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Abundance of plasma proteins in response to divergent ratios of dietary ω6:ω3 fatty acids in gestating and lactating sows using a quantitative proteomics approach

Thi Xuan Nguyen [a,b,e,*], Alessandro Agazzi [a], Suzanne McGill [b], Stefan Weidt [b], Quang Hanh Han [b,e], Andrea Gelemanović [c], Mark McLaughlin [b], Matilde Piñeiro [d], Giovanni Savoini [a], Peter David Eckersall [b], Richard Burchmore [b]

[a] Università degli Studi di Milano, Via dell'Università, 6, 26900 Lodi, Italy
[b] University of Glasgow, Bearsden Rd, G61 1QH, United Kingdom
[c] Mediterranean Institute for Life Sciences (MedILS), Meštrovićevo šetalište 45, 21000 Split, Croatia
[d] Acuvet Biotech, C/Bari, 25 dpdo, 50197 Zaragoza, Spain
[e] Vietnam National University of Agriculture, Hanoi, Viet Nam

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ABSTRACT

This study aimed to investigate the characteristic proteomic pattern of plasma from sows supplemented with low dietary ω6:ω3 fatty acids (FAs) ratio during gestation and lactation. Two dietary treatments (n = 8 each) comprised either a control ratio of ω6:ω3 FAs (CR, 13:1 during gestation and 10:1 during lactation) or a low ratio (LR, 4:1 during gestation and lactation) by adding soybean oil or linseed oil, respectively. High-resolution mass spectrometry-based quantitative proteomics was applied on plasma (n = 5 each) at day 108 of gestation (G108) and at the end of lactation (L-End), and a total of 379 proteins and 202 master proteins were identified. Out of these, four differentially abundant proteins between LR and CR samples at G108 may relate to serine-type endopeptidase inhibitor activity. Differentially abundant proteins in L-End versus G108 (12 up-regulated and 10 down-regulated) were positively correlated with the events that regulate plasma lipoproteins, stimulus- and defence-responses. These findings demonstrate the benefit of increased dietary ω3 FAs in modifying proteins involved in protective mechanisms against increased stresses in key life cycle phases in pigs. In addition, proteome changes from late gestation to late lactation disclosed the underlying mechanism of pigs in response to reproduction-related stimuli.

Significance: This study aimed to provide a proteomics insight into the beneficial effects of maternal diet supplementation with a low ω6:ω3 fatty acids ratio, based on previously reported performance and zootechnical data. The results suggest that a low dietary ω6:ω3 fatty acids ratio could enhance the cellular defence mechanisms against increased stresses and in particular to oxidative stress in sows during gestation and lactation, as reflected in proteomic changes of haptoglobin (HP), alpha-1-antitrypsin (SERPINA1) and serum amyloid P-component (APCS). Furthermore, significantly changed proteome profiles in sow plasma between late gestation and lactation phases have been revealed for the first time. This finding identified the adaptation mechanisms of sows to changing physiological events during reproduction.

Abbreviations: CR, control ratio of ω6:ω3 fatty acids; LR, low ratio of ω6:ω3 fatty acids; G108, day 108 of gestation; L-End, the end of lactation; PUFAs, polyunsaturated fatty acids; TMT, Tandem Mass Tag; IS, internal standard; PSM, peptide spectrum match; DAPs, differentially abundant proteins; PPI, protein-protein interaction; GO, Gene Ontology; BP, Biological Process; MF, Molecular Function; CC, Cellular Component; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride; APP, acute-phase protein.

* Corresponding author at: Università degli Studi di Milano, Via dell'Università, 6, 26900 Lodi, Italy.
E-mail address: thixuan.nguyen@unimi.it (T.X. Nguyen).

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1. Introduction

Nowadays, the pig diet has an imbalance in the ratio of omega-6 and omega-3 (ω6:ω3) polyunsaturated fatty acids (PUFAs) (ranging from 10:1 to 15:1) [1] because they consume an increased amount of ω6 FAs from corn and other grains [2] and a noticeably decreased amount of ω3 FAs such as from fish meal or linseed oil. Given the opposing physiological functions of ω6 and ω3 PUFAs [3], elevated intake of ω6 and poor intake of ω3 PUFAs can give rise to systemic inflammation and metabolic and inflammatory disorders [3]. Therefore, supplementation of low-ratio ω6:ω3 PUFAs, especially in the long-term, can reduce risk factors for inflammation-related diseases [4,5]. Nevertheless, there is no consensus on the optimal ratio of ω6:ω3 PUFAs for good health despite decades of research [5,6]. Several studies suggested that a ratio of ω6:ω3 PUFAs above 3:1 up to 6:1 is optimal for decreasing oxidative stress and lipoperoxidation [7]. However, other studies have proposed that a suitable ratio for reducing inflammation levels can range from 1:1 to 5:1 [4] or from 1:1 to 6:1 [8].

From a physiological and nutritional aspect, pregnancy is the most sensitive phase in the female life cycle [9,10] for both humans and animals. During pregnancy, sows undergo complex physiological changes such as weight gain, fetal and placental development [9]. Nutrient exchange between mother and fetus is only possible through the placenta - their temporary and unique metabolic link [10]. After birth, the neonates interact with their mother via breastfeeding, and maternal diet through gestation and lactation plays a significant role in early and long-term offspring's life growth, development, and health. In pigs, maternal nutrient requirements change according to the reproduction phases and massive metabolic alterations, such as increased oxidation levels, can occur in many tissues [11]. Antioxidant levels in sow diets should be re-assessed to avert elevated oxidative damage during late gestation and lactation and to improve sow productivity and health [12].

In the context of biological processes, the ω3 PUFAs are widely known for their antioxidant and anti-inflammatory properties by regulating the antioxidant signalling pathway and modulating inflammation [13]. Further, they are involved in regulating platelet activity and hepatic lipid metabolism [5]. An updated meta-analysis of human studies revealed that low-ratio ω6:ω3 PUFAs supplementation remarkably decreased serum inflammatory factors, including tumour necrosis factor (TNF)-alpha and IL-6 levels [4]. This result was further confirmed in a mouse study [8]. Moreover, although the biological properties of ω3 PUFAs are well-recognized, the link between ω6 and ω3 PUFAs, their bioactive lipid metabolites and inflammation has not yet been entirely elucidated [5].

Proteomic studies in pigs have expanded in recent years [14,15]. Several studies have focused on proteins abundance in sows, such as changes in uterine endometrium [16,17], adipose tissue of fetuses [18], colostrum [19], and mammary tissue [20]. These reports disclosed the mechanisms underlying the molecular responses of sows to the internal and external stressors during gestation. However, inadequate attention has been given to the lactation stage in the sow. Therefore, an enhanced understanding of the protein profile from late gestation to late lactation would explain the molecular mechanisms contributing to a successful reproductive outcome. Furthermore, although numerous studies have investigated the beneficial effects of PUFAs supplementation during gestation and lactation stages [7] [21–28], the underlying molecular mechanism of these effects remains to be elucidated. Furthermore, there is no available report in pigs focused on plasma proteome changes between late gestation and late lactation stages.

The present study tested the hypothesis that a low dietary ω6:ω3 fatty acid ratio could affect the plasma proteome during late pregnancy and lactation, two critical periods that reflect physiological adaptations of the sows to ensure maternal health, fetal and postnatal growth. Using a tandem mass tag (TMT)-based proteomics approach, this study provides evidence explaining the mechanism for enhanced survival rate and weight gain observed in piglets born from the mothers fed a low ω6:ω3 ratio in the diets [29]. Additionally, these data demonstrate the benefit of increased ω3 fatty acid in pig feed, which can be used as a complementary resource for human nutrition research.

2. Materials and methods

2.1. Animals and sample collection

The sow experiment was performed on a private swine farm (Arioli and Sangalli Agricultural Company S.S., Genzone, Italy). The experimental animal protocol was approved by the Ethical Committee of the University of Milan (OPBA 67/2018) and the Italian Ministry of Health (authorization n. 168/2019 PR). The study design with the results of performance, colostrum and milk fatty acid profiles, and oxidative status were previously described in detail in a report on the in vivo study results [29].

Sixteen sows were artificially inseminated at the 13th month of age (second parity) and delivered piglets at around 17th month of age. They had the similar bodyweight (202.57 ± 7.16 kg, mean ± SEM) and body condition score (2.36 ± 0.12, mean ± SEM) at the beginning of feeding trial period. Sows were randomly assigned to two isonitrogenous and isoenergetic diets (Table S1), containing either a control ratio of ω6:ω3 PUFAs (CR, 13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation) or a low ratio of ω6:ω3 PUFAs (LR, 4:1, from G28 until the end of lactation (L-End)) (Table S2). They were housed in groups of 8 sows from 7 days after artificial insemination before moving to individual farrowing crates at day 108 of gestation and stayed there until the end of lactation.

Blood samples were collected at G108 and L-End before fresh feed was provided in the afternoon. Ethylenediaminetetraacetic acid (EDTA) tubes (K3E K3EDTA, 9 mL, REF 455036, Greiner Bio-One GmbH) were used for plasma collection. Blood samples were centrifuged (15 min; 3000 ×*g*; room temperature). The EDTA plasma was aspirated and transferred to microtubes (2.0 ml, Sarstedt AG & Co., Nümbrecht, Germany) before being stored at −80 °C until analysis.

2.2. Protein identification and quantification using the TMT approach

2.2.1. Protein quantification

The plasma samples were quantified for the total protein concentration by Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories GmbH, Müchen, Germany) using bovine serum albumin as standard. Samples were kept at −80 °C until analyses.

2.2.2. Protein digestion and Isobaric labelling – tandem mass tag

Samples were analyzed for proteome profiling by applying a quantitative Tandem Mass Tagging (TMT) approach as previously described [30]. In general, 100 μg of total plasma protein per sample were converted to peptides using an in-solution digestion method (Filter-aided sample preparation, FASP) with 10 kDa cut-off filters. Briefly, samples (~1.20 μL) were mixed with SDT buffer (4% (*w*/*v*) sodium dodecyl sulfate(SDS), 0.1 M Tris/HCL pH 7.6, 0.1 M dithiothreitol (DTT) (Sigma Aldrich, St. Louis, MO, USA), alkylated by adding 0.05 M iodoacetamide (IAA) solution (20 min, room temperature in the dark) (Sigma Aldrich, St. Louis, MO, USA), and depleted of detrimental low-molecular-weight components by filtration in 8 M urea-containing solution (0.1 M Tris/HCL pH 8.5). Protein was digested overnight using 1 μg of trypsin (20 μg/mL, Promega, Madison, WI, USA) resuspended in 0.05 M ammonium bicarbonate (NH4HCO3) buffer, at 1:37 (*w*/w), at 37 °C. The released peptides were dissolved in 10% acetonitrile (ACN)(in water and acidified by 1% trifluoroacetic acid (TFA, CF_3COOH) before vacuum centrifuged at 45 °C until completely dry.

A pooled internal standard (IS) was prepared with equal amounts of peptides from all samples to normalize the data. Subsequently, the peptides (lyophilized protein pellets) from samples and the IS were diluted using 0.1 M triethylammonium bicarbonate(TEAB, Thermo

Scientific, Rockford, USA) before being tagged using a freshly prepared TMT11plex Label Reagent set (Thermo Scientific, Rockford, IL, USA). An amount of 25 μL of the appropriate TMT Label Reagent was added to each sample (50 μg protein pellets) for the labelling reaction (60 min, room temperature), and then 8 μL of 5% (w/v) hydroxylamine (Sigma Aldrich, St. Louis, MO, USA) was added to stop the reaction (45 min). Next, ten TMT-modified peptide samples were combined with the IS (labelled with TMT 126 *m/z*) in equal amounts into a new micro-centrifuge tube, aliquoted, dried, and stored at −80 °C until analysis in the mass spectrometer. Two TMT11plex experiments were performed for a total of 20 samples (5 animals per group x 2 groups × 2-time points).

2.2.3. Liquid chromatography tandem mass spectrometer (LC-MS/MS)

Labelled dry peptides residues were solubilized in 20 μL of 5% (*v*/v) ACN with 0.5% (v/v) formic acid using the auto-sampler of a nanoflow uHPLC system (Thermo Scientific RSLCnano). Peptide ions were detected online by electrospray ionisation (ESI) mass spectrometry MS/MS with an Orbitrap Elite MS (Thermo Scientific). LC eluent was ionized by interfacing the LC coupling device to a NanoMate Triversa (Advion Bioscience) with an electrospray voltage of 1.7 kV. An injection volume of 5 μL of peptides was desalted and concentrated for 12 min on the trap column (0.3 × 5 mm) using a flow rate of 25 μL/min with 1% ACN and 0.1% formic acid.

Subsequently, the peptide was separated on a Pepmap C18 reversed-phase column (50 cm × 75 μm, particle size 3 μm, pore size 100 Å, Thermo Scientific) using a solvent gradient at a fixed solvent flow rate of 0.3 μL/min for the analytical column. The solvent compositions contain A) 0.1% formic acid in water and B) 0.08% formic acid in 80% ACN and 20% water. The solvent gradient was 4% (v/v) solvent B for 10 min, 4–60% (v/v) solvent B for 170 min, 60–99% (v/v) solvent B for 15 min, held at 99% (v/v) solvent B for 5 min. Then, the column was re-equilibrated at starting conditions (4% (v/v) solvent B for 10 min) before the next injection.

The mass spectrometer was operated in the Orbitrap Elite, using full-scan MS spectra (m/z 380–1800) at a high resolution of 60,000 RP. Ion fragmentation was performed with collision-induced dissociation (CID) and the top three precursor ions from the MS scan were detected in the linear ion trap. The three precursor ions were also subjected to high energy collision dissociation (HCD) in the HCD collision cell and were detected at a resolution of 30,000 (full-width half-maximum (FWHM) or mass resolving power defined at 400 m/z). Single-charged ions were omitted, and selected precursors ions were added to a dynamic exclusion list for 180 s.

2.3. MS/MS data processing

Raw MS/MS spectra were processed, and databases were searched to identify and quantify proteins using the Proteome Discoverer (version 2.4, Thermo Fisher Scientific) with Sequest HT algorithm against *Sus scrofa* FASTA files (downloaded from UniProtKB database on 18/02/2021; 104,940 sequences). The database search was performed with the parameters as follows: two trypsin missed cleavage sites, precursor mass tolerance threshold of 10 ppm and fragment tolerance of 0.02 Da, carbamidomethyl (C) fixed peptide modification; oxidation (M), deamidation (N, Q) and TMT six-plex (K, peptide N-terminus) variable modifications. The significant threshold of the peptide identification was calculated based on a false discovery rate (FDR) of ≤ 1% using the Percolator algorithm based on processing against a decoy database. Proteins were confidently identified with at least two peptides and 5% FDR. Only master proteins were considered for statistical analysis. Significantly altered proteins were defined at an adjusted p-value < 0.05, and suggestive altered proteins were defined at a p-value < 0.05. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via PRIDE [31] partner repository with the dataset identifier PXD030880.

2.4. Statistical and bioinformatics analysis

2.4.1. Statistical analysis for proteomics data

The data at peptide spectrum match (PSM) level were exported from Proteome Discoverer and statistically analysed based on linear mixed-effects models with Empirical Bayes moderation using MSstatsTMT package version 2.0.0 [32] in R version 4.1.0 [33]. Proteins were quantified using only unique peptides and summarized using the median polish method. Each protein was normalized with reference channel (or pooled internal standard channel). The Benjamini-Hochberg procedure was employed to adjust for multiple pairwise comparisons for significant differential abundance of proteins ($p < 0.05$).

The volcano plots, heatmaps, and PCA plots were created by applying packages *ggplot2* version 3.3.3 [34] and *ggrepel* version 0.9.1, *ggplot2* version 3.3.3 [34], and *pheatmap* version 1.0.12, respectively. Venn diagrams were created using web tool Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/) [35].

2.4.2. Bioinformatics analysis

The accession numbers of master protein were converted into the corresponding gene symbols using the online converter of UniProt database. Undefined proteins were replaced with the best match (i.e. largest overlap) on *Sus scrofa* orthologous genes of minimum 70% identity using SmartBLAST tool (https://blast.ncbi.nlm.nih.gov/smartblast/). The protein-protein interaction (PPI) network; Gene Ontology (GO) functional enrichment analysis covering three sub GOs: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC), were retrieved by STRING database version 11.5 [36] using default settings. Interactions between desired GO-BP pathways and proteins with significantly different abundances between comparisons were mapped through Cytoscape software v3.8.2 [37]. Significantly enriched (FDR < 0.05) GO terms were submitted into the REVIGO [38] web server (revigo.irb.hr) to omit dispensable terms, using the following settings: whole UniProt as the database, SimRel as semantic similarity measure considering a small similarity threshold of 0.5 at which the term was removed from the list and assigned to a cluster.

2.4.3. Statistical analysis for validation data

The proteomic validations for APOA1, HP, and ITIH4 (pig-MAP) were statistically analysed and compared with the proteomic results. Data were checked for normal distribution by the Shapiro-Wilk normality test and homogeneity of variance by Levene's test using R package *car* version 3.0–10. Differences between groups were determined using the linear mixed model by package *lme4* version 1.1–27, multiple pairwise comparisons were performed by the Tukey's post-hoc method and p-values were corrected for multiple testing by applying the Benjamini Hochberg method by *lsmeans* package version 2.30–0, separately for each biochemical assay. Fixed effects were sow diet, time, and their interactions. The random effect was an individual animal. Pearson correlation analysis was performed to assess the association between proteomics and validation results for HP and ITIH4 (pig-MAP) concentrations using R package *ggpubr* version 0.4.0. Spearman correlation analysis was applied for APOA1 because western blot results did not show a normal distribution.

The experimental design, pipeline for proteomics analysis (containing sample preparation, TMT labelling, and LC-MS/MS, data analysis, and quantitation), and validation of proteomics results are illustrated in Fig. 1.

2.5. Validation of proteomics results

Proteomics results were validated by determining alteration in the non-depleted plasma contents of APOA1, HP, and ITIH4 (pig-MAP), which had shown significantly changed abundances by TMT proteomic analysis to explore the biological significance of the alterations in abundance. Antibody availability was also crucial in using these targets

in the validation which uses the samples of the same animals as in proteomic analysis. As APOA1 was not the only apolipoprotein to be altered in abundance and in respect of lipoprotein metabolism of the sows during gestation and lactation, relevant lipid profile parameters in plasma, notably total cholesterol, HDL and LDL cholesterol, and triglyceride, were also assessed.

The plasma level of APOA1 was determined using Western blot, as previously described [39]. Briefly, 10 μg of proteins of each plasma sample (5 samples/group/each time point) was denatured at 95 °C for 4 min in 4× Laemmli buffer and 1 M dithiothreitol (DTT) and separated by electrophoresis on 4–12% Bis-Tris precast Criterion XT gels (Bio-Rad, USA). Resolved proteins were transferred to nitrocellulose membranes which were then stained with 0.1% Ponceau S to confirm equal loading among samples. The membranes were blocked for 1 h with 5% (w/v) skim milk powder in 0.1% (v/v) Tween 20 in Tris-buffered saline (T-TBS) and then immersed overnight at 4 °C with rabbit polyclonal antibody to APOA1 at 1:20,000 dilution (PAA519Po01, Cloud-Clone Corp, USA). Then, the blot was incubated at room temperature with secondary antibody to rabbit IgG conjugated to horseradish peroxidase (HRP, 1:10,000; ab6721, Abcam Ltd. UK) for 1 h. Western blot signal was detected using the enhanced chemiluminescent (ECL) reaction (Thermo Fisher Scientific™, Meridian Rd., Rockford, USA) and visualized using radio-graphic film. The intensity of the protein bands was quantified using Image J NIH software (https://imagej.nih.gov/ij/).

The quantification of HP and ITIH4 (pig-MAP) was performed by ELISA using species-specific kits, as described by the manufacturer (ACUVET ELISA pig-HP and ACUVET ELISA pig-MAP, Acuvet Biotech, Zaragoza, Spain). HP assay used polyclonal antibodies specific for pig HP. The pig-MAP assay was based on two monoclonal antibodies [40]. Both assays use pig-specific standards and are calibrated according to the European Reference Serum for pig acute-phase proteins (European Concerted Action QLK5-CT-1999-0153).

Total cholesterol and triglyceride concentrations were measured in the Veterinary Diagnostic Services, School of Veterinary Medicine, University of Glasgow using polychromatic and bichromatic endpoint techniques, respectively, using the biochemical autoanalyser (Dimension® Xpand Plus™, Siemens Inc., USA). The HDL cholesterol level was determined using the HDL-cholesterol assay kit (ab65390; Abcam, Cambridge, UK) following the manufacturer's instructions, which included initial separation of HDL by precipitation from other lipoproteins followed by a colorimetric assay for cholesterol contained by the HDL. The level of LDL-cholesterol in plasma was determined using the Friedewald equation: LDL cholesterol = Total cholesterol − HDL cholesterol − Triglyceride/5 [41].

3. Results

3.1. Proteomic analysis

The TMT-based quantitative proteomics approach matched 1149 peptides, which represented 379 proteins and 202 "master" proteins. These terms will be explained as follows for a more comprehensive understanding of the results obtained. Each protein is denoted in UniProt by a distinguish accession number based on its isoform, and a protein can have many isoforms originating from the same gene, which are referred to a diverse set of protein sequences with different numbers of amino acids for the protein compared to the base primary gene (canonical) sequence [42]. The "master" protein is the primary translation product of the coding sequence and expresses at least one of the known protein isoforms, coded by the canonical sequence [43] - the most extended sequence that is most common and similar to orthologous sequences in different species [44]. Proteins are digested into peptides that could be unique for a specific protein or are shared between multiple proteins [45]. A unique peptide is defined as a different combination of amino acid sequence and modifications, irrelevant to the charge state of the precursor ions [46].

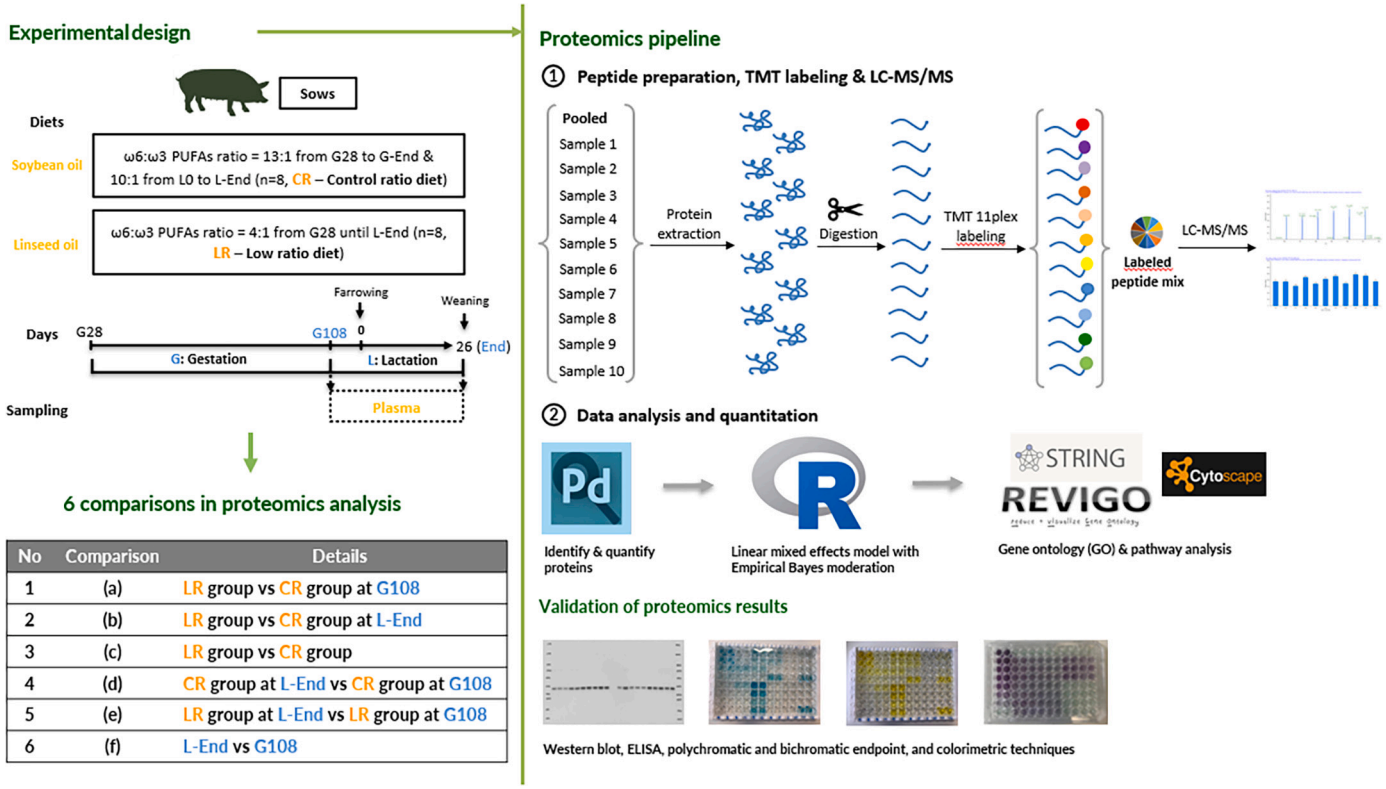

Fig. 1. The experimental design, proteomics pipeline and validation of proteomics results. Proteomics pipeline contains: (1) Peptide preparation, TMT labelling and LC-MS/MS analysis, (2) Data analysis and quantitation: protein identification and quantitation using Proteome Discoverer, statistical analysis based on the linear mixed-effects model with empirical Bayes moderation (R package MSstatsTMT), bioinformatics analysis (Gene Ontology (GO) enrichment analysis).

Identified proteins were excluded if they have no unique peptide, only one peptide, and singly charged ions. After filtering, 87 "master" proteins from all replicates and samples were quantified and remained for statistical analysis (Supplementary material 1_List of 87 "master" proteins).

The principal component analysis (PCA) score plot showing the clustering of samples from four groups of sows are represented in Fig. 2, containing CR group at G108 (CR-G108); CR group at L-End (CR- LEnd); LR group at G108 (LR-G108); and LR group at L-End (LR- LEnd). The analysis of the score plots showed that clusters of CR and LR groups at L-End were differentiated from the samples of both groups at G108, with the first two PCs explaining 60.69% of the total variance (Fig. 2). Nevertheless, mixed distributions were shown for samples in LR and CR groups at each sampling time. In addition, the differentially abundant proteins (DAPs) were examined via six comparisons in Table 1. Details of these comparisons are described in Fig. 1.

3.1.1. DAPs in the comparisons between LR and CR groups

Comparison (a): Plasma from low dietary ω6:ω3 ratio (LR) group at G108 showed 4 DAPs; 3 increased and one decreased) when compared to plasma from the control dietary ω6:ω3 ratio (CR) group at G108 (Table 1, $p < 0.05$). In comparison (b), plasma from LR sows at L-End versus CR sows at L-End gave no DAP. For comparison (c), plasma from LR sows versus CR sows regardless of sampling time yielded 5 DAPs (2 increased and 3 decreased) (Table 1, $p < 0.05$). Of these proteins, hemopexin (HPX) and inter-alpha-trypsin inhibitor heavy chain H1 (ITIH1) were increased, and haptoglobin (HP) was decreased in both (a) and (c) comparisons ($p < 0.05$, Tables 1 and 2). Alpha-1-antitrypsin (SERPINA1) was up-regulated in comparison (a) and serum amyloid P-component (APCS) was down-regulated in comparison (c) ($p < 0.05$, Table 1). The separating and overlapping DAPs between comparisons (a) and (c) are shown in the Venn diagram (Fig. 3A) and heatmap (Fig. 4).

The DAPs between LR and CR groups at G108 - comparison (a) are shown in a volcano plot (Supplementary Fig. S1-A). A mixed distribution with some overlaps of the samples based on the DAPs of this comparison is visualized in the PCA, with a high explained variance of 88.11% (Supplementary Fig. S1—B), and the DAPs are shown in the hierarchically clustered heatmaps (Supplementary Fig. S1—C).

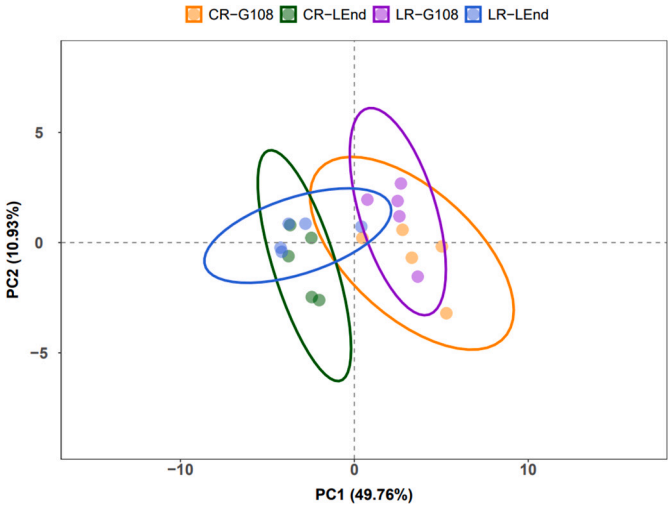

Fig. 2. Principal component analysis (PCA) score plots showing the clustering of samples from four groups of sows: CR group at G108 (CR-G108, orange dots); CR group at L-End (CR- LEnd, green dots); LR group at G108 (LR-G108, purple dots); LR group at L-End (LR- LEnd, blue dots). The ellipses were drawn at 95% confidence interval of standard error. CR: sow diet with ω6:ω3 ratio = 13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: sow diet with ω6:ω3 ratio = 4:1 from G28 until the end of lactation (L-End). G108 = day 108 of gestation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.1.2. DAPs in the comparisons between L-End and G108 sampling time

Looking at the sampling time, comparison (d) examined CR plasma at L-End compared to CR plasma at G108 and showed 17 DAPs (9 increased and 8 decreased) (Table 1, $p < 0.05$). Comparison (e) examined LR plasma at L-End versus LR plasma at G108 and yielded 15 DAPs (11 increased and 4 decreased) (Table 1, $p < 0.05$). Comparison (f) determined plasma from L-End versus G108 regardless of dietary treatment and found 22 DAPs (12 increased and 10 decreased) (Table 1, $p < 0.05$). Of these proteins, there are 8 proteins increased, and 4 proteins decreased in both (d) and (e); 10 proteins increased, and 4 proteins decreased in both (e) and (f); 9 proteins increased, and 7 proteins decreased in both (d) and (f) (Table 2). In all three comparisons, several proteins were all up-regulated such as apolipoprotein A1, A2, and C3 (APOA1, APOA2, APOC3); complement C8 alpha chain (C8A), fetuin-B isoform 1 (FETUB), and histidine-rich glycoprotein (HRG) and some proteins were all down-regulated including CD5 antigen-like precursor (CD5L) and SERPIN domain-containing proteins (SERPINF2 and SERPIN (LOC100156325)). The overlapped DAPs among these three comparisons are represented in the Venn diagram (Fig. 3B) and heatmap (Fig. 4).

The DAPs in comparisons (d), (e), and (f) are displayed in the volcano plots (Supplementary Figs. S2-A, S3-A, and S4-A). The PCA plots show clear separations between samples in (d), (e), and (f), with the explained variances of 76.98% (Supplementary Fig. S2—B), 75.95% (Supplementary Fig. S3—B), and 62.63% (Supplementary Fig. S4—B), respectively. The distinct proteome profiles of these comparisons are shown in the hierarchically clustered heatmaps (Supplementary Figs. S2—C, S3—C, and S4—C, respectively).

3.1.3. Gene ontology (GO) enrichment of all identified proteins

The protein-protein interaction (PPI) network of all 87 master proteins kept in this work comprises 59 nodes and 628 edges ($p < 1.0e-16$) (Supplementary Fig. S5). The functional enrichments showed 49, 19, and 8 GO terms on BP, MF, and CC, respectively (Supplementary Table S3). The DAPs were primarily involved in response to stimulus [GO:0065007], response to stress [GO:0044238], and organonitrogen compound metabolic process [GO:1905907]. The main functions of these DAPs were serine-type endopeptidase activity [GO:0004252], enzyme regulator activity [GO:0030234], and lipoprotein particle receptor binding [GO:0070325]. They were primarily localized in the cellular anatomical entity [GO:0110165], extracellular space [GO:0005615], and protein-containing complex [GO:0032991]. The top 20, 10, and 5 GO terms in BP, MF, and CC categories, respectively, are illustrated in Supplementary Fig. S6.

3.1.4. Gene ontology enrichment in the comparisons between LR and CR groups

The DAP PPI network between LR and CR plasma at G108 - comparison (a) contains 4 nodes and 5 edges ($p = 8.93e-10$). Their list of enriched GO terms is presented in Supplementary Table S4 and Supplementary Fig. S7. Regardless of sampling time, the PPI network in the DAPs between LR and CR plasma - comparison (c) contains 5 nodes and 3 edges ($p = 1.68e-06$), but no significant pathway enrichment was observed. GO analysis on the DAPs in comparison (a) disclosed the interaction between SERPINA1 and ITIH1 in the extracellular region and their contributions to the inhibitor activity of serine-type endopeptidase.

3.1.5. Gene ontology enrichment in the comparisons between L-End and G108 sampling times

The PPI network of comparison (d) (CR at L-End versus CR at G108), comparison (e) (LR at L-End versus LR at G108), and comparison (f) (L-End versus G108) contains 16 nodes and 43 edges, 15 nodes and 41 edges, and 21 nodes and 59 edges ($p < 1.0e-16$). The annotated GO terms corresponding to the DAPs of comparisons (d), (e), and (f) is presented in Supplementary Table S5, S6, and S7 (comparisons (d) and

Table 1
The differentially abundant proteins (DAPs) in sow plasma of six comparisons: (a) - LR group versus CR group at G108; (b) - LR group versus CR group at L-End; (c) - LR group versus CR group; (d) - CR group at L-End versus CR group at G108; (e) - LR group at L-End versus LR group at G108; (f) - L-End versus G108. Protein names marked with * were shown as "Uncharacterized protein" in the UniProt *Sus scrofa* database and thus were substituted with the best match on *Sus scrofa* database, using SMARTBLAST tool.

Protein name	Gene names	Accession number[1]	Number of peptides	log2FC[2]	*P*-value	Adjusted *p*-value
Comparison (a) - LR group versus CR group at G108						
Hemopexin	HPX	P50828	14	0.53	0.018	0.708
Alpha-1-antitrypsin/ Serpin Family A Member 1	SERPINA1	P50447	9	0.24	0.043	0.708
Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	A0A480SQD8	10	0.19	0.026	0.708
Haptoglobin	HP	Q8SPS7	21	−0.32	0.032	0.708
Comparison (b) - LR group versus CR group at L-End: no DAPs						
Comparison (c) - LR group versus CR group						
Hemopexin	HPX	P50828	14	0.49	0.002	0.214
Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	A0A480SQD8	10	0.12	0.019	0.480
Haptoglobin	HP	Q8SPS7	21	−0.33	0.042	0.734
Serum amyloid P-component	APCS	O19063	3	−0.53	0.022	0.480
SERPIN domain-containing protein	LOC100156325	A0A4X1SGC8	10	−1.23	0.021	0.480
Comparison (d) - CR group at L-End versus CR group at G108						
Fetuin-B isoform 1	FETUB	A0A480SUZ7	2	1.03	<0.001	0.013
Histidine-rich glycoprotein	HRG	A0A481B9A6	12	0.80	<0.001	0.023
Apolipoprotein C-III	APOC3	A0A5G2QLU1	3	0.68	0.006	0.070
Transthyretin	TTR	A0A5G2QIE9	2	0.56	0.041	0.211
Apolipoprotein A-I	APOA1	K7GM40	27	0.47	0.001	0.023
Plasma kallikrein *	KLKB1	A0A4X1VWY2	3	0.47	0.016	0.110
Apolipoprotein A-II	APOA2	A0A4X1VK69	3	0.46	0.008	0.083
Complement C8 alpha chain	C8A	F1S788	3	0.30	0.012	0.091
Transferrin	TF	P09571	54	0.17	0.037	0.204
Comparison (d) - CR group at L-End versus CR group at G108 (*cont.*)						
Complement C4 gamma chain	C4A	A0A4X1VBD2	18	−0.20	0.038	0.204
SERPIN domain-containing protein	SERPINF2	A0A4X1U6E7	2	−0.31	<0.001	0.013
Haptoglobin	HP	Q8SPS7	21	−0.40	0.009	0.083
CD5 antigen-like precursor *	CD5L	A0A4X1VVV3	4	−0.44	0.011	0.091
IgM heavy chain constant region *	IGHM	A0A287ALC1	22	−0.55	0.028	0.171
Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	A0A4X1VPB7	18	−0.80	0.005	0.070
SERPIN domain-containing protein	LOC100156325	A0A4X1SGU6	10	−1.06	0.006	0.070
SERPIN domain-containing protein		A0A4X1SGE8	11	−1.10	0.017	0.110
Comparison (e) - LR group at L-End versus LR group at G108						
Fetuin-B isoform 1	FETUB	A0A480SUZ7	2	1.10	<0.001	0.012
Apolipoprotein C-III	APOC3	A0A5G2QLU1	3	0.66	0.016	0.143
Histidine-rich glycoprotein	HRG	A0A481B9A6	12	0.60	0.010	0.135
Apolipoprotein A-II	APOA2	A0A4X1VK69	3	0.57	0.016	0.143
Apolipoprotein A-I	APOA1	K7GM40	27	0.50	<0.001	0.012
Transthyretin	TTR	A0A5G2QIE9	2	0.48	0.011	0.135
Afamin	AFM	F1RUM1	3	0.45	0.024	0.158
Plasma kallikrein *	KLKB1	A0A4X1VWY2	3	0.42	0.003	0.071
Complement C8 alpha chain	C8A	F1S788	3	0.40	<0.001	0.012
SERPIN domain-containing protein	SERPIND1	A0A4X1UYS0	3	0.24	0.036	0.224
Hemopexin	HPX	P50828	14	0.22	0.005	0.095
SERPIN domain-containing protein	SERPINF2	A0A4X1U6E7	2	−0.23	0.048	0.279
CD5 antigen-like precursor *	CD5L	A0A4X1VVV3	4	−0.49	0.022	0.158
IgM heavy chain constant region *	IGHM	A0A287ALC1	22	−0.51	0.019	0.151
SERPIN domain-containing protein	LOC100156325	A0A4X1SGU6	10	−0.61	0.016	0.143
Comparison (f) - L-End versus G108						
Fetuin-B isoform 1	FETUB	A0A480SUZ7	2	1.08	<0.001	<0.001
Histidine-rich glycoprotein	HRG	A0A481B9A6	12	0.70	<0.001	<0.001
Apolipoprotein C-III	APOC3	A0A5G2QLU1	3	0.69	<0.001	0.001
Transthyretin	TTR	A0A5G2QIE9	2	0.52	<0.001	0.006
Apolipoprotein A-II	APOA2	A0A4X1VK69	3	0.52	<0.001	0.002
Apolipoprotein A-I	APOA1	K7GM40	27	0.50	<0.001	<0.001
Plasma kallikrein *	KLKB1	A0A4X1VWY2	3	0.46	<0.001	0.001
Afamin	AFM	F1RUM1	3	0.37	0.006	0.033
Complement C8 alpha chain	C8A	F1S788	3	0.36	<0.001	<0.001
Apolipoprotein-E *	APOE	A0A4X1W1F9	8	0.30	0.023	0.099
SERPIN domain-containing protein	SERPIND1	A0A4X1UYS0	3	0.17	0.014	0.070
Transferrin	TF	P09571	54	0.16	0.007	0.038
C3/C5 convertase (Complement factor B)	CFB	K7GPT9	8	−0.15	0.020	0.093
SERPIN domain-containing protein	SERPINF2	A0A4X1U6E7	2	−0.26	<0.001	0.001
Ficolin-2	FCN2	A0A4X1SUF1	5	−0.36	0.033	0.130
Haptoglobin	HP	Q8SPS7	21	−0.41	0.009	0.045
CD5 antigen-like precursor *	CD5L	A0A4X1VVV3	4	−0.46	<0.001	0.002

(*continued on next page*)

Table 1 (*continued*)

Protein name	Gene names	Accession number[1]	Number of peptides	log2FC[2]	*P*-value	Adjusted *p*-value
IgM heavy chain constant region *	IGHM	A0A287ALC1	22	−0.53	0.001	0.007
Joining chain of multimeric IgA and IgM	JCHAIN	A0A287BQC8	2	−0.55	0.029	0.119
Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	A0A4X1VPB7	18	−0.60	0.001	0.007
SERPIN domain-containing protein	LOC100156325	A0A4X1SGU6	10	−0.84	<0.001	0.001
SERPIN domain-containing protein		A0A4X1SGE8	11	−1.00	0.004	0.027

[1] Accession number from UniProt protein database for *Sus scrofa*.

[2] log2FC is base 2 logarithm transformed of fold change value which represents the ratio of abundance levels in the first-mentioned group vs second-mentioned group. CR: sow diet with ω6:ω3 ratio = 13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: sow diet with ω6:ω3 ratio = 4:1 from G28 until the end of lactation (L-End). G108 = day 108 of gestation.

Table 2
The differentially abundant proteins (DAPs) in sow plasma among five comparisons.

Gene	Description	log2FC				
		Comparison (a)	Comparison (c)	Comparison (d)	Comparison (e)	Comparison (f)
		LR-G108 vs CR-G108	LR vs CR	CR-LEnd vs CR-G108	LR-LEnd vs LR-G108	LEnd vs G108
AFM	Afamin	/	/	/	0.45	0.37
APCS	Serum amyloid P-component	/	−0.53	/	/	/
APOA1	Apolipoprotein A-I	/	/	0.47	0.5	0.5
APOA2	Apolipoprotein A-II	/	/	0.46	0.57	0.52
APOC3	Apolipoprotein C-III	/	/	0.68	0.66	0.69
APOE	Apolipoprotein-E *	/	/	/	/	0.3
C4A	Complement C4 gamma chain	/	/	−0.2	/	/
C8A	Complement C8 alpha chain	/	/	0.3	0.4	0.36
CD5L	CD5 antigen-like precursor *	/	/	−0.44	−0.49	−0.46
CFB	C3/C5 convertase (Complement factor B)	/	/	/	/	−0.15
FCN2	Ficolin-2	/	/	/	/	−0.36
FETUB	Fetuin-B isoform 1	/	/	1.03	1.1	1.08
HP	Haptoglobin	−0.32	−0.33	−0.4	/	−0.41
HPX	Hemopexin	0.53	0.49	/	0.22	/
HRG	Histidine-rich glycoprotein	/	/	0.8	0.6	0.7
IGHM	IgM heavy chain constant region *	/	/	−0.55	−0.51	−0.53
ITIH1	Inter-alpha-trypsin inhibitor heavy chain H1	0.19	0.12	/	/	/
ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4	/	/	−0.8	/	−0.6
JCHAIN	Joining chain of multimeric IgA and IgM	/	/	/	/	−0.55
KLKB1	Plasma kallikrein *	/	/	0.47	0.42	0.46
SERPIN (A0A4X1SGE8)	SERPIN domain-containing protein	/	/	−1.1	/	−1
SERPIN (LOC100156325)	SERPIN domain-containing protein	/	−1.23	−1.06	−0.61	−0.84
SERPINA1	Alpha-1-antitrypsin/ Serpin Family A Member 1	0.24	/	/	/	/
SERPIND1	SERPIN domain-containing protein	/	/	/	0.24	0.17
SERPINF2	SERPIN domain-containing protein	/	/	−0.31	−0.23	−0.26
TF	Transferrin	/	/	0.17	/	0.16
TTR	Transthyretin	/	/	0.56	0.48	0.52

log2FC is base 2 logarithm transformed of fold change value which represents the ratio of abundance levels in the first-mentioned group vs second-mentioned group. CR: sow diet with ω6:ω3 ratio = 13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: sow diet with ω6:ω3 ratio = 4:1 from G28 until the end of lactation (L-End). G108 = day 108 of gestation.

(e) do not have GO term in the BP category). The top 20, 5, and 3 leading GO terms in BP, MF, and CC categories, of the PPI network of DAPs in comparison (f), are illustrated in Supplementary Fig. S8.

Briefly, GO enrichment analysis on the DAPs in comparisons (d), (e), and (f) highlighted the role of APOA1 in the receptor-binding activity of high-density lipoprotein through its functionally linked to APOC3 in the extracellular space. GO analysis of comparison (f) underlined the interaction between APOA1, APOC3, APOE, complement factor B (CFB), HP, ITIH4 (pig-MAP), and transthyretin (TTR) and their participation in the metabolic process of the organonitrogen compound. APOA1 and APOC3 also contributed to chemical homeostasis through their interactions with transferrin (TF). Furthermore, CFB, HP, and ITIH4 (pig-MAP) play a role in defence response via their interactions with Ficolin-2 (FCN2); and the response to stimulus through their communication with FCN2, APOC3, and TTR. Besides, the contribution of HP and ITIH4 in the acute-phase response was shown.

To give an overview of the interaction between the DAPs and significantly enriched GO-BP terms, a network among three main comparisons (i.e. (a), (d), and (e)) was designed (Fig. 5). In this network, up- and down-regulated proteins separately in each comparison and shared between two of them were mapped with their associated enriched GO terms.

3.2. Validation of proteomics results

Results of validation assays for apolipoprotein A1 (APOA1), haptoglobin (HP), and ITIH4 (pig-MAP), together with their respective protein abundance values determined by proteomics; is presented in Table 3 and visualized in Supplementary Fig. S9. In addition, plasma levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride in four groups of sows are also shown in Supplementary Fig. S9.

3.2.1. Apolipoprotein A1

Western blot images of APOA1 in plasma samples from two groups of sows at two sampling times are presented in Fig. S9-A1. Clear bands corresponding to APOA1 (Mw 26 kDa) were detected in all samples. Statistical analysis of the band intensity for each group showed significant increases of APOA1 at L-End compared to G108 sampling time (Table 3, $p = 0.001$). However, there was no significant difference in

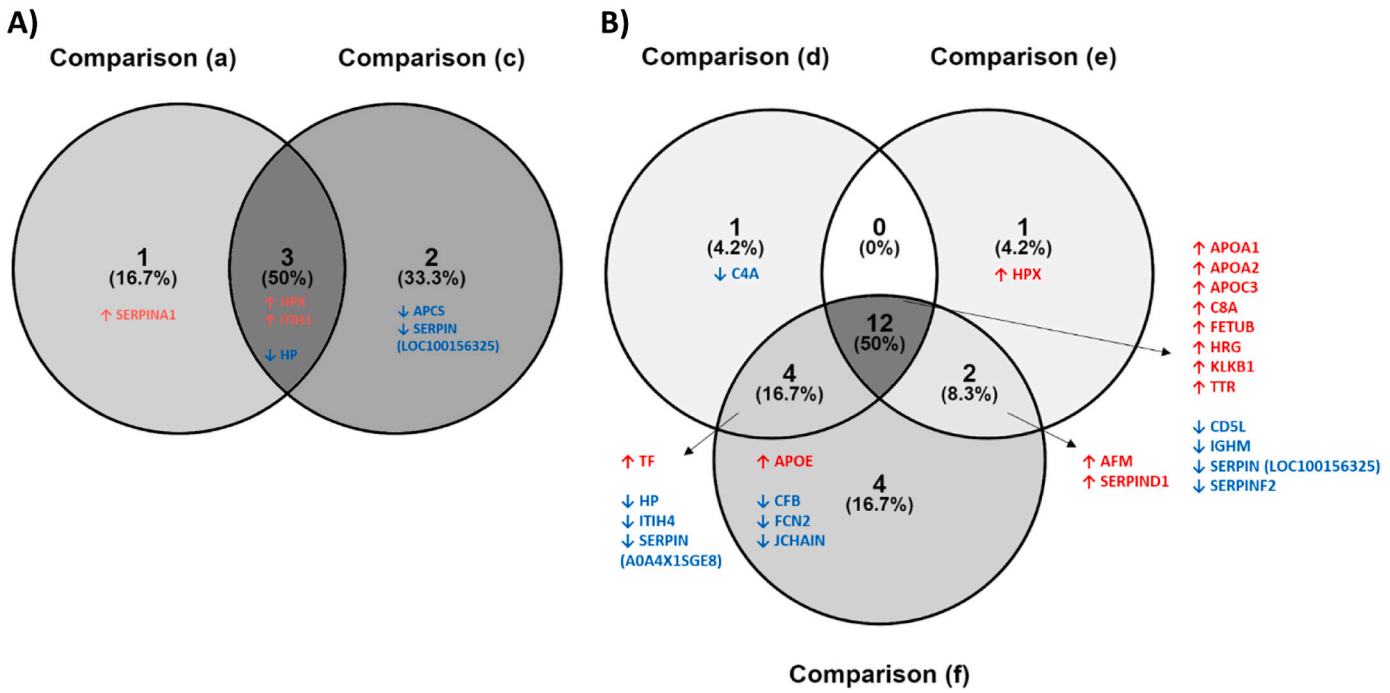

Fig. 3. Venn diagram representing separated and overlapped differentially abundant proteins (DAPs) among: A) Comparison (a) - LR group versus CR group at G108 and comparison (c) - LR group versus CR group; and B) Comparison (d): CR group at L-End versus CR group at G108, comparison (e): LR group at L-End versus LR group at G108, and comparison (f): both CR and LR groups at L-End versus both groups at G108. CR: sow diet with ω6:ω3 ratio = 13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: sow diet with ω6:ω3 ratio = 4:1 from G28 until the end of lactation (L-End). G108 = day 108 of gestation.

APOA1 concentration between LR plasma compared to CR plasma. Moreover, no effect of interaction between sow diet and time was found. A significantly higher concentration of APOA1 was determined at L-End compared to G108 within CR group (Table 3, $p = 0.011$), within LR group (Table 3, $p = 0.037$); in LR compared to CR groups and vice versa (Table 3, both $p = 0.011$). The relative abundance quantified by proteomics showed a similar increasing pattern of APOA1 at L-End compared to G108 (Supplementary Fig. S9-A2, $p < 0.001$), as observed in validated APOA1 concentration. The overall evaluation found a strong correlation between validated APOA1 concentration and APOA1 proteomics relative abundance (Supplementary Fig. S10-A, $R = 0.68$, $p = 0.0013$).

3.2.2. Haptoglobin (HP) and ITIH4 (pig-MAP)

HP and ITIH4 concentrations were significantly decreased at L-End compared to G108 (Supplementary Fig. S9-B1 and S9-C1, respectively, both $p = 0.01$). A significant decrease of HP concentration from G108 to L-End was found when compared CR group at G108 to LR group at L-End (Table 3, $p = 0.015$). The proteomics relative abundance showed a similar decreasing pattern of HP and ITIH4 at L-End compared to G108 (Supplementary Fig. S9-B2 and S9-C2, $p = 0.0009$ and $p = 0.0003$, respectively); as shown in their validated concentrations. The proteomics abundance pattern of HP was supported by a strong correlation between HP abundance and validated HP concentration (Supplementary Fig. S10—B, $R = 0.85$, $p = 2.3e\text{-}06$). Similarly, validated ITIH4 concentration was strongly correlated to ITIH4 proteomics abundance (Supplementary Fig. S10—C, $R = 0.96$, $p = 4.2e\text{-}11$). No effect of either sow diet or sow diet - time interaction was revealed.

3.2.3. Total cholesterol, HDL- and LDL-cholesterol, and triglyceride

Total cholesterol level significantly increased at L-End compared to G108 sampling time (Supplementary Fig. S9-D1, $p = 0.0001$). A highly significant increase of total cholesterol at L-End compared to G108 was observed in the CR group ($p = 0.002$), in the LR group ($p = 0.006$), in LR compared to CR groups and vice versa (both $p = 0.002$). Effects of sow diet and sow diet-time interaction on total cholesterol level were not found. HDL-cholesterol significantly increased at L-End compared to G108 sampling time (Supplementary Fig. S9-D2, $p = 1.353e\text{-}06$). A highly significant increase of HDL-cholesterol at L-End compared to G108 was observed in the CR group (Table 3, $p = 0.001$), in the LR group (Table 3, $p = 0.001$); in LR compared to CR groups, and vice versa (Table 3, both $p < 0.001$). Additionally, HDL-cholesterol level in plasma was not affected by either sow diet or sow diet-time interaction. Moreover, there were no significant differences between groups or sampling times for LDL-cholesterol and triglyceride (Supplementary Fig. S9-E1 and S9-E2, respectively).

4. Discussion

While the dietary effects on sows have been well-studied [21–29,47] there have been a few previous studies on the plasma proteome beyond gestation, and this is the first report describing plasma proteome changes between late gestation and late lactation stages in sows supplemented with low dietary ω6:ω3 ratio. For several proteins, highly significant differences in abundance between G108 and L-End were observed; however other more modest differences (non-adjusted p-value < 0.05) may still be considered relevant, especially for guiding future research where larger group size or targeted quantitative proteomics would confirm significant differences.

4.1. Effect of sow dietary treatments: LR versus CR during G108 and L-End

Investigation of the effect of sows' diet, 4 and 5 proteins were changed in abundance following the change in the ratio between ω6 and ω3 PUFAs at G108 and regardless of time, respectively. Hemopexin (HPX) and alpha-1-antitrypsin (SERPINA1), implicated in inflammatory response regulation, and (SERPINA1 and Inter-alpha-trypsin inhibitor heavy chain H1 (ITIH1), implicated in protease inhibition, were upregulated. Haptoglobin (HP) and Serum amyloid P-component (APCS), implicated in immune and inflammatory responses, were down-

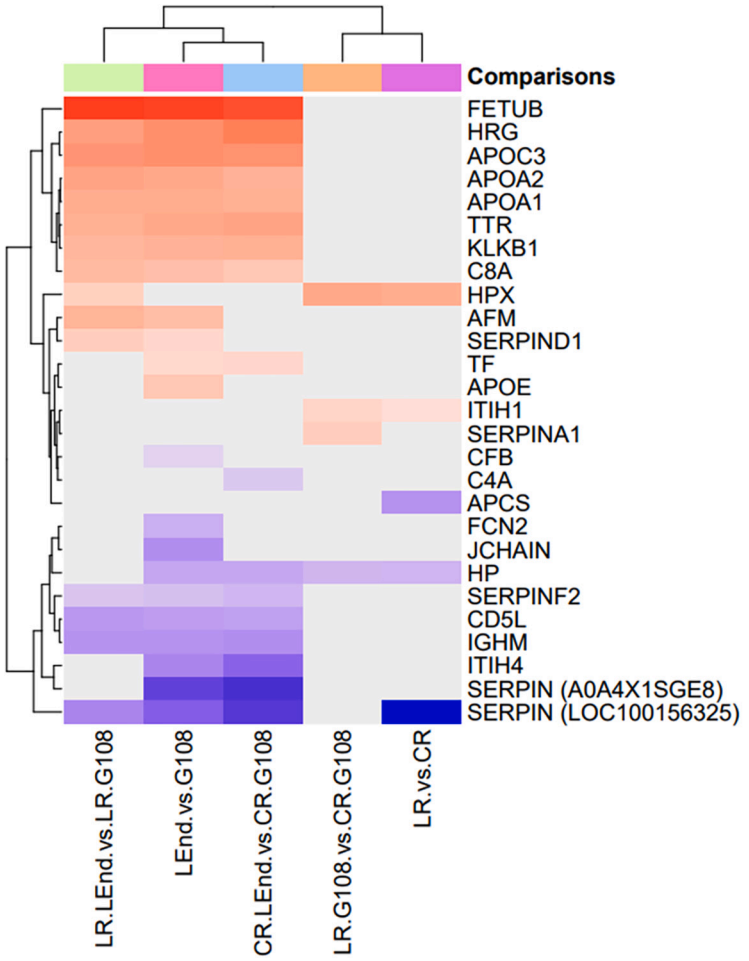

Fig. 4. Heatmap of the 27 significant differentially abundant proteins (DAPs) among five comparisons: (a) - LR group versus CR group at G108 (cerulean bar); (c) - LR group versus CR group (purple bar); (d) - CR group at L-End versus CR group at G108 (arctic bar); (e) - LR group at L-End versus LR group at G108 (pink bar); (f) - L-End versus G108 (yellow bar). CR: sow diet with ω6:ω3 ratio = 13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: sow diet with ω6:ω3 ratio = 4:1 from G28 until the end of lactation (L-End). G108 = day 108 of gestation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

regulated. These observations support the hypothesis that low dietary ω6:ω3 fatty acids can modify the protein profile of plasma in sows during late gestation and lactation.

Haptoglobin (HP) is a specific positive acute-phase protein (APP) with normal plasma levels ranging from 0.3 to 3 mg/mL [48]. HP primarily acts as an antioxidant and anti-inflammatory molecule by binding to free hemoglobin (HB) to detoxify HB and prevent its peroxidative side reactions to tissues [49]. In addition, HP functions as an immunomodulator in innate and adaptive cellular and humoral immune responses [50] by modulating helper T cell and lymphocyte function (that assists wound repair with minor cellular damage), inhibiting Th2 cytokine (anti-inflammatory) release, and weakly inhibiting Th1 cytokine (pro-inflammatory) release [51]. The ELISA assay validated the decreased abundance of HP in the plasma of LR-fed sows and showed a strong correlation with HP abundance quantified by proteomics.

In an earlier report in humans, the consumption of a low ω6:ω3 ratio was shown to lower plasma concentrations of HP and APCS, implying the activating effect of a low ω6:ω3 ratio intake on anti-inflammatory mechanisms [52]. Consistent with this observation, we also noticed the downregulation of HP and APCS in LR sows, suggesting that the anti-inflammatory mechanism was activated in these animals by the effect of a low ω6:ω3 ratio.

In contrast to the downregulation of HP and APCS, we found upregulation of SERPINA1 in the plasma of LR sow. SERPINA1 is also an acute-phase protein that inhibits proteases and stimulates the inflammatory response [53]. The increased abundance of SERPINA1 in plasma could be considered as a defence mechanism against excessive tissue damage at the final weeks of pregnancy.

4.2. Effect of time point: L-End versus G108 for LR and CR, separately and overall

The protein profile of sow plasma was measured in late gestation when the metabolic rate and energy expenditure in pregnant females is highest to meet the highest metabolic demand in the fetus [54], and proteome changes were found to move towards increased adipose tissue metabolism [18]. The lactating sows undergo accelerated catabolism because of the continued growth of mammary glands to produce a high milk yield, which can be a source of excessive oxidative stress [12]. Generally, sows in their late pregnancy and lactation suffer decreased antioxidative capacity and increased damage to immune cells due to excessive DNA oxidative attack [12]. An early study suggested that supplementing antioxidants can help fight against elevated oxidative stress and inflammation and restore the antioxidant defences of gestating and lactating sows [55].

Significant shifts were found over the late gestation-late lactation period affecting the plasma lipoproteins and the acute-phase proteins. Related to the former, apolipoprotein A-1 (APOA1), A-2 (APOA2), and C-3 (APOC3) were all increased by L-End. They are soluble-multifunctional proteins that play a crucial role in lipid metabolism through regulating critical enzymes activity and acting as ligands to lipoprotein receptors [56]. Over the same period, positive acute-phase proteins in pigs, haptoglobin (HP), and ITIH4 (or pig-MAP) were decreased in abundance. These findings imply that the end of pregnancy is associated with an acute-phase reaction demonstrated by the rise in the abundance of positive acute-phase proteins and a decrease in negative acute-phase proteins such as APOA1, while by the end of lactation, this acute-phase reaction has dissipated. Western blot or

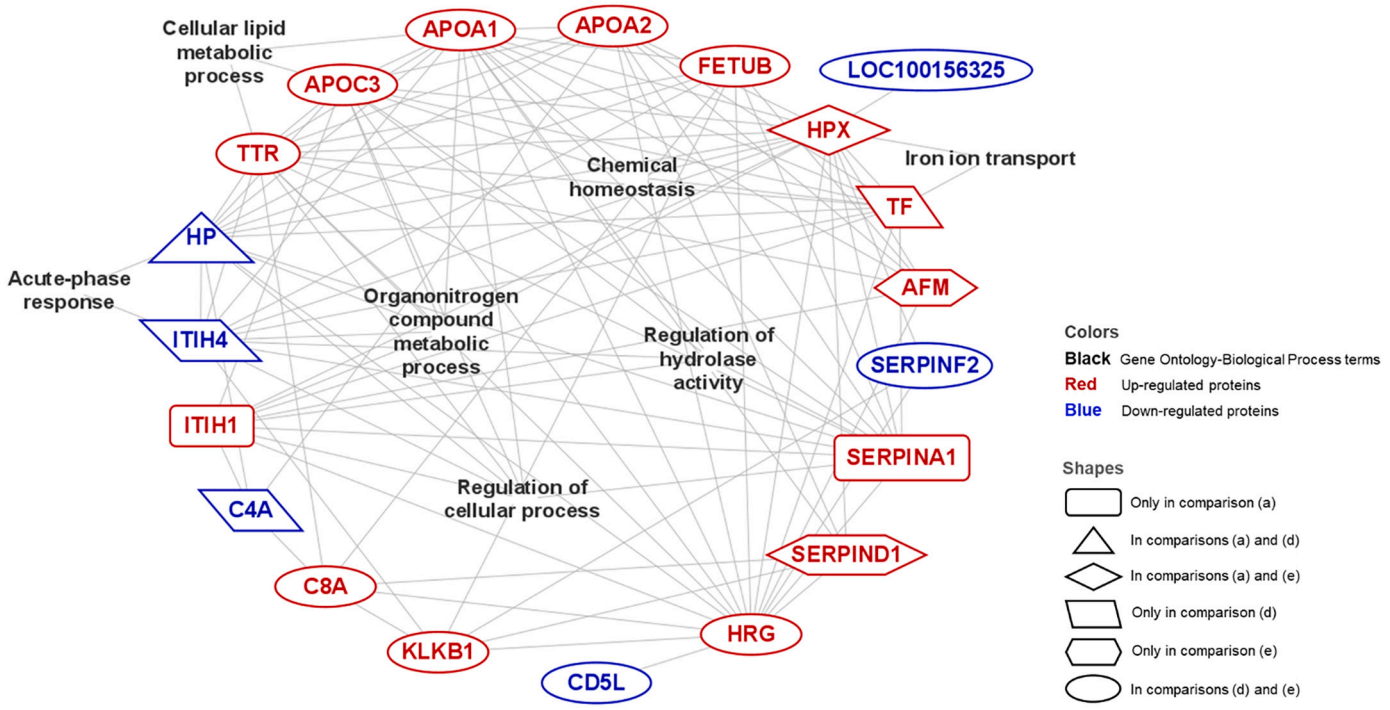

Fig. 5. Network of the significant differentially abundant proteins (DAPs) among three main comparisons with significantly enriched Gene Ontology-Biological Process terms, containing comparison (a) - LR group versus CR group at G108; comparison (d) - CR group at L-End versus CR group at G108; and comparison (e) - LR group at L-End versus LR group at G108. CR: sow diet with ω6:ω3 ratio = 13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: sow diet with ω6:ω3 ratio = 4:1 from G28 until the end of lactation (L-End). G108 = day 108 of gestation.

immunoassay confirmed the changes of the plasma proteins in these groups. These findings support the hypothesis that plasma proteome profiles in pigs could be significantly altered during late gestation and lactation.

Apolipoproteins A1 (APOA1) and C3 (APOC3) are closely linked together because they are both in the same gene cluster, which was proposed to regulate the immune response to inflammation in pigs [57]. This effect is expressed by the decrease in HDL and the increase in TG and LDL [57]. Although no changes in TG and LDL were observed for sows at late gestation in our study, the mechanism of this action was partly shown because there was a decrease in plasma total cholesterol and HDL- cholesterol level in late gestating sows compared to late lactating sows. Indeed, this finding is further supported by the Gene Ontology enrichment analysis in which the interaction between APOA1 and APOC3 was shown in the HDL particle's receptor-binding activity.

APOA1 is a major negative APP in pigs due to its decreased level when the host is infected with bacteria [58], and this decrease is associated with decreased HDL levels in different acute-phase responses (APR) [57]. Similar behavior of APOA1 during acute inflammation has been reported in humans [59], mice [60], and cows [61], suggesting APOA1 contribution in modulating some of the host acute-phase reactions [59]. A chronic APR is caused by improper nutrient management and tissue stress or malfunction [62]. In the present work, a reduction of sow plasma HDL, APOA1, and APOA2 (the second most abundant HDL protein) was observed in late gestation versus late lactation, suggesting that sows had undergone elevated chronic systemic inflammation, oxidative damage, and lipid oxidation in late gestation versus late lactation. These observations support the hypothesis that the inflammatory responses changed in sows during different stages of reproduction. A Western blot assay confirmed the alteration in the proteomics result of APOA1, and the HDL-cholesterol assay showed a similar changing pattern on HDL cholesterol level.

APOC3 is primarily presented on the surface of chylomicrons, very low-density proteins (VLDL), and HDL [63], so it participates in regulating triglycerides (TG)-rich lipoprotein [64] and plasma VLDL metabolism, also act as a pro-inflammatory factor in vivo [65,66]. Comparing to plasma APOC3 levels in humans (~6 mg/dL) [67], mice (~4–5 mg/dL) [68] and cows (~6 mg/dL, in non-lactating stage); the pig has an unusual low level of ~50 μg/dL and consequently, a low circulating TG level [57]. Although the concentration of APOC3 was not measured in this study, HDL measurement showed a significantly lower level of ~7.7 mg/dL at G108 compared to ~20 mg/dL at L-End. HDL can promote cholesterol efflux and act as an antioxidant molecule in plasma, although this contribution is relatively small (1–2%) to the plasma's total antioxidant capacity [71]. HDL also has anti-inflammatory properties [71]. Therefore, this study's higher HDL level in late lactation contributes to decreased oxidative stress and inflammation in lactating sows compared to gestating sows.

Acute-phase proteins, notably HP and ITIH4, were higher in abundance in plasma at G108 than L-End, and Gene Ontology enrichment analysis confirmed an interaction between these two main positive APPs. They both contributed to the organization of the extracellular region and participated in several biological processes, particularly acute-phase and defence responses of the host to stimulus. Indeed, a significant correlation between plasma HP and ITIH4 concentrations was found ($R = 0.62$, $p = 0.0037$ for proteomics abundance; and $R = 0.68$, $p = 0.001$ for validated concentration, Supplementary Fig. S11-A and B, respectively). The validation showing elevated HP and ITIH4 concentrations in the sows, particularly at late gestation, supports previous studies on the porcine APP [69]. The validated concentrations of HP and ITIH4 had strong correlations with the proteomics abundance, providing a reference for further research examining the role of the acute-phase response in swine reproduction.

Previous studies reported the crucial role of HP in modulating

Table 3
Results of validation assays for the concentrations of apolipoprotein A1 (APOA1), haptoglobin (HP), and pig-MAP (ITIH4) are presented with their respective protein abundance values determined by proteomics. In addition, plasma levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride in four groups of sows are shown. Difference between groups was analysed separately for each tested protein and validation method.

Sow diet (S)		G108		L-End		SEM	*P*-value								
Time point (T)		CR (LSM)	LR (LSM)	CR (LSM)	LR (LSM)		S	T	S*T	LR-G108 vs CR-G108	CR-LEnd vs CR-G108	LR-LEnd vs CR-G108	CR-LEnd vs LR-G108	LR-LEnd vs LR-G108	LR-LEnd vs CR-LEnd
APOA1	Proteomics (relative abundance)	6.92^{a}	7.02^{a}	7.47^{b}	7.47^{b}	0.11	0.616	<0.001	0.626	0.583	0.018	0.012	0.018	0.026	0.992
	Western blot assay (optical density – arbitrary units)	9884^{a}	9695^{a}	17882^{b}	21076^{b}	713.00	0.890	0.001	0.412	0.626	0.011	0.011	0.011	0.037	0.591
HP	Proteomics (relative abundance)	7.53^{c}	7.21^{bc}	7.13^{ab}	6.78^{a}	0.12	0.064	<0.001	0.894	0.109	0.016	0.007	0.652	0.016	0.109
	ELISA assay (mg/mL)	1.70^{b}	1.28^{ab}	1.15^{ab}	0.96^{a}	0.15	0.080	0.010	0.400	0.110	0.061	0.015	0.550	0.211	0.448
pig-MAP (ITIH4)	Proteomics (relative abundance)	7.34^{b}	7.13^{b}	6.54^{a}	6.73^{a}	0.16	0.964	<0.001	0.071	0.394	0.002	0.031	0.031	0.031	0.394
	ELISA assay (mg/mL)	1.35^{a}	1.15^{a}	0.70^{a}	0.85^{a}	0.18	0.902	0.010	0.274	0.520	0.114	0.182	0.182	0.327	0.549
Total cholesterol	Total cholesterol measurement (mmol/L)	1.14^{a}	1.28^{a}	2.19^{b}	2.10^{b}	0.15	0.885	<0.001	0.423	0.615	0.002	0.002	0.002	0.006	0.661
HDL cholesterol	HDL cholesterol assay (mmol/L)	0.43^{a}	0.42^{a}	1.10^{b}	1.13^{b}	0.09	0.926	<0.001	0.842	0.940	0.001	<0.001	<0.001	0.001	0.940
LDL cholesterol	LDL cholesterol (mmol/L)	0.64^{a}	0.79^{a}	1.01^{a}	0.89^{a}	0.13	0.951	0.058	0.238	0.524	0.233	0.454	0.454	0.524	0.524
Triglyceride	Triglyceride measurement (mmol/L)	0.31^{a}	0.36^{a}	0.42^{a}	0.43^{a}	0.07	0.662	0.229	0.788	0.742	0.742	0.742	0.742	0.742	0.904

CR: sow diet with ω6:ω3 ratio = 13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: sow diet with ω6:ω3 ratio = 4:1 from G28 until the end of lactation (L-End). G108 = day 108 of gestation. LSM = least square mean, SEM = standard error of the mean.

gamete interaction and early embryo development because it is up-regulated in the female reproductive fluid of pigs (in vitro study) [70], cows [71], and humans [72]. A recent study on sows confirmed the vital role of HP in early embryo development: it is excreted by the epithelium lining the oviduct and uterus throughout different stages of the oestrus cycle [73]. Linking HP function on lymphocyte modulation, it's increase in the oviduct and uterus throughout the luteal phase (and reaching a maximum on d 12 of pregnancy) implies HP's vital role in regulating maternal-embryonic communication [73]. These could be the underlying mechanism and explain the higher abundance of HP in late gestation compared to late lactation in this study. This finding is further validated by ELISA assay showing a similar altering pattern and a strong correlation with the proteomics result.

ITIH4 has an antiprotease activity and is also involved in the modulation of cell migration and proliferation [74], increasing rapidly in stressful conditions such as an injection, surgical trauma, or transportation [75,76]. In addition, ITIH4 may play a role in immune protection within the pig uterus and, thus, in pregnancy establishment [77]. These roles could explain a higher abundance of sow plasma ITIH4 in late gestation compared to late lactation in the present work. Moreover, ITIH4 concentrations of sows in this study agree with previous studies. ITIH4 concentration in healthy sows was 0.5 mg/mL (average at ~0.8 mg/mL [69]) and will be increased to 1.25 mg/mL at parturition; which are all below acute pathology level (6.11 mg/mL) [75].

5. Conclusions

The results revealed that low ω6:ω3 fatty acids ratio altered the plasma levels of several acute-phase proteins in late gestating and late lactating sows. HP, SERPINA1, and APCS might be involved in protective mechanisms against accelerated oxidative impairment at later stages of gestation and lactation. Furthermore, the plasma proteome profile in sows was changed between late gestation and late lactation periods, in particular the relative abundance of apolipoproteins such as APOA1, APOA2, and APOC3 and positive acute-phase proteins such as HP and ITIH4 (pig-MAP). These findings provide novel insights into animal adaptation's molecular mechanisms to changing stressors in different reproduction stages.

Author contributions

Conceptualization and design of the study: TXN, AA, GS, PDE, and RB; funding acquisition and project administration: TXN, RB, DE, GS; methodology: TXN, SM, SW, QHH, AG, MM, MP, RB, DE; acquisition of data: TXN, RB, DE; formal analysis and interpretation of data: TXN, AG; drafting the manuscript: TXN; critical revising the manuscript: TXN, AA, QHH, AG, MM, MP, GS, DE, and RB; approval of the final version to be submitted: TXN, AA, SM, SW, QHH, AG, MM, MP, GS, DE, and RB.

Declaration of Competing Interest

The authors have no competing interests. The funder of the study had no role in study design, data collection, analyses, and interpretation; manuscript preparation, or decision to publish the results.

Data availability

No

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Appendix A. Supplementary data

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