Journal of Proteomics 304 (2024) 105232

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Conjugated linoleic acid (CLA) modulates bovine peripheral blood mononuclear cells (PBMC) proteome *in vitro*

G. Ávila [a], F. Ceciliani [a,*], D. Viala [b,c], S. Dejean [d], G. Sala [a], C. Lecchi [a], M. Bonnet [b]

[a] Department of Veterinary Medicine and Animal Science, Università Degli Studi di Milano, Via dell'Università 6, 26900 Lodi, Italy
[b] INRAE, Université Clermont Auvergne, Vetagro Sup, UMRH, 63122 Saint-Genès-Champanelle, France
[c] INRAE, Metabolomic and Proteomic Exploration Facility (PFEM), F-63122 Saint-Genès-Champanelle, France
[d] Institut de Mathématiques de Toulouse, Université de Toulouse, CNRS, UPS, UMR 5219, 31062 Toulouse, France

ARTICLE INFO

Keywords:
Conjugated linoleic acid
Bovine PBMC proteome
Antiapoptotic
Immunomodulation
SERPINA3–7
ITIH4
HMOX1

ABSTRACT

Conjugated linoleic acid (CLA) is a group of natural isomers of the n-6 polyunsaturated fatty acid (PUFA) linoleic acid, exerting biological effects on cow physiology. This study assessed the impact of the mixture 50:50 (vol:vol) of CLA isomers (cis-9, trans-11 and trans-10, cis-12) on bovine peripheral blood mononuclear cells (PBMC) proteome, identifying 1608 quantifiable proteins. A supervised multivariate statistical analysis, sparse variant partial least squares – discriminant analysis (sPLS-DA) for paired data identified 407 discriminant proteins (DP), allowing the clustering between the CLA and controls. The ProteINSIDE workflow found that DP with higher abundance in the CLA group included proteins related to innate immune defenses (PLIN2, CD36, C3, C4, and AGP), with antiapoptotic (SERPINF2 and ITIH4) and antioxidant effects (HMOX1). These results demonstrated that CLA modulates the bovine PBMC proteome, supports the antiapoptotic and immunomodulatory effects observed in previous *in vitro* studies on bovine PBMC, and suggests a cytoprotective role against oxidative stress. *Significance:* In this study, we report for the first time that the mixture 50:50 (vol:vol) of cis-9, trans-11, and trans-10, cis-12-CLA isomers modulates the bovine PBMC proteome. Our results support the immunomodulatory and antiapoptotic effects observed in bovine PBMC *in vitro*. In addition, the present study proposes a cytoprotective role of CLA mixture against oxidative stress. We suggest a molecular signature of CLA treatment based on combining a multivariate sparse discriminant analysis and a clustering method. This demonstrates the great value of sPLS-DA as an alternative option to identify discriminant proteins with relevant biological significance.

1. Introduction

Conjugated linoleic acid (CLA) is a group of naturally occurring positional and geometrical isomers of the essential n-6 polyunsaturated fatty acid (PUFA) linoleic acid, characterized by conjugated double bonds in either cis or trans configuration. The cis-9,trans-11 (c9,t11), and the trans-10,cis-12 (t10,c12) are the two most abundant and biologically relevant CLA isomers [1–4]. In ruminants, the concentration of CLA in blood has been recently measured as 0.54 mg/L, primarily made of c9,t11–18:2 [5,6]. Like other species, the cis-9,trans-11 (c9,t11) and the trans-10,cis-12 (t10,c12) are the most abundant isomers [6]. CLA plasma and milk concentrations in dairy cows have been shown to increase due to grass and lipid-based diets [7]. In dairy cows, CLA increases milk yield and reduces milk fat, thus enhancing whole-body energy utilization and increasing reproductive performance in early lactation [8–10] and the transition period [11]. A potential role of CLA in mitigating the pro-inflammatory status associated with oxidative stress around calving in dairy cows has been proposed [12], as shown for other PUFA. Each isomer exerts differential biological effects when used separately, but enhanced and synergic effects are demonstrated when combined in equal amounts [4–6].

In vitro, *ex vivo,* and *in vivo* studies have also demonstrated that CLA isomers separately or in a mixture can modulate the bovine immune response by affecting peripheral blood mononuclear cells (PBMC) proliferation and cytokine expression, PBMC stimulation ability and increasing different PBMC subsets (*e.g.*, T and B cells) in mesenteric lymph nodes and ileal lamina propria, respectively [15–17]. In addition, in our previous *in vitro* study, only the CLA 50:50 (vol:vol) mixture decreased bovine monocyte apoptosis and increased Reactive Oxygen Species (ROS) production under experimentally induced pro-

* Corresponding author at: Department of Veterinary Medicine, Università Degli Studi di Milano, Via dell'Università 6, 26900 Lodi, Italy.
E-mail address: fabrizio.ceciliani@unimi.it (F. Ceciliani).

https://doi.org/10.1016/j.jprot.2024.105232
Received 20 April 2024; Received in revised form 16 June 2024; Accepted 20 June 2024
Available online 22 June 2024

inflammatory conditions [18]. On the contrary, the individual isomer activity is much weaker or absent. The exact molecular mechanisms underlying these immunomodulatory effects have not yet been identified.

OMIC technologies – the set of high-throughput technologies that detect genes (genomics), RNA (transcriptomics), proteins (proteomics), lipids (lipidomics), and metabolites (metabolomics) – have become a widely used approach in livestock nutrition as they help to understand the molecular effects of novel feed additives in a comprehensive, holistic and systematic way [19]. Specifically, proteomics demonstrated that supplementing CLA affects dairy cows' plasma and liver proteomes and promotes the cross-talk between lipid metabolism and immunity [20–23].

Although a recent study determined the *ex vivo* bovine PBMC proteome of postpartum dairy cows supplemented with omega-3 fatty acids [24], still, to our knowledge, the *in vitro* impact of CLA, especially of the CLA (50:50) mixture on bovine PBMC proteome, has not been assessed yet.

To cover this gap, this study, using an untargeted proteomics approach, aimed to evaluate the capacity of CLA (50:50) mixture in modulating bovine PBMC proteome *in vitro*.

2. Materials and methods

2.1. Purification of bovine PBMC from blood

Bovine PBMC were isolated from peripheral blood through Ficoll density gradient centrifugation, as previously described [18], with minor modifications. Briefly, peripheral blood from 7 healthy pluriparous late lactation Holstein-Friesian cows was collected during routine slaughtering procedures at a local slaughterhouse in sterile flasks containing 1.8 mg K_2EDTA per mL of blood as an anticoagulant. Blood was first centrifuged (without breaks) to collect the buffy coat that was then diluted with sterile PBS without Ca^{2+} and Mg^{2+} containing 2 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA), layered carefully on Ficoll (1.077 g/mL; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and centrifuged again without breaks. The PBMC ring was recovered at the interface, washed with sterile PBS without Ca^{2+} and Mg^{2+} containing 2 mM EDTA, and treated with red blood cell lysis buffer (Roche Diagnostics GmbH, Mannheim, Germany) for red blood cell elimination. Finally, the PBMC were counted, their viability assessed with trypan blue exclusion (>90%) and resuspended at the desired concentration in complete medium (RPMI 1640 Medium +25 mM HEPES + L-Glutamine, supplemented with 1% of Non-essential Amino Acid Solution 100× and 1% Penicillin Streptomycin Solution 100× and 1% FBS (Sigma-Aldrich)).

2.2. Bovine PBMC stimulation with CLA (50:50) mixture

Firstly, the two CLA isomers 9(E),11(*Z*)-octadecadienoic acid (c9, t11) and 10(E),12(Z)-octadecadienoic acid (t10,c12) (Matreya LLC, State College, PA, USA) were used to prepare the CLA (50:50) mixture as previously described for bovine monocytes [18] with some minor modifications. Briefly, the mixture containing both CLA isomers in a 50:50 proportion at a final concentration of 50 μM was prepared in a complete medium (1% FBS) when needed for each experiment and set as the working concentration for *in vitro* stimulation of bovine PBMC. Secondly, a total of 15×10^6 bovine PBMC (1.5 mL) was seeded in triplicates in Corning® tissue-culture treated culture 60 mm dishes (Corning Inc., Costar, Kennebunk, ME, USA) and incubated with 50 μM of CLA (50:50) mixture (100 μL) or 0.014% ethanol (vehicle) as a control - this being the same concentration of ethanol found in the 50 μM CLA (50:50) mixture that was used for the two isomers solubilization - overnight at 39 °C in a humified atmosphere with 5% CO_2. Complete medium (1% FBS) was then added to each dish to reach a final volume of 3 mL. Cells treated with only complete medium (1% FBS) (untreated cells) were also used as a negative control to evaluate the effects of 0.014% ethanol on the cells' proteome. Finally, according to the results of our previous study, ethanol at this concentration did not affect the viability of the cells as assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetra-zolium bromide (MTT)-based assay [18]. Seven biological replicates (animals) were used for the experiment, and the cells from each animal were seeded and treated in three technical replicates.

2.3. Bovine PBMC collection and protein extraction

The cells' supernatant (3 mL) containing the cells in suspension (lymphocytes) was collected into two tubes of 1.5 mL. Cells were centrifuged at 500 xg for 7 min at 4 °C to pellet the lymphocytes, which were then washed twice with 500 μL sterile-PBS without Ca^{2+} and Mg^{2+} (room temperature). In the meantime, three washes with 2 mL of sterile-PBS without Ca^{2+} and Mg^{2+} (room temperature) were performed in the 60 mm dishes to wash the adhered monocytes, remove dead cells, and rest of the contaminant medium and FBS. When both the lymphocytes and monocytes were washed, 500 μL of lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA and EGTA, 100 mM NaF, 4 mM $Na_4P_2O_7{\cdot}10\ H_2O$, 2 mM Na_3VO_4, 1% Triton X100, 0.5% IGEPAL CA-630 (Sigma-Aldrich), 1 pill for 50 mL of total volume of Protease Inhibitor Cocktail (Roche)) were added to the lymphocytes pellet, the cells were resuspended and then collected and added directly to the monocytes in the 60 mm dishes. The dishes were then carefully mixed to ensure the surface was covered with Igepal buffer and incubated for 1 h at 4 °C with regular shaking to lyse the cells. After incubation, the cells were scraped using sterile Corning Cell scrapers (blade L 1.8 cm, handle L 25 cm Corning Inc.), the cell lysate was collected in 1.5 mL tubes and sonicated for 10 min in an ultrasonic bath (Branson 2200, Danbury, CT, USA) to complete the cells' lysis and homogenization. Finally, the tubes with the cell lysate were centrifuged for 10 min at 6000 xg at 4 °C to remove the cell debris, and the supernatant containing the extracted proteins of all 3 technical replicates for each biological replicate was pooled in 1.5 mL tubes, aliquoted and stored at −80 °C for further analyses.

2.4. Sample preparation for proteomic analysis

Total protein concentration was first determined with the Pierce Bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), following the manufacturer's instructions. For peptide preparation, extracted proteins were digested and concentrated using the Filter Aided Sample Preparation (FASP) method, following the manufacturer's instructions with minor modifications. Briefly, fifty μg of protein lysate were put into Milipore 10 kDa MWCO filters, and 100 μL of Urea 8 M in 0.1 M Tris-HCl pH 8.5 were added for solubilization. The detergent and other contaminant components within the protein lysate were removed by repeated filtrations with centrifugation. After discarding the flow-through, proteins were reduced with 100 μl DTT (10 mM) for 45 min and centrifuged at 14000 rpm for 10 min. Then, 200 μL of 25 mM iodoacetamide solution were added to the filters and incubated for 20 min at room temperature for protein alkylation. After performing several washes of the filters with Urea 8 M and 50 mM of ammonium bicarbonate, proteins were digested with 200 μL of 0.25 mg/mL trypsin (50:1 ratio of protein:enzyme) and mixed at 600 rpm in a thermomixer for 1 min. Filters were transferred to new collection tubes and incubated overnight in a wet chamber at 37 °C. Finally, peptides were eluted by adding 200 μL of 50 mM of ammonium bicarbonate, and the filtrate was lyophilized in a SpeedVac and then suspended in 100 μL of 1% formic acid.

2.5. Nano-LC-MS/MS analysis

For each sample, 50 μg of proteins were used according to the FASP preparation. The final volume was adjusted exactly to 300 μL with a recovery solution (H_2O/ACN/TFA – 94.95/5/0.05). After passing

through the ultrasonic bath (10 min), the supernatant was transferred to an HPLC vial before LC-MS/MS analysis. Peptide mixtures were analyzed by nano-LC-MS/MS (ThermoFisher Scientific) using an Ultimate 3000 system coupled to a QExactive HF-X mass spectrometer (MS) with a nanoelectrospray ion source. Five µL of hydrolyzate was first preconcentrated and desalted at a flow rate of 30 µL/min on a C18 precolumn 5 cm length x 100 µm (Acclaim PepMap 100 C18, 5 µm, 100 A nanoViper) equilibrated with Trifluoroacetic Acid 0.05% in water. After 6 min, the concentration column was switched online with a nanodebit analytical C18 column (Acclaim PepMap 100–75 µm inner diameter × 25 cm length; C18–3 µm -100 Å - SN 20106770) operating at 400 nL/min equilibrated with 96% solvent A (99.9% H2O, 0.1% formic acid). The peptides were then separated according to their hydrophobicity thanks to a gradient of solvent B (99.9% acetonitrile, 0.1% formic acid) of 4 to 25% in 60 min. For MS analysis, eluted peptides were electrosprayed in positive-ion mode at 1.6 kV through a Nano electrospray ion source heated to 250 °C. The mass spectrometer operated in data-dependent mode: the parent ion is selected in the orbitrap cell (FTMS) at a resolution of 60,000. Each MS analysis is succeeded by 18 MS/MS with analysis of the MS/MS fragments at a resolution of 15,000).

2.6. Processing of raw mass spectrometry data

At the end of the LC-MS/MS analysis, for raw data processing, an MS/MS ion search was carried out with Mascot v2.5.1 (http://www.matrixscience.com) against the bovine database (*i.e.* ref_bos_taurus 20,210,114–37,512 sequences). The following parameters were used during the request: precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da, a maximum of two missed cleavage sites of trypsin, carbamidomethylation (C), oxidation (M) and deamidation (NQ) set as variable modifications. Protein identification was validated when at least two peptides originating from one protein showed statistically significant identity above Mascot scores with a False Discovery Rate of 1%. Ions score is −10 log(P), where P is the probability that the observed match is random. For the bovine proteome, the Mascot score was 32, with a False Discovery Rate (FDR) of 1%. The adjusted *p*-value was 0.02561. Finally, LC-Progenesis QI software (version 4.2, Nonlinear Dynamics, Newcastle upon Tyne, UK) was used for label-free protein quantification analysis with the same identification parameters described above with the phenotypic data among all matrices. All unique validated peptides of an identified protein were included, and the total cumulative abundance was calculated by summing the abundances of all peptides allocated to the respective protein. LC-Progenesis analysis yielded 1608 unique quantifiable proteins, with at least two and two unique peptides. Finally, it was impossible to quantify any proteins for sample 1 (corresponding to animal 1) from the untreated group, so this sample was eliminated for further statistical and bioinformatic analyses.

2.7. Statistical analyses

2.7.1. Univariate analysis

To identify the differentially abundant proteins, a univariate statistical approach was performed on all 1608 quantified proteins, using an R package for proteomic analysis (available on GitHub with the DOI https://doi.org/10.5281/zenodo.2539329) as previously described [25]. Briefly, data normality distribution was assessed by the Shapiro-Wilk-Test. Then, either a paired Student's *t*-test (normally distributed data) or a paired Wilcoxon test (not normally distributed data) was applied to determine the *p*-value between the different treatment groups comparisons (CLA *vs.* Vehicle, CLA *vs.* Untreated, and Vehicle *vs.* Untreated). Finally, the obtained *p*-values were corrected for multiple tests using different adjustment methods (*e.g.*, Benjamini & Hochberg or FDR, Hochberg, and Bonferroni). For all of these analyses, R version 4.2.2 was used.

2.7.2. Multivariate analysis

As no differentially abundant proteins were obtained after performing the p-value corrections at the issue of the univariate analysis, an alternative multivariate analysis was performed to discriminate the three groups thanks to several proteins (rather than with a unique protein considered in univariate analysis). A sparse variant partial least square discriminant analysis (sPLS-DA) was applied to select discriminative proteins (DP) among the identified proteome, according to a supervised method (DA among the three groups) for repeated measures (multilevel option), using the mixOmics package in R version 4.2.2 [26]. The multilevel sPLS-DA enables the selection of the most DP to classify the samples between the three different treatment groups [27]. With this supervised method and the mixOmics function plotLoadings() – Loadings Bar Plot, it was also possible to identify the DP that presented higher abundances (median) in each treatment group. However, only the proteins from the sPLS-DA component 1 that strongly correlated to one another (data given by the mixOmics function: plotVar() – Correlation Circle Plot) with the highest abundance in each treatment group were used to perform further bioinformatic analyses (GO enrichment analyses). Two strategies were used to visualize the abundance of the selected proteins. First, the average log fold-change (log FC) of the raw abundances of all the 407 DP from the CLA mixture samples was calculated with respect only to those of the vehicle group and plotted using R version 4.2.2. Secondly, a hierarchical clustering heat map analysis was performed to illustrate the relative abundance of all the 407 DP selected by sPLS-DA using the clustered image maps (cim) function from the mixOmics package in R (http://mixomics.org/graphics/cim/). Hierarchical clustering of both proteins and samples was performed. Dendrograms were used along the left and upper axes to show how each protein and sample clusters based on their similarity and the hierarchical clustering method used. Euclidean and Ward methods were used as the distance and cluster methods, respectively. The `cim()` clustering is a great complementary tool to the correlation circle plot (`plotVar()`). The correlation between subsets of variables from each dataset can be observed to help interpret the sPLS-DA results.

2.8. Bioinformatic analysis of discriminant proteins selected by sPLS-DA

First, before performing the bioinformatic and functional annotation analyses, the accession numbers from all 1608 quantified proteins were converted into Gene ID using the UniProt retrieve/ID mapping online tool. The Gene ID of human orthologs of bovine proteins was assigned for undefined proteins. Second, for the selected discriminant proteins, gene ontology (GO) and KEGG pathways enrichment analyses focused on the Biological Processes (BP) were performed using ProteINSIDE online tool version 2.0 (available at https://www.proteinside.org/), as previously described [28], and using *Bos Taurus* as reference species. Only significant (*p*-value FDR < 0.05) GO BP terms or KEGG pathways were considered enriched and were used for further interpretation and figure creation. The enriched GO BP terms or KEGG pathway were summarized. Their enrichments were expressed as -log10 (p-value FDR) for visualization on horizontal bar graphs plotted using GraphPad Prism 9.1.2 for Mac OS X, GraphPad Software (San Diego, California, USA). Selected discriminant proteins were also subjected to Protein-Protein interaction (PPI) analyses using the ProteINSIDE online tool version 2.0, as previously described [28] to identify the proteins that have been experimentally detected to interact with each other. The PPI databases used were BAR; DIP; EBI-GOA-nonIntAct; HPIDb; IMEx; IntAct; MBInfo; MINT; MPIDB; MatrixDB; Reactome; UniProt; VirHostNet; bhf-ucl; mentha and tfact2gene.

3. Results

3.1. The proteome of bovine PBMC and identification of the molecular signature of CLA (50:50) mixture

A total of 1608 proteins, with at least two unique peptides, were identified and quantified in bovine PBMC (Supplementary Table S1). Of these, 407 proteins have contributed to a good separation of the three treatment groups on the most "explicative" component by the PLS-DA (Fig. 1). As revealed by the mixOmics loadings bar plot function, from the 407 DP, a total of 85 proteins had the highest abundance in the CLA group, while 96 and 27 in the vehicle (ethanol) and untreated groups, respectively (Supplementary Table S2). Fig. 2 presents the plot of the changes (*logFC*) in the abundance of all the 407 DP between the CLA (50:50) mixture and vehicle group. The protein with the highest change in abundance in the CLA treated group (logFC >20) was PLIN2, followed by CD36 (logFC >5), VCAN, SERPINA3–7, SDS, and LUM (logFC >2). On the other hand, proteins such as APOA2, PNKP, GBP2, and RBM1B showed the lowest abundance in the CLA group (logFC <1). The 407 DP were annotated by 237 enriched (FDR < 0.05) GO terms within the Biological Process (BP) category (Supplementary Table S3). The main enriched BP were related to cellular RNA splicing (GO:0008380), mRNA processing (GO:0006397), organonitrogen compound metabolic process (GO:1901564), negative regulation of proteolysis (GO:0045861), metabolic process (GO:0008152), mitochondrial electron transport, NADH to ubiquinone (GO:0006120), immune system process (GO:0002376), negative regulation of endopeptidase (GO:0010951) and catalytic activity (GO:0043086) (Fig. 3). The BP annotated by the highest number of proteins were: metabolic process (269), organonitrogen compound metabolic process (91), immune system process (86) and negative regulation of catalytic activity (34).

3.2. Hierarchical clustering heatmap analysis of all the discriminant proteins identified with sPLS-DA

A hierarchical clustering heatmap analysis was built to illustrate the relative abundance of all 407 DP (Fig. 4). Both proteins and samples were clustered by similarity, as indicated by the left and upper dendrograms, respectively. Samples were sorted into three main clusters (upper dendrogram). The first cluster contained samples from the CLA group (green), while the second and third clusters contained all the samples from untreated (blue) and vehicle (orange) groups, respectively. Moreover, the 407 DP proteins were also sorted into three clusters (left dendrogram). Cluster 1 (light blue) had 164 DP (data not shown), mainly showing higher abundances in untreated and vehicle groups and low abundance in the CLA group. Instead, cluster 2 (red) contained 173 DP (data not shown) that showed higher abundances, mainly in the vehicle group and some samples in the CLA group. Cluster 3 (purple) had 70 DP (data not shown), which presented higher abundances in the CLA group and low abundances in the vehicle and untreated groups. Altogether, these results showed that 164 (cluster 1, light blue) and 70 (cluster 3, purple) were under and over-abundant in the CLA group compared to the vehicle group.

3.3. Identification of overlapping proteins from the DP with higher abundance in each group selected by sPLS-DA and DP clustered by hierarchical clustering heat map analysis

A total of 67 proteins out of the 85 DP with the highest abundance in the CLA group were also found among the DP found in cluster 3 (purple), as shown in the Venn Diagram (Fig. 5A). On the other hand, a total of 94 proteins out of the 96 DP with the highest abundance in the vehicle group were grouped in cluster 1 (light blue) (Fig. 5B); while all the 27 DP with the highest abundance in the untreated group were also among the ones grouped in cluster 1 (Fig. 5C).

3.4. GO and KEGG enrichment analyses and PPI for the identified overlapping proteins with higher abundance in each group

The GO enrichment analyses revealed that the overlapping 67 DP over-abundant in the CLA group and identified both by the multivariate and clustering methods were annotated by a total of 239 enriched (FDR < 0.05) BP GO terms and 6 enriched (FDR < 0.05) KEGG pathways (Supplementary Table S4. The enriched GO terms were mainly related to: wound healing (GO:0042060), blood coagulation (GO:0007596), complement activation (GO:0006956), immune system process (GO:0002376), inflammatory response (GO:0006954), lipid metabolic process (GO:0006629), negative regulation of proteolysis (GO:0045861) and regulation of cytokine production (GO:0001817) (Fig. 6A). The enriched KEGG pathways were mainly related to Complement and

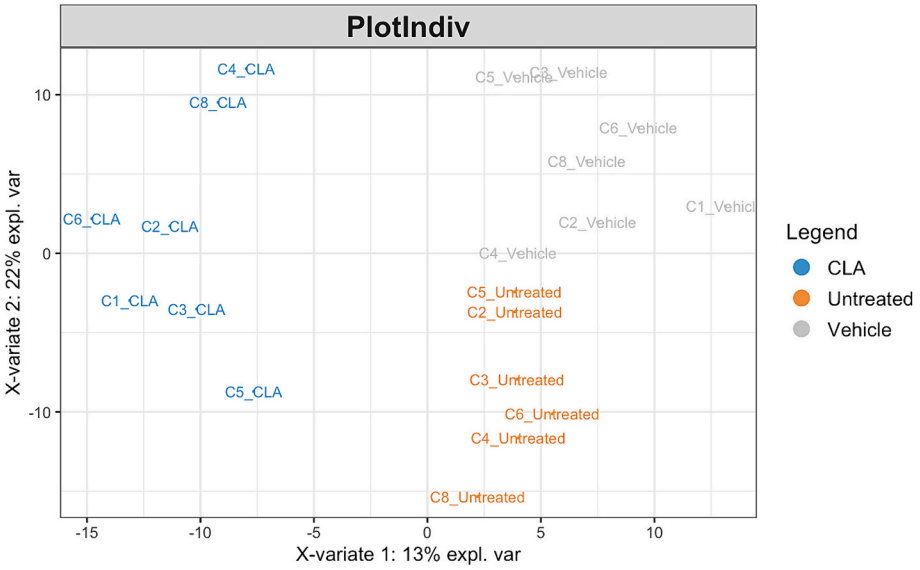

Fig. 1. Individual plot from a multilevel sparse partial least squares discriminant analysis (sPLS-DA). The individual plot shows the similarities and relationships between samples of the conjugated linoleic acid (CLA) mixture (50:50) (blue), vehicle (ethanol) (gray), and untreated (orange) treatment groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

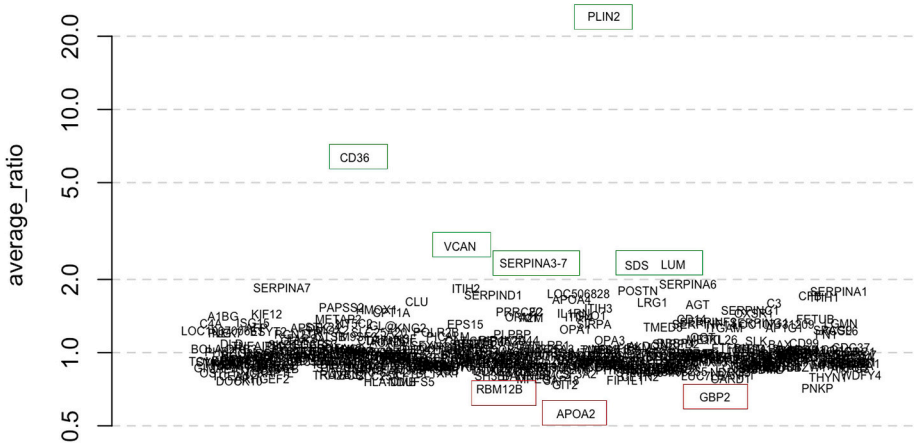

Fig. 2. Average fold-change of protein raw abundances of the 407 discriminant proteins (DP) selected by sPLS-DA. The y-axis shows the average log fold-change (average ratio) of the raw abundances from all seven different biological replicates (samples) after CLA (50:50) mixture treatment, with respect to the vehicle group. In green are the proteins that are upregulated more than logFC>2. In red are the proteins that are upregulated les that logFC <1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

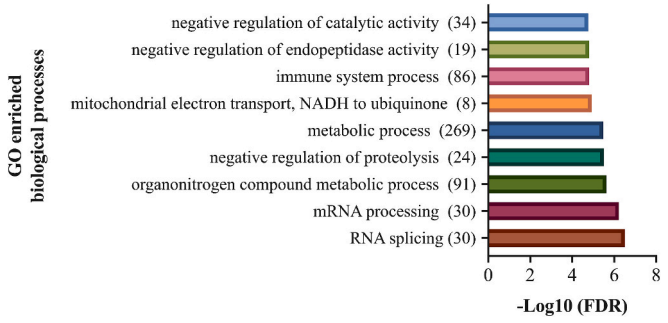

Fig. 3. Global enriched ($P < 0.05$) Gene ontology (GO) terms within the biological processes (BP) category that has annotated all 407 discriminant proteins (DP). GO terms enrichments are expressed as -log10 (FDR) for visualization on graphs. The number of proteins annotated by GO BP terms is shown in brackets.

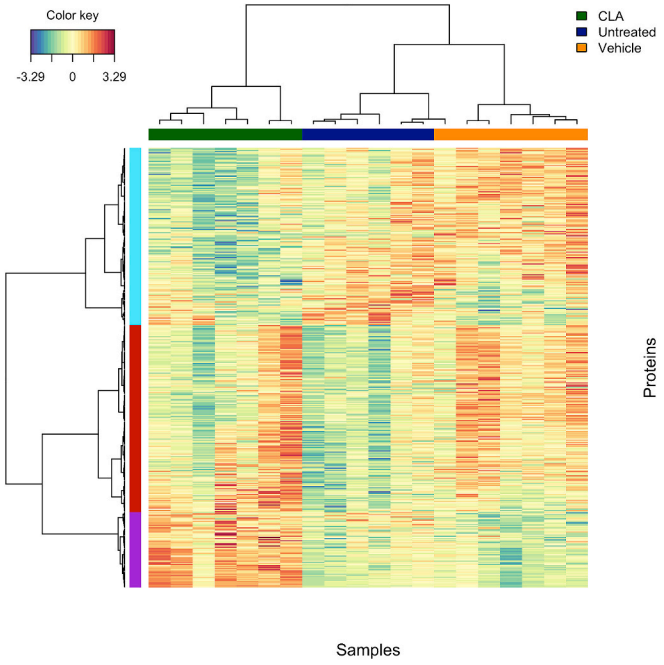

Fig. 4. Clustered image map analysis of discriminant proteins (DP) selected by sPLS-DA. Rows and columns are sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms. Red and blue represent increased and decreased protein abundance, respectively. Samples from the three treatment groups are presented with different colors: green = CLA (50:50) mixture, orange = vehicle, and blue = untreated. A total of 173 DP was sorted in cluster 1 (light blue), while 164 DP in cluster 2 (red) and 70 DP in cluster 3 (purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

coagulation cascades (KEGG:04610), PPAR signaling pathway (KEGG:03320), Cholesterol metabolism (KEGG:04979), and some diseases more related to human than bovine immunity (KEGG:05133, Pertussis; KEGG:05150, *Staphylococcus aureus* infection; KEGG:04936, Alcoholic liver disease). Of the 67 DP proteins, 9 were involved in 3 PPI networks (Fig. 6A).

The overlapping 94 DP relative to the vehicle group and underabundant in the absence of CLA were annotated by a total of 36 enriched (FDR < 0.05) BP GO terms and 13 enriched (FDR < 0.05) KEGG pathways (Supplementary Table S5), and those terms were mainly related to: cellular amide metabolic process (GO:0043603), cytokine production (GO:0001816), negative regulation of protein metabolic process (GO:0051248), alternative mRNA splicing, *via* spliceosome (GO:0000380), negative regulation of nitrogen compound metabolic process (GO:0051172), translation (GO:0006412), regulation of interleukin-6 production (GO:0032675), and negative regulation of proteolysis (GO:0045861) among others (Fig. 6B). Of the KEGG pathways not related to human diseases, mRNA surveillance pathway (KEGG:03015), Pyruvate metabolism (KEGG:00620), Cysteine and methionine metabolism (KEGG:00270), Intestinal immune network for IgA production (KEGG:04672) were related to proteins listed among the 94 DP relative to the vehicule group. Of these, 5 proteins were involved in 2 PPI networks (Fig. 6B).

Finally, the overlapping 27 DP mainly related to the untreated group were annotated only by 5 enriched (FDR < 0.05) BP GO terms (Supplementary Table S6, which were linked to: regulation of RNA splicing (GO:0043484), snoRNA localization (GO:0048254), positive regulation of double-strand break repair *via* nonhomologous end joining (GO:2001034), cellular nitrogen compound metabolic process (GO:0034641) and regulation of double-strand break repair *via* nonhomologous end joining (GO:2001032) (Fig. 6C). No enriched KEGG pathways or PPI were identified for these 27 DP.

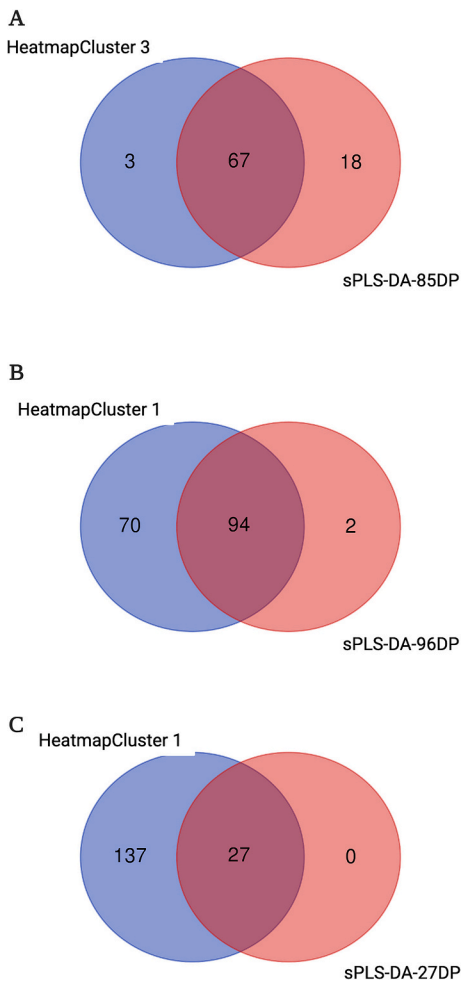

Fig. 5. Venn diagram represents common (overlapping) discriminant proteins (DP), which showed higher abundance in each treatment group according to the sPLS-DA and the clusters generated in the hierarchical clustering heat map analysis. A. Common DP between the 85 DP - selected by sPLS-DA - with the highest abundance in conjugated linoleic acid (CLA) mixture (50:50) group and cluster 3. B. Common DP between the 96 DP - selected by sPLS-DA - with the highest abundance in vehicle (ethanol) group and cluster 1. C. Common DP between the 27 DP - selected by sPLS-DA - with the highest abundance in the untreated group (only medium-treated cells) and cluster 1.

4. Discussion

Conjugated linoleic acid isomers are routinely used as a feed supplement for dairy cows to improve energy balance, increase milk production, and reduce metabolic-related diseases [10,29–31]. Although several studies in human species suggested specific activities of the individual isomers [3], in dairy cows, individual isomers are much less effective, and the best effects are elicited by combining at a ratio of 50:50 two CLA isomers: cis-9, trans-11, and trans-10, cis-12 [13,14]. Previous studies on bovine apoptosis confirmed only the CLA mixture but not the individual cla isomers [18]. Moreover, only the 50:50 CLA mixture could increase the extracellular respiratory burst [18].

This study provides a molecular background to previous findings by demonstrating that CLA can modulate proteolytic and inflammatory pathways at the proteome level. The present results also support what was previously reported by other studies on CLA immunomodulatory activity [32,33]. We propose a molecular signature of CLA treatment based on combining a multivariate sparse discriminant analysis and a clustering method with a high overlap between the DP identified by both methods. Our results show that the key DP overabundant in bovine PBMC treated with a CLA mixture has anti-apoptotic and anti-inflammatory activity.

After CLA treatment, the DP proteins with the highest abundances are perilipin-2 (PLIN2) and platelet glycoprotein 4 (scavenger receptor-class B - CD36). PLIN2 is a lipid droplet-associated protein [34] involved in innate immunity against bacterial infections and chemokine production in monocytes [35,36]. CD36 is a scavenger receptor that plays a role in the uptake or import of long-chain fatty acids, with similar expression regulatory mechanisms of PLIN2 [37,38].

Fatty acids regulate the expression of both PLIN2 and CD36 *via* peroxisome proliferator-activated receptors α (PPARα), γ (PPARγ), and δ (PPARδ) [37–41]. CLA is among the most potent PPAR agonists, especially PPARα and PPARγ, primarily active in macrophages [42]. Several of CLA's immunomodulatory and anti-inflammatory effects are mediated by the PPAR signaling pathways [42,43]. In *in vivo* studies, the t10, c12 CLA isomer increased the gene expression of PLIN2 and CD36 in the liver of CLA-fed rats [44], which supports our findings on bovine PBMC that 5 proteins (ACSL6, LOC518526 CD36, CPT1A, PLIN2) are part of the PPAR signaling KEGG pathway.

The specific role of the DP with the highest abundance in each group was elucidated by performing separate GO enrichment analyses on the overlapping proteins from the DP with higher abundance in each group selected by sPLS-DA and DP clustered by hierarchical clustering heat map analysis.

The GO analysis of the overlapping 67 DP with the highest abundance in the CLA (50:50) mixture group shows a negative regulation of proteolysis, critical for the immune response and elimination of invading pathogens and apoptosis regulation [45,46], explaining the antiapoptotic effects of the CLA mixture that we previously observed on bovine monocytes *in vitro* [18]. DP included SERPINF2, SERPINA6, SERPINC1, SERPIND1, and SERPING1, serine proteases' inhibitors belonging to the Serpin superfamily [47]. Serpins are involved in multiple inflammatory processes, such as blood coagulation [48], immune and inflammatory response [49], and apoptosis [50]. Also, the inter-alpha trypsin inhibitor heavy chain 4 (ITIH4) was found to be annotated. ITIH4, a plasma serine endopeptidase inhibitor contributing to immune response, inflammation, and apoptosis, was also overabundant [51,52].

Further annotated proteins include Acute Phase Proteins (APPs) proteins like alpha-1-acid glycoprotein (AGP), prothrombin (F2), complement 3 (C3), complement component 4 A (C4A), and component factor B (CFB). APPs play different important roles in immune defense, including immune cell recruitment, proteolytic enzyme inactivation, complement activation, opsonization, and limiting tissue damage by scavenging free radicals and promoting the resolution of inflammation [53,54]. Our results agree with previous studies in pigs supplemented with CLA diets, which increased APP abundance, such as AGP [55]. This suggests that CLA plays an essential role in the bovine immune and inflammatory response, likely involving interactions between proteins of the serpin family and APP, as suggested by the PPI network.

The study also identifies additional enriched BP associated with the immune system, such as wound healing and blood coagulation.

Heme oxygenase 1 (HMOX1) is annotated in wound healing, immune system process, and inflammatory response BP. HMOX1 is a microsomal enzyme with antiapoptotic and cytoprotective effects in macrophages *in vivo* and *in vitro*, directly affecting the oxidative stress response to counteract or reduce inflammation [56,57]. The increase in abundance of antioxidant, antiapoptotic, and cytoprotective proteins against oxidative stress is remarkable and supports our previous *in vitro* study on bovine monocytes treated with a CLA (50:50) mixture, in which an increase in ROS production was observed [18]. Therefore, CLA might not only modulate the activation of the immune response but also help in the resolution phase of inflammation, specifically in tissue repair.

Further enriched processes are lipid metabolism, as determined by the change in the abundance of proteins involved in fatty acid beta-oxidation, lipid binding, transport, lipid absorption, and cholesterol

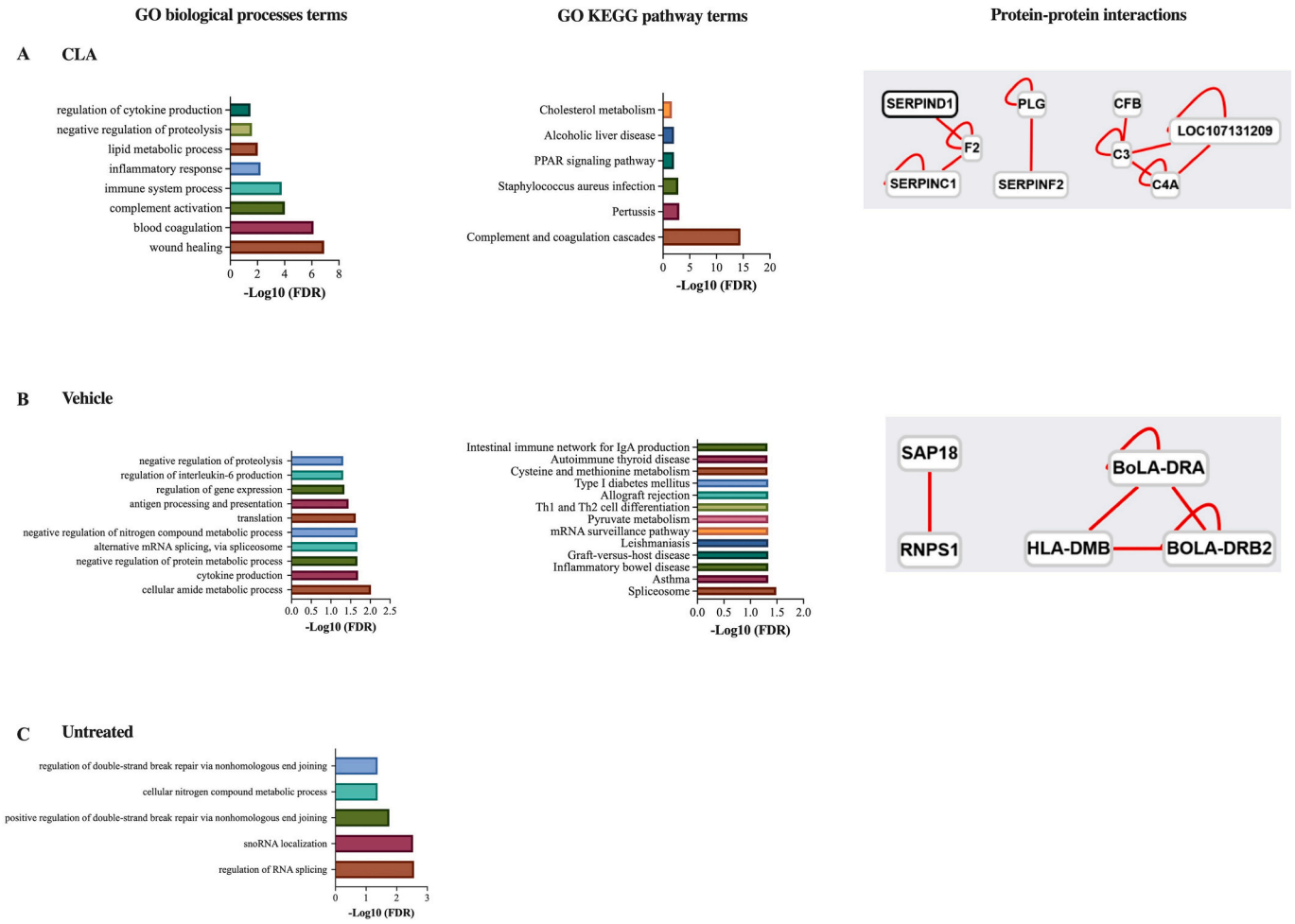

Fig. 6. Enriched (P < 0.05) Gene ontology (GO) terms within the biological processes category, as well as KEGG pathways enrichment that have annotated the overlapping discriminant proteins (DP), which show higher abundance in each treatment group according to both the sPLS-DA and the clusters generated in the hierarchical clustering heat map analysis. A. Enriched biological processes (BP) or KEGG pathways and PPI for the overlapping 67 DP with higher abundance in conjugated linoleic acid (CLA) mixture (50:50) group. B. Enriched biological processes (BP) or KEGG pathways and PPI for the overlapping 94 DP with higher abundance in the vehicle (ethanol) group. C. Enriched biological processes (BP) or KEGG pathways and PPI for the overlapping 27 DP with higher abundance in the untreated group (only medium-treated cells). GO terms and KEGG pathways enrichments are expressed as -log10 (FDR) for visualization on graphs.

metabolism, including the very long-chain specific acyl-CoA dehydrogenase, mitochondrial (ACADVL), ACSL6 (Long-chain-fatty-acid-CoA ligase), carnitine O-palmitoyltransferase (CPT1A), apolipoprotein A-IV (APOA4), apolipoprotein E (APOE) and catalase (CAT) [58–61].

These results suggest that CLA (50:50) mixture might exert anti-apoptotic, immunoregulatory, antioxidant, and cytoprotective effects on bovine PBMC. Moreover, these results demonstrated that the sPLS-DA and hierarchical clustering heat map analysis are adequate for identifying the proteins with the highest abundance in each treatment group. They mostly coincide in this identification, as the GO enrichment analyses revealed.

4.1. Overabundant DP in bovine PBMC in the absence of CLA mixture treatment

The overlapping 94 and 27 DP with the highest abundances in the vehicle and untreated groups are underabundant in the CLA group. These proteins are primarily annotated by shared or similar enriched BP, which include unspecific and basic GO terms and KEGG pathways (only identified for the 94 DP relative to the vehicle group) related mainly to RNA splicing and metabolic processes. This is also confirmed by the PPI analysis, which shows the interaction between Histone deacetylase complex subunit (SAP18) and RNA-binding protein with serine-rich domain 1 (RNPS1), involved in mRNA transcription and splicing. This suggests that both control treatments had a similar effect on bovine PBMC. However, interleukin-6 (IL-6) production regulation in the vehicle-treated group was enriched. IL-6 is a cytokine with pleiotropic functions in inflammation and immune response. One of the proteins annotated by this BP is the allograft inflammatory factor 1 (AIF1), a cytoplasmic protein involved mainly in activating macrophages and enhancing the production of pro-inflammatory cytokines [62]. In human PBMC and murine macrophages, AIF1 expression has been positively correlated with IL-6 and chemokines secretion, suggesting an essential role in these cells' activation and inflammatory response [63,64].

Our results agree with those reported in other models, such as in microglia cells – the only macrophage population in the central nervous system – from ethanol-treated mice, where an increase in the mRNA levels of AIF1 was found [65]. Undoubtedly, the enrichment of the negative regulation of proteolysis also in the overlapping 94 DP with higher abundance in the vehicle group was unexpected due to its enrichment in the CLA group. The enrichment of this biological pathway could be due to the impairment that alcohol causes on the proteolytic system, often leading to inflammation and cell death [66].

In conclusion, these results made evident that after CLA (50:50) mixture treatment, there was a functional switch from very basic BP to

others highly related to different immune-related processes, and that even though in both vehicle and untreated groups mostly redundant functions from the DP were found, ethanol treatment did cause a minimal differential effect on bovine PBMC immune response.

5. Conclusions

The results of this study demonstrate for the first time that the mixture (50:50) of the two main CLA isomers (c9, t11, and t10, c12) modulate bovine PBMC proteome *in vitro* and elucidate the molecular signature of CLA treatment. The GO analysis revealed that the CLA (50:50) mixture caused an enrichment in biological processes related mainly to the negative regulation of proteolysis, which is a critical biochemical feature of apoptosis, complement activation, inflammatory response, regulation of cytokine production, and wound healing, confirming the antiapoptotic and immunomodulatory effects observed in our previous *in vitro* study on bovine monocytes. Moreover, our results suggest that the CLA (50:50) mixture might also exert potential cytoprotective effects against oxidative stress by increasing the abundance of proteins with antioxidant capacity. By revealing some of the molecular mechanisms underlying the immunomodulatory effects of CLA mixture, we pretend to have a clearer idea of its potential biological significance *in vivo,* thus allowing better management for its inclusion in nutrition strategies to enhance the animals' immune and health status.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2024.105232.

Ethics statement

The procedures for the blood collection were carried out during routine slaughtering procedures.

Funding

This study was supported by the European Union's Horizon 2020 research and innovation program H2020-MSCA- ITN-2017- EJD: Marie Skłodowska-Curie Innovative Training Networks (European Joint Doctorate) [Grant agreement n°: 765423, 2017] – MANNA.

CRediT authorship contribution statement

G. Ávila: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Conceptualization. **F. Ceciliani:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **D. Viala:** Writing – original draft, Software, Resources, Methodology, Formal analysis, Data curation. **S. Dejean:** Writing – review & editing, Software, Resources, Formal analysis, Data curation. **G. Sala:** Resources, Methodology. **C. Lecchi:** Writing – review & editing, Validation. **M. Bonnet:** Writing – review & editing, Writing – original draft, Supervision, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper's content.

Data availability

Data will be made available on request.

Acknowledgments

The authors acknowledge A. Delavaud (INRAE, Herbivore Research Unit) for his technical assistance in protein extraction, quantification, and concentration for mass spectrometry analyses, and also Arnaud Cougoul, Jeremy Tournayre and Céline Boby (INRAE, Herbivore Research Unit) for their assistance in the statistical and bioinformatic analyses, respectively; We acknowledge Valentina Zamarian (Department of Veterinary Medicine and Animal Science, Università Degli Studi di Milano, Lodi, Italy) for her valuable support in the proteins' extraction from bovine PBMC.

References

[1] A. Bhattacharya, J. Banu, M. Rahman, J. Causey, G. Fernandes, Biological effects of conjugated linoleic acids in health and disease, J. Nutr. Biochem. 17 (2006) 789–810, https://doi.org/10.1016/j.jnutbio.2006.02.009.
[2] M. Viladomiu, R. Hontecillas, J. Bassaganya-Riera, Modulation of inflammation and immunity by dietary conjugated linoleic acid, Eur. J. Pharmacol. 785 (2016) 87–95, https://doi.org/10.1016/j.ejphar.2015.03.095.
[3] S. Li, L. Xu, J. Qing, X. Wu, H. Li, H. Chen, X. Liu, Multiple biological activities and biosynthesis mechanisms of specific conjugated linoleic acid isomers and analytical methods for prospective application, Food Chem. 409 (2023) 135257, https://doi.org/10.1016/j.foodchem.2022.135257.
[4] M.W. Pariza, Y. Park, M.E. Cook, The biologically active isomers of conjugated linoleic acid, Prog. Lipid Res. 40 (2001) 283–298, https://doi.org/10.1016/S0163-7827(01)00008-X.
[5] M.N. Lahlou, R. Kanneganti, L.J. Massingill, G.A. Broderick, Y. Park, M.W. Pariza, J.D. Ferguson, Z. Wu, Grazing increases the concentration of CLA in dairy cow milk a, Animal 8 (2014) 1191–1200, https://doi.org/10.1017/S1751731114000998.
[6] A. Veshkini, F. Ceciliani, M. Bonnet, H.M. Hammon, Review: effect of essential fatty acids and conjugated linoleic acid on the adaptive physiology of dairy cows during the transition period, Animal 17 (Suppl. 2) (2023) 100757, https://doi.org/10.1016/j.animal.2023.100757.
[7] L. Bernard, M. Bonnet, C. Delavaud, M. Delosière, A. Ferlay, H. Fougère, B. Graulet, Milk fat globule in ruminant: major and minor compounds, nutritional regulation and differences among species, Eur. J. Lipid Sci. Technol. 120 (2018), https://doi.org/10.1002/ejlt.201700039, 1700039.
[8] E. Galamb, V. Faigl, M. Keresztes, Z. Csillik, A. Tröscher, P. Elek, M. Kulcsár, G. Huszenicza, H. Fébel, F. Husvéth, Effect of pre- and post-partum supplementation with lipid-encapsulated conjugated linoleic acid on milk yield and metabolic status in multiparous high-producing dairy cows, J. Anim. Physiol. Anim. Nutr. (Berl). 101 (2017) 1026–1035, https://doi.org/10.1111/jpn.12544.
[9] K.T. Selberg, A.C. Lowe, C.R. Staples, N.D. Luchini, L. Badinga, Production and metabolic responses of periparturient Holstein cows to dietary conjugated linoleic acid and trans-octadecenoic acids, J. Dairy Sci. 87 (2004) 158–168, https://doi.org/10.3168/jds.S0022-0302(04)73153-7.
[10] M.J. de Veth, D.E. Bauman, W. Koch, G.E. Mann, A.M. Pfeiffer, W.R. Butler, Efficacy of conjugated linoleic acid for improving reproduction: A multi-study analysis in early-lactation dairy cows, J. Dairy Sci. 92 (2009) 2662–2669, https://doi.org/10.3168/jds.2008-1845.
[11] A. Veshkini, F. Ceciliani, M. Bonnet, H.M. Hammon, Review: effect of essential fatty acids and conjugated linoleic acid on the adaptive physiology of dairy cows during the transition period, Animal 100757 (2023), https://doi.org/10.1016/j.animal.2023.100757.
[12] N. Hanschke, M. Kankofer, L. Ruda, M. Höltershinken, U. Meyer, J. Frank, S. Dänicke, J. Rehage, The effect of conjugated linoleic acid supplements on oxidative and antioxidative status of dairy cows, J. Dairy Sci. 99 (2016) 8090–8102, https://doi.org/10.3168/jds.2015-10685.
[13] H.-J. Song, I. Grant, D. Rotondo, I. Mohede, N. Sattar, S.D. Heys, K.W.J. Wahle, Effect of CLA supplementation on immune function in young healthy volunteers, Eur. J. Clin. Nutr. 59 (2005) 508–517, https://doi.org/10.1038/sj.ejcn.1602102.
[14] L. Renner, J. Pappritz, R. Kramer, S. Kersten, G. Jahreis, S. Dänicke, Fatty acid profile and proliferation of bovine blood mononuclear cells after conjugated linoleic acid supplementation, Lipids Health Dis. 11 (2012) 63, https://doi.org/10.1186/1476-511X-11-63.
[15] L. Renner, S. Kersten, A. Duevel, H.J. Schuberth, S. Dänicke, Effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid, linoleic acid, phytanic acid and the combination of various fatty acids on proliferation and cytokine expression of bovine peripheral blood mononuclear cells, Nutrients 5 (2013) 2667–2683, https://doi.org/10.3390/nu5072667.
[16] S. Dänicke, J. Kowalczyk, L. Renner, J. Pappritz, U. Meyer, R. Kramer, E.-M. Weber, S. Döll, J. Rehage, G. Jahreis, Effects of conjugated linoleic acids fed to dairy cows during early gestation on hematological, immunological, and metabolic characteristics of cows and their calves, J. Dairy Sci. 95 (2012) 3938–3953, https://doi.org/10.3168/jds.2011-4879.
[17] W. Liermann, T. Viergutz, K.L. Uken, L. Vogel, M. Gnott, D. Dannenberger, A. Tuchscherer, H. Kienberger, M. Rychlik, A. Tröscher, H.M. Hammon, Influences of maternal conjugated linoleic acid and essential fatty acid supply during late pregnancy and early lactation on T and B cell subsets in mesenteric lymph nodes and the small intestine of neonatal calves, Front. Vet. Sci. 7 (2020).
[18] G. Ávila, C. Catozzi, D. Pravettoni, G. Sala, P. Martino, G. Meroni, C. Lecchi, F. Ceciliani, In vitro effects of conjugated linoleic acid (CLA) on inflammatory functions of bovine monocytes, J. Dairy Sci. 103 (2020) 8554–8563, https://doi.org/10.3168/jds.2020-18659.

[19] J.D. Lippolis, E.J. Powell, T.A. Reinhardt, T.C. Thacker, E. Casas, Symposium review: omics in dairy and animal science—promise, potential, and pitfalls*, J. Dairy Sci. 102 (2019) 4741–4754, https://doi.org/10.3168/jds.2018-15267.
[20] A. Pardanani, E.D. Wieben, T.C. Spelsberg, A. Tefferi, Primer on Medical Genomics Part IV: Expression Proteomics, in: Mayo Clin. Proc, Elsevier, 2002, pp. 1185–1196.
[21] A. Veshkini, H.M. Hammon, L. Vogel, M. Delosière, D. Viala, S. Dèjean, A. Tröscher, F. Ceciliani, H. Sauerwein, M. Bonnet, 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, J. Proteome 252 (2022) 104436, https://doi.org/10.1016/j.jprot.2021.104436.
[22] A. Veshkini, H.M. Hammon, H. Sauerwein, A. Tröscher, D. Viala, M. Delosière, F. Ceciliani, S. Déjean, M. Bonnet, Longitudinal liver proteome profiling in dairy cows during the transition from gestation to lactation: investigating metabolic adaptations and their interactions with fatty acids supplementation via repeated measurements ANOVA-simultaneous component analy, J. Proteome 252 (2022) 104435, https://doi.org/10.1016/j.jprot.2021.104435.
[23] A. Veshkini, H.M. Hammon, L. Vogel, D. Viala, M. Delosière, A. Tröscher, S. Déjean, F. Ceciliani, H. Sauerwein, M. Bonnet, Plasma proteomics reveals crosstalk between lipid metabolism and immunity in dairy cows receiving essential fatty acids and conjugated linoleic acid, Sci. Rep. 12 (2022) 5648, https://doi.org/10.1038/s41598-022-09437-w.
[24] G. Kra, N. Nemes-Navon, J.R. Daddam, L. Livshits, S. Jacoby, Y. Levin, M. Zachut, U. Moallem, Proteomic analysis of peripheral blood mononuclear cells and inflammatory status in postpartum dairy cows supplemented with different sources of omega-3 fatty acids, J. Proteome 246 (2021) 104313, https://doi.org/10.1016/j.jprot.2021.104313.
[25] J. Bazile, B. Picard, C. Chambon, A. Valais, M. Bonnet, Pathways and biomarkers of marbling and carcass fat deposition in bovine revealed by a combination of gel-based and gel-free proteomic analyses, Meat Sci. 156 (2019) 146–155, https://doi.org/10.1016/j.meatsci.2019.05.018.
[26] F. Rohart, B. Gautier, A. Singh, K.-A., Lê Cao, mixOmics: an R package for 'omics feature selection and multiple data integration, PLoS Comput. Biol. 13 (2017) e1005752.
[27] K.-A. Lê Cao, S. Boitard, P. Besse, Sparse PLS discriminant analysis: biologically relevant feature selection and graphical displays for multiclass problems, BMC Bioinformatics. 12 (2011) 253, https://doi.org/10.1186/1471-2105-12-253.
[28] N. Kaspric, B. Picard, M. Reichstadt, J. Tournayre, M. Bonnet, ProteINSIDE to easily investigate proteomics data from ruminants: application to mine proteome of adipose and muscle tissues in bovine Foetuses, PLoS One 10 (2015) e0128086, https://doi.org/10.1371/journal.pone.0128086.
[29] J.W. Perfield, A.L. Lock, J.M. Griinari, A. Sæbø, P. Delmonte, D.A. Dwyer, D. E. Bauman, Trans-9, cis-11 conjugated linoleic acid reduces milk fat synthesis in lactating dairy cows, J. Dairy Sci. 90 (2007) 2211–2218, https://doi.org/10.3168/jds.2006-745.
[30] L. Basiricò, P. Morera, D. Dipasquale, A. Tröscher, U. Bernabucci, Comparison between conjugated linoleic acid and essential fatty acids in preventing oxidative stress in bovine mammary epithelial cells, J. Dairy Sci. 100 (2017) 2299–2309, https://doi.org/10.3168/jds.2016-11729.
[31] Z. Csillik, V. Faigl, M. Keresztes, E. Galamb, H.M. Hammon, A. Tröscher, H. Fébel, M. Kulcsár, F. Husvéth, G. Huszenicza, W.R. Butler, Effect of pre- and postpartum supplementation with lipid-encapsulated conjugated linoleic acid on reproductive performance and the growth hormone–insulin-like growth factor-I axis in multiparous high-producing dairy cows, J. Dairy Sci. 100 (2017) 5888–5898, https://doi.org/10.3168/jds.2016-12124.
[32] J.J. Gross, L. Grossen-Rösti, R. Héritier, A. Tröscher, R.M. Bruckmaier, Inflammatory and metabolic responses to an intramammary lipopolysaccharide challenge in early lactating cows supplemented with conjugated linoleic acid, J. Anim. Physiol. Anim. Nutr. (Berl). 102 (2018) e838–e848, https://doi.org/10.1111/jpn.12843.
[33] A. Veshkini, M. Gnott, L. Vogel, C. Kröger-Koch, A. Tuchscherer, A. Tröscher, U. Bernabucci, E. Trevisi, A. Starke, M. Mielenz, L. Bachmann, H.M. Hammon, Abomasal infusion of essential fatty acids and conjugated linoleic acid during late pregnancy and early lactation affects immunohematological and oxidative stress markers in dairy cows, J. Dairy Sci. 106 (2023) 5096–5114, https://doi.org/10.3168/jds.2022-22514.
[34] H. Itabe, T. Yamaguchi, S. Nimura, N. Sasabe, Perilipins: a diversity of intracellular lipid droplet proteins, Lipids Health Dis. 16 (2017) 83, https://doi.org/10.1186/s12944-017-0473-y.
[35] K.A. Mattos, F.A. Lara, V.G.C. Oliveira, L.S. Rodrigues, H. D'Avila, R.C.N. Melo, P. P.A. Manso, E.N. Sarno, P.T. Bozza, M.C.V. Pessolani, Modulation of lipid droplets by mycobacterium leprae in Schwann cells: a putative mechanism for host lipid acquisition and bacterial survival in phagosomes, Cell. Microbiol. 13 (2011) 259–273.
[36] M.M. Wurfel, W.Y. Park, F. Radella, J. Ruzinski, A. Sandstrom, J. Strout, R. E. Bumgarner, T.R. Martin, Identification of high and low responders to lipopolysaccharide in Normal subjects: an unbiased approach to identify modulators of innate immunity, J. Immunol. 175 (2005), https://doi.org/10.4049/jimmunol.175.4.2570, 2570 LP – 2578.
[37] H. Vosper, L. Patel, T.L. Graham, G.A. Khoudoli, A. Hill, C.H. Macphee, I. Pinto, S. A. Smith, K.E. Suckling, C.R. Wolf, C.N.A. Palmer, The Peroxisome Proliferator-activated Receptor δ Promotes Lipid Accumulation in Human Macrophages *, J. Biol. Chem. 276 (2001) 44258–44265, https://doi.org/10.1074/jbc.M108482200.
[38] C. Buechler, M. Ritter, C.Q. Duong, E. Orso, M. Kapinsky, G. Schmitz, Adipophilin is a sensitive marker for lipid loading in human blood monocytes, Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1532 (2001) 97–104, https://doi.org/10.1016/S1388-1981(01)00121-4.
[39] K.T. Dalen, S.M. Ulven, B.M. Arntsen, K. Solaas, H.I. Nebb, PPARα activators and fasting induce the expression of adipose differentiation-related protein in liver, J. Lipid Res. 47 (2006) 931–943, https://doi.org/10.1194/jlr.M500459-JLR200.
[40] M. Schubert, S. Becher, M. Wallert, M.B. Maeß, M. Abhari, K. Rennert, A.S. Mosig, S. Große, R. Heller, M. Grün, S. Lorkowski, The Peroxisome Proliferator–Activated Receptor (PPAR)-γ Antagonist 2-Chloro-5-Nitro-N-Phenylbenzamide (GW9662) Triggers Perilipin 2 Expression via PPARδ and Induces Lipogenesis and Triglyceride Accumulation in Hum, Mol. Pharmacol. 97 (2020), https://doi.org/10.1124/mol.119.117887, 212 LP – 225.
[41] S. Tian, P. Lei, C. Teng, Y. Sun, X. Song, B. Li, Y. Shan, Targeting PLIN2/PLIN5-PPARγ: Sulforaphane disturbs the maturation of lipid droplets, Mol. Nutr. Food Res. 63 (2019) 1900183.
[42] E. Stachowska, M. Baśkiewicz-Masiuk, V. Dziedziejko, I. Gutowska, I. Baranowska-Bosiacka, M. Marchlewicz, B. Dołegowska, B. Wiszniewska, B. Machaliński, D. Chlubek, Conjugated linoleic acid increases intracellular ROS synthesis and oxygenation of arachidonic acid in macrophages, Nutrition 24 (2008) 187–199, https://doi.org/10.1016/j.nut.2007.10.018.
[43] S. McClelland, C. Cox, R. O'Connor, M. de Gaetano, C. McCarthy, L. Cryan, D. Fitzgerald, O. Belton, Conjugated linoleic acid suppresses the migratory and inflammatory phenotype of the monocyte/macrophage cell, Atherosclerosis 211 (2010) 96–102, https://doi.org/10.1016/j.atherosclerosis.2010.02.003.
[44] J. Li, S. Viswanadha, J.J. Loor, Hepatic metabolic, inflammatory, and stress-related gene expression in growing mice consuming a low dose of Trans-10, cis-12-conjugated linoleic acid, J. Lipids. 2012 (2012) 571281, https://doi.org/10.1155/2012/571281.
[45] L. Combaret, D. Taillandier, C. Polge, D. Béchet, D. Attaix, Chapter 3 - Cellular Mechanisms of Protein Degradation Among Tissues, in: D.B.T.-T.M.N. of A.A. and P. Dardevet, Academic Press, Boston, 2016, pp. 27–37, https://doi.org/10.1016/B978-0-12-802167-5.00003–7.
[46] S. Maschalidi, S. Hässler, F. Blanc, F.E. Sepulveda, M. Tohme, M. Chignard, P. van Endert, M. Si-Tahar, D. Descamps, B. Manoury, Asparagine endopeptidase controls anti-influenza virus immune responses through TLR7 activation, PLoS Pathog. 8 (2012) e1002841.
[47] A. Sánchez-Navarro, I. González-Soria, R. Caldiño-Bohn, N.A. Bobadilla, An integrative view of serpins in health and disease: the contribution of SerpinA3, Am. J. Physiol. Physiol. 320 (2020) C106–C118, https://doi.org/10.1152/ajpcell.00366.2020.
[48] A.H. Schmaier, Serpin targets in hemostasis/kinin formation, Blood 134 (2019) 1566–1568, https://doi.org/10.1182/blood.2019002713.
[49] C. Heit, B.C. Jackson, M. McAndrews, M.W. Wright, D.C. Thompson, G. A. Silverman, D.W. Nebert, V. Vasiliou, Update of the human and mouse SERPINgene superfamily, Hum. Genomics 7 (2013) 22, https://doi.org/10.1186/1479-7364-7-22.
[50] M. Mokhber, M. Moradi-Shahrbabak, M. Sadeghi, H. Moradi-Shahrbabak, A. Stella, E. Nicolzzi, J. Rahmaninia, J.L. Williams, A genome-wide scan for signatures of selection in Azeri and Khuzestani buffalo breeds, BMC Genomics 19 (2018) 449, https://doi.org/10.1186/s12864-018-4759-x.
[51] M.S. Lord, J. Melrose, A.J. Day, J.M. Whitelock, The inter-α-trypsin inhibitor family: versatile molecules in biology and pathology, J. Histochem. Cytochem. 68 (2020) 907–927, https://doi.org/10.1369/0022155420940067.
[52] A. Hamm, J. Veeck, N. Bektas, P.J. Wild, A. Hartmann, U. Heindrichs, G. Kristiansen, T. Werbowetski-Ogilvie, R. Del Maestro, R. Knuechel, E. Dahl, Frequent expression loss of inter-alpha-trypsin inhibitor heavy chain (ITIH) genes in multiple human solid tumors: a systematic expression analysis, BMC Cancer 8 (2008) 25, https://doi.org/10.1186/1471-2407-8-25.
[53] F. Ceciliani, J.J. Ceron, P.D. Eckersall, H. Sauerwein, Acute phase proteins in ruminants, J. Proteome 75 (2012) 4207–4231, https://doi.org/10.1016/j.jprot.2012.04.004.
[54] C. Lecchi, G. Avallone, M. Giurovich, P. Roccabianca, F. Ceciliani, Extra hepatic expression of the acute phase protein alpha 1-acid glycoprotein in normal bovine tissues, Vet. J. 180 (2009) 256–258, https://doi.org/10.1016/j.tvjl.2007.12.027.
[55] J. Bassaganya-Riera, R. Hontecillas-Magarzo, K. Bregendahl, M.J. Wannemuehler, D.R. Zimmerman, Effects of dietary conjugated linoleic acid in nursery pigs of dirty and clean environments on growth, empty body composition, and immune competence, J. Anim. Sci. 79 (2001) 714–721, https://doi.org/10.2527/2001.793714x.
[56] I. Petrache, L.E. Otterbein, J. Alam, G.W. Wiegand, A.M.K. Choi, Heme oxygenase-1 inhibits TNF-α-induced apoptosis in cultured fibroblasts, Am. J. Physiol. Cell. Mol. Physiol. 278 (2000) L312–L319, https://doi.org/10.1152/ajplung.2000.278.2.L312.
[57] S. Brouard, L.E. Otterbein, J. Anrather, E. Tobiasch, F.H. Bach, A.M.K. Choi, M. P. Soares, Carbon monoxide generated by Heme oxygenase 1 suppresses endothelial cell apoptosis, J. Exp. Med. 192 (2000) 1015–1026, https://doi.org/10.1084/jem.192.7.1015.
[58] C.Y. Park, S.N. Han, Chapter 8 - Lipid Pathway in Liver Cells and Its Modulation by Dietary Extracts, in: V.B.B.T.-T.M.N. of F. Patel, Academic Press, 2019, pp. 103–116, https://doi.org/10.1016/B978-0-12-811297-7.00008-1.
[59] J. Qu, C.-W. Ko, P. Tso, A. Bhargava, Apolipoprotein A-IV: A multifunctional protein involved in protection against atherosclerosis and diabetes, Cells 8 (2019) 319, https://doi.org/10.3390/cells8040319.

[60] J.R. Pérez-Estrada, D. Hernández-García, F. Leyva-Castro, J. Ramos-León, O. Cuevas-Benítez, M. Díaz-Muñoz, S. Castro-Obregón, R. Ramírez-Solís, C. García, L. Covarrubias, Reduced lifespan of mice lacking catalase correlates with altered lipid metabolism without oxidative damage or premature aging, Free Radic. Biol. Med. 135 (2019) 102–115, https://doi.org/10.1016/j.freeradbiomed.2019.02.016.

[61] Y. Huang, R.W. Mahley, Apolipoprotein E: structure and function in lipid metabolism, neurobiology, and Alzheimer's diseases, Neurobiol. Dis. (2014) 3–12, https://doi.org/10.1016/j.nbd.2014.08.025, 72 Pt A.

[62] K. Piotrowska, S. Słuczanowska-Głabowska, M. Kurzawski, V. Dziedziejko, P. Kopytko, E. Paczkowska, D. Rogińska, K. Safranow, B. Machaliński, A. Pawlik, Over-expression of allograft inflammatory Factor-1 (AIF-1) in patients with rheumatoid arthritis, Biomolecules 10 (2020) 1064, https://doi.org/10.3390/biom10071064.

[63] M. Kadoya, A. Yamamoto, M. Hamaguchi, H. Obayashi, K. Mizushima, M. Ohta, T. Seno, R. Oda, H. Fujiwara, M. Kohno, Y. Kawahito, Allograft inflammatory factor-1 stimulates chemokine production and induces chemotaxis in human peripheral blood mononuclear cells, Biochem. Biophys. Res. Commun. 448 (2014) 287–291, https://doi.org/10.1016/j.bbrc.2014.04.106.

[64] K. Watano, K. Iwabuchi, S. Fujii, N. Ishimori, S. Mitsuhashi, M. Ato, A. Kitabatake, K. Onoé, Allograft inflammatory factor-1 augments production of interleukin-6, −10 and −12 by a mouse macrophage line, Immunology 104 (2001) 307–316, https://doi.org/10.1046/j.1365-2567.2001.01301.x.

[65] B. Dukay, F.R. Walter, J.P. Vigh, B. Barabási, P. Hajdu, T. Balassa, E. Migh, A. Kincses, Z. Hoyk, T. Szögi, E. Borbély, B. Csoboz, P. Horváth, L. Fülöp, B. Penke, L. Vígh, M.A. Deli, M. Sántha, M.E. Tóth, Neuroinflammatory processes are augmented in mice overexpressing human heat-shock protein B1 following ethanol-induced brain injury, J. Neuroinflammation 18 (2021) 22, https://doi.org/10.1186/s12974-020-02070-2.

[66] T.M. Donohue Jr., N.A. Osna, Intracellular proteolytic systems in alcohol-induced tissue injury, Alcohol Res. Health 27 (2003) 317–324.