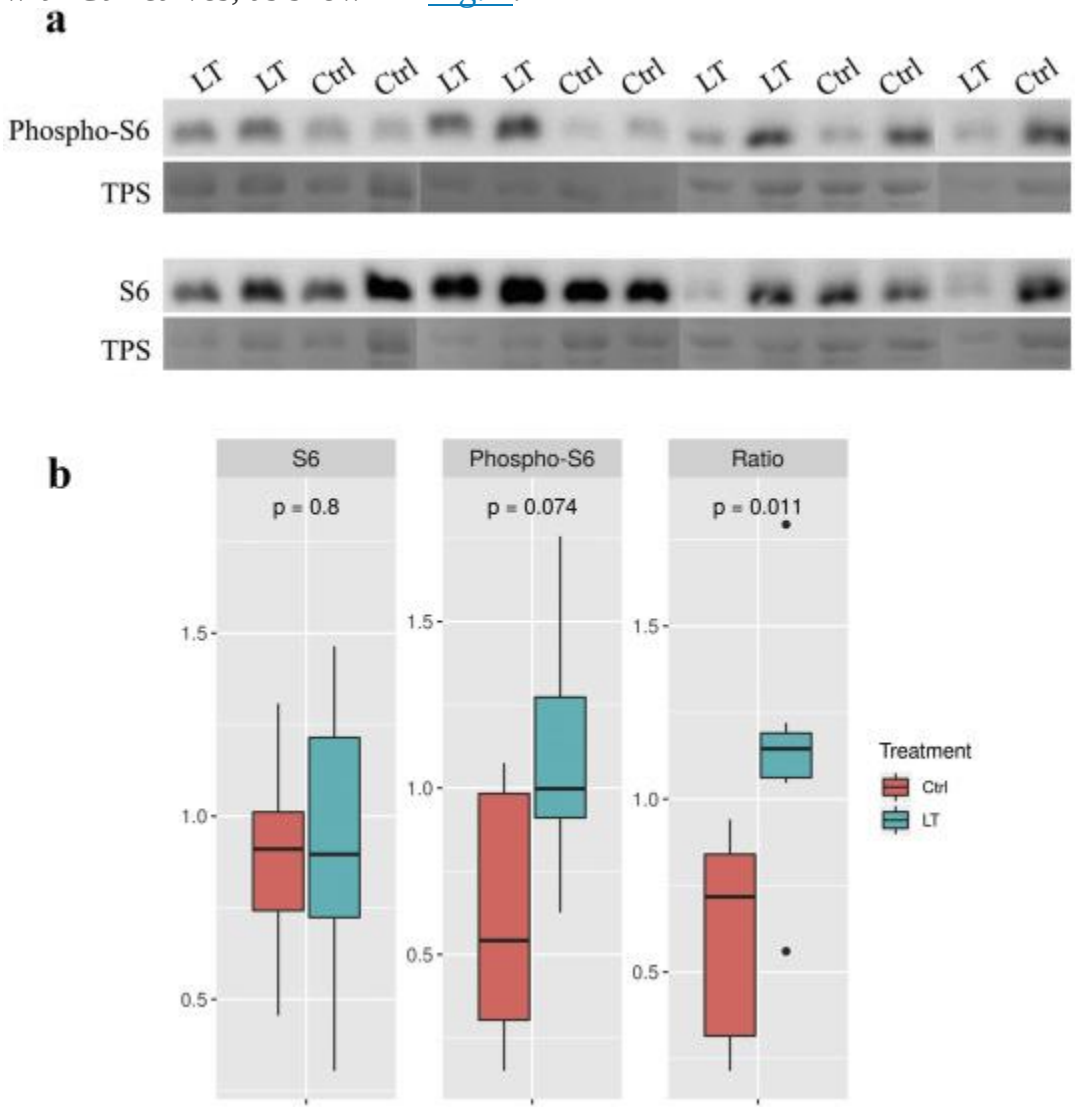

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Fig. 1. a. Western blot of Phospho-S6 and S6 with their corresponding total protein staining in each animal; b. Boxplots of S6, Phospho-S6 and the ratio of Phospho-S6/S6 with comparison *p*-values between Ctrl and LT. Mean intensities were normalized and scaled to 1.

3.5. Metabolomics in skeletal muscle and serum

Quantified metabolites of muscle and serum with significant differences are shown in Table 4, Table 5, respectively.

Table 4. Metabolites (mean ± SE, μMol/100 g muscle) with significantly differential concentration in skeletal muscle identified in Ctrl and LT groups.

Metabolite	Ctrl	LT	*p*-Value
2-Oxoglutarate	0.80 ± 0.07	1.22 ± 0.10	0.005
Acetate	1.08 ± 0.12	1.45 ± 0.13	0.052
Acetoacetate	0.52 ± 0.04	0.67 ± 0.05	0.035
GTP	0.50 ± 0.03	0.62 ± 0.02	0.005
Leucine	0.51 ± 0.05	0.70 ± 0.07	0.042
NADH	0.21 ± 0.04	0.35 ± 0.05	0.049
Valine	0.94 ± 0.06	1.30 ± 0.11	0.013

Table 5. Metabolites (mean ± SE, μM) with significantly differential concentration in serum identified in Ctrl and LT groups.

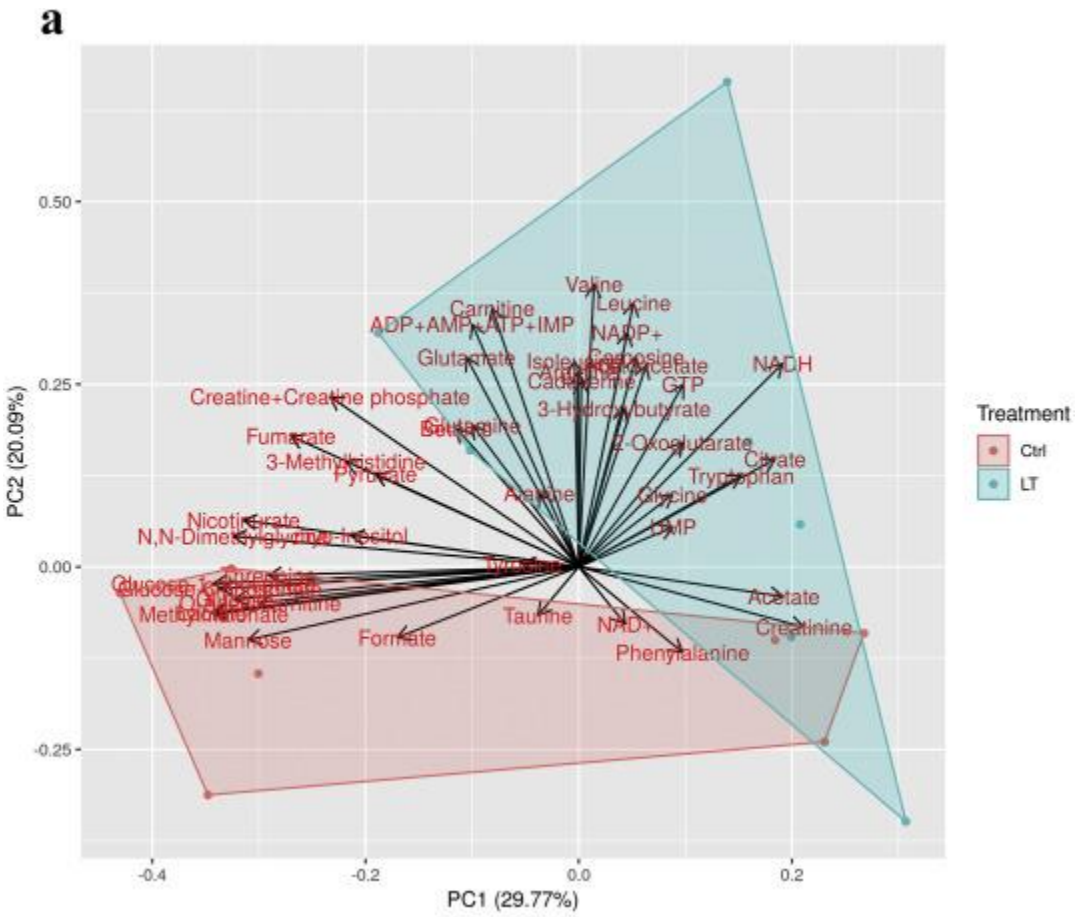
Metabolite	Ctrl	LT	*p*-Value
2-Oxobutyrate	6.93 ± 0.71	10.00 ± 1.15	0.042
2-Oxoisocaproate	4.09 ± 0.26	5.66 ± 0.30	0.002
3-Hydroxybutyrate	44.73 ± 4.11	71.01 ± 9.57	0.021
3-Hydroxyisobutyrate	9.34 ± 0.79	11.69 ± 0.57	0.034
Asparagine	25.24 ± 1.42	30.17 ± 1.37	0.028
Creatinine	15.86 ± 1.40	19.57 ± 1.15	0.064
Formate	35.59 ± 2.37	30.60 ± 1.46	0.08
Fructose	119.11 ± 10.24	83.50 ± 11.51	0.039
Histidine	10.87 ± 1.21	16.70 ± 1.88	0.023
Isoleucine	39.70 ± 2.24	50.31 ± 3.32	0.021
Leucine	64.06 ± 3.96	81.19 ± 6.02	0.035
Proline	53.79 ± 1.60	64.51 ± 2.40	0.002

Metabolite	Ctrl	LT	*p*-Value
Serine	51.36 ± 2.40	68.80 ± 4.81	0.007
Threonine	66.43 ± 5.00	78.94 ± 3.60	0.065
Valine	59.11 ± 3.58	77.06 ± 6.35	0.03
Carnitine	5.76 ± 0.25	8.10 ± 0.48	0.001
Citrulline	39.16 ± 3.10	49.77 ± 1.39	0.009
Methylsuccinate	4.46 ± 0.40	5.51 ± 0.32	0.061

In muscle, two BCAA (leucine and valine), two citrate cycle-related metabolites (2-oxoglutarate and GTP) as well as NADH and acetoacetate were significantly increased in LT animals. Furthermore, acetate showed a tendency for increase.

In serum, seven proteinogenic AA (asparagine, histidine, isoleucine, leucine, proline, serine and valine) and one AA derivative (citrulline) were significantly increased in LT animals. Threonine showed a tendency to increase. Furthermore, catalytic metabolites derived from leucine (2-oxoisocaproate), threonine (2-oxobutyrate) and valine (3-hydroxyisobutyrate) as well as carnitine and 3-hydroxybutyrate, two metabolites related to lipid degradation, were also elevated.

The PCA for the quantified metabolites with their corresponding loadings are shown in Fig. 2 for muscle (a) and serum (b), respectively. Verification of consistency with the original data was performed by matching PCA of chemometrics from both biological samples (Supplementary material Fig. S1).
Correlation plots for all metabolites and proteins in muscle and serum are shown in Fig. 3a and b, respectively ($p < .05$). In muscle, leucine showed significant correlation with acetoacetate, carnitine as well as the other BCAA (isoleucine and valine). Also a positive correlation appeared between the proteins related to mitochondria and OXPHOS (COX2, COX5A, VCP and NDUFAB1), and between leucine and HADHA. In serum, the correlation plot revealed several positive correlations between leucine and/or threonine and several AA. SerpinA3.1, SerpinA3.7, PON1, CRP and APOC3 also clustered together showing a positive correlation, as well as SerpinA3.5, SerpinA3.6, SerpinA3.8, ITIH4 and APOA2. These two groups of proteins correlated negatively one to the other.


a
Valine
Leucine
Carnitine
ADP+AMP
ATP+IMP
NADP+
Glutamate
NADH
Creatine+Creatine phosphate
GTP
3-Hydroxybutyrate
Fumarate
2-Oxoglutarate
3-Methylhistidine
Pyruvate
Citrate
Tryptophan
Alanine
Glycine
Nicotinurate
DMP
Acetate
Taurine
NAD+
Creatinine
Mannose
Formate
Phenylalanine
PC2 (20.09%)
PC1 (29.77%)
0.50
0.25
0.00
-0.25
-0.4
-0.2
0.0
0.2
Treatment
Ctrl
LT

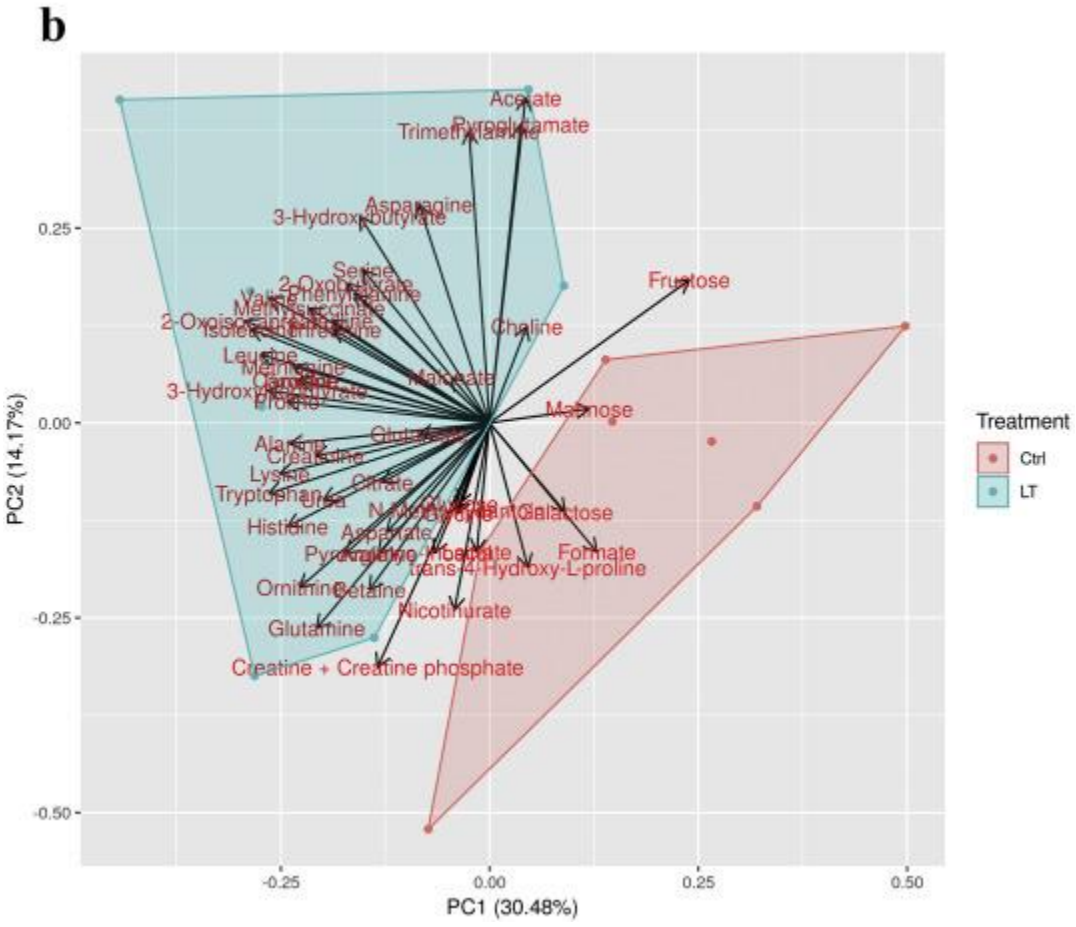

b
Acetate
Pyroglutamate
Asparagine
3-Hydroxybutyrate
Fructose
Choline
Mannose
Glutamine
Creatine + Creatine phosphate
Nicotinurate
Ornithine
Formate
Galactose
Histidine
PC2 (14.17%)
PC1 (30.48%)
0.25
0.00
-0.25
-0.50
-0.25
0.00
0.25
0.50
Treatment
Ctrl
LT

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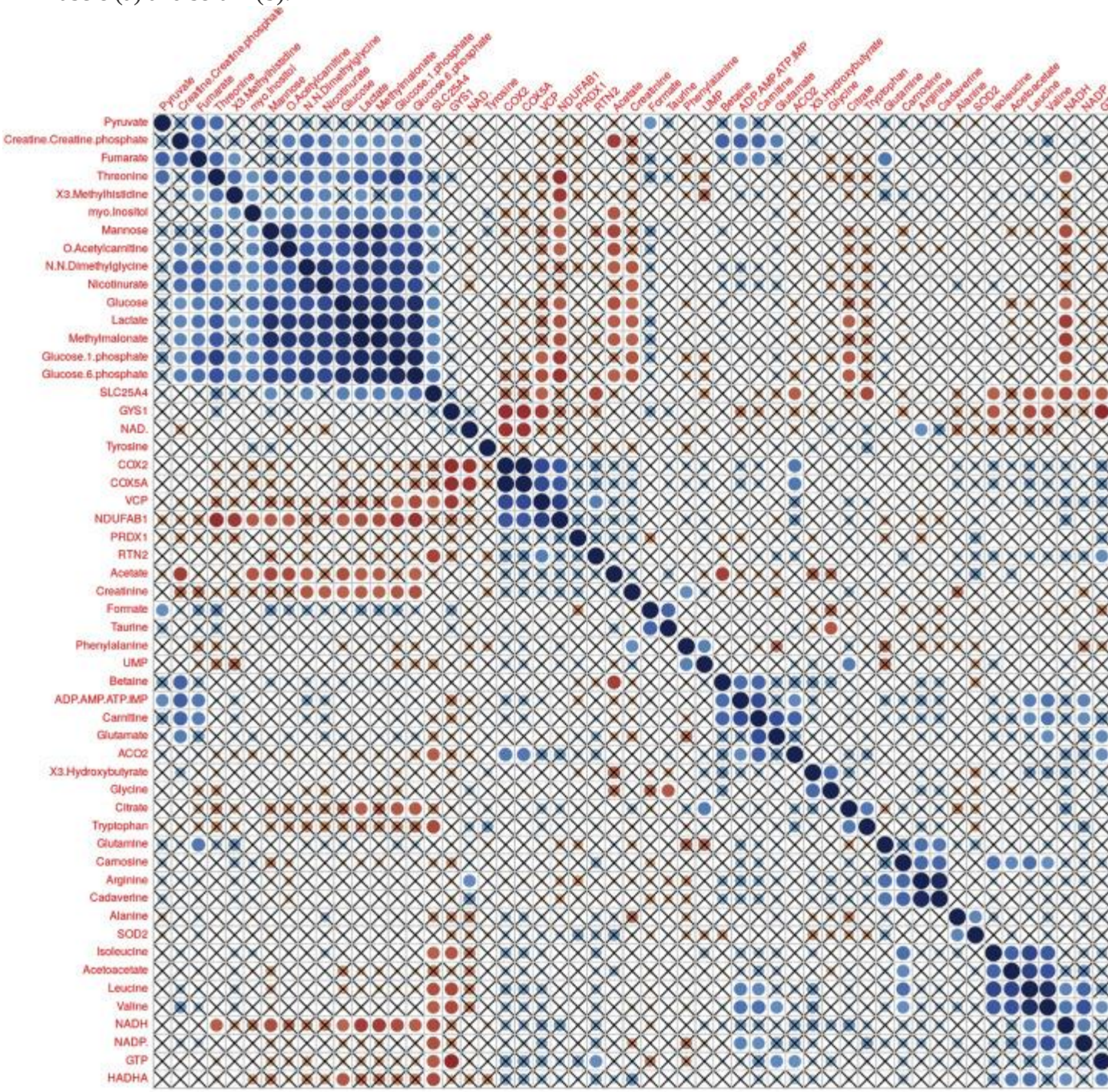
Fig. 2. Principal component analyses (PCA) with loadings of quantified metabolites from muscle (a) and serum (b).



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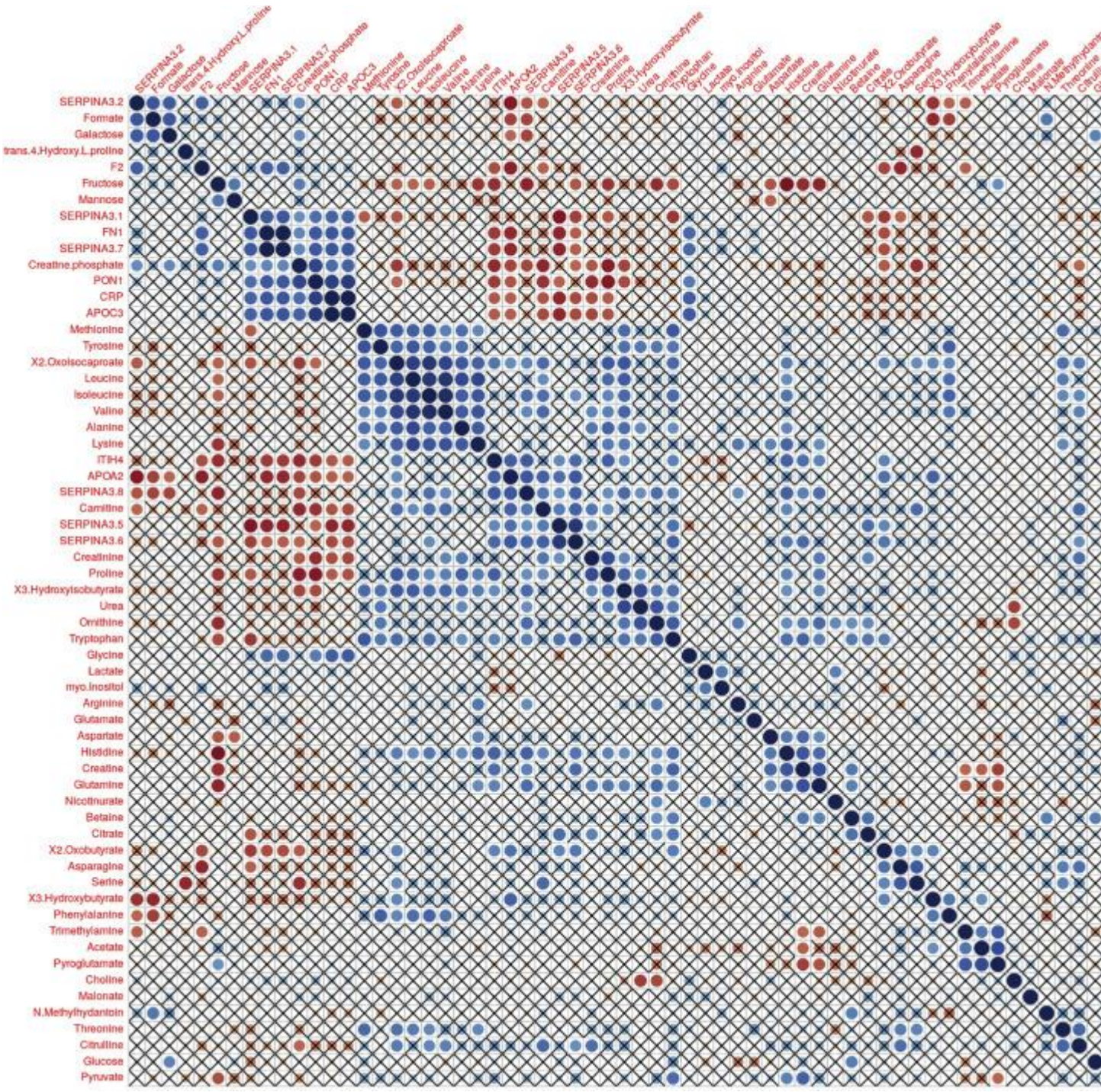

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Fig. 3. Correlation plots of quantified metabolites in muscle (a) and serum (b). Dark blue and dark red colors indicate stronger positive and negative correlation, respectively. Pearson r coefficients were calculated and significant level was set at $p < .05$. The larger size of the circle represents a high correlation coefficient (approaching to 1) whereas

smaller size of the circle indicate the opposite (approaching to 0). Non-significant correlations are depicted with a cross on top of the circle. Compounds are grouped by hierarchical clustering order. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The analysis of the correlation between metabolites in serum and muscle (Supplementary material Fig. S2) showed that muscular leucine and valine positively correlated with muscular and serum carnitine, and with serum citrulline and amino acids as isoleucine and lysine. Muscular NADP also correlated with several serum AA (isoleucine, leucine, lysine, proline, tryptophan, valine, phenylalanine). Negative correlations were observed between serum acetate and muscular glucose, glucose-1P and pyruvate; 2-oxobutyrate showed negative correlations with glucose, glucose-1P, glucose-6P and lactate; and serum lactate negatively correlated with muscular acetate.

3.6. Pathway analysis

The Reactome pathway for protein analysis yielded several significant pathways, in accordance with the GO ontology. The Reactome analysis for small molecules (KEGG nomenclature) also presented coherent results (Supplementary material Table S9).

In muscle, citrate cycle and respiratory electron transport, mitochondrial fatty acid beta-oxidation and detoxification of reactive oxygen species were identified with proteomic data. Among the most relevant for metabolites, BCAA catabolism (R-HSA-70895), metabolism of amino acids and derivatives (R-HSA-71291), synthesis of ketone bodies (R-HSA-77111), tRNA modification in the nucleus and cytosol (R-HSA-6782315) and citrate cycle (R-HSA-71403) were observed.

In plasma/serum, chylomicron assembly and remodeling (R-HSA-8963888 and R-HSA-8963901) and lipoprotein assembly and remodeling (R-HSA-8963898 and R-HSA-8963899) were identified with proteomic data and tRNA aminoacylation (R-HSA-379724) and metabolism of AA and derivatives (R-HSA-71291) with small compounds.

The joint pathway analysis of increased abundance proteins and metabolites in blood and muscle developed with MetaboAnalyst are shown in Table 6. Four pathways (valine, leucine and isoleucine degradation; butanoate metabolism, aminoacyl-tRNA

metabolism; and citrate cycle) were found with significant change, furthermore coherent with the Reactome analysis.

Table 6. Joint pathway analyses of upregulated proteins and metabolites in muscle and blood using MetaboAnalyst.

Pathway	Muscle (9 proteins and 9 metabolites)								Blood (5 proteins an			
	Protein	Metabolite	hit	*p*-value	-log(*p*)	Holm *p*	FDR	Impact	Protein	Metabolite	hit	*p*-valu
Valine, leucine and isoleucine degradation	HADHA	leucine, acetoacetate, valine, isoleucine	5/82	6.69E-05	9.6	0.005	0	0.18	–	leucine, isoleucine, valine, 2-oxoisocaproate, 3-hydroxy isobutyrate	5/82	5.66E 04
Butanoate metabolism	HADHA	2-oxoglutarate, acetoacetate	3/47	0.003	5.9	0.21	0.07	0.34	–	–	–	
Aminoacyl-tRNA metabolism	–	leucine, isoleucine, valine	3/87	0.015	4.2	1	0.3	0.04	–	leucine, isoleucine, alanine, valine, serine, proline, histidine, asgaragine	8/87	1.74E 07
Citrate cycle	ACO2	2-oxoglutarate	2/50	0.039	3.2	1	0.62	0.3	–	–	–	

p-value, *p*-values of significant pathways in enrichment analysis; Impact, impact of the pathway according to the pathway topology analysis. Higher -log(*p*) means more significant pathway; higher impact means a higher percentage of hit compounds.

4. Discussion

Schematics of the putative metabolic effects caused by leucine/threonine supplementation is shown in Fig. 4. Detailed explanation is given below.

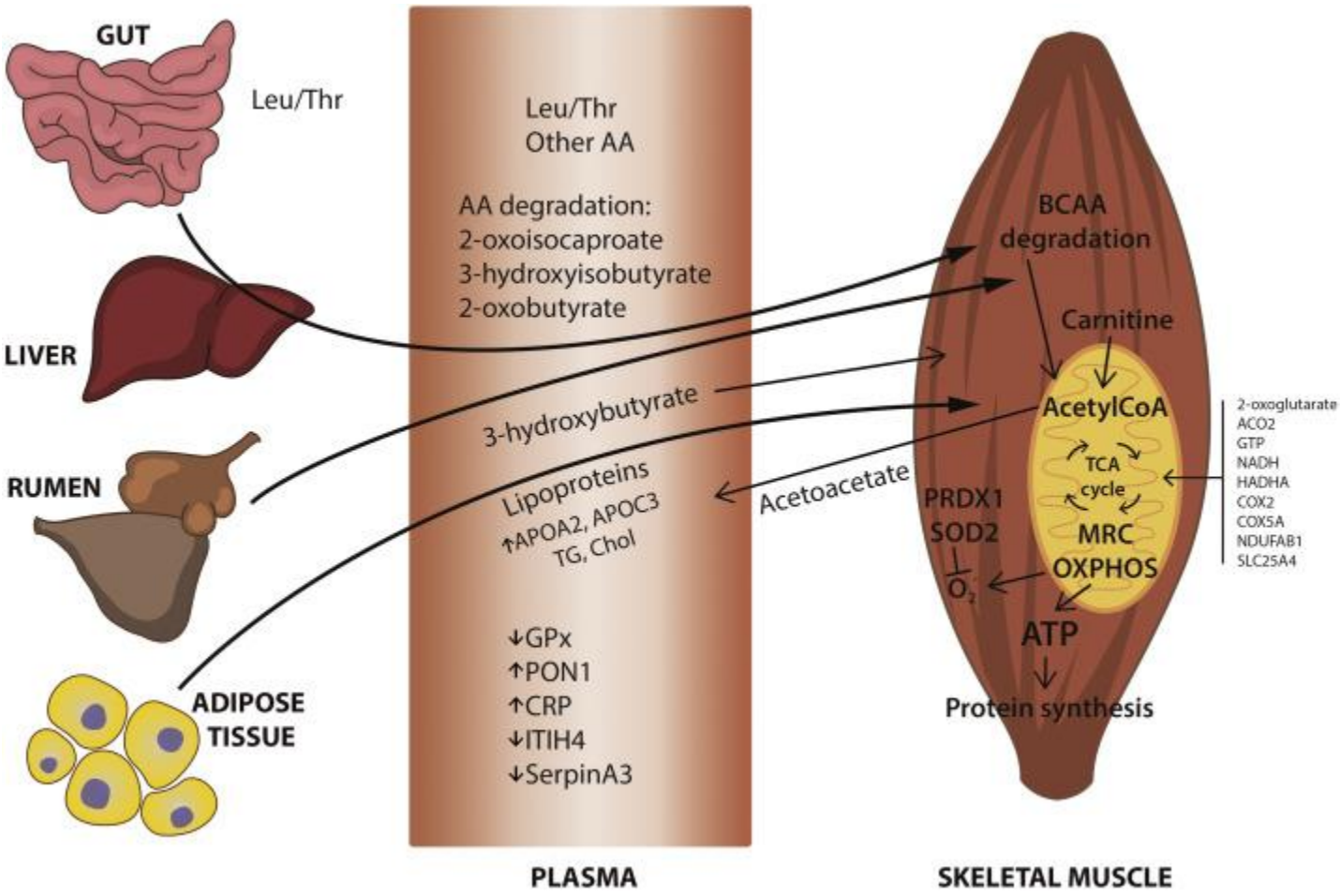

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Fig. 4. Schematic representation of the putative metabolic effects exerted by leucine/threonine supplementation in the MR in pre-weaned calves.

4.1. Supplementation with leucine and threonine increases the concentration of BCAA and their derivative metabolites

Metabolomic analysis indicated increases in the three BCAA in serum and muscle, which implies that leucine reached the blood and the muscle, and that a metabolic connection among BCAA exists. On the other hand, no increase in threonine neither in blood nor muscle was observed, despite being supplemented at the same level as leucine. Serine was greatly increased, thus this may be explained by the rapid conversion of threonine to serine.

Most AA are first metabolized in the liver and turned into ketoacids by transamination. In contrast, BCAA are not initially metabolized in the liver, due to low hepatic activity of branched-chain-amino-acid aminotransferase (BCAT). Thus, BCAA increase rapidly in the plasma after protein intake and are readily available to extrahepatic tissues [28]. Most of the BCAA are initially catabolized in the skeletal muscle, due to its high BCAT activity, which can reversibly transfer the alpha-amino group in BCAA to 2-oxoglutarate (α-KG) to form glutamate and the corresponding branched-chain keto acids (BCKA): 2-oxo-isocaproate (ketoleucine), 2-oxo-3-methylvalerate (ketoisoleucine) and 2-oxo-isovalerate (ketovaline). Glutamate can further form alanine or glutamine to be released in the bloodstream whereas BCKA are catabolized mainly in the liver [29]. Since the three BCAA have parallel pathways for degradation, the finding of higher concentrations of 2-oxoisocaproate and 3-hydroxyisobutyrate in serum in the LT group indicates that these AA are indeed catabolized [30]. These AA and their metabolites (α-ketoisocaproic acid and β-hydroxy-β-methylbutyrate) have been reported to play important roles in enhancing muscle growth during the neonatal period [31]. Positive correlation between muscular leucine, isoleucine and valine with serum citrulline and aspartate, both intermediates of the urea cycle, may indicate a higher degradation of amino acids as a general event of the organism, in the last stage of metabolism of the amino group in the urea cycle taking place in the liver.

4.2. Supplementation with leucine and threonine affects lipid metabolism

Results from the proteomic analysis indicated an increase in plasma apolipoproteins APOA2 and APOC3, which are involved in lipid and cholesterol transport (GO:2000910 and GO:0060621) in the LT group. Furthermore, PON1, an enzyme that is involved in detoxification, is bound to high-density lipoproteins (HDL) in plasma [32]. The increase in plasma apolipoproteins is consistent with the increase in TGs and cholesterol observed after conventional clinical chemistry assays. Regarding the increase in serum 3-hydroxybutyrate, it could likely be consequence of an increase in the production of butyrate in the rumen, since these young animals have already a partially functional rumen at the end of the experiment.

4.3. Supplementation with leucine and threonine affects energy metabolism in skeletal muscle

The proteomic and metabolomic data obtained from skeletal muscle biopsies highlighted other two main metabolic adaptations. On one hand, the pathway analysis showed an increase in oxidative metabolism as components of NADH dehydrogenase (Complex I) (NADH and NDUFAB1) and cytochrome *c* oxidase (Complex IV) (COX2 and COX5A) [33] were increased in the LT group, indicating an increase in the activity of the mitochondrial respiratory chain and oxidative phosphorylation (OXPHOS). OXPHOS would be fueled by a higher rate of the citrate cycle, as suggested by an upregulation of enzymes (ACO2) and metabolites (2-oxoglutarate, GTP and NADH). The ADP/ATP carrier protein SLC25A4, involved in the exchange of cytoplasmic ADP with mitochondrial ATP across the mitochondrial inner membrane, was also found at higher abundance. It is interesting to note that these proteins (COX2, COX5A and NDUFAB1, as well as VCP) clustered positively in the correlation analysis in muscle (Fig. 3A). The citrate cycle would be more active due to the fuel provided by BCAA, however it has been shown that it could also be fueled by an increase in fatty acid degradation, as carnitine, involved in the transport of fatty acids to the mitochondria, and HADHA, involved in fatty acid beta-oxidation, had both higher abundances in the LT group. Positive correlation between muscle leucine with carnitine in muscle and serum, and with HADHA in muscle, may also be an indicative of this metabolic adaptation. The metabolites acetoacetate and 3-hydroxybutyrate also indicated an increase in the metabolism of Acetyl CoA. Nutrients (BCAA, butyrate and fats) are metabolized to acetyl-CoA, which subsequently enters the citrate cycle to finally produce ATP as energy for cellular processes [34]. Consequently, increased ATP availability leads to increased ATP transfer from mitochondria (upregulation of SLC25A4) as well as transport across membranes in other organelles (upregulation of the ATPase VCP).
On the other hand, the increased abundance of PRDX1 and SOD2 in the muscle tissue pointed towards the activation of superoxide radicals removal (GO:0019430) [35]. Since OXPHOS is not completely efficient and not every oxygen receives four electrons, a greater amount of superoxide anions and peroxides are likely produced

under OXPHOS activation. The increase in antioxidant defenses may be a compensatory mechanism for the increase in oxygen radicals.

Another potential effect of leucine is its ability to increase protein synthesis through its effect on the mTOR complex 1 (mTORC1) which is known for its central regulation of cell metabolism, growth, proliferation, and survival [36]. There is evidence that leucine [37] as well as its metabolite 2-oxoisocaproate are able to activate mTOR [31,38]. Herein, we use the phosphorylation of the ribosomal protein S6, downstream of the mTORC1 signaling pathway, as an indicator of the mTORC1 function, directly related to the upregulation of protein synthesis [39]. In our case, the ratio of phospho-S6/S6 indicated a significant upregulation of mTORC1 in the LT group. Our results suggest that the supplemented leucine stimulated the mTORC1 pathway and protein synthesis, which may have contributed to the slight but significant increase in the protein percentage observed in LT calves after CT-imaging performed at the same time than biopsies (manuscript in preparation).

Our present hypothesis is that supplementation with Leu-Thr would positively affect skeletal muscle growth (increase of muscular mass) by affecting two mechanisms: on one side, improving ribosomal protein synthesis through the phosphorylation of S6 (putatively via mTOR) and, on the other side, by providing enough energy (ATP) to drive protein synthesis, which is energy-consuming.

4.4. Supplementation with leucine and threonine affects plasma composition

The metabolic effects were also visualized in plasma, due to the appearance of two oxidation/citrate cycle components (carnitine and 2-oxoglutarate), ketone bodies (3-hydroxybutyrate and acetoacetate) and, as mentioned above, BCAA derivatives (2-oxoisocaproate and 3-hydroxyisobutyrate).

Changes in the plasma proteome indicated that leucine/threonine supplementation may have complex effects in the defense mechanisms of the organism (oxidative stress and native immune system): haptoglobin was not altered (Table S4), CRP and PON1 increased, ITIH4 decreased. PON1 is involved in detoxification and binds to HDL, preventing and delaying the oxidation of HDL and low-density lipoprotein (LDL) and therefore inhibiting the onset and progression of oxidative stress [40], whereas CRP and ITIH4 are acute phase proteins upregulated in acute phase response

[41], although CRP is considered a minor acute phase protein in bovine [42]. On the other hand, a decrease in several serpin 3A isoforms was observed. Serpin A3 belongs to the serpin superfamily of protease inhibitors and it is encoded by a cluster of eight closely related genes in cattle [43]. The functionality of serpins remains unclear, but they may be involved in blood coagulation, fibrinolysis, cell migration, and hormone transport [44,45]. A hint on the potential physiological roles of different serpins may be inferred from the correlation analysis, since some serpins (A3.1, A3.7) positively correlated with PON1, CRP and APOC3, whereas other serpins (A3.5, A3.6 and A3.8) clustered together with ITIH4 and APOA2. Curiously, these two groups of proteins correlated negatively one to the other. In any case, further research is needed to clarify whether these processes can be affected by leucine supplementation and/or a metabolically tensed status exist in these animals.

5. Conclusions

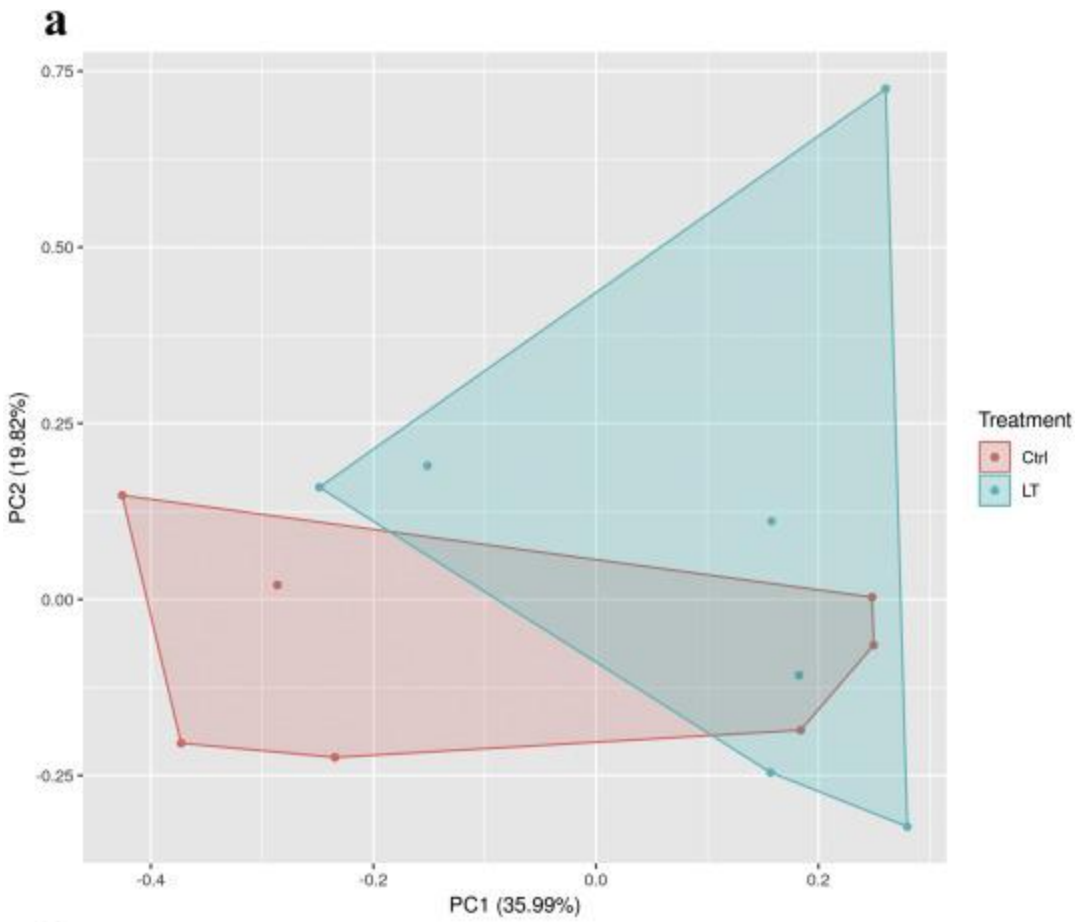
Supplementation with leucine and threonine increased oxidative metabolism in muscle through an increase in citrate cycle, the mitochondrial respiratory chain and OXPHOS. The energy substrates were the AA, which were catabolized to ketoacids mainly in the muscle and may yield acetyl-CoA to enter the citrate cycle. Other substrates were short fatty acids and/or products of lipid mobilization, which were also converted into acetyl-CoA to be oxidized. The generation of oxidative stress in the mitochondria was balanced by the upregulation of antioxidant proteins. Lastly, a complex readjustment of the plasma defense systems also occurred.

In conclusion, an integrative approach by combining metabolomics, proteomics and clinical chemistry allowed to characterize metabolic adaptations occurring in these animals, even in the absence of macroscopic changes in productive parameters. More studies to analyze the role of the rumen, adipose tissue and liver, and the metabolomic study of hydrophobic compounds would extend our understanding about the metabolism in dairy calves and may help to optimize their nutritional management.

The following are the supplementary data related to this article. Download : **Download spreadsheet (11KB)**

Table S1. Diet management of Ctrl and LT groups during the experiment. Download : **Download spreadsheet (9KB)**

Table S2. Starter concentrate feeding program.
Download : **Download spreadsheet (12KB)**
Table S3. Performance and food intake of calves fed a milk replacer supplemented with 0.3% leucine plus 0.3% threonine (LT) or a non-supplemented milk replacer.
Download : **Download spreadsheet (16KB)**
Table S4. Concentration of serum biochemical analytes and AA profile.
Download : **Download spreadsheet (293KB)**
Table S5. Raw proteomic data of muscle samples.
Download : **Download spreadsheet (110KB)**
Table S6. Raw proteomic data of serum samples.
Download : **Download spreadsheet (12KB)**
Table S7. PANTHER GO-Slim analysis of biological process and molecular functions of the differential proteins between Ctrl and LT groups identified in skeletal muscle.
Download : **Download spreadsheet (18KB)**
Table S8. PANTHER GO-Slim analysis of biological process and molecular functions of the differential proteins between Ctrl and LT groups identified in plasma.
Download : **Download spreadsheet (114KB)**
Table S9. Reactome Pathway Analysis for the differential proteins and metabolites identified in skeletal muscle and serum between Ctrl and LT groups.


a
0.75
0.50
0.25
0.00
-0.25
PC2 (19.82%)
-0.4
-0.2
0.0
0.2
PC1 (35.99%)
Treatment
Ctrl
LT

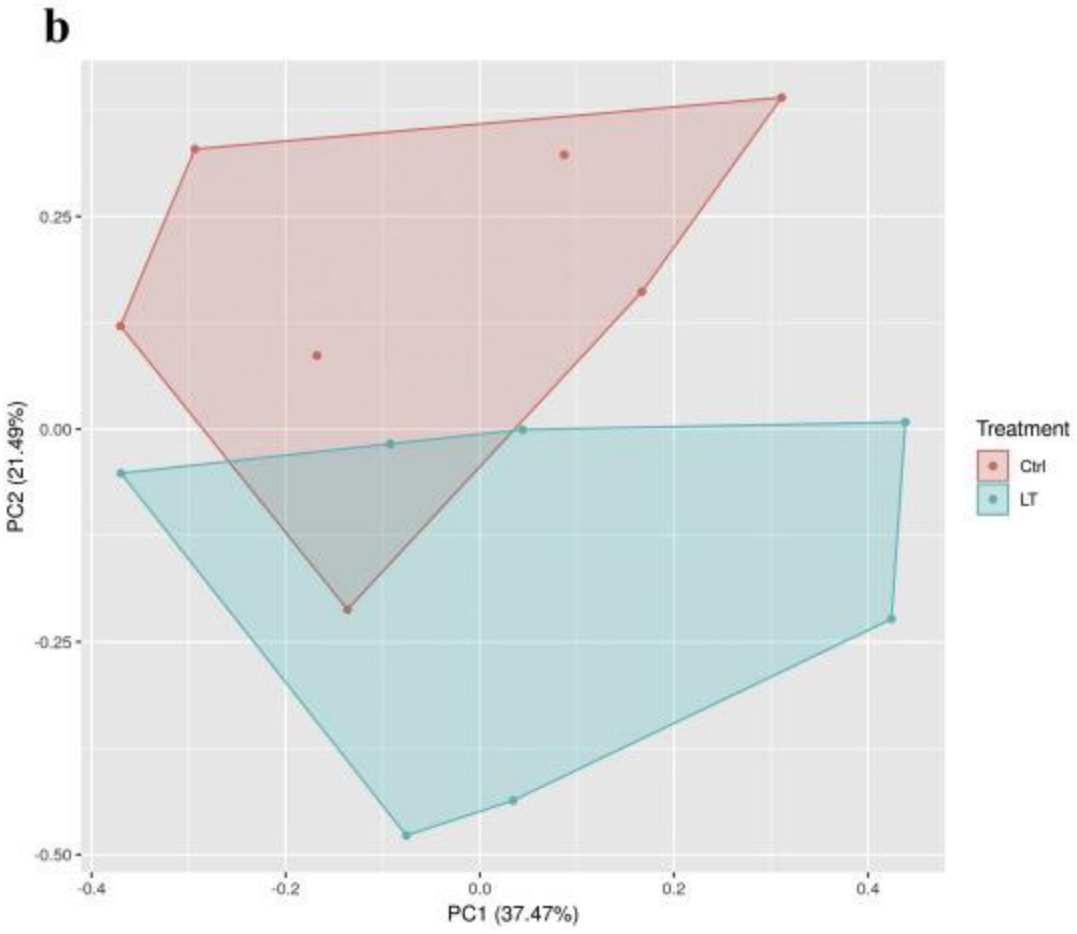

b
0.25
0.00
-0.25
-0.50
PC2 (21.49%)
-0.4
-0.2
0.0
0.2
0.4
PC1 (37.47%)
Treatment
Ctrl
LT

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Fig. S1. Principal component analyses (PCA) of muscle (a) and serum (b) chemometrics data.

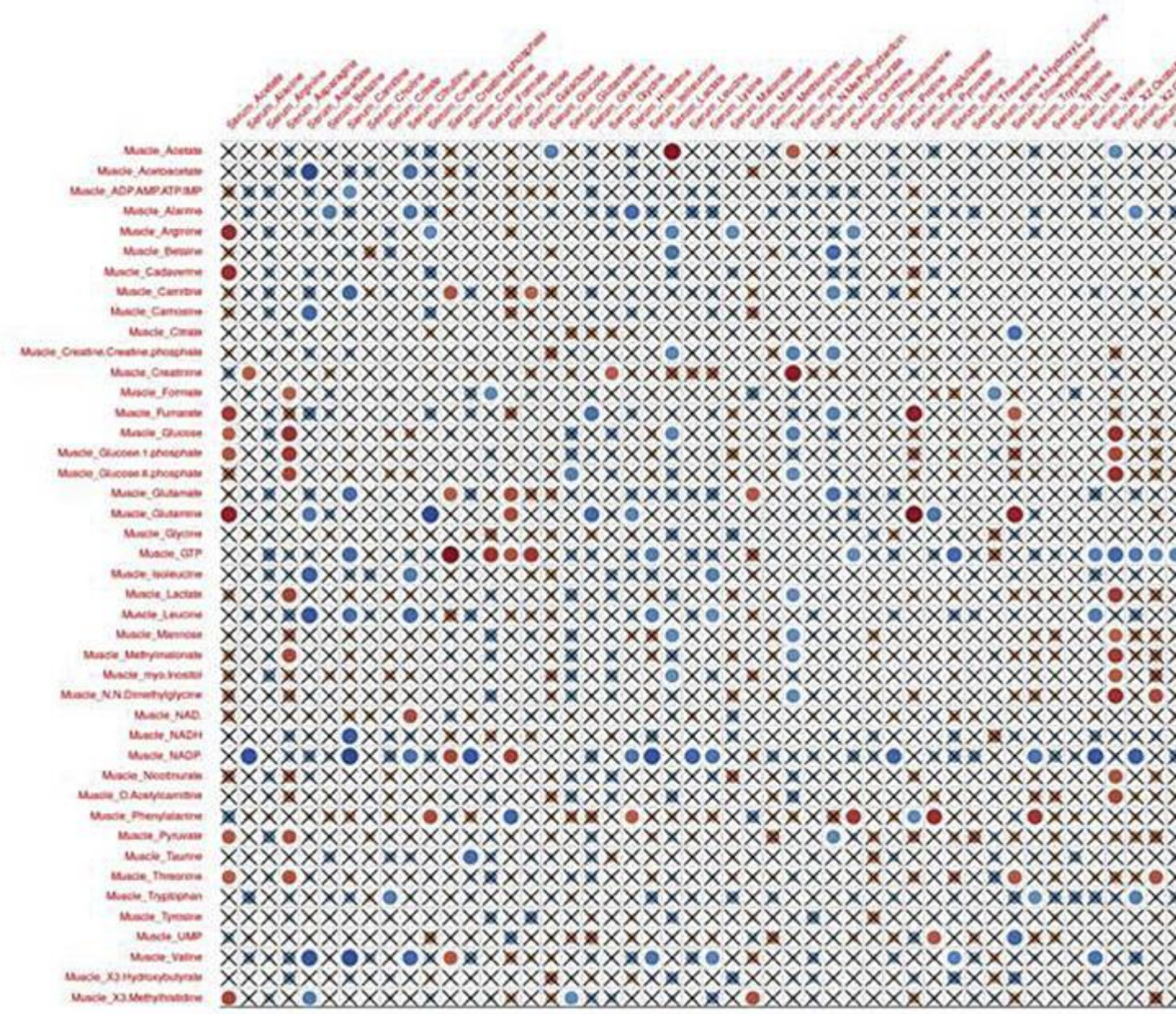

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Fig. S2. Correlation plots of quantified metabolites in muscle versus serum. Dark blue and dark red colors indicate stronger positive and negative correlation, respectively. Pearson r coefficients were calculated and significant level was set at $p < .05$. The larger

size of the circle represents a high correlation coefficient (approaching to 1) whereas smaller size of the circle indicate the opposite (approaching to 0). Non-significant correlations are depicted with a cross on top of the circle.

Availability of data and materials

The datasets during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Author contributions

K.Y. performed the experimental sessions in metabolomics and proteomics, data analysis, bioinformatics, statistics and manuscript writing. M.M. (manuscript, metabolomics and data analysis), A.H. (manuscript, proteomics and data analysis), M.T. (experimental design, manuscript, sampling, data analysis and statistics), A. Bach (experimental design, manuscript revision), J.K. (manuscript and proteomics), N.Y., L.A., E.R. and R.P (analytical work), N.G. (analytical work and data analysis), A.A. and P.D. E. (manuscript revision), A. Bassols (experimental design, analytical work supervision, manuscript writing and revision). All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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