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Liver proteome profiling in dairy cows during the transition from gestation to lactation: Effects of supplementation with essential fatty acids and conjugated linoleic acids as explored by PLS-DA

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1 **Liver proteome profiling in dairy cows during the transition from gestation to**
2 **lactation: Effects of supplementation with essential fatty acids and conjugated**
3 **linoleic acids as explored by PLS-DA**

4

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23

24 **Highlights**

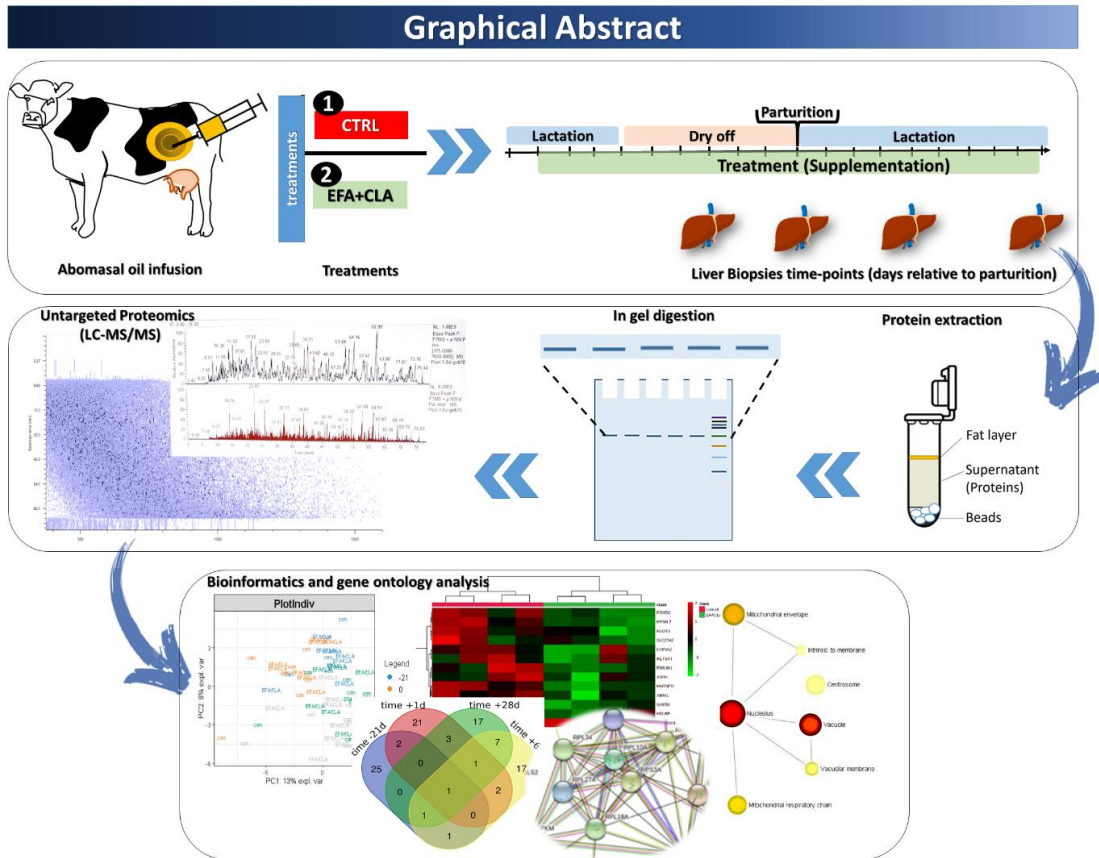
25 1. Supplementation with fatty acids affected the liver proteome in dairy cows

26 2. Out of 1680 proteins identified, 96 were differentially abundant

27 3. The key pathways involved were Cytochrome P450 and ω -oxidation of fatty acids

28 4. Specific cytochrome P450 (CYP) enzymes were identified at each time point

29 **Graphical abstract**



30
31

32 **Abstract**

33 This study aimed at investigating the synergistic effects of essential fatty acids (EFA) and conjugated linoleic acids
 34 (CLA) on the liver proteome profile of dairy cows during the transition to lactation. 16 Holstein cows were infused
 35 from 9 wk antepartum to 9 wk postpartum into the abomasum with either coconut oil (CTRL) or a mixture of EFA
 36 (linseed + safflower oil) and CLA (EFA+CLA). Label-free quantitative proteomics was performed in liver tissue
 37 biopsied at days -21, +1, +28, and +63 relative to calving. Differentially abundant proteins (DAP) between treatment
 38 groups were identified at the intersection between a multivariate and a univariate analysis. In total, 1680 proteins were
 39 identified at each time point, of which between groups DAP were assigned to the metabolism of xenobiotics by
 40 cytochrome P450, drug metabolism - cytochrome P450, steroid hormone biosynthesis, glycolysis/gluconeogenesis,
 41 and glutathione metabolism. Cytochrome P450, as a central hub, enriched with specific CYP enzymes comprising:
 42 CYP51A1 (d -21), CYP1A1 & CYP4F2 (d +28), and CYP4V2 (d +63). Collectively, supplementation of EFA+CLA
 43 in transition cows impacted hepatic lipid metabolism and enriched several common biological pathways at all time
 44 points that were mainly related to ω -oxidation of fatty acids through the Cytochrome p450 pathway.

45

46 **Keywords:** Liver Proteome, negative energy balance, postpartum, cytochrome p450, fatty acid oxidation, gene
 47 ontology

48 **Significance**

49 In three aspects this manuscript is notable. First, this is among the first longitudinal proteomics studies in nutrition of
50 dairy cows. The selected time points are critical periods around parturition with profound endocrine and metabolic
51 adaptations. Second, our findings provided novel information on key drivers of biologically relevant pathways
52 suggested according to previously reported performance, zootechnical, and metabolism data (already published
53 elsewhere). Third, our results revealed the role of cytochrome P450 that is hardly investigated, and of ω -oxidation
54 pathways in the metabolism of fatty acids with the involvement of specific enzymes.

55 **1. Introduction**

56 Most mammals enter a state of negative energy balance (NEB) at the onset of lactation when the needs for lactation
57 and maintenance cannot be met by feed intake. This metabolic status leads to mobilization of body reserves, mainly
58 from adipose tissue in the form of non-esterified fatty acids (NEFA) to meet the energy requirements for lactation [1].
59 In high-yielding dairy cows, the liver plays a crucial role in metabolic homeostasis and energy production by
60 metabolizing NEFA via precisely regulated signaling and cellular pathways [2]. However, hepatic lipid metabolism
61 is impaired at the onset of lactation when uptake of NEFA by the liver exceeds their oxidation and the export capacity
62 via lipoproteins and may thus result in a fatty liver syndrome [3].

63 Essential fatty acids (EFA), including linoleic acid (LA, 18:2 n-6) and α -linolenic acid (ALA, 18:3 n-3), affect the
64 energy and FA metabolism, inflammation, and immune responses through activation of nuclear receptors [4-6].
65 Conjugated Linoleic Acids (CLA) which are stereo-isomers of LA have been reported to induce milk fat depression
66 (MFD), thus partitioning energy by sparing milk energy for other organs [7, 8]. Energy spared from reduced milk fat
67 synthesis was shown to affect energy partitioning, as toward adipose tissue fat stores [9, 10] and consequently to
68 decrease plasma NEFA concentration and the risk for fatty liver [11]. The shift in dairy farming towards modern
69 indoor production systems went along with a change from using pasture (grass) to feed rations that are largely based
70 on so-called total mixed rations (TMR), in which the roughage component is mainly corn silage in many countries.
71 The decreased or lacking consumption of fresh grass leads to a drop in the intake of ω -3 FA and CLA production [12-
72 14]. A large body of work has highlighted the increased body deposition of n-3 FA and CLA in dairy cows fed with
73 fresh grass in comparison to corn silage (for example [15]).

74 Assessing the effects of specific FA in different feeding practices is complex. Using an experimental model in which
75 dairy cows receiving a corn-silage-based ration without any grass, the EFA and CLA's effects were tested by abomasal
76 supplementation avoiding microbial degradation in the forestomachs [11, 16, 17]. The results showed that the FA
77 marginally improved metabolic health by induction of MFD, which increased energy balance and reduced plasma
78 concentration of triglycerides and NEFA. In addition, paraoxonase, a hepatic antioxidant enzyme, was elevated
79 postpartum (PP) by the FA application. Although some of these impacted metabolites and proteins were directly or
80 indirectly related to the liver, EFA and CLA-driven hepatic responses remain to be investigated.

81 Improvements in proteomics in the last decade have increased our understanding of the biological pathways impacted
82 by various physiological conditions and diseases [18]. Characterization and comprehensive proteome profiling of the
83 liver as a central organ in energy and lipid metabolism could open up new insights into the regulatory metabolic
84 pathways influenced by different nutritional supplements. Proteomics results allow better understanding and
85 predicting the metabolism and help define rapid biomarkers for use in the early diagnosis of steatosis or other
86 metabolic diseases associated with liver metabolic health [19]. In this regard, there are several studies in dairy cows
87 entailing the liver proteome for investigating feed efficiency [20], fatty liver [21], and heat stress [22, 23]. In the
88 current study, untargeted proteomics was applied on liver samples from dairy cows supplemented or not with EFA
89 and CLA to investigate metabolic responses during several critical time points around parturition. To the best of our
90 knowledge, this is the first proteomics report considering the longitudinal response of EFA and CLA in dairy cows
91 during the transition from late pregnancy to early lactation.

92 **2. Material and methods**

93 **2.1. Animals, Treatments, and Experimental Design**

94 The trial was carried out as described previously [11] with 16 multiparous (second lactation) German Holstein cows
95 at the Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany. The experimental
96 animal procedures were evaluated and approved by the German Animal Welfare Act (Landesamt für Landwirtschaft,
97 Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, Germany; LALLF M-V/TSD/7221.3-1-038/15).
98 More details on housing, feeding, feed intake, performance, and milk production of studied cows were presented
99 earlier [11]. Briefly, dairy cows housed in a free-stall and abomasally injected with 1-control, the coconut oil (CTRL,
100 n = 8; Bio-Kokosöl #665, Kräuterhaus Sanct Bernhard, KG, Bad Ditzgenbach, Germany) or 2- EFA+CLA, a
101 combination of linseed oil (DERBY® Leinöl #4026921003087, DERBY Spezialfutter GmbH, Münster, Germany),
102 safflower oil (GEFRO Distelöl, GEFRO Reformversand Frommlet KG, Memmingen, Germany) and Lutalin® (CLA,
103 n = 8; cis-9, trans-11, 10 g/d trans- 10, cis-12 CLA, BASF SE, Ludwigshafen, Germany) for 18 weeks started from d
104 63 antepartum (AP) until d 63 PP (Figure 1 A). Supplements were injected twice daily at 0700 and 1630 h in equal
105 portions through abomasal infusion lines (Teflon tube [i. d. 6 mm] with 2 perforated Teflon flanges [o.d. 120 mm],
106 placed in rumen cannulas (#2C or #1C 4", Bar Diamond Inc., Parma, ID). The amount and FA composition of the
107 lipid supplements is given in Supplementary, Table S1.

108 The cows were fed a conventional corn silage-based total mixed ration (TMR), formulated using the equation
109 published by the German Society for Nutrition Physiology (2001 [24], 2008 [25], 2009 [26]) and Deutsche
110 Landwirtschaftliche Gesellschaft (DLG, 2013) [27], for AP and PP. The basal diet was provided ad libitum at 0600 h,
111 with free access to water and trace-mineralized salt blocks. The ingredients and chemical composition of the
112 experimental diets are presented in Supplementary Table S2.

113

114 **2.2. Liver biopsies**

115 Liver tissue samples were obtained using a biopsy needle (outer diameter of 6 mm) under local anesthesia on d -21
116 AP, d 1 and d 28 PP, and after slaughtering the cows on d 63 PP as previously described [28] (Figure 1 A). The
117 specimens were immediately frozen in liquid nitrogen and stored at -80 °C until protein extraction.

118

119 **2.3. Liver Preparation for Proteomics Analysis**

120 Frozen samples were first ground mechanically using a mortar and pestle chilled in liquid nitrogen. Eighty mg of
121 tissue powder were placed in a reinforced 2-mL tube containing six ceramic beads (Dutscher, United Kingdom) and
122 mixed with 1 mL of freshly prepared Laemmli sample buffer (50 mM Tris pH 6.8, 2% SDS, 5% glycerol, 2 mM DTT,
123 2.5 mM EDTA, 2.5 mM EGTA, H₂O 920 µLl, 2x phosphatase inhibitors tablets (Perbio, Thermo Fischer, Hercules,
124 California, USA), 1x protease inhibitor (Roche, Boulogne-Billancourt, France), 4 mM sodium orthovanadate, and 20
125 mM sodium fluoride). Subsequently, liver tissue was homogenized in a Precellys® 24 homogenizer (PEQLAB
126 Biotechnology GmbH, Erlangen, Germany) at 6800 rpm, 3 x 30 sec (30-sec break between each cycle) at room
127 temperature (RT). Immediately after the homogenization step, tubes were boiled for 10 min in 100 °C boiling water,

128 followed by centrifugation for 15 min at 16000 g at RT. The supernatant was carefully separated and stored at -80°C
129 until proteomics analysis. An aliquot of the lysate was used to measure the total protein concentration using the
130 bicinchoninic acid (BCA, Pierce, Rockford, IL) assay. For peptide preparation, 100 μg of protein were first
131 concentrated in 1D SDS-PAGE gel containing 5-15% acrylamide for stacking and resolving gel, respectively. Once
132 the proteins enter the resolving gel, the electrophoresis was stopped and a small piece of gel containing a major band
133 was cut. After reduction and alkylation, proteins were subjected to in-gel digestion with 10 $\text{ng}/\mu\text{L}$ porcine trypsin
134 (Promega, Madison, Wisconsin, United States) overnight (Figure 1 B).

135

136 **2.4. Nano-LC-MS/MS Analysis**

137 After digestion, the liver peptides mixture was analyzed using nano-scaled liquid chromatography (LC) in Ultimate
138 3000 RSLCnano system (Dionex) coupled to an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher
139 Scientific) for mass spectrometry (MS), adopting the methods previously described by [29]. To reduce between-group
140 variability, the LC-MS/MS was performed on all 64 samples consecutively and samples were randomly injected
141 without any order related to time or treatment.

142 Briefly, a reversed-phase LC was carried out by loading 1 μL of the resuspended peptide mixture onto a trapping
143 column (pre-column 5 mm length X 300 μm ; Acclaim PepMap C18, 5 μm , 100 \AA) equilibrated with trifluoroacetic
144 acid 0.05% in water, at a flow rate of 30 $\mu\text{L}/\text{min}$. After 6 min, the pre-column was switched in-line with the analytical
145 column (Acclaim PepMap 100 - 75 μm inner diameter \times 25 cm length; C18 - 3 μm -100 \AA , Dionex), equilibrated with
146 96% solvent A (99.5% H_2O , 0.5% formic acid) and 4% solvent B (99.5% ACN, 0.5% formic acid).

147 Peptides were eluted at a 400 nL/min flow rate according to their hydrophobicity using a 4 to 20% gradient of solvent
148 B for 60 min. Briefly, the analytical column was first equilibrated with 96% A solvent and 4% B solvent for 6 min,
149 followed by a gradual increase of the B solvent to 20% for 70 min. Then, to clear the system from hydrophobic
150 peptides, the B gradient rose from 20 to 80% in one min (at 77 min) and remained constant for further 5 minutes.
151 Subsequently, the concentration of solvent B was decreased to 4% within 0.1 min and kept constant for 8 min to
152 prepare the system for the next injection.

153 The nano-electrospray ion source (Proxeon) was used as a connector between the LC and Q Exactive HF-X mass
154 spectrometer (Thermo Scientific). Eluates of LC step electro sprayed in positive-ion mode at 1.6 kV through a
155 nano-electrospray ion source heated to 250°C . The Orbitrap Q Exactive HF-X MS used in HCD top 18 modes (i.e. 1
156 full scan MS and the 18 major peaks in the full scan selected for MS/MS). The mass spectrometry method duration
157 was set to 79 min, the polarity was positive, and the default charge was 2.

158 On the MS1 scan, the parent ions were selected in the orbitrap Fourier transform mass spectrometry (FTMS) at the
159 following parameters: a resolution of 60,000, an injection time of 50 ms. mass ranges from 375 to 1600 m/z and the
160 Automatic gain control (AGC) target is set on 3×10^6 ions. Each MS analysis was followed by 18 data-dependent
161 MS2 scans with an analysis of MSMS fragments at a resolution of 15,000, 1×10^5 AGC, and an injection time of 100
162 ms. The HCD collision energy set to 28% NCE, and ~ 15 s dynamic exclusion.

163

164 **2.5. Processing of raw mass spectrometry data**

165 The processing of raw Peptide MS/MS spectra was performed in Progenesis QI software (version 4.2, Nonlinear
166 Dynamics, Newcastle upon Tyne, UK) using automatic alignment to the reference sample automatically defined by
167 the software with the default parameter settings (maximum allowable ion charged set to 5 and Ions ANOVA p-value
168 < 0.05). The mass generating function (mgf) list containing the detected and the quantified peptide ions were directly
169 exported to MASCOT (version 2.5.1) interrogation engine and searched against a *Bos taurus* decoy database (Uniprot,
170 download date: 2019/11/07, a total of 37,513 entries). The search criteria were set as follows: an enzyme digest of a
171 protein set to trypsin, tryptic specificity required (cleavage C-terminal after lysine or arginine residues); 2 missed
172 cleavages were allowed; carbamidomethylation (C) and oxidation (M) set as variable modification. The mass tolerance
173 was set to 10 ppm for precursor ions, 0.02 Da for fragment ions, and FDR < 0.01. The identified peptides from the
174 database search were imported back to Progenesis QI, and the corresponding proteins were identified and quantified
175 based on the intensities of the specific validated peptides. Strict exclusion criteria (deamidated, carbamidomethyl, and
176 oxidation contaminant proteins, having at least two peptides and two unique peptides, and presence in at least 50% of
177 the samples in each treatment group/time point) were applied before analysis.

178

179 **2.6. Data pre-processing**

180 Statistical analyses were performed using the normalized intensity values combined with some in-house developed,
181 EnhancedVolcano, MetaboAnalystR 3.0, and mixOmics R-packages in R statistical software (R version 4.0.0). Before
182 the analyses, the following modifications were applied to proteins, in very severe filtrations: proteins with less than
183 two unique peptides or having zero values in more than 50% of the replicates were not included in the analysis. After
184 filtration, the log10 transformation and auto-scaling (z-transformation), which is mean-cantered and divided by the
185 standard deviation of each variable applied to normalized intensities. The missing or zero values (indicated the peak
186 did not reach the detectable thresholds) were imputed and replaced with the small values (half of the smallest positive
187 value in the dataset). The PCA scatter plot was used to visualize the 2-D cross-section of hyperspace between samples
188 and to distinguish the samples located far away from the treatment clusters (potential outliers). One cow (from the
189 CTRL group in time point -21d AP) considered an outlier by both principal component analysis and hierarchical
190 clustering was removed from the analysis

191

192 **2.7. Statistical analyses**

193 The selection of the most important proteins (VIP) involved in the discrimination of the CTRL and EFA+CLA groups
194 at each time point was based on the intersection of two complementary analyses.

195

196 **2.7.1 Multivariate analysis**

197 Firstly, PCA analysis was done to reduce the dimension of data and to visualize clustering of samples regardless of
198 treatment groups. Partial Least Square Discriminant Analysis (PLS-DA) analysis (mixOmics package in R) ranked
199 proteins importance in projection scores of the first two components (PC1 and PC2) in each time point. This step aims
200 to rank the most discriminative proteins that contribute to cluster separation between treatment groups. A permutation
201 test (defined to 100 random computations) was applied to disprove the over-fitting of the PLS-DA model. Since the

202 permutation test indicated over-fitting in all time points, we performed the second filtration step according to
203 univariate analysis. Although this study aimed to compare different treatments, not assessing populations parameter
204 or identifying predictive model, therefore, permutation test's significance was not the case.

205 2.7.2 Univariate analysis

206 Secondly, from those proteins that were top VIP-ranked (score > 1.5), only ones with P-value < 0.05, and log₂ (fold
207 change) >1.3 (metaboanalyst R package) were considered as differentially abundant proteins (DAP) for further
208 analysis. The P-value was assessed either by Student's t-test (parametric) or Wilcoxon Mann-Whitney test (non-
209 parametric), according to the normality distribution of each protein (Shapiro-Wilk-Test) as previously described [30].

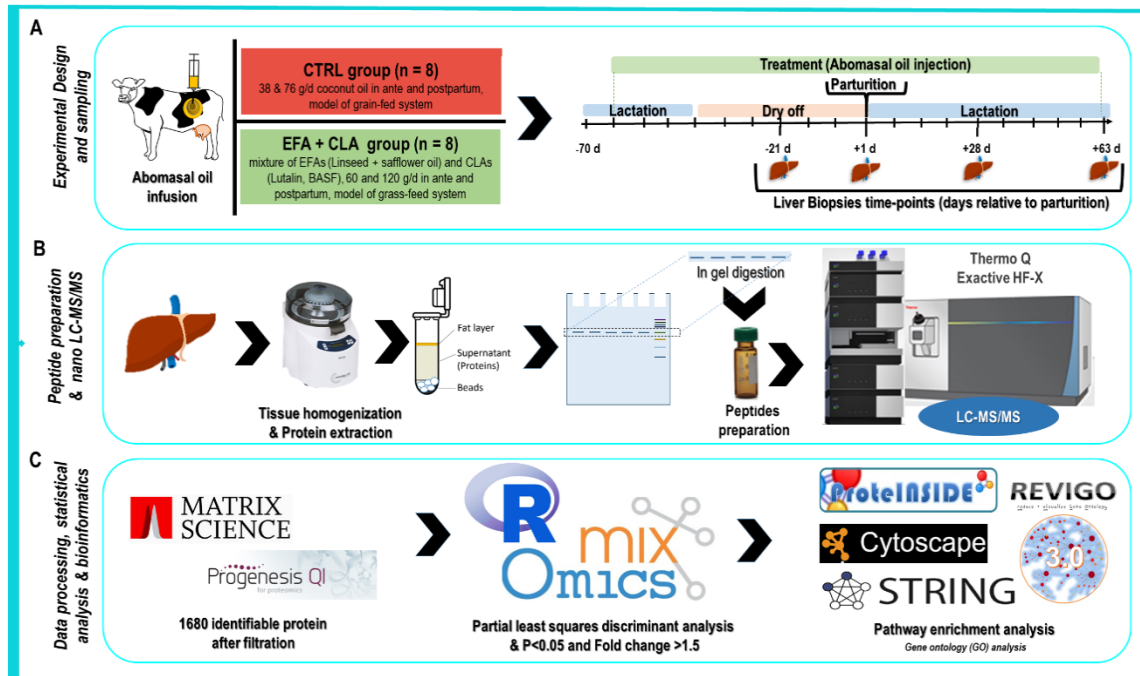
210 2.7.3 Intersection between multivariate and univariate analyses to identify discriminative and 211 differentially abundant proteins (DAP)

212 The intersection between the results from the two methods was chosen to reduce the list of relevant proteins involved
213 in the treatment effect. Thus, we considered two filters, and we selected the proteins that passed through both by
214 choosing the intersection between the two complementary methods. Hierarchical clustering Heat map analysis was
215 performed to approve and visualize DAP (Figure 1 C).

216

217 2.8. Bioinformatics analysis of differentially abundant proteins

218 Before bioinformatics analysis, proteins' accession was converted into Gene ID using the UniProt (retrieve/ID
219 mapping) database conversion tool, and undefined proteins were blasted and replaced with their Gene ID in *Bos taurus*
220 and *Homo sapiens*. Then, the gene ontology (GO) analysis containing Biological Process (BP), Molecular Function
221 (MF), and Cellular Component (CC), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome pathways
222 enrichment analysis of the DAP were performed in STRING web tool version 11.0 in Cytoscape and ProteINSIDE
223 (version 1.0) constructed specifically under *B. taurus* interactions map. Only pathways with adjusted P-value < 0.05
224 (corrected to false discovery rate with Benjamini-Hochberg method) and having at least two hits in each pathway were
225 considered as significantly enriched (Figure 1 C). REVIGO web server (<http://revigo.irb.hr/>) was used to summarize
226 BP terms. Generated GO terms were submitted to Cytoscape version 3.8.2 and Networkanalyst.ca version 3.0 to build
227 the interaction networks. Protein protein interaction networks was constructed by inputting the DAP in each time point
228 to STRING and visualized in cytoscape software, in which nodes and edges represent proteins and their interactions,
229 respectively [31].



230
 231 Figure 1) Schematic diagram of the (A) study design, (B) proteomics workflow, and (C) bioinformatics pipeline. (A) Timeline of treatments
 232 supplementation (from -63d ante to +63d postpartum) and liver biopsy collection (-21 d, +1 d, +28 d, and +63 d relative to parturition).
 233 Bold lines indicate liver biopsy sampling timepoints. (B) Protein extraction, purification, reduction, alkylation, and digestion; peptides
 234 were analysed by high-resolution LC-MS/MS, (C) Peptides alignment (progenesis), and protein identification (mascot) procedure were
 235 performed by Progenesis software coupled with the Mascot search engine, statistical analysis was based on Partial least squares
 236 discriminant analysis (PLS-DA) merged with $P < 0.05$ and Fold change > 1.5 , followed by bioinformatics analysis (protein-protein
 237 interaction and Gene Ontology (GO) enrichment analysis).

238

239 3. Results

240 3.1. Cows performance data

241 A summary of cows performance and plasma metabolites data from the CTRL and EFA+CLA group was extracted
 242 from [11, 16, 17] and provided in supplementary S3 and S4. In brief, EFA+CLA supplementation increased plasma
 243 concentration of these FA, decreased PP NEFA and TG content, induced MFD, increased energy balance, and slightly
 244 affected markers of ketogenesis and hepatic inflammation (i.e., haptoglobin and paraoxonase). Dry matter intake, body
 245 weight, milk yield, and net energy intake were not affected by treatment.

246

247 3.2. Liver proteome profile

248 Out of 2720 identified proteins, a total of 1680 proteins at each time point were maintained for statistical analysis after
 249 applying the exclusion criteria [31]. Of the 1680 proteins, 1614 proteins were annotated by GO terms related to 907
 250 BP, as well as 111 KEGG, and 270 Reactome pathways that covered a diverse range of metabolic pathways related to
 251 metabolism (carbohydrate, energy, lipid, nucleotide, amino acid, glycan, vitamin, and xenobiotic metabolism), genetic

252 information processing (translation and folding, sorting and degradation), cellular process (transport and catabolism
253 and cell growth and death), and organismal systems (immune system and endocrine system).

254

255 **3.3. Differentially abundant proteins and functional enrichment at day 21 antepartum (Figure 2 A),**

256 From the total identified proteins, 29 proteins were differentially abundant on 21 d AP (Table 1), in which the relative
257 abundance of 19 proteins was increased with a fold change that ranged from 1.43 - 3.92 (P-value < 0.05), and ten
258 proteins were decreased (ranging from 0.38 - 0.70 fold, P-value < 0.05) in the EFA+CLA group when compared to
259 the CTRL group. The DAP were further approved by clustered Heat map and are presented in Figure 2 (A, B, and C).

260 The overabundant proteins were annotated by GO terms related to “cholesterol biosynthetic process (GO:0006695)”
261 and “lipid metabolic process (GO:0006629)” (Figure 2 D, details in [31]). Underabundant proteins were not annotated
262 by any GO terms.

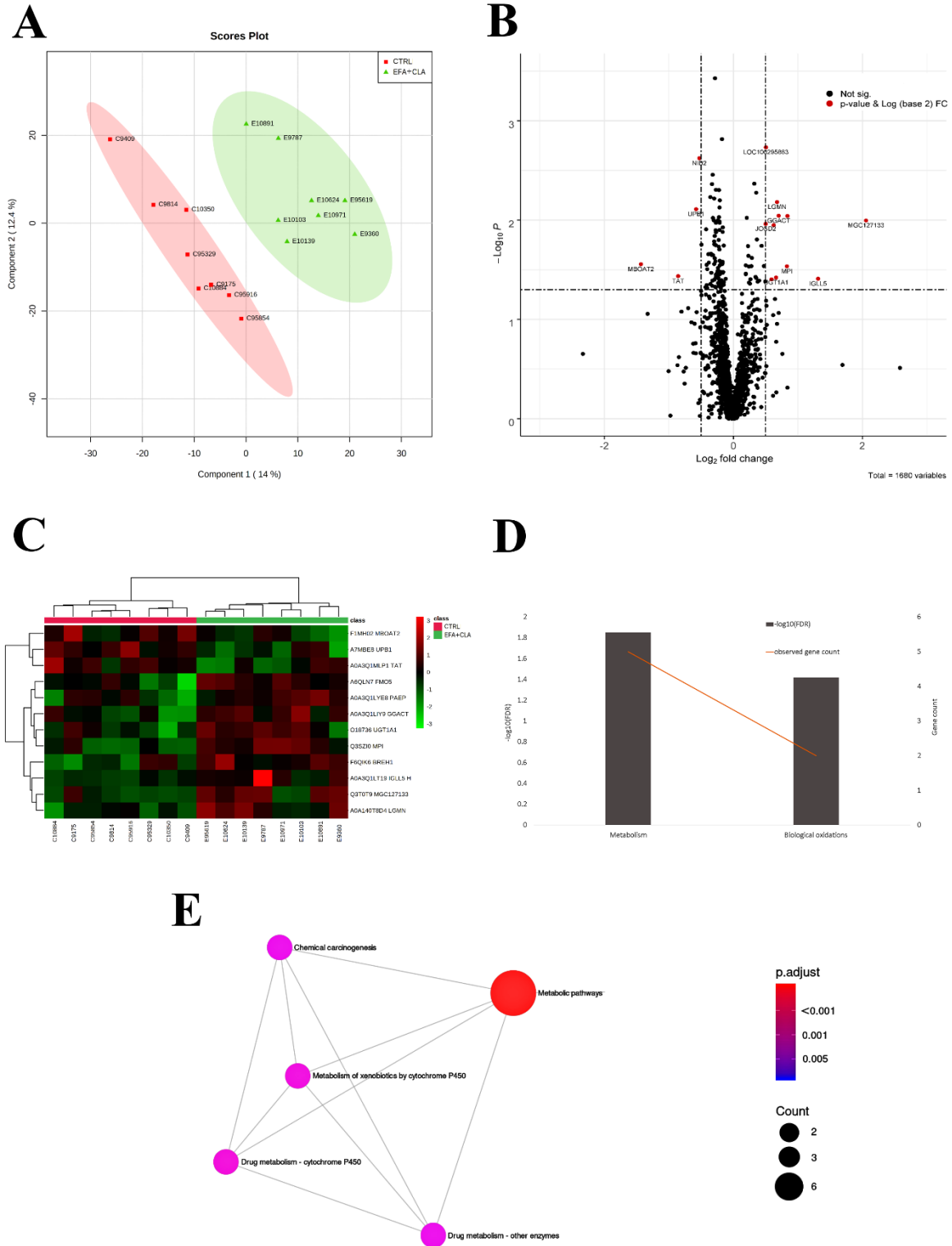
263 Considering all DAP, “steroid biosynthesis (bta00100)”, “metabolism of xenobiotics by cytochrome P450
264 (bta00980)”, “drug metabolism - cytochrome P450 (bta00982)”, “retinol metabolism (bta00830)”, “metabolic
265 pathways (bta01100)” were mapped to KEGG metabolic pathways (Figure 2 E, details in [31]).

272 abundant proteins (DAP). Fold enrichment (Bars, $-\log_{10}$ (adjusted P-value)) refers to the number of relevant gene names represented in each
273 category relative to random expression of all genes in the *Bos taurus* genome. The line between pathways represents their dependence. E. KEGG
274 pathways map of DAP. The colour of the nodes represents the $-\log_{10}$ (adjusted P-value); the size of the dots represents the number of DAP in the
275 pathway. The line between pathways represents their dependence.

276
277 **3.4. Differentially abundant proteins, interaction network, and functional enrichment of day 1 postpartum,**

278 On the day after parturition, 12 proteins were differentially abundant between treatment groups (Table 1), including
279 nine increased proteins (with a fold change that ranged from 1.50 - 4.16, P-value < 0.05), and three decreased proteins
280 (ranging from 0.37 - 0.67) in the EFA+CLA group. The DAP are shown in a Volcano plot, and their expression was
281 plotted by heat maps (Figure 3 A, B, C).

282 Also, the DAP were annotated by KEGG pathways, including “drug metabolism - cytochrome P450 (bta00982)” and
283 “metabolism of xenobiotics by cytochrome P450 (bta00980)” (Figure 3 D) and Reactome pathway “metabolism of
284 lipids (bta556833)” (Figure 3 E, details in [31]).
285



286
 287
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 291

Figure 3) A. Partial least squares discriminant analysis (PLS-DA) score plot of CTRL (red squares) and EFA+CLA (green triangle) in day 1 of postpartum. B. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group, increased (top right) and decreased (top left) proteins were highlighted in red ($P < 0.05$ and fold change > 1.5). C. Hierarchical clustering heat map analysis of differentially abundant proteins, Rows and columns are respectively sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms, red and green represent CTRL and EFA+CLA, respectively. D. Reactome enrichment analysis (x-axis), fold enrichment (bars, left y-axis); the number of

292 significant genes in each pathway ($-\log_{10}$, adjusted P-value) is represented by the lines on the right y-axis) represent. E. KEGG pathways map of
293 differentially abundant proteins (DAP). The colour of the nodes represents the $-\log_{10}$ (adjusted P-value); the size of the dots represents the number
294 of DAP in the pathway. The line between pathways represents their dependence.
295

296 **3.5. Differentially abundant proteins, interaction network, and functional enrichment at day 28** 297 **postpartum,**

298 At this time point, the relative abundance of 27 proteins was different between treatments (Table 1), of which 21
299 proteins were increased (with a fold change that ranged from 1.50 - 4.70, P-value < 0.05) and 6 proteins decreased
300 (ranging from 0.57 - 0.66) in the EFA+CLA group as compared to the control group (Figure 4 A, B, C). Twenty-three
301 BP have annotated (adjusted P-value < 0.05) by increased proteins, of which “cellular iron ion homeostasis
302 (GO:0006879)”, “apoptotic mitochondrial changes (GO:0008637)”, “mitochondrial transport (GO:0006839)”,
303 “regulation of lipid metabolic process (GO:0019216)”, “membrane organization (GO:0061024)”, “apoptotic process
304 (GO:0006915)”, and “regulation of cellular process (GO:0050794)” (Figure 4 D, details in [31]). Moreover, the GO
305 term “ferric iron-binding (GO:0008199)” in the MF category has been annotated. The proteins were localized in the
306 “mitochondrial intermembrane space (GO:0005758)”, “lysosome (GO:0005764)”, and “cytoplasm (GO:0005737)”,
307 respectively ([31]).

308 Also, the KEGG pathways were linked to “ferroptosis (bta04216)”, “mineral absorption (bta04978)”, “porphyrin and
309 chlorophyll metabolism (bta00860)”, “drug metabolism - cytochrome P450 (bta00982)”, “metabolism of xenobiotics
310 by cytochrome P450 (bta00980)”, “chemical carcinogenesis (bta05204)”, “arachidonic acid metabolism (bta00590)”,
311 and “metabolic pathways (bta01100)” (Figure 4 E).

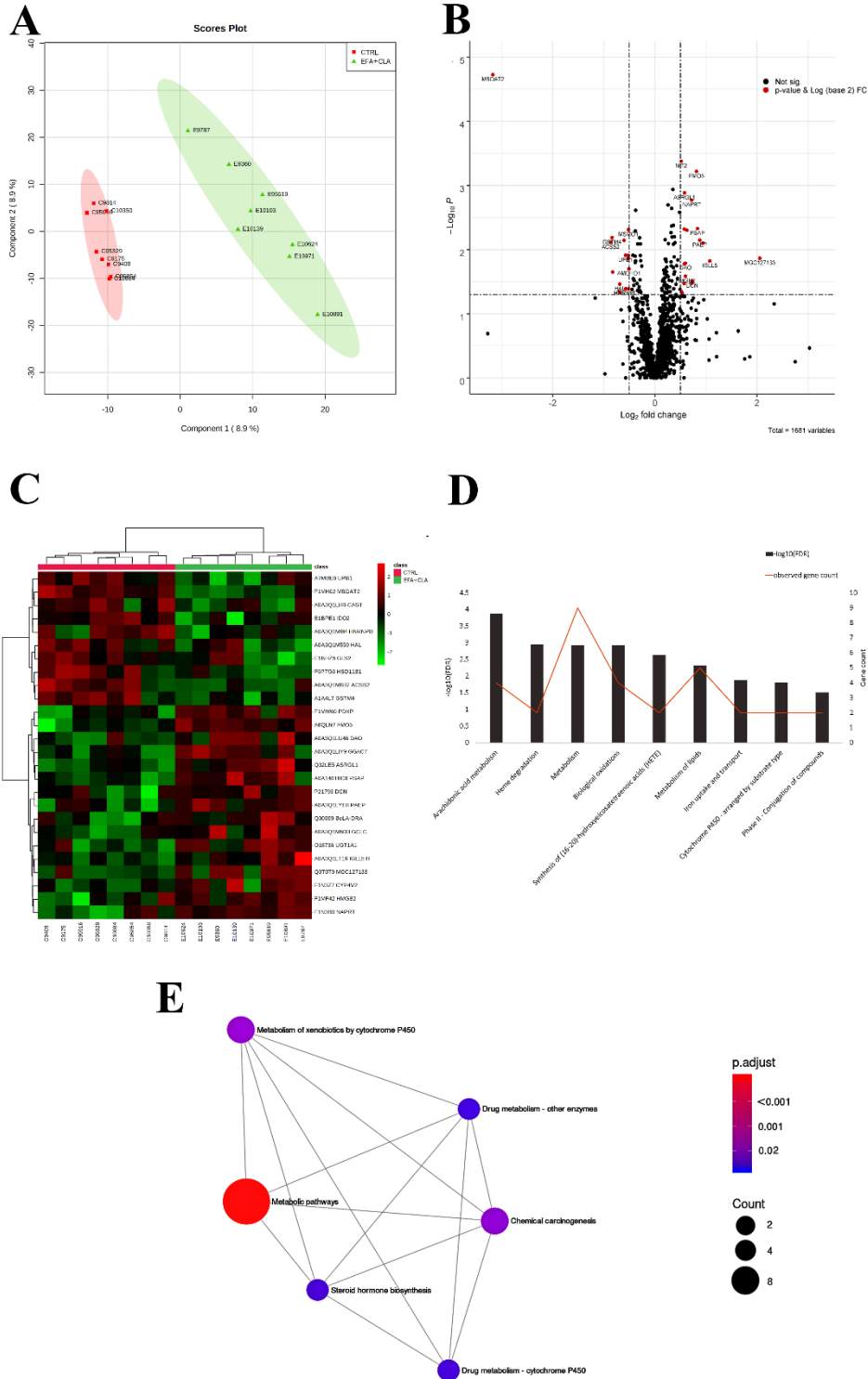
312 Decreased proteins were annotated by KEGG pathways related to “steroid hormone biosynthesis (bta00140)”,
313 “metabolism of xenobiotics by cytochrome P450 (bta00980)”, and “chemical carcinogenesis (bta05204)” ([31]).
314 Reactome enriched pathways included “arachidonic acid metabolism BTA-2142753”, “cytochrome P450 - arranged
315 by substrate type BTA-211897” and “metabolism of lipids BTA-556833” (Figure 4 F).

322 (adjusted P-value) is coloured in red according to the degree of significance, refers to the number of relevant gene names represented in each
323 category relative to random expression of all genes in the *Bos taurus* genome. The line between pathways represents their dependence. E. KEGG
324 pathways map of DAP. The colour of the nodes represents the $-\log_{10}$ (adjusted P-value); the size of the dots represents the number of DAP in the
325 pathway. The line between pathways represents their dependence.

326
327 **3.6. Differentially abundant proteins, interaction network, and functional enrichment at day 63**
328 **postpartum,**

329 At the last time-point, 26 proteins were considered as DAP (Table 1), among which 16 proteins were upregulated
330 (with a fold change ranging from 1.49 - 4.16, P-value < 0.05), and 10 proteins were downregulated (ranged from 0.11-
331 0.67) in the treatment group as compared to the CTRL group (Figure 5 A, B, C).

332 The decreased proteins annotated by KEGG pathways belong to “drug metabolism - cytochrome P450 (bta00982)”,
333 “metabolism of xenobiotics by cytochrome P450 (bta00980)”, “chemical carcinogenesis (bta05204)”, and “metabolic
334 pathways (bta01100)” (Figure 5 D, details in [31]). Interestingly, the same pathways were also enriched by the
335 upregulated proteins (details in [31]). Moreover, DAP were annotated by Reactome terms to “metabolism BTA-
336 1430728”, “Phase II - conjugation of compounds BTA-156580”, “glutathione conjugation BTA-156590” (Figure 5
337 E).

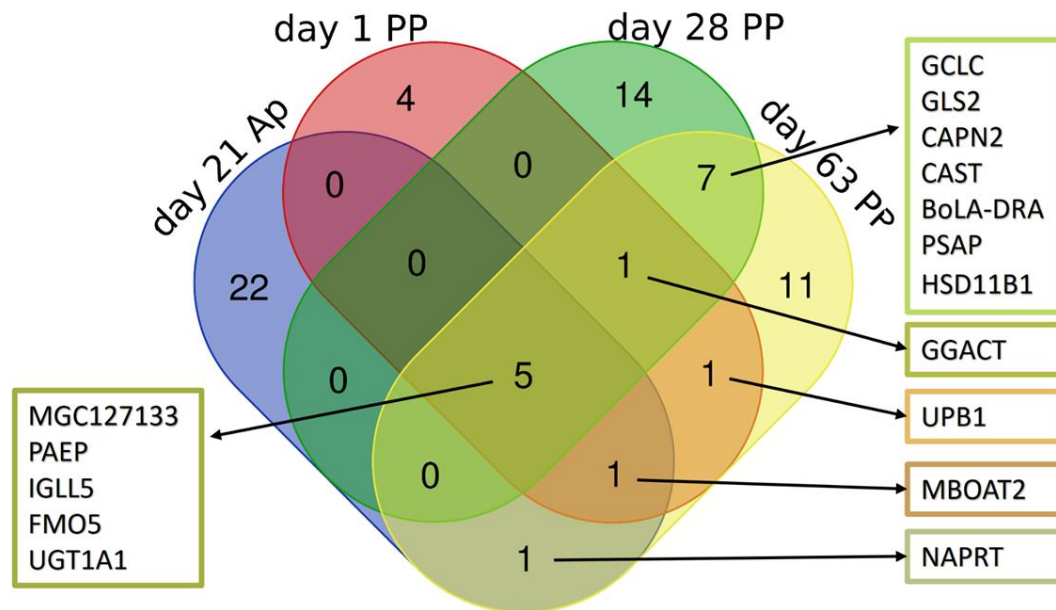


338
 339 Figure 5) A. Partial least squares discriminant analysis (PLS-DA) score plot of CTRL (red squares) and EFA+CLA (green triangle) in day 63 of
 340 postpartum. B. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group, increased (top right) and decreased
 341 (top left) proteins were highlighted in red ($P < 0.05$ and fold change > 1.5). C. Hierarchical clustering heat map analysis of differentially abundant
 342 proteins, Rows and columns are respectively sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms, red and green
 343 represent CTRL and EFA+CLA, respectively. D. Reactome enrichment analysis (x-axis), fold enrichment (bars, left y-axis); the number of

344 significant genes in each pathway ($-\log_{10}$, adjusted P-value) is represented by the lines on the right y-axis) represent. E. KEGG pathways map of
 345 differentially abundant proteins (DAP). The colour of the nodes represents the $-\log_{10}$ (adjusted P-value); the size of the dots represents the number
 346 of DAP in the pathway. The line between pathways represents their dependence.
 347

348 3.7. Common differentially abundant proteins along time

349 As illustrated in the Venn diagram (Figure 6), the DAP pattern was time-specific, probably due to substrates (i.e.
 350 supplemented FA, NEFA, and accumulated intermediates) abundance. The relative abundance of 5 common proteins
 351 including 20-beta-hydroxysteroid dehydrogenase-like (Q3T0T9, GN: MGC127133), lipocln_cytosolic_FA-bd_dom
 352 domain-containing protein (A0A3Q1LYE8, GN: PAEP), Ig-like domain-containing protein (A0A3Q1LT19, GN:
 353 IGLL5), dimethylaniline monooxygenase [N-oxide-forming] (A6QLN7, GN: FMO5), and UDP-
 354 glucuronosyltransferase (O18736, GN: UGT1A1) were affected by EFA+CLA treatment during all time points (Figure
 355 5). Moreover, seven common proteins including glutamate-cysteine ligase catalytic subunit (A0A3Q1MN33, GN:
 356 GCLC), glutaminase 2 (E1BHZ6, GN: GLS2), calpain-2 catalytic (A0A3Q1LRZ7, GN: CAPN2), calpastatin
 357 (A0A3Q1LI46, GN: CAST), boLA-DR-alpha (Q30309, GN: BoLA-DRA), prosaposin (A0A140T8C6, GN: PSAP),
 358 and hydroxysteroid 11-beta dehydrogenase 1 (F6PTG3, GN: HSD11B1) were affected by EFA+CLA treatment on
 359 days 28 and 63.
 360



361
 362 Figure 6) Venn diagram represent common and specific differentially abundant proteins identified in -21, +1, +28, and +63 days relative to
 363 parturition.
 364
 365
 366
 367

368 **4. Discussion**

369 This study aimed to investigate the metabolic adaptation in dairy cows supplemented with a combination of EFA and
370 CLA during the transition from pregnancy to lactation by applying proteomics in liver tissue samples. The synergistic
371 effect of these two FA on performances and “classical” parameters including energy metabolism, the somatotrophic
372 axis signaling pathway, plasma fatty acids profile, and markers of inflammation was recently presented [11, 16, 17].
373 The present study complements previously published works on the hepatic metabolic adaptations as it pointed out
374 proteins and pathways that are part of the molecular signatures elicited by supplementation with EFA and CLA, the
375 latter representing a model for feeding on grass.

376

377 **4.1. Common pathways identified antepartum and postpartum**

378 The relative abundance of MGC127133, PAEP, IGLL5, FMO5, and UGT1A1 was affected by EFA+CLA regardless
379 of time (Figure 6). These proteins were annotated by KEGG pathways related to drug metabolism - cytochrome P450,
380 metabolism of xenobiotics by cytochrome P450, and retinol metabolism, all belonging to the lesser-studied
381 “cytochrome P450 epoxidation/hydroxylation” pathways involved in ω -oxidation of FA. Unfortunately, less
382 information is available regarding these enzymes’ specific functions or their associated pathways in dairy cows,
383 especially in *in vivo* models. Nevertheless, in many species, particularly humans and mice, cytochrome P450 and
384 xenobiotic metabolism regulate the cross-talk between the immune system and metabolism [32].

385 Cytochrome (CYP) refers to a superfamily of heme-containing membrane-associated enzymes, regulating several
386 functions related to cholesterol and FA metabolism, detoxification of xenobiotic substances, steroid metabolism, drug
387 and pro-carcinogen deactivation, and catabolism of exogenous compounds, located primarily in the liver, but also in
388 all other tissues [33]. In this context, along with the α - and β -oxidation of FA, hepatic ω -oxidation of FA (CYP P450)
389 can help utilize PUFA and prevent hepatic lipid overload [34]. ω -oxidation of FA is an alternative pathway when
390 mitochondrial β -oxidation is deficient and involves the oxidation of the ω -carbon of FA in the endoplasmic reticulum
391 to provide succinyl-CoA [35]. CYP isoforms may have different functions, activities, and substrates [36]; therefore,
392 their inhibition and induction are regulated indirectly by ligand activation of xenobiotics to nuclear receptors, such as
393 peroxisome proliferator-activated receptors (PPARs) [37] and pregnane X receptor (PXR) [38]. In this respect,
394 xenobiotics are defined as natural components such as diet-derived compounds (e.g., lipids) or synthetic drugs
395 considered foreign to the body and therefore being subjected to the liver metabolism primarily to increase their polarity
396 and make them easier to excrete [39].

397 Previously, in a precise activity-based protein profiling technique, it has been shown that a commercial high-fat diet
398 (based on lard) decreased P450 activity in mouse liver, which led to obesity, obesity-induced chronic inflammation,
399 increased risk for hepatotoxicity, and metabolic disease [40]. Herein, we suppose that EFA and CLA or their
400 intermediates acted as xenobiotic substances and oxidized through cytochrome P450 pathways. It is worth pointing
401 out that any alteration to the average physiological level of CYP activities may cause disease, being their activity
402 required to detoxify drugs, neutral components, or biochemical intermediates to avoid impeding critical metabolic
403 pathways. Taken together, the low level of PUFA or n-3 to n-6 ratio in the CTRL group may negatively influence the

404 functional capacity of xenobiotic-metabolizing P450. Herein, the results indicated specific and different isoforms
405 (isoform-specific manner) of CYP affected by treatment during the transition period.

406 Recent studies using knockout *Fmo5*^{-/-} mice revealed that FMO5 not only functions as a xenobiotic-metabolizing
407 enzyme but also has been implicated as a regulator of glucose and lipid homeostasis, metabolic ageing, and insulin
408 sensitivity [41, 42]. In addition, FMO5 acts as NADPH oxidase, lowering NADPH which is the electron source in
409 lipid and cholesterol biosynthesis. In this regard, downregulation of FMO5 in mice has been associated with reduced
410 fat deposition and lower plasma cholesterol [41, 42]. Thus, the increased expression of this protein is probably induced
411 by a xenobiotic-like function of supplemented FA.

412

413 **4.2. Metabolic adaptation in the antepartum period**

414 On d 21 AP, 4 proteins were annotated by enriched GO term related to cholesterol metabolism. In addition to FMO5
415 which is a DAP identified at all time points, squalene monooxygenase (A5D9A8, GN: SQLE, unreviewed proteins in
416 *Bos taurus*) and squalene synthase (Q6IE76, GN: FDFT1, unreviewed proteins in *Bos taurus*) and CYP51A1 were
417 increased in the EFA+CLA group. Previously in a human study, a significant association of CYP51A1 gene expression
418 with lower blood total cholesterol and LDL cholesterol levels, but not with TG and HDL-cholesterol, has been reported
419 in women in their second trimester of pregnancy [43]. The CYP51 protein is very conserved between species (NCBI
420 homology, <https://www.ncbi.nlm.nih.gov/>); therefore, the same function of this protein in dairy cows can be supposed.
421 However, in this study, total cholesterol and LDL cholesterol concentrations were not affected by treatment in the AP
422 period (Figure S4).

423 Moreover, SQLE, FDFT1, and CYP51A1 are all involved in the cholesterol biosynthesis pathways through the Sterol
424 regulatory element-binding proteins (SREBP)-activated mevalonate pathway [44, 45]. In this pathway, FDFT1
425 initiates the conversion of farnesyl-pyrophosphate to squalene, which is the first stage of liver cholesterol synthesis
426 [46], followed by the synthesis of lanosterol from squalene catalyzed by SQLE, and the final step is the conversion of
427 lanosterol to cholesterol by the action of CYP51A [47]. Cholesterol homeostasis is crucial for normal cellular and
428 physiological functions and is strictly controlled by nuclear receptors, mammalian target of rapamycin
429 (mTOR)/SREBP2 pathway [48] and Liver X Receptors (LXR) [49] which induce and inhibit its synthesis,
430 respectively. In dairy cows, insufficiency of cholesterol metabolism and acceleration of body fat degradation before
431 parturition was reported to be associated with developing ketosis PP [50]. On the other side, chronic hepatic expression
432 of SREBP2 and excessive cholesterol storage has been shown to cause fatty liver disease (steatosis),
433 hypertriglyceridemia, and insulin resistance in non-ruminant species [51]. Fortunately, no differences were observed
434 in the plasma concentration of total cholesterol, TG, LDL, HDL (Figure S4), and hepatic expression of HMGCS2
435 between treatment groups before parturition [11, 17], which possibly points towards the feedback regulation that
436 synthesized cholesterol was used to maintain its homeostasis crucial in pregnant cows. Indeed, as a structural
437 component of the cellular membrane and precursor for steroid hormones, cholesterol esters, and bile acids (BA),
438 cholesterol is essential for the normal development of the dam and the fetus. In humans and rodents with a hemochorial
439 or hemoendothelial placenta type, the fetus depends on exogenous cholesterol sources obtained from the maternal
440 circulation transported across the placenta, mainly through lipoproteins [52]. It is not known whether this applies for

441 species with an epitheliochorial placenta type, such as most farm animals. Also, BA are incorporated into lipoproteins
442 and may induce hepatocytes to secrete and export the accumulated lipids from the liver (for review [53]). Intrahepatic
443 cholestasis and elevated BA and/or transaminases are considered as a liver disease [54].
444 Moreover, antepartum and around parturition, the membrane-bound O-acyltransferase 2 (F1MH02, GN: MBOAT2,
445 also known as lysophosphatidylcholine acyltransferase 4), a newly discovered member of the MBOAT family [55]
446 was decreased in the EFA+CLA group. This conserved enzyme catalyzes the production of glycerophospholipids in
447 the mammalian cell membrane, particularly phosphatidylcholine and phosphatidylethanolamine, which determine
448 membrane intrinsic curvature and fluidity [56]. This is the first study reporting the expression of MBOAT2 in dairy
449 cows' hepatocytes, and it is probably involved in modulating the ratio of PUFA in cellular membranes.

450

451 **4.3. Metabolic adaptation in lactation**

452 The day after parturition, along with cytochrome P450 pathways, the catabolic process and proteolysis, and bile
453 secretion KEGG pathways were annotated by DAP in the EFA+CLA group (identified by PLS-DA analysis). The
454 upregulated solute carrier organic anion transporter family member 1B3 (F1MYV0, GN: SLC01B3) enzyme not only
455 incorporates with activation of BA secretion [57] but also in the uptake of endogenous and xenobiotic compounds
456 [58]. Apart from already discussed mechanisms, BA has been reported to play novel roles as signaling molecules
457 regulating energy homeostasis, TG concentrations, and glucose [59-61]. In this regard, in a transcriptomic study, the
458 BA synthesis pathway reduction was reported in dairy cows with severe compared to mild negative energy
459 balance[62].

460 The liver is the main site regulating BA synthesis [40], primarily through the cholesterol/lipid homeostasis pathway
461 [63]. The activated mevalonate pathway thereby increased cholesterol synthesis that was discussed for the last time-
462 point, probably induced the downstream pathway, BA synthesis, and may explain why cholesterol concentration was
463 not different between treatments. More interestingly, converting cholesterol to BA, is regulated by cytochrome P450
464 (CYP7a1 and CYP8b1) pathways [40], although neither CYP7a1 abundance nor CYP8b1 were affected by treatment.
465 This may propose other pathways besides cytochrome P450 to regulate this conversion in dairy cows. Nevertheless,
466 no remarkable differences in performance and metabolite were observed between treatments. The difference in energy
467 balance between treatment groups [11] may indicate that the more negative energy balance in the CTRL group had
468 impaired cholesterol and BA synthesis.

469 On day 28 PP, cytochrome P450 family 4 subfamily F member 2 (A0A3S5ZPG5, GN: CYP4F2) and cytochrome
470 P450 family 1 subfamily A member 1 (F1MM10, GN: CYP1A1) had higher and lower abundance in the EFA+CLA
471 group, respectively. In this regard, a study in mice reported decreased CYP4F2 protein in the liver upon feeding a
472 high-fat diet associated with impaired hepatic lipid metabolism α -tocopherol pathways [64]. In general, the CYP4
473 members are tissue-specific and involved in FA metabolism, maintaining the concentration of FA and FA-derived
474 bioactive molecules within a normal physiological range [65]. CYP4F2 [66] and CYP4V2 [67] are two important
475 members of this family and are highly abundant in the liver. Arachidonic acid, lauric acid, vitamin K, and leukotriene
476 are the specific substrates for the CYP4F2 enzyme [68, 69]. We observed a significant difference in the plasma
477 concentration of FA on day 28 PP with lesser values in the EFA+CLA group. The greater FA concentration in the

478 CTRL group may have impaired mitochondrial function, reduced ATP synthesis, and potentially triggered lipotoxicity
479 [70]. On the other hand, an overabundance of CYP4F2 in the EFA+CLA group has been reported in humans to amplify
480 the capacity of hepatocytes to oxidize excess FA [71], which may support our proteomic results. Induction of CYP4F2
481 expression is proposed to be mediated by the ligand activation of nuclear receptors with supplemented FA and in
482 response to activated AMPK and SREBP pathways, which then augment the capacity of cytochrome P450 to oxidize
483 xenobiotics [71]. However, regulation may be at the level of enzyme activity rather than of protein abundance, since
484 enrichment of these two pathways was not observed in the present study. In other words, during the negative energy
485 balance, when the liver is stressed by the excessive FA supply from lipogenesis that may cause lipotoxicity, the
486 activation of CYP4F2, which removes FA, is logic and may explain how the EFA+CLA group accomplish the
487 inhibition of steatosis.

488 On the other side, the members of the CYP1 family use endogenous sex hormones such as progesterone and
489 testosterone, amine hormones like melatonin, vitamins, FA such as linoleic acid, and phospholipids as substrates [72],
490 which under specific circumstances activate compounds that react with DNA leading to an imitation of the mutagenic
491 process [73]. Furthermore, it has been reported both in *in vivo* [74] and *in vitro* [75-77] studies that CYP1A1 is
492 involved in PUFA metabolism.

493 Previously, the xenobiotic-like potential of fish oil in the induction of CYP1A1 mRNA expression in primary cultured
494 bovine hepatocytes was reported [78]. Also, there is emerging evidence that induction of CYP1A1 leads to non-
495 alcoholic fatty liver disease and the development of oxidative stress in humans, which is another molecular support
496 for hepatic metabolic imbalance in our CTRL group [79]. The exact mechanism of how CYP1A1 was inhibited in
497 EFA+CLA is not yet precisely known, although based on a study in mice [80], it could be speculated that
498 transcriptional regulation of CYP450 through activation of PPAR α is likely a possible pathway.

499 During the PP period (d +28 and +63), 11 β -hydroxysteroid dehydrogenase type 1 (F6PTG3, GN: HSD11B1) and
500 glutamate-cysteine ligase catalytic subunit (A0A3Q1MN33, GN: GCLC) increased, and phosphate-activated
501 mitochondrial glutaminase (E1BHZ6, GN: GLS2) decreased in EFA+CLA (Figure 5). Among them, GCLC and GLS2
502 are involved in the glutamine and glutamate metabolic processes and the glutathione (GSH) system. In GSH
503 biosynthesis, GLS2 catalyzes the conversion of glutamine to glutamate [81], and GCLC is a rate-limiting enzyme in
504 converting glutamate to GSH [82]. The combination of the above-noted enzymatic changes would be expected to
505 result in glutamate regulation. Glutamate, as one of the most abundant amino acids in the liver, is considered to be at
506 the crossroads of hepatic metabolism, where it is mainly involved in the TCA cycle, gluconeogenesis, FA oxidation
507 [83], and electron transport from the cytoplasm into the mitochondria via the malate-aspartate shuttle [84].

508 The HSD11B1 is an endoplasmic reticulum-located reductase that activates cortisone to cortisol, thereby modulating
509 hepatic gluconeogenesis [85]. It also plays a crucial role in glucocorticoid receptor (GR) activation, which in turn is
510 involved in the regulation of anti-stress and anti-inflammatory pathways [86]. It has been previously shown that liver
511 synthesized BA inhibit the HSD11B1 [87], which may be related to the downregulation of HSD11B1.

512 On day 63 PP, cytochrome P450 family 4 subfamily V member 2 (F1N3Z7, GN: CYP4V2) was more abundant in the
513 EFA+CLA than in the CTRL group. CYP4V2 has the same characteristic as the CYP4 classes but preferably
514 metabolizes arachidonic acid, lauric acid, eicosapentaenoic acid, docosahexaenoic acid, and medium-chain FA as

515 substrates [67, 88, 89]. The greater abundance of different CYP isomers between d 28 and 63 PP, probably related to
516 FA concentration, may compete with EFA and CLA for ligand activation of nuclear receptors (substrate dependent).
517 At this time point that coincides with returning to positive EB, the previously enriched cytochrome P450 pathways
518 and steroid hormone biosynthesis were affected by both downregulations of HSD11B1, glutathione S-transferase Mu
519 4 (A1A4L7, GN: GSTM4), and upregulation of MGC127133 and UGT1A1. Moreover, enrichment of several KEGG
520 pathways in the EFA+CLA group was observed by PLS-DA-identified DAP related to pentose and glucuronate
521 interconversions, starch and sucrose metabolism, pyruvate metabolism, glutamate metabolic process, and
522 glycolysis/gluconeogenesis. These pathways are intimately interconnected and are associated with energy metabolism.
523 Therefore, we considered these alterations to restore metabolic adaptation to the normal metabolism in positive EB
524 status. The EFA+CLA cows turned back to a positive EB around 21 days earlier than the CTRL group [11]. Therefore,
525 the activated metabolic adaptive processes in response to the NEB were also switched off or returned to normal
526 functions faster.

527

528 **5. Conclusion**

529 The results indicated that EFA+CLA supplementation altered the proteome profile of the liver in transition dairy cows.
530 Bioinformatics analysis of DAP revealed enriched pathways related to hepatic cholesterol biosynthesis, drug
531 metabolism - cytochrome P450, metabolism of xenobiotics by cytochrome P450, chemical carcinogenesis,
532 arachidonic acid metabolism, TCA cycle, and BA synthesis. Furthermore, in each time point, the relative abundance
533 of CYP enzymes affected by EFA+CLA supplementation in a time-dependant manner slightly impacted the capacity
534 of hepatic ω -oxidation. The results also suggest that EFA+CLA supplementation might be in support of preventing
535 hepatic steatosis during the transition period. Altogether, these findings provided novel information regarding the
536 underlying molecular mechanism by which hepatic metabolism responds to supplemented FA. Nonetheless, further
537 investigation with more accurate measures of hepatic steatosis is needed to replicate these findings in different
538 populations and physiological statuses.

539 **Funding**

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543

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546 concentration for mass spectrometry analyses.

547

548 **Declaration of Competing Interest**

549 Authors declare no conflict of interests.

550 **Figures legends**

551 Figure 1) Schematic diagram of the (A) study design, (B) proteomics workflow, and (C) bioinformatics pipeline. (A) Timeline of treatments
552 supplementation (from -63d ante to +63d postpartum) and liver biopsy collection (-21 d, +1 d, +28 d, and +63 d relative to parturition). Bold lines
553 indicate liver biopsy sampling time points. (B) Protein extraction, purification, reduction, alkylation, and digestion; peptides were analysed by high-
554 resolution LC-MS/MS, (C) Peptides alignment (progenesis), and protein identification (mascot) procedure were performed by Progenesis software
555 coupled with the Mascot search engine, statistical analysis was based on Partial least squares discriminant analysis (PLS-DA) merged with $P < 0.05$
556 and Fold change > 1.5 , followed by bioinformatics analysis (protein-protein interaction and Gene Ontology (GO) enrichment analysis.

557
558
559 Figure 2) A. Partial least squares discriminant analysis (PLS-DA) score plot of CTRL (red squares) and EFA+CLA (green triangle) on day 21
560 antepartum. B. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group, increased (top right) and decreased
561 (top left) proteins were highlighted in red ($P < 0.05$ and fold change > 1.3). C. Hierarchical clustering heat map analysis of differentially abundant proteins; Rows and columns are sorted by similarity as indicated by the left (proteins) and
562 top (samples) dendrograms, red and green represent CTRL and EFA+CLA, respectively. D. Biological Process Ontology for the differentially
563 abundant proteins (DAP). Fold enrichment (Bars, $-\log_{10}$ (adjusted P-value)) refers to the number of relevant gene names represented in each
564 category relative to random expression of all genes in the *Bos taurus* genome. The line between pathways represents their dependence. E. KEGG
565 pathways map of DAP. The colour of the nodes represents the $-\log_{10}$ (adjusted P-value); the size of the dots represents the number of DAP in the
566 pathway. The line between pathways represents their dependence.

567
568
569 Figure 3) A. Partial least squares discriminant analysis (PLS-DA) score plot of CTRL (red squares) and EFA+CLA (green triangle) in day 1 of
570 postpartum. B. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group, increased (top right) and decreased
571 (top left) proteins were highlighted in red ($P < 0.05$ and fold change > 1.5). C. Hierarchical clustering heat map analysis of differentially abundant
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573 represent CTRL and EFA+CLA, respectively. D. Reactome enrichment analysis (x-axis), fold enrichment (bars, left y-axis); the number of
574 significant genes in each pathway ($-\log_{10}$, adjusted P-value) is represented by the lines on the right y-axis) represent. E. KEGG pathways map of
575 differentially abundant proteins (DAP). The colour of the nodes represents the $-\log_{10}$ (adjusted P-value); the size of the dots represents the number
576 of DAP in the pathway. The line between pathways represents their dependence.

577
578
579 Figure 4) A. Partial least squares discriminant analysis (PLS-DA) score plot of CTRL (red squares) and EFA+CLA (green triangle) in day 28 of
580 postpartum. B. Volcano plot represents differentially abundant proteins between CTRL and EFA+CLA group, increased (top right) and decreased
581 (top left) proteins were highlighted in red ($P < 0.05$ and fold change > 1.5). C. Hierarchical clustering heat map analysis of differentially abundant
582 proteins, Rows and columns are respectively sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms, red and green
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595 differentially abundant proteins (DAP). The colour of the nodes represents the $-\log_{10}$ (adjusted P-value); the size of the dots represents the number
of DAP in the pathway. The line between pathways represents their dependence.

596
597
598

Figure 6) Venn diagram represent common and specific differentially abundant proteins identified in -21, +1, +28, and +63 days relative to parturition.

599 **Table heading**

600 Table 1. The differentially abundant proteins identified between CTRL and EFA+CLA in -21, +1, +28, and +63 days relative to parturition and
 601 their associated gene names.

Num.	Protein	Associated gene name	Time point
1	20-beta-hydroxysteroid dehydrogenase-like	MGC127133	1, 2, 3, 4*
2	Progestagen Associated Endometrial Protein	PAEP	1, 2, 3, 4
3	Dimethylaniline monooxygenase [N-oxide-forming]	FMO5	1, 2, 3, 4
4	Immunoglobulin Lambda Like Polypeptide 5	IgLL5	1, 2, 3, 4
5	UDP-glucuronosyltransferase	UGT1A1	1, 2, 3, 4
6	Membrane bound O-acyltransferase domain containing 2	MBOAT2	1, 2, 4
7	Gamma-glutamylaminocyclotransferase	GGACT	2, 3, 4
8	Nicotinate phosphoribosyltransferase	NAPRT	1, 4
9	Beta-ureidopropionase 1	UPB1	2, 4
10	Glutamate-cysteine ligase catalytic subunit	GCLC	3, 4
11	Glutaminase 2	GLS2	3, 4
12	Calpain-2 catalytic subunit	CAPN2	3, 4
13	Calpastatin	CAST	3, 4
14	BoLA-DR-alpha	BoLA-DRA	3, 4
15	Prosaposin	PSAP	3, 4
16	Hydroxysteroid 11-beta dehydrogenase 1	HSD11B1	3, 4
17	Squalene epoxidase	SQLE	1
18	FDFT1 protein	FDFT1	1
19	Lanosterol 14-alpha demethylase	CYP51A1	1
20	Cytochrome P450 4A25-like	LOC784417	1
21	Inter-Alpha-Trypsin Inhibitor Heavy Chain 2	ITIH2	1
22	Peptidylprolyl Isomerase A	PPIA	1
23	Aldo-keto reductase family 1, member C5	AKR1C5	1
24	Putative glycerol kinase 5	GK5	1
25	Shootin 1	SHTN1	1
26	RNA Transcription, Translation And Transport Factor	RTRAF	1
27	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	1
28	EF-hand domain-containing protein D2	EFHD2	1
29	Nucleoside diphosphate kinase	NME3	1
30	Aldo_ket_red domain-containing protein	LOC788425	1
31	Melanoma inhibitory activity protein 2	MIA2	1
32	RNA-binding protein 14	RBM14	1
33	Rab GDP dissociation inhibitor	GDI2	1
34	Ribosomal Protein S14	RPS14	1
35	Hydroxyacid-oxoacid transhydrogenase, mitochondrial	ADHFE1	1
36	Collagen Type X Alpha 1 Chain	COL10A1	1
37	Ras-related protein Rab-10	RAB10	1
38	Cingulin like 1	CGNL1	1
39	Legumain	LGMN	2

40	Tyrosine aminotransferase	TAT	2
41	Mannose-6-phosphate isomerase	MPI	2
42	Carboxylic ester hydrolase	BREH1	2
43	Cytochrome P450 Family 1 Subfamily A Polypeptide 1	CYP1A1	3
44	Ferritin light chain	FTL	3
45	Mediator Of Cell Motility 1	MEMO1	3
46	Ferritin	FTH1	3
47	Cathepsin C	CTSC	3
48	Heme oxygenase 1	HMOX1	3
49	Fatty acid amide hydrolase	FAAH	3
50	Thioesterase Superfamily Member 4	THEM4	3
51	Cytochrome P450 Family 4 Subfamily F Member 2	CYP4F2	3
52	Cytokine Induced Apoptosis Inhibitor 1	CIAPIN1	3
53	Reticulon-4-interacting protein 1, mitochondrial	RTN4IP1	3
54	Stomatin (EPB72)-like 2	STOML2	3
55	Queuosine salvage protein	C8H9orf64	3
56	Glutathione S-transferase Mu 1	GSTM4	3
57	Acyl-CoA synthetase short chain family member 2	ACSS2	4
58	Pyridoxal phosphate phosphatase	PDXP	4
59	Histidine ammonia-lyase	HAL	4
60	D-amino acid oxidase	DAO	4
61	Cytochrome P450 Family 4 Subfamily V Member 2	CYP4V2	4
62	High Mobility Group Box 2	HMGB2	4
63	Indoleamine 2,3-dioxygenase 2	IDO2	4
64	Decorin	DCN	4
65	Heterogeneous nuclear ribonucleoprotein D	HNRNPD	4
66	Asparaginase And Isoaspartyl Peptidase 1	ASRGL1	4
67	VPS35 Retromer Complex Component	VPS35	4

*1, 2, 3, and 4 correspond to days -21, +1, +28, and +63 relative to parturition, respectively.

602
603

604 **Supplementary Material**

605

606 **Supplementary S1**

607 Table S1. Amounts of daily abomasally infused supplements¹.

Supplementation	treatment			
	CTRL ²	EFA+CLA		
	Coconut oil ³	Linseed oil ⁴	Safflower oil ⁵	Lutalin® ⁶
Daily infused oils (g/d)				
Dosage lactation	76	78	4	38
Dosage dry period	38	39	2	19
Daily infused fatty acids (g/d) at the lactation dosage ⁷				
18:3 cis-9, cis-12, cis-15	0.00	39.9	0.01	0.00
18:2 cis-9, cis-12	1.39	12.4	2.48	1.34
18:2 cis-9, trans-11	0.00	0.00	0.01	10.3
18:2 trans-10, cis-12	0.00	0.02	0.01	10.2

608 1Cows were supplemented daily with coconut oil (CTRL), or a mixture of linseed, safflower oil (EFA), and Lutalin® (CLA, c9, t11 and t10,
609 c12), (EFA+CLA).

610 2Addition of vitamin E (0.06 g/d), Covitol 1360 (BASF, Ludwigshafen, Germany), to compensate for the vitamin E in linseed oil (0.07%) and
611 safflower oil (0.035%).

612 3Sanct Bernhard, Bad Ditzgenbach, Germany

613 4DERBY, Derby Spezialfutter GmbH, Münster, Germany

614 5GEFRO, Memmingen/Allgäu, Germany

615 6BASF, Ludwigshafen, Germany

616 7The lactation dosage was halved during the dry period.

617

618

619 **Supplementary S2**

620 Table S2. Ingredients and chemical compositions of the diets.

Item (g/kg of DM)	Diet	
	Dry period ¹	Lactation
Ingredients	421	457
Corn silage	223	97
Straw		
Compound feed DEFA ² (granulated)	-	446
Dried sugar beet pulp	163	-
Extracted soybean meal	99	-
Grain of rye	75	-
Mineral-vitamin mixture ³	10	-
Urea ⁴	9	-
Chemical composition		
NEL (MJ/kg DM) ⁵	6.2	7.1
Crude fat	21	23
Crude fiber	219	173
Crude protein	141	146
Utilizable protein ⁵	141	143
NFC	379	432
NDF	423	346
ADF	249	197
RNB ^{5,6}	0.0	0.5

621 ¹ The dry period diet was fed from wk 6 to wk 1 before calving.
622 ² Ceravis AG, Malchin, Germany Ingredients: 46.5% dried sugar beet pulp, 25.3% extracted soybean meal, 23.8% grain of rye, 1.4% urea, 1.1%
623 premix cow, 1.00% calcium, 0.37% phosphorus, 0.42% sodium, vitamins A, D3, E, copper, ferric, zinc, manganese, cobalt, iodine, selenium
624 Chemical composition: 44.4% NFC, 24.1% crude protein, 21.6% NDF, 12.4% ADF, 9.3% crude fiber, 8.2% crude ash, 1.8% crude fat, 7.9 MJ
625 NEL/kg DM
626 ³ KULMIN®MFV Plus (Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG, Kulmbach, Germany): 8.5% magnesium, 7.5% phosphorus,
627 6.5% sodium, 3.5% HCl insoluble ash, 1.5% calcium, additives: vitamins A, D3, E, B1, B2, B6, B5, B3, B12, B9, H, zinc, manganese, copper,
628 cobalt, iodine, selenium, and Saccharomyces cerevisiae
629 ⁴ Piarumin® (SKW Stickstoffwerke Piesteritz GmbH, Lutherstadt Wittenberg, Germany): 99% urea, 46.5% total nitrogen
630 ⁵ Society of Nutrition Physiology (GfE, 2001, 2008, 2009) and Deutsche Landwirtschaftliche Gesellschaft (DLG, 2013)
631 ⁶ RNB = ruminal nitrogen balance
632
633

634 **Supplementary S3.**

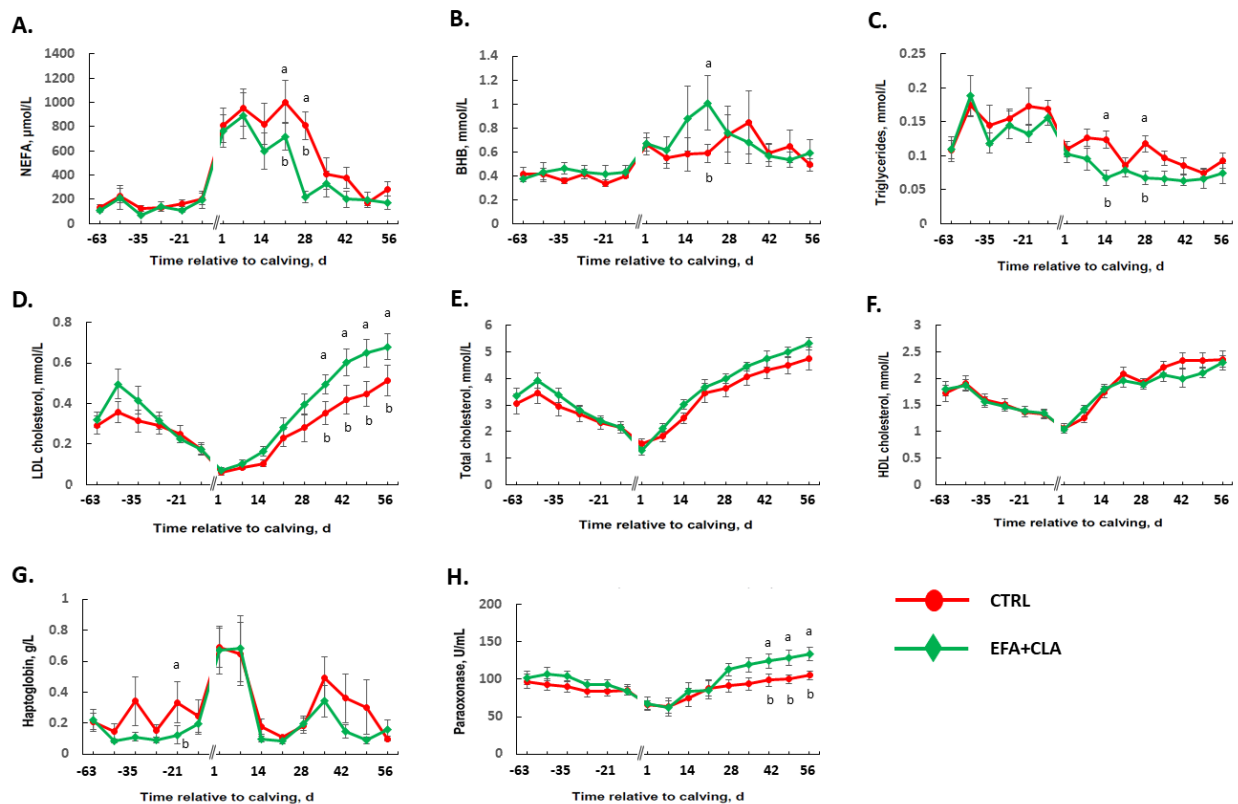
635 Table S3. Performance data of day 21 ante, and days +1, +28, and +63 postpartum of cows supplemented abomasally with coconut oil (CTRL; n =
636 8), or the combination of linseed and safflower oil (EFA) and conjugated linoleic acid (CLA) (EFA+CLA; n=8) from wk 9 antepartum until wk 9
637 postpartum, Adapted from [11].
638

			treatment		Fixed effect, P-value		
			CTRL	EFA+ CLA	EFA+ CLA	time	EFA+CLA*t ime
NEL intake, MJ NEL/d	late lactation		120.2 ± 4.6	113.8 ± 3.9	0.7	0.12	
	Dry period		80.6 ± 3.1	84.1 ± 2.8	0.8	0.001	
	Transition period		93.9 ± 3.3	93.4 ± 2.9	0.7	0.001	
	Postpartum		120.8 ± 3.8	115 ± 3.5	0.3	0.001	
	Entire Study		106.6 ± 3.3	104 ± 2.9	0.6	0.001	
FEMY, kg milk/kg DMI	late lactation		0.96 ± 0.11	0.98 ± 0.09	0.19	0.001	
	Early lactation		2.25 ± 0.1	2.43 ± 0.09	0.7	0.001	
FEECM, kg ECM/kg DMI	late lactation		1.08 ± 0.1	0.95 ± 0.09	0.2	0.001	
	Early lactation		2.31 ± 0.11	1.95 ± 0.1	0.5	0.001	
BW, kg	late lactation		701 ± 21	670 ± 19	0.5	0.001	
	Dry period		742 ± 22	718 ± 20	0.2	0.001	
	Transition period		690 ± 20	672 ± 18	0.3	0.001	
	Postpartum		634 ± 18	621 ± 17	0.4	0.001	
	Entire Study		685 ± 20	665 ± 18	0.3	0.001	
BCS	late lactation		3.62 ± 0.11	3.29 ± 0.1	0.7	0.001	
	Dry period		3.72 ± 0.12	3.62 ± 0.11	0.9	0.001	
	Transition period		3.54 ± 0.12	3.5 ± 0.11	1	0.001	
	Postpartum		3.12 ± 0.11	3.1 ± 0.1	0.8	0.001	
	Entire Study		3.43 ± 0.11	3.31 ± 0.1	0.8	0.001	
BFT, mm	late lactation		13.4 ± 1	11.3 ± 0.9	0.8	0.001	
	Dry period		15.3 ± 1.1	14.6 ± 1	0.9	0.001	
	Transition period		14.7 ± 1.1	14.5 ± 1	0.8	0.001	
	Postpartum		12.1 ± 1	12.6 ± 0.9	0.8	0.001	
	Entire Study		13.5 ± 1	13 ± 0.9	0.9	0.001	

639 1Values are presented as the LSM \pm SE.
 640 2FEMY = feed efficiency for milk production; FEECM = feed efficiency for ECM production; BFT = back fat thickness.
 641

642 **Supplementary S4.**

643 Plasma concentrations of (A) non-esterified fatty acids (NEFA), (B) β -hydroxybutyrate (BHB), (C) triglycerides, (D) low-density
 644 lipoprotein (LDL), (E) total cholesterol, (F) high-density lipoprotein (HDL), (G) haptoglobin, and (H) paraxonase from 83 d before
 645 until 63 d after calving in cows supplemented daily with coconut oil (\circ CTRL; n = 8), or a combination of linseed and safflower
 646 oil and Lutalin (cis-9,trans-11 and trans-10,cis-12 CLA; BASF, Ludwigshafen, Germany; \blacklozenge EFA+CLA; n = 8). Changes in plasma
 647 metabolites concentrations were analyzed using the MIXED procedure by repeated-measures ANOVA. Data are presented as the
 648 least squares means (LSM) and their standard errors (SE) (LSM \pm SE), LSM with different superscripts (a, b) differ ($P < 0.05$) at
 649 the respective time point. Statistically significant ($P < 0.05$) effects for (A) NEFA concentration during the entire study (time;
 650 EFA+CLA \times time interaction). Statistically significant ($P < 0.05$) effect for (B) BHB, (C) triglycerides, (D) LDL, (E) total
 651 cholesterol, (F) HDL, (G) haptoglobin, and (H) paraxonase concentration during the time. Adapted from [11, 16, 17].
 652
 653



654
 655
 656 **Supplementary files**
 657 The data and related analyses are available through the link <https://doi.org/10.15454/5U5WQS>.
 658

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