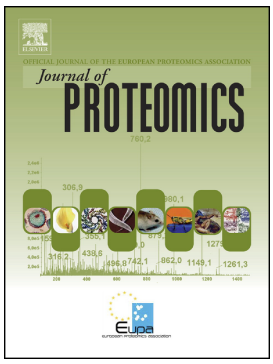

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Proteomic changes associated with maternal dietary low ω6:ω3 ratio in piglets supplemented with seaweed Part I: Serum proteomes

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Abstract

This study examines whether maternal low ω6:ω3 ratio diet and offspring SW supplementation can improve offspring immunity and performance by elucidating the effects on piglet serum proteome. A total of 16 sows were given either a standard (CR, 13:1) or low ω6:ω3 ratio diet (LR, 4:1) during pregnancy and lactation and their male weaned piglets were supplemented with SW powder (4 g/kg, SW) or not (CT) in a 21-day post-weaning (PW) diet. Four PW piglet groups were then identified based on dam and piglet treatment, namely CRCT, CRSW, LRCT, and LRSW (n = 10 each). Piglet serum collected at weaning and d21 PW were analysed (n = 5 each) using TMT-based quantitative proteomics and validated by appropriate assays. The differentially abundant proteins (n = 122) displayed positive effects of maternal LR diet on anti-inflammatory properties and innate immune stimulation. Progeny SW diet activated the innate immunity and enhance the host defense during inflammation. These data demonstrate the value of decreasing ω6:ω3 ratio in maternal diet and SW supplementation in PW piglet's diet to boost their immunity and anti-inflammation properties.

Highlights

* Maternal low ω6:ω3 ratio (4:1) and offspring seaweed supplementation affected the serum proteome in post-weaned piglets.

* Transferrin was altered in comparison between two maternal dietary treatments and between weaning versus day 21 post-weaning.

* Serum acute phase proteins and apolipoproteins were altered between weaning versus day 21 post-weaning.

Significance

This novel proteomic study in post-weaned piglets addresses the interplay between maternal and offspring nutritional interventions in a context of rapid and dynamic alterations in piglet metabolic status around weaning. Decreasing ω6:ω3 ratio in maternal diet and SW supplementation in PW piglet's diet can boost their immunity and anti-inflammation properties. This study also provides new insights into piglet serum proteome regulation during post-weaning, a critical development period in swine.

Keywords: pig proteome, ω6:ω3 fatty acids, seaweed, acute phase proteins, apolipoproteins

1 Introduction

Weaning is a critical familiarisation (adaptive) period in a pig's life to grow and develop fully in adulthood [1]. During weaning at an early age of ~3-4 weeks [2], piglets get through a transition period to build a gradual adaptation to the introduction of solid feed [3], new social relationships, and a changing housing environment [4]. This permits adaptation of physiological and behavioural responses against different stressors and can thus ameliorate the effects of stress. During this sensitive growth stage, invading pathogens and inflammatory stimuli can negatively affect the biological system, so the host defence responses play a critical role in maintaining the balance of biological pathways [5]. The host defence consists of activated functions of non-specific innate and specific adaptive immune cells to preserve tissue homeostasis in response to inflammatory reactions caused by various stimuli [5]. Pathogens and tissue injuries from birth can stimulate innate immune cells [6–9] while long-lasting immune responses are maintained thanks to adaptive immune cells [10]. Nutritional intervention such as supplementation with antioxidant compounds might reduce weaning-related stress and intestinal barrier dysfunction in pigs [11], thus supporting the immune system.

Maternal dietary treatments drastically affect the growth and metabolic patterns of the offspring's prenatal, early postnatal, and juvenile growth and, in turn, mature phenotype [12,13]. Many studies in humans have shown the benefits of a low dietary ratio of omega 6:omega 3 (ω6:ω3) polyunsaturated fatty acids (PUFAs) on diminishing inflammation severity, particularly in the long-term [14,15], due to the anti-inflammatory and antioxidant properties of ω3 PUFAs [16,17]. Lowering the dietary ratio of ω6:ω3 in sows through increasing ω3 PUFAs from linseed oil has been reported to enhance their piglet's growth performance [18], levels of plasma immunoglobulins and hepatic gene expression [19].

In piglets, diet at weaning serves an essential role in controlling gut function, shaping the adaptive immune response, assisting the host defence against stimuli, and consequently contributing to the overall performance and health status [20]. In recent years, supplementing piglet diet with in-feed antibiotics alternatives from sustainable sources has been rapidly developed [20]. *Ascophyllum nodosum* is a brown seaweed (SW) species, rich in bioactive ingredients such as phlorotannins and polyphenols [21–24]; and polysaccharides (laminarin, fucoidans and alginates [21], and ascophyllan [25]). Thus, it is a natural dietary source of antioxidants [26], antimicrobials and prebiotics [21,27], anti-inflammatory and immune regulators [28,29]. *A. nodosum* supplementation at 3, 6, 9, and 10 g/kg has been reported for its health benefits in pigs, mainly through its enhancement of gut health against pathogens and, consequently, has been proposed as an alternative to nutritional (in-feed) antibiotics [21,26,30].

However, the mechanism underlying the benefits of supplementing SW in pig diet has not been addressed so far.

An extensive review on the effects of alternatives to in-feed antibiotics in post-weaned piglets suggested that this type of study should pay equal attention to both physiological - functional approaches and gene expression [20]. Moreover, further investigations on the long-term effects of early- (prenatal to early postnatal or pre-weaning) and later- (post-weaning) nutritional programming are required to understand the underlying mechanisms of these dietary treatments in weaned piglet health and growth [20]. Studies considering the interplay between early- and later-life nutritional interventions will add to our understanding of the critical role of maternal diet in offspring growth and development [31].

This novel study describes the interplay between maternal low ω6:ω3 ingestion and offspring seaweed supplementation on offspring's serum (Part I) and ileal protein profiles (Part II, submitted) by quantitative proteomics, a method increasingly being used in studies of pig nutrition [32–34] here using the tandem mass tagged (TMT) approach. In reporting these studies, we have kept these two parts separate because of the complex nature of the study design and as there were no shared proteins between the serum and ileum proteome studies. This study aims to provide a reference for future research on improving the life-long immune system of pigs. It is hypothesized that the mother's low ω6:ω3 diet and offspring seaweed supplementation diet will be suggestive of an additive effect on the offspring's immunity and growth. This paper is Part I of an interconnected study and covers the protein profiles in serum of weaned piglets in exploring the underlying mechanisms, while Part 2 examines changes in the ileum proteome in the same piglets.

2. Materials and methods

2.1 Animals and sample collection

The feeding trial on piglets was conducted at the Animal Production Research and Teaching Centre of the Department of Veterinary Medicine and Animal Science, University of Milan (Lodi, Italy). The study 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 experimental design, growth performance, and blood oxidative status results were detailly described [35].

Briefly, a total of 40 male weaned piglets (Large White x (Landrace x Large White)) were designated from different mothers within a group, amongst two maternal dietary treatments containing either a control

ratio of ω6:ω3 PUFAs (CR, 13:1 during gestation and 10:1 during lactation) or a low ratio of ω6:ω3 PUFAs (LR, 4:1, during gestation and lactation). Piglets were weaned at day 26 (± 1.76) of age with an average body weight of 6.46 kg (± 0.15) (mean ± SE). Piglets were fed a meal-based commercial diet (Supplementary Table S1) and supplemented with or without 4 g seaweed powder *(A. nodosum;* Prodotti Arca S.r.l, Monza, Italy) per kg of feed, namely SW and CT groups, respectively. Four groups (*n* = 10 each) were formed: CRCT; CRSW; LRCT; and LRSW.

Blood samples were collected from the jugular vein from each piglet on weaning (d0) and day 21 post-weaning (d21 PW) using BD Vacutainer tubes (10 mL, REF 367896, BD-Plymouth, PL678P, UK). Blood samples were left to clot for a minimum of 60 min and a maximum of 120 min at room temperature, centrifuged (15 min; 3000× g; room temperature), and subsequently kept at −80 °C until analysis.

2.2 Protein identification and quantification using the TMT approach

Protein quantification

Total protein concentration of the serum samples was measured by Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories GmbH, Müchen, Germany) using bovine serum albumin as standard.

Protein digestion and Isobaric labelling – tandem mass tag

The proteomic profiling of serum samples was performed using Tandem Mass Tag (TMT) labelling quantitative approach as described earlier [36]. For each sample, 37 µg protein was digested with trypsin using filter-aided sample preparation (FASP) [37] with 10 kDa molecular weight cut-off filters. Digesting proteins into peptides was executed 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 remaining peptides were collected using 10% acetonitrile (ACN) in water. Trypsin activity was inhibited by acidification with 1% trifluoroacetic acid (TFA, CF_3COOH) before entirely drying at 45 °C under a high vacuum.

Next, the peptides pellets obtained were diluted using 0.1 M triethylammonium bicarbonate (TEAB, Thermo Fisher Scientific, Rockford, USA) before being tagged using a freshly prepared TMT10plex Label Reagent set followed manufacturers instructions (Thermo Fisher Scientific, Rockford, IL, USA). All TMT-modified peptide samples were combined in equal amounts into a new microcentrifuge tube, aliquoted,

SpeedVac lyophilized, and kept at -80 °C before tandem mass spectrometry analysis. Besides, an equal quantity of peptides from every sample was pooled and labelled by a TMT tag before adding to each TMT multiplexed sample as an internal standard (or linker) between multiple TMT sets. Five TMT10plex experiments were performed for a total of 40 samples (5 animals per group x 4 groups x 2 time points).

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

Before analysis, peptides were dissolved 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 Fisher Scientific RSLCnano, Horsham, UK). Peptide ions were detected and characterized using electrospray ionisation (ESI) mass spectrometry MS/MS with an Orbitrap Elite MS (Thermo Fisher Scientific, Horsham, UK). LC eluent was ionized by interfacing the LC coupling device to a NanoMate Triversa (Advion Bioscience, Harlow, UK) with an electrospray voltage of 1.7 kV. Online desalting and trapping of peptides (5 µL) were performed 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.

After desalting, the purified peptide was further separated on a Pepmap C18 reversed-phase column (50 cm × 75 µm, particle size 3 µm, pore size 100 Å, Thermo Fisher Scientific, Horsham, UK) using mobile phase A and B at a fixed flow rate of 0.3 µL/min for the analytical column. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.08% formic acid in 80% ACN and 20% water. The peptides were fractionated with a series of mobile phase B gradients: 4% v/v for 10 min, 4-60% v/v for 170 min, 60-99% v/v for 15 min, and held at 99% v/v for 5 min. The column was adjusted to the initial conditions and re-equilibrated under that condition for 10 min before the next sample injection.

Eluting peptides were analysed on an Orbitrap Elite mass spectrometer. Each MS scan (380 – 1800 m/z) was acquired at a resolution of 60000 FWHM, followed by CID fragmentation and detection of the top three precursor ions from the MS scan in the linear ion trap. The top three precursor ions are also subjected to HCD in the HCD collision cell, followed by detection in the Orbitrap at a resolution of 30000 FWHM, as defined at 400 m/z. Selected precursors were added to a dynamic exclusion list for 180s and single-charged ions were omitted from selection.

2.3 MS/MS data processing

Raw MS/MS spectra were processed in Proteome Discoverer (version 2.4, Thermo Fisher Scientific). The UniProtKB database was searched to identify and quantify proteins using the Sequest HT algorithm against *Sus scrofa* FASTA files (104,940 sequences; downloaded on 18/02/2021). Precursor ion mass

tolerance of 10 ppm and fragment tolerance of 0.02 Da were applied. Trypsin was selected as the enzyme with the option of two missed cleavage sites. Carbamidomethyl (C) was stated as the fixed modification. The dynamic modifications contain oxidation (M), deamidation (N, Q), and TMT six-plex (K, peptide N-terminus) were specified. The peptides were identified using the Percolator algorithm based on the search results against a decoy database. A false discovery rate (FDR) was set at 1% on the peptide level. At least two peptides and 5% FDR were required to report confidently identified proteins. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via PRIDE [38] partner repository with the dataset identifier PXD032327.

2.4 Validation of proteomics results

For a comprehensive validation of proteomics results, alterations in the non-depleted sample contents of APOA1, HDL-cholesterol, and SAA were determined. These proteins were selected based on their significant alterations in abundance analysis by TMT-based proteomic, their known biological significance, and as methods for use in their validation were available. Depletion of high-abundance proteins was not performed to avoid the potential depletion bias in shotgun proteomics related to the unintended removal of some potentially important non-targeted proteins such as albumin, antitrypsin, transferrin and haptoglobin [39].

The concentration of **APOA1** in serum was measured using Western blot according to a previous method [40]. In brief, 10 µg of proteins of each sample (5 samples/group/time point) was denatured, followed by electrophoresis separation before transferring into nitrocellulose membranes. Membranes were then blocked in blocking solution and probed with rabbit polyclonal antibody to porcine APOA1 at 1:20,000 dilution (PAA519Ho01, Cloud-Clone Corp, USA) by overnight incubation at 4 °C. This primary antibody was later probed with secondary antibody HRP conjugated anti-rabbit IgG to horseradish peroxidase (1:10,000; ab6721, Abcam Ltd. UK) in T-TBS containing 5% milk powder for 1 h at room temperature. ECL substrate was added for protein detection (Thermo Fisher Scientific™, Meridian Rd., Rockford, USA) and ECL image was visualized using radiographic film (Hyperfilm ECL, Amersham Biosciences). The protein band intensity was measured using Image J NIH software (https://imagej.nih.gov/ij/).

HDL-cholesterol level was quantitatively analysed using the HDL-cholesterol assay kit (ab65390; Abcam, Cambridge, UK) following the manufacturer's instructions. The HDL component was preliminarily

separated using chemical precipitation from other lipoproteins. Then, the cholesterol contained by the HDL was determined using a colorimetric assay.

The quantification of **SAA** was implemented by ELISA using species-specific kits (Life Diagnostics pig SAA ELISA, Life Diagnostics Inc., West Chester, US). The assay uses peptide-specific pig SAA antibody for solid-phase immobilization. Pig SAA antibody conjugated with horseradish peroxidase (HRP) was used for detection.

2.5 Statistical and Bioinformatics Analysis

Statistical analysis for proteomics data

The statistical analyses were performed using peptide spectrum match (PSM)-level data based on linear mixed-effects models with Empirical Bayes moderation using MSstatsTMT package version 2.0.0 [41] in R version 4.1.0 [42]. Proteins were quantified using unique peptides and summarized using the median polish method. Individual protein was normalized with the pooled internal standard channel. The Benjamini-Hochberg method was used to correct multiple pairwise comparisons (p < 0.05). The statistical analysis used master proteins only. Significantly changed proteins were determined at an adjusted p-value < 0.05, and possibly changed proteins were determined at a p-value < 0.05.

The volcano plots were generated using packages *ggplot2* version 3.3.3 [43] and *ggrepel* version 0.9.1, PCA plots used *ggplot2* version 3.3.3 [43], and heatmaps used *pheatmap* version 1.0.12. Venn diagrams were generated using web tool Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/) [44].

Bioinformatics analysis

The accession numbers of identified master protein were converted into the corresponding gene ID using the UniProt retrieve/ID mapping tool. Undefined proteins were replaced with the best match on *Sus scrofa* orthologue annotated genes of minimum 70% identity using SmartBLAST tool (https://blast.ncbi.nlm.nih.gov/smartblast/). STRING database version 11.5 [45] was used to retrieve the protein-protein interaction (PPI) network, Reactome pathways, Gene Ontology (GO) analysis containing Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). Enriched GO terms were further filtered using the REVIGO [46] webserver (revigo.irb.hr) to remove the redundant terms, applying 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. Interactions between desired Reactome pathways and proteins with significantly different abundances between comparisons were mapped through Cytoscape software v3.8.2 [47].

Statistical analysis for validation data

The APOA1 and SAA validation data were analysed and compared with results measured by proteomics. Before the data analysis, the normality of the data distribution for each variable was examined using the Shapiro-Wilk test and homogeneity of variance was checked using Levene's test. Difference between groups was investigated applying the linear mixed model, and multiple pairwise comparisons were corrected using the Benjamini-Hochberg false discovery control procedure. Fixed effects were sow diet, piglet diet, time, and their interactions. The random effect was an individual animal. Pearson correlation analysis was applied to assess the association between proteomics and validation results for APOA1 concentrations. Spearman correlation was used for the validation data of SAA (data was normally distributed, but the variances were not equal).

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 Figure 1.

3. Results

3.1. Proteomic analysis

The TMT mass spectrometry-based relative quantitation found 12832 features and mapped 2675 unique peptides, which represented 489 proteins and 260 master proteins. Proteins matched with no unique peptide or only one peptide were excluded. The master protein - primary translation product of the coding sequence and expressed at least one of the known protein isoforms, coded by the canonical sequence [48], was used for statistical analysis. As a result, 122 master proteins have been quantified and remained for statistical analysis (Supplementary material_List of 122 master proteins).

Individual principal component analysis (PCA) score plot displays the clustering of samples from each combination of groups of piglets were presented in Supplementary Figures S1 to S7.

The differentially abundant proteins (DAPs) in 7 comparisons are summarised in Table 1 (details are presented in Supplementary Table S2) including (a) - LR vs CR at d0; (b) - LR vs CR at d21 PW; (c) - LR vs CR; (d) - SW vs CT at d21 PW; (e) - CR at d21 PW vs CR at d0; (f) - LR at d21 PW vs LR at d0; and (g) - d21 PW vs d0.

DAPs in LR vs CR groups (comparisons (a), (b), (c))

On d0, serum in LR piglets gave 7 DAPs (3 increased and 4 decreased) when compared to CR piglets (comparison (a), Table 1, $p < 0.05$), while on d21 PW, LR piglets exhibited 10 DAPs (6 increased and 4 decreased) when compared to CR piglets (comparison (b)). Irrespective of sampling time, LR piglets generated 13 DAPs (5 increased and 8 decreased) if compared to CR piglets (comparison (c), Table 1, $p < 0.05$). Among these proteins, transferrin (TF, B3CL06) was decreased in all three comparisons (a), (b), and (c), while TF (P09571) was increased in (b) and (c). Antithrombin-III (SERPINC1) was increased in both (a) and (c). The DAPs separated and overlapped among three comparisons (a), (b), and (c) are represented in the Venn diagram (Figure 3-A) and heatmap (Figure 4).

A mixed distribution with more overlaps of the samples between CR and LR groups towards the end of the post-weaning period is presented in the PCA of comparison (a), (b), and (c) (Supplementary Figure S1-A, S2-A, and S3-A, respectively). The total variance explained in the PCA procedure was gradually decreased from weaning (70.51%) to d21 PW (62.78%) and regardless of sampling time (50.30%). The volcano plots of DAPs in (a), (b), and (c) are shown in Supplementary Figures S1-B, S2-B, and S3-B, respectively. The hierarchical cluster heatmaps of the DAPs clearly showed a mixed distribution between LR and CR serum in (a), (b), and (c) (Supplementary Figure S1-C, S2-C, and S3-C, respectively).

DAPs in SW vs CT groups (comparisons (d))

Taking into account the effect of the piglet diet, comparison (d) examined SW serum vs CT serum at d21 PW, and it yielded 4 DAPs ($p < 0.05$, Table 1 and Supplementary Table S2). As shown in Supplementary Figure S4-B, the volcano plot indicates three upregulated proteins comprising Complement C5a anaphylatoxin (C5), kininogen-1 (KNG1), and ceruloplasmin (CP), and one downregulated protein, IgM heavy chain constant region (IGHM). The PCA analysis showed a mixed cluster between SW and CT samples, with the first two components explaining a high rate (78.06%) of data variation (Supplementary Figure S4-A). This mixed cluster is also shown in the hierarchical cluster heatmaps of the DAPs between SW and CT serum (Supplementary Figure S4-C). Investigation of the groups before having diets supplemented by SW identified 7 DAPs that were up or down-regulated in a CRCT-d0 vs CRSW-d0 comparison and 5 DAPs up or down-regulated comparing LRCT-d0 vs LRSW-d0 (Supplementary Table S3).

DAPs in d21 PW vs d0 (comparisons (e), (f), (g))

Considering the sampling time, comparison (e) investigated CR serum at d21 PW versus CR serum at d0 and disclosed 9 increased and 23 decreased DAPs (Table 1, $p < 0.05$). Comparison (f) analysed LR serum at d21 PW versus LR serum at d0 and found 13 increased and 18 decreased DAPs (Table 1, $p < 0.05$). Irrespective of dietary treatment, comparison (g) determined serum from d21 PW versus d0 and revealed 15 increased and 27 decreased DAPs (Table 1, $p < 0.05$). A heatmap (Figure 4) showed that, these three comparisons (e, f, g) share similar increased-abundant proteins such as transferrin (TF, P09571), histidine-rich glycoprotein (HRG), fetuin-B isoform 1 (FETUB), immunoglobulin (IGHA1, IGHG) and similar decreased-abundance proteins such as serum amyloid A (SAA), serpin-containing proteins; apolipoprotein A-IV, B and E (APOA4, APOB, APOE). The Venn diagram also illustrated the overlapped DAPs among these three comparisons (Figure 3-B).

As shown in the PCA plots, the serum at d0 and d21 PW were distinctly clustered in comparisons (e), (f), and (g), with the explained variances of 68.56% (Supplementary Figure S5-A), 55.66% (Supplementary Figure S6-A), and 57.30% (Supplementary Figure S7-A) respectively. The hierarchically clustered heatmaps also displayed the distinct proteome profiles of these comparisons (Supplementary Figures S5-C, S6-C, and S7-C, respectively). The increased- and decreased- abundance proteins in (e), (f), and (g) are highlighted in the volcano plots (Supplementary Figures S5-B, S6-B, and S7-B, respectively).

GO enrichment of all identified serum proteins

The analysis of protein-protein interaction (PPI) enrichment illustrates an association among 122 master proteins with 78 nodes and 1171 edges ($p < 1.0e-16$) (Supplementary Figure S8). The functional enrichments highlighted 99, 28, and 19 GO terms on biological processes (BP), molecular function (MF), and cellular components (CC), respectively (Supplementary Table S4). The top three enriched GO-BP terms were regulation of proteolysis [GO:0030162], vesicle-mediated transport [GO:0016192], and regulation of immune effector process [GO:0002697]. The top three enriched GO-MF terms were enzyme regulator activity [GO:0030234], heparin-binding [GO:0008201], and lipid binding [GO:0008289]. The top three enriched GO-CC terms were blood microparticle [GO:0072562], extracellular space [GO:0005615], and vesicle [GO:0031982]. The top 20, 10, and 5 GO terms on BP, MF, and CC, respectively, are shown in Supplementary Figure S9.

GO and Reactome pathway enrichment in LR vs CR groups (comparisons (a), (b), (c))

The DAP PPI network between LR and CR serum at d0 - comparison (a) contains 5 nodes and 6 edges ($p = 7.7e-09$). No enriched GO terms on BP and MF were found. The list of enriched GO terms on CC and

Reactome pathways is presented in Supplementary Table S5. The PPI network in the LR serum compared to CR serum at d21 PW - comparison (b) comprises 8 nodes and 7 edges (p = 6.81e-08). The list of enriched GO terms on CC and Reactome pathways is presented in Supplementary Table S6. Regardless of sampling time, the PPI network in the DAP between LR and CR serum - comparison (c) contains 9 nodes and 7 edges (p = 1.31e-05). No enriched GO terms on BP and Reactome pathways were observed. The list of enriched GO terms on MF and CC are presented in Supplementary Table S7.

Reactome pathway analysis in comparison (a) highlighted the interaction among C3, SERPINC1, and TF in the regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs) [HSA-381426], and post-translational protein phosphorylation [HSA-8957275]. In comparison (b), three Reactome pathways were enriched containing haemostasis [HSA-109582], binding and uptake of ligands by scavenger receptors [HSA-2173782], and scavenging by class B receptors [HSA-3000471].

GO and Reactome pathway enrichment in SW vs CT groups (comparisons (d))

The expanded DAP PPI network with maximum 5 interactors shown in the first shell, between SW and CT serum at d21 PW, - comparison (d) contains 13 nodes and 17 edges (p = 3.75e-05). Moreover, their list of enriched GO terms on BP, CC, and Reactome pathways are presented in Supplementary Table S8 underlined the role of C5 in regulating complement cascade [HSA-977606] and terminal pathway of complement [HSA-166665].

GO and Reactome pathway enrichment in d21 PW vs d0 (comparisons (e), (f), (g))

The PPI network in the DAPs of CR group at d21 PW versus d0 - comparison (e) contains 23 nodes and 148 edges (p < 1.0e-16). The PPI network in the DAPs of LR group at d21 PW versus d0 - comparison (f) comprises 23 nodes and 116 edges (p < 1.0e-16). Regardless of dietary treatments, the PPI network in d21 versus d0 serum - comparison (g) contains 31 nodes and 243 edges (p < 1.0e-16). The list of enriched GO terms and Reactome pathways corresponding to the DAPs of comparisons (e), (f), and (g) is presented in Supplementary Table S9, S10, and S11, respectively. The top 10 leading GO terms on BP, MF, and CC, and Reactome pathways of the PPI network of DAPs in (e), (f), and (g); are illustrated in Supplementary Figure S10, S11, S12, respectively.

Briefly, the pathways analysis in comparisons (e), (f), and (g) underscored the interactions of DAPs in multiple Reactome pathways such as regulation of Insulin-like growth factor (IGF) transport and uptake

by Insulin-like growth factor binding proteins (IGFBPs) [HSA-381426], innate immune system [HSA-168249], and metabolism of proteins [HSA-392499].

Furthermore, a network among three main comparisons (i.e. (c), (d), and (g)) gives an overview of the interaction between the DAPs and significantly enriched Reactome pathways (Figure 5). In this network, the up- and down-regulated proteins with and between each comparison are shown with their related enriched Reactome pathways such as plasma lipoprotein remodelling [HSA-8963899], post-translational modification [HSA-597592], and complement cascade [HSA-166658].

3.2. Validation of proteomics results

Results of validation assays for apolipoprotein A1 (APOA1) and serum amyloid A (SAA) in serum samples, together with their respective protein abundance data determined by proteomics, are shown in Supplementary Figure S13. Furthermore, serum levels of high-density lipoprotein (HDL) cholesterol in 4 groups of piglets at d0 and d21 PW are displayed in the same Supplementary Figure.

Apolipoprotein A1 (APOA1)

Western blot analysis of APOA1 in serum is displayed in Supplementary Figure S13. Clear bands at 26 kDa indicating the presence of APOA1 were detected in all samples. Protein band intensity was higher at d0 than at d21 PW ($p < 0.001$). There were no significant effects of dietary treatments on the sows and piglets or their interactions with sampling times on APOA1 concentration. A significantly lower concentration of APOA1 was determined at d21 PW for all four groups (CRCT, CRSW, LRCT, and LRSW) compared to CRCT, LRCT, and LRSW at d0 ($p < 0.001$). At d0, LRSW serum had a higher APOA1 concentration than CRSW serum ($p = 0.026$). The proteomics relative abundance exhibited a similar increasing pattern of APOA1 at d21 PW compared to that at d0 ($p = 0.03$), as shown by the validated APOA1 concentration. The overall evaluation found a moderate positive linear correlation between validated APOA1 concentration and APOA1 proteomics relative abundance (Supplementary Figure S14-A, $R = 0.47$, $p = 0.0021$).

Serum amyloid A (SAA)

Concentrations of SAA was significant decreased at d21 PW compared to d0 (Supplementary Figure S13-B1, $p < 0.001$). Furthermore, the LR diet in sows increased the SAA concentration compared to the CR diet (Supplementary Figure S13-B1, $p = 0.031$). Regarding the interaction between piglet diets and sampling time, the concentration of SAA was decreased over time after weaning in both CT and SW

groups (Supplementary Figure S13-B1, p = 0.044). A significant decrease of SAA concentration from d0 to d21 PW was shown when comparing CRCT and CRSW at d21 to LRCT and LRSW at d0 ($p < 0.05$). The relative proteomics abundance presented a similar decreasing pattern of SAA at d21 compared to d0 (Supplementary Figure S13-B2, $p < 0.001$), especially when considering the effect of piglet diet over time (Supplementary Figure S13-B2, p = 0.02); as observed in the validated SAA concentration. The proteomics abundance pattern of SAA was supported by a significant correlation with validated SAA concentration (Supplementary Figure S14-B, R = 0.7, p = 1.1e-06).

HDL-cholesterol

HDL-cholesterol level significantly decreased at d21 (~0.94 mmol/L) compared to d0 (~1.20 mmol/L) (Supplementary Figure S13-D, p = 0.002). However, the level of HDL-cholesterol in piglet serum was not affected by either sow diet, piglet diet, or their interaction with sampling time.

4. Discussion

Maternal diet is critical to offspring growth, immune development, and intestinal functions [49–51]. During pregnancy and lactation, the nutritional program creates early-life adaptation and development and has long-term (permanent) effects on adult life [52]. Maternal micronutrients can function as enzyme substrates or cofactors responsible for the maintenance of genome stability, and macronutrients can affect the DNA-damage response through physiological regulation [51]. This is the first proteomics study of newly weaned (d0) and post-weaned (d21 PW) piglet serum, influenced by the interplay between maternal nutrition (a marker of early-life programming) and offspring nutrition (later-life programming). Although many DAPs with adjusted p-values < 0.05 were highlighted, DAPs have more modest alterations with raw p-values < 0.05 may still be relevant for guiding further research if a larger sample size or targeted quantitative proteomics would prove significant alterations.

Effect of sow dietary treatments: LR vs CR groups (comparisons (a), (b), (c))

At weaning (d0), the relative abundance of complement component 3 (C3) - a critical component in the complement system, was influenced by maternal LR diet. Complement C3 is well-established for its pro-inflammatory property, therefore, contributing to the innate and adaptive inflammation in response to stimuli [53]. The concentration of C3 was increased in most inflammatory conditions [54]; hence, the down-regulated of C3 in LR versus CR piglets at weaning in this study may relate to the anti-inflammatory property of low ω6:ω3 ratio in their mother's diet. The up-regulation of protein C3 in CR piglets may contribute to some key features of inflammation, such as hypo-fibrinolysis and hyper-

coagulation [55]. Indeed, a short-term increased pig C3 serum level is an immune response during inflammation's sub-acute phase, such as after vaccinations/immunizations [56].

Addressing how maternal diet can affect piglet's immunity, acute phase proteins (APPs) are suitable parameters to consider because they regulate the acute phase response (APR) - the cornerstone of the innate immune system to restore homeostasis and promote the healing process [57]. Compared to two main positive APPs in pigs, i.e., pig-MAP and HP, SAA is fast but less prolonged released in the innate defence system against infection [57–59]. SAA can activate innate immune sensors such as Toll-like receptors 2 and 4 (TLR2 and TLR4), leading to activation of innate immunity and suppression of excessive inflammation and tissue damage [60–62]. In the current work, a significantly high level of SAA in LR piglets at d21 PW reflects that the innate defence system is strongly activated and participates in the inflammatory response. In line with the results of the current study, Cai et al. (2005) and Sack Jr (2018) reported that SAA can bind to the scavenger receptor class B type 1 (SR-B1) and participate in the selective uptake of high-density lipoprotein (HDL). During an APR in humans, hepatocytes' SAA synthesis is quickly released into the bloodstream, associated with HDL [64], and involved in lipid metabolism [60]. Acute phase SAA becomes the major apolipoprotein on HDL by displacing the APOA1 sub-unit, compromising HDL's role to protect against oxidation and prevent cholesterol accumulation (by promoting reverse cholesterol transport) [65]. Following maternal LR diet, serum level of SAA was increased in piglets at d21 PW compared to piglets on the CR diet and contributed to the host protective mechanism.

On d21 PW, another APP that participates in innate immune and inflammation regulation is transferrin (TF), an iron-carrier glycoprotein from the intestine to proliferative cells through the body [66]. In this study, although two accession numbers of TF were identified, only P09571 was kept for discussion due to the strong correlation between its relative abundance measured by proteomics and band intensity validated by SDS-PAGE (Supplementary Figure S14-C1, R = 0.63, p = 1.5e-05). At the same time, there was no similar correlation in B3CL06 (Supplementary Figure S14-C2, R = -0.011, p = 0.96).

Piglets are born with a low serum level of TF, ~1.8 mg/mL [67,68], and usually faced with an iron deficiency state [69]. Piglet TF level can increase to ~6.1 mg/mL at day 42 post-birth [67,68] before staying constant. The gradual increase of TF was observed in the current study in piglets from weaning (d0 PW or d26 of life) to d21 PW (or d46 of life), and in LR versus CR piglets at d21 PW, reflecting increased TF synthesis in the liver and total body iron stores. By being actively involved in the active iron sites, TF is considered as an integral part of the protection system together with hemoglobin-

haptoglobin complexes [70]. Besides, TF is also regarded as a negative APP in pigs [57,71,72] and chickens [73]. The concentration of serum TF was decreased following inflammation to stimulate host defence and immune regulatory mechanisms [73]. Transferrin can act as an antioxidant and antimicrobial agent, protecting the host against tissue lipid peroxidation and pathogenic microorganisms, respectively [68,74]. Furthermore, TF is considered as a growth-promoting factor because it participates in DNA transcription and supports cell growth and proliferation [75]. Taken together, a higher level of TF in LR than CR piglets suggested the role of maternal diet in regulating the host response against inflammation, but its contribution to the growth rate in piglets has not been observed.

Regardless of sampling time, maternal LR diet affect the relative abundance of proteins that regulate peptidase activity such as PZP, FN1, SERPINC1, and SERPINA3.

Alpha-2-Macroglobulin (or Pregnancy-zone protein, PZP), an endopeptidase inhibitor, was up-regulated in LR versus CR group but down-regulated in d21 PW versus d0 in this study. The down-regulation of PZP in piglet serum from weaning to d21 PW in this study may follow the decrease in PZP concentration in their maternal blood during the lactation period [76]. Rubio-Aliaga et al. [77] reported that PZP might involve body weight regulation; however, the mechanism of this effect needs to be further studied. The concentration of fibronectin (FN1) – a positive APP was down-regulated in LR versus CR serum, contributing to the enhanced host protective response [78] that is affected by low ω6:ω3 ratio in the maternal diet.

Two members of the serpins (serine proteinase inhibitors) family, Alpha-1-Antichymotrypsin (SERPINA3) was decreased while Antithrombin-III (SERPINC1) was increased in abundance in LR versus CR serum. Serpins are diverse in their functions but highly structurally conserved (similar) group of proteins [79] that are most abundant in humans [80] and higher organisms [81] such as swine, as shown in this study (Table 1). Apart from the central function in cellular homeostasis maintenance [80], serpins also have non-inhibitory roles related to various proteolytic pathways such as inflammation, coagulation, tissue remodelling, and angiogenesis [79,82,83]. SERPINA3 seems to be increased, and SERPINC1 decreased with the inflammation conditions [80]. So the down-regulation of SERPINA3 and up-regulation of SERPINC1 in the serum of LR piglets suggest the anti-inflammatory effect of low ω6:ω3 ratio in the maternal diet. Notably, SERPINA3 was strongly decreased in abundance on d21 PW versus d0, confirming that piglets at weaning time undergone higher inflammation state compared to the later stage of life, which is in agreement with previous observations [2].

Effect of piglet dietary treatments: SW vs CT groups (Comparison (d))

On d21 PW, the relative abundance of C5 was increased in the SW-fed piglets. The activated complement system produced anaphylatoxins – proinflammatory agents such as C5a that can activate innate immune cells, i.e., monocytes and neutrophils, and mediated endothelial permeability [84]. Additionally, C5a can trigger the endothelium to release inflammatory mediators such as Interleukin-6 (IL-6), thereby in-directly stimulating the innate immunity [85,86]. The enrichment of C5a in SW piglets at d21 PW in the current study suggests the essential role of C5a as a bridge to connect innate and adaptive immunity to enhance the host defence during inflammation conditions [87]. Interestingly, there were 12 proteins which different between groups assigned to the study of SW supplementation on d0 of the diet, likely due to biological variation, but none of these proteins were shown to be DAPs on d21 of the diet.

Effect of time point: d21 vs d0 (Comparisons (e), (f), (g))

The protein profiles at d21 PW and those at d0 were distinguished (as shown in the PCA score plot, Supplementary Figure S7-A), indicating the alterations in the proteomic patterns of the post-weaned piglets. The day 21 PW, serum lipoproteins (e.g., APOA1, APOB, APOE); immunoglobulin (e.g., IGHA1, IGHG) and acute phase proteins (e.g., HP, ITIH4, TF, SAA, SERPINA1) were changed in their relative abundance, compared to d0. These DAPs were annotated by 25 Reactome pathways; mostly referred to the metabolism of proteins, and Insulin-like Growth Factor (IGF), and innate immune system; regardless of maternal or offspring dietary treatments.

Apolipoproteins play a critical role in lipid transport throughout the lymphatic system, which regulates adaptive immunity and inflammation [88,89], particularly during the critical metabolic adaptation transition periods from gestation to lactation [88]. The decreased concentration of APOA1 and APOE in late pregnancy and increased concentration during lactation were reported in dairy cows [90] and sows - piglets' mothers of this study (unpublished data). Consequently, suckling piglets gained relatively high levels of apolipoproteins such as APOA1, APOB, and APOE from their mother; however, these concentrations fell considerably in post-weaning, as shown in the current work. Indeed, the changes in the apolipoprotein concentrations during weaning reflect piglet adaptation to dynamic and complex metabolic processes [91]. Apolipoproteins A1 containing lipoproteins (HDL) and APOB containing lipoproteins (chylomicrons, very-low density lipoprotein or VLDL, and low-density lipoprotein or LDL) are interrelated in lipoprotein metabolism [92]. Specifically, APOB containing lipoproteins transport lipids

(primarily triglycerides (TG) and cholesterol) to provide energy from the intestine and liver to other tissues, while APOA1 containing lipoproteins eliminate excess lipids (mainly cholesterol) from tissues and transport them to the liver [92]. Since HDL has multiple biological functions (anti-inflammatory, antioxidant, anti-thrombotic, and anti-apoptotic) [93]; a higher level of both APOA1 and HDL-cholesterol in piglets at weaning compared to d21 PW, as observed in this study, has shown that these changes are two of the host protective activities against weaning stress. In this study, while numerous apolipoproteins (APOA1, APOA4, APOB, APOC3, APOE, and APOF) were found to be altered in abundance between d0 and d21, validation was performed on APOA1 due to antibody availability, but the level of HDL was estimated via HDL-cholesterol assay. These methods confirmed that the concentrations of APOA1 and HDL were decreased towards d21 PW when a higher amount of adipose tissue was deposited [94].

The high levels of positive APPs (e.g., HP, ITIH4, SAA) and low levels of negative APPs (e.g., TF, TTR, and SERPINA1) in piglet serum at d0 were observed as a consequence of stress stimuli following separation from the mother, transportation, change to a new environment and social mixing (regrouping). These results match those observed in an earlier study [95]. Weaning stress induces appetite and feed intake suppression [95]. The stress may arise from accelerating catabolism [95], pushing animals into a state of immune challenge, negative energy balance, and reduced weight gain [96,97].

Immunoglobulins are the main components of adaptive immunity; they protect the body against pathogens and maintain animals' health and growth [98,99]. Previous studies have confirmed that serum level of immunoglobin such as IgA was significantly higher in post-weaned piglets than in those piglets at weaning [100,101]. In this study, post-weaned pigs had higher abundances of serum immunoglobulin than newly weaned pigs, indicating an improved immune function over time via increasing immunoglobulin production of their body.

The data in this study demonstrated a significant difference in DAPs as early-onset physiological mechanisms to promote adaptation to inflammatory signalling [102]. Fetuin-B, a protease inhibitor that can regulate insulin-like and hepatocyte growth factors (which in turn are IGF and HGF) following systemic inflammation [103], was up-regulated in d21 PW versus d0 in both CR and LR groups. Another group of protease inhibitors such as Inter-alpha-trypsin inhibitor heavy chain (i.e., ITIH3, mainly) and Alpha-1-microglobulin (AMBP) is also associated with the inflammatory response with either inflammatory or anti-inflammatory properties, especially acute inflammation triggered by infection [104]. Indeed, the down-regulation of ITIH3 and AMBP in d21 PW versus d0 in this study was related to

multiple biological processes such as vesicle-mediated transport and the regulation of proteolysis, localization, organonitrogen compound metabolic process. Coagulation factor XII (F12) was activated in LR versus CR serum at d0 to quickly create enzymatic activity on the surface of pathological cells in response to inflammation [105]. Protein F12 also contributes to developing the protective system in weaning piglets by regulating the cross-talk that links coagulation pathways and inflammation, particularly in innate immunity [105].

5. Conclusions

The present study provides evidence that nutritional intervention strategies, such as reducing the ω6:ω3 ratio in maternal diet, can promote transmission of protective factors to the offspring, leading to better anti-inflammatory activities, activation of innate immunity, and contributing to the enhanced host protective response. Supplementing SW in post-weaned piglets stimulated the connection between innate and adaptive immunity to enhance the host defense during inflammation. The altered serum proteome profile between weaning and d21 post-weaning was related to the activation of the innate immune system. Particularly striking were the decreases in abundance of acute phase proteins such as serum amyloid and the increases in apolipoprotein and HDL between weaning and d21 post-weaning. These findings collectively demonstrate the efficiency of reducing ω6:ω3 ratio (to 4:1) in maternal diet and SW supplementation in the diet of post-weaned piglets to boost offspring's immunity and anti-inflammation properties. Early- and later-life nutrition during critical periods in a pig's life would drive pig production towards sustainable development.

Supplementary data

Supplementary data to this article can be found at attached files.

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, RB, DE; acquisition of data: TXN, RB, DE; formal analysis and interpretation of data: TXN; drafting the manuscript: TXN; critical revising the manuscript: TXN, AA, QHH, AG, GS, DE, and RB; approval of the final version to be submitted: TXN, AA, SM, SW, QHH, AG, MM, GS, DE, and RB.

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Declaration of conflicts of 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.

Table 1. The differentially abundant proteins (DAPs) in piglet serum among seven comparisons: (a) LR vs CR at weaning (d0); (b) LR vs CR at day 21 post-weaning (d21 PW); (c) - LR vs CR; (d) - SW vs CT at d21 PW; (e) - CR at d21 PW vs CR at d0; (f) - LR at d21 PW vs LR at d0; and (g) - d21 PW vs d0.

Protein name	**Gene names**	**log2FC (with p-value < 0.05)**						
		Comparisons						
		(a)	**(b)**	**(c)**	**(d)**	**(e)**	**(f)**	**(g)**
		LR-d0 vs CR-d0	**LR-d21 vs CR-d21**	**LR vs CR**	**SW-d21 vs CT-d21**	**CR-d21 vs CR-d0**	**LR-d21 vs LR-d0**	**d21 vs d0**
Alpha-1-microglobulin	AMBP	/	/	/	/	-0.29	/	-0.21
Apolipoprotein A-I	APOA1	/	/	/	/	-0.18	/	-0.17
Apolipoprotein A-IV*	APOA4	/	/	/	/	-1.36	-1.21	-1.29
Apolipoprotein B	APOB	/	0.19	/	/	-1.02	-0.66	-0.84
Apolipoprotein C-III	APOC3	/	/	/	/	-0.76	/	-0.63
Apolipoprotein-E*	APOE	/	/	/	/	-0.75	-0.53	-0.64
Apolipoprotein F precursor*	APOF	/	/	/	/	/	/	-0.33
Complement C3	C3	-0.20	/	/	/	/	/	/
Complement C4A	C4A	/	/	/	/	-0.63	-0.57	-0.60
Complement C5a anaphylatoxin	C5	/	/	/	0.24	/	/	/
CD5 Molecule Like*	CD5L	/	/	/	/	/	-0.48	-0.36
Complement factor H isoform a	CFH	-0.37	/	-0.20	/	-0.24	/	/
C-type lectin domain family 3 member B	CLEC3B	/	/	/	/	/	-0.47	-0.42
Ceruloplasmin*	CP	/	/	/	0.16	-0.52	-0.24	-0.38
Coagulation factor XII	F12	0.23	/	/	/	/	/	/

Coagulation factor II	F2	/	/	/	/	-0.31	/	-0.23
Ficolin-1	FCN 1	/	/	-1.67	/	/	/	/
Fetuin-B isoform 1	FETUB	/	/	/	/	0.97	1.15	1.07
Fibronectin	FN1	/	/	-0.21	/	/	/	/

log2FC is base 2 logarithm transformed of fold change value which represents the ratio of expression levels in the first-mentioned group vs second-mentioned group (positive values - upregulated or increased abundant proteins, negative values - downregulated or decreased abundant proteins). 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. CR: piglet born from sow fed diet with ω6:ω3 ratio=13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: piglet born from sow fed diet with ω6:ω3 ratio=4:1 from G28 until the end of lactation (L-End). G108: day 108 of gestation.

Table 1. The differentially abundant proteins (DAPs) in piglet serum among seven comparisons *(cont., 1).*

Protein name	**Gene names**	**log2FC (with p-value < 0.05)**						
		Comparisons						
		(a)	**(b)**	**(c)**	**(d)**	**(e)**	**(f)**	**(g)**
		LR-d0 vs CR-d0	**LR-d21 vs CR-d21**	**LR vs CR**	**SW-d21 vs CT-d21**	**CR-d21 vs CR-d0**	**LR-d21 vs LR-d0**	**d21 vs d0**
Glutathione peroxidase 3 precursor*	GPX3	/	0.46	/	/	/	/	0.81
Gelsolin	GSN	/	/	/	/	/	0.29	/
Haemoglobin subunit alpha	HBA	/	/	-0.56	/	/	/	/
Haptoglobin	HP	/	/	/	/	/	/	-0.79
Histidine-rich glycoprotein	HRG	/	/	/	/	1.51	1.67	1.60
IgA heavy chain constant region*	IGHA1	0.47	0.74	0.61	/	0.89	1.16	1.03
IgG heavy chain	IGHG	-0.79	/	-0.58	/	0.68	0.73	0.71
Ig-like domain-containing protein		/	/	/	/	0.65	0.90	0.78
IgM heavy chain constant region*	IGHM	/	/	/	-0.22	/	-0.34	-0.24
Immunoglobulin kappa chain**		/	/	/	/	/	/	0.18
Immunoglobulin kappa variable region*	IGKV1-5	/	/	/	/	1.41	/	1.11
Immunoglobulin lambda-like	IGLL5	/	/	/	/	0.61	0.64	0.62

polypeptide 5 isoform 1*								
Inter-Alpha-Trypsin Inhibitor Heavy Chain 3*	ITIH 3	/	/	/	/	-0.73	-0.47	-0.60
Inter-alpha-trypsin inhibitor heavy chain H4 isoform 1	ITIH 4	/	/	/	/	-1.45	/	-1.05
Joining chain of multimeric IgA and IgM	JCH AIN	/	0.35	0.32	/	/	/	/
Plasma kallikrein*	KLK B1	/	-0.27	/	/	/	/	/
Kininogen 1*	KNG 1	/	/	/	0.18	/	/	/
Galectin-3-binding protein	LGA LS3B P	/	-0.57	/	/	/	/	/

log2FC is base 2 logarithm transformed of fold change value which represents the ratio of expression levels in the first-mentioned group vs second-mentioned group (positive values - upregulated or increased abundant proteins, negative values - downregulated or decreased abundant proteins). 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. Proteins ** were shown as "Uncharacterized protein" in the UniProt Sus scrofa database and cannot find the best match on Sus scrofa database, thus were substituted with the best match on Homo sapiens database, using SMARTBLAST tool. CR: piglet born from sow fed diet with ω6:ω3 ratio=13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: piglet born from sow fed diet with ω6:ω3 ratio=4:1 from G28 until the end of lactation (L-End). G108: day 108 of gestation.

Table 1. The differentially abundant proteins (DAPs) in piglet serum among seven comparisons *(cont., 2).*

Protein name	Gene names	log2FC (with p-value < 0.05)						
		Comparisons						
		(a)	(b)	(c)	(d)	(e)	(f)	(g)
		LR-d0 vs CR-d0	LR-d21 vs CR-d21	LR vs CR	SW-d21 vs CT-d21	CR-d21 vs CR-d0	LR-d21 vs LR-d0	d21 vs d0
SERPIN domain-containing protein	LOC100156325	/	/	/	/	-0.84	-1.00	-0.92
Ig-like domain-containing protein	LOC100523213	/	/	/	/	/	/	-0.67
SERPIN domain-containing protein	LOC100650547	/	/	/	/	-1.72	-1.30	-1.51
Amine oxidase	LOC110256000	/	/	-0.26	/	/	/	/
SERPIN domain-containing protein	LOC396684	/	/	/	/	-1.05	-0.76	-0.90
Alpha-1 acid glycoprotein	ORM1	/	/	/	/	/	0.40	0.35
Plasminogen	PLG	/	/	/	/	-0.36	/	-0.40
Perilipin 5	PLIN5	/	/	/	/	-0.55	-0.54	-0.55
Alpha-2-Macroglobulin	PZP	/	/	1.23	/	-0.66	-0.40	-0.49
Serum amyloid A	SAA	/	1.18	/	/	-3.08	-1.95	-2.52
Alpha-1-antitrypsin/Serpin Family A Member 1	SERPINA1	/	/	/	/	/	0.29	0.21
Serpin A3-8*	SERPINA3	/	-0.24	-0.21	/	-2.14	-1.35	-1.74
Alpha-1-antichymotrypsin 2	SERPINA3-2	/	/	/	/	-0.32	/	/
Serpin A3-5*	SERPINA3-5	/	/	/	/	-0.84	-0.83	-0.84
Antithrombin-III	SERPINC1	0.26	/	0.20	/	/	/	/
SERPIN domain-containing protein	SERPIND1	/	/	/	/	/	0.29	0.20
Serpin family G member 1	SERPING1	/	/	/	/	-0.37	-0.36	-0.36
Transferrin (B3CL06)	TF	-3.14	-3.32	-3.33	/	/	/	/

Transferrin (P09571)	TF	/	0.78	0.79	/	1.43	1.41	1.42
Transthyretin	TTR	/	/	/	/	0.67	0.97	0.83
Vitronectin	VTN	/	/	/	/	/	0.32	0.28

log2FC is base 2 logarithm transformed of fold change value which represents the ratio of expression levels in the first-mentioned group vs second-mentioned group (positive values - upregulated or increased abundant proteins, negative values - downregulated or decreased abundant proteins). 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. CR: piglet born from sow fed diet with ω6:ω3 ratio=13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: piglet born from sow fed diet with ω6:ω3 ratio=4:1 from G28 until the end of lactation (L-End). G108: day 108 of gestation.

Figures

Figure 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) and Reactome pathway enrichment analysis).

Figure 2. Venn diagram representing separated and overlapped differentially abundant proteins (DAPs) among six comparisons of piglet serum: A) Comparison (a) - LR vs CR at weaning (d0); Comparison (b) - LR vs CR at day 21 post-weaning (d21 PW); and Comparison (c) - LR vs CR; B) Comparison (e) - CR at d21 PW vs CR at d0; Comparison (f) - LR at d21 PW vs LR at d0; and Comparison (g) – d21 PW vs d0. CR: piglet born from sow fed diet with ω6:ω3 ratio=13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: piglet born from sow fed diet with ω6:ω3 ratio=4:1 from G28 until the end of lactation. SW: piglet diet with seaweed supplementation, CT: piglet diet without seaweed supplementation.

Figure 3. Heatmap of the significant differentially abundant proteins (DAPs) among seven comparisons of piglet serum: (a) - LR vs CR at d0; (b) - LR vs CR at d21 PW; (c) - LR vs CR; (d) - SW vs CT at d21 PW; (e) - CR at d21 PW vs CR at d0; (f) - LR at d21 PW vs LR at d0; and (g) - d21 PW vs d0. CR: piglet born from sow fed diet with ω6:ω3 ratio=13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: piglet born from sow fed diet with ω6:ω3 ratio=4:1 from G28 until the end of lactation. SW: piglet diet with seaweed supplementation, CT: piglet diet without seaweed supplementation.

Figure 4. Network of the significant differentially abundant proteins (DAPs) among three main comparisons of piglet serum with significantly enriched Reactome pathways, containing comparison (c) - LR vs CR; (d) - SW vs CT at day 21 post-weaning; and (g) - day 21 post-weaning versus weaning day. CR: piglet born from sow fed diet with ω6:ω3 ratio=13:1 during gestation, starting from day 28 (G28) and 10:1 during lactation; LR: piglet born from sow fed diet with ω6:ω3 ratio=4:1 from G28 until the end of lactation. SW: piglet diet with seaweed supplementation, CT: piglet diet without seaweed supplementation.

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Declaration of conflicts of 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.

Graphical Abstract

Highlights

* Maternal low ω6:ω3 ratio (4:1) and offspring seaweed supplementation affected the serum proteome in post-weaned piglets.

* Transferrin was altered in comparison between two maternal dietary treatments and between weaning versus day 21 post-weaning.

* Serum acute phase proteins and apolipoproteins were altered between weaning versus day 21 post-weaning.

Significance

This novel proteomic study in post-weaned piglets addresses the interplay between maternal and offspring nutritional interventions in a context of rapid and dynamic alterations in piglet metabolic status around weaning. Decreasing ω6:ω3 ratio in maternal diet and SW supplementation in PW piglet's diet can boost their immunity and anti-inflammation properties through contributing to the cross-talk between coagulation cascade and inflammation. This study also provides new insights into piglet serum proteome regulation during post-weaning, a critical development period in swine.