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Liquid chromatography mass spectrometry plasma proteome analysis in nursery pigs fed diets enriched with native chicory inulin

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ABSTRACT. Despite the extensive evidence of health benefits associated with prebiotics, their effects on the haemostatic system remain largely unexplored. Hence, this study aimed to assess the influence of incorporating 1% (T1 diet) or 3% (T2 diet) native chicory inulin into the diet of nursery pigs on plasma proteome changes, focusing particularly on proteins associated with haemostasis and the complement system. Piglets were fed inulin-enriched diets from 10 days of age. Piglets were sacrificed at 50 days of age and blood samples were collected for liquid chromatography-mass spectrometry (LC-MS) analysis. Gene Ontology, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway and protein-protein interaction analyses of differentially expressed plasma proteins indicated that the direction of changes in proteins related to coagulation and fibrinolysis likely reflected a shift in haemostatic balance towards a more prothrombotic state. Quantitative changes in the expression of several other plasma proteins further supported the well-established anti-inflammatory, immunomodulatory and lipid lowering properties of inulin. In conclusion, inulin effectively coordinated the crosstalk between lipid metabolism and the inflammatory cascade, as reflected by the direction of changes in serum clusterin (Apo J), C-reactive protein and paraoxonase/arylesterase 1 expression levels.

Introduction

Inulin (IN), a naturally occurring linear biopolymer found in various edible plants, including chicory (*Cichorium intybus* L.), is regarded as one of the most extensively studied and well-established prebiotic carbohydrates. Given the specific chemical structure of IN, consisting of a complex mixture of β-2,1 linear fructans, it reaches the intestines in a virtually unaltered form, where it is fermented by microbial flora (Hughes et al., 2022). This process enhances beneficial bacterial populations and improves small intestinal architecture in weaning piglets (Uerlings et al., 2021). Short-chain fatty acids

(SCFAs), mainly butyrate, acetate and propionate, produced through anaerobic bacterial fermentation of inulin, are considered critical regulators of host energy balance and fuel homeostasis not only locally in the gut (Herosimczyk et al., 2018; 2020), but also directly affecting signalling and metabolic pathways in various peripheral tissues in nursery pigs (Herosimczyk et al., 2017; Robak et al., 2019). Apart from the large array of well-established health-modulating mechanisms of SCFAs (Nogal et al., 2021), their impact on the haemostatic system still remains poorly understood. Our previous 2-DE-based proteomic study showed that a diet supplemented with 2% IN induced simultaneous alterations in the expression of serum proteins involved in blood coagulation, fibrinolysis, and the complement system (Herosimczyk et al., 2015). The observed changes in the expression of two key proteins, vitronectin (VN) and plasminogen (Plg), drew particular attention. It was found that Plg expression was nearly 2-fold lower, while VN levels were increased 3-fold. The somatomedin B domain of VN is known to bind and stabilise plasminogen activator inhibitor-1 (PAI-1), i.e. the main inhibitor of plasminogen conversion to plasmin. Thus, this protein is considered a key regulator of the fibrinolytic system acting as a thrombus stabiliser (Zhou et al., 2003). Moreover, *in vitro* studies suggest that VN also contains binding sites on Plg surface (Yasar Yildiz et al., 2014), indicating its multifunctional role in this process. VN both enhances the inhibitory activity of PAI-1 and regulates the availability of free plasminogen. Interestingly, VN has also been shown to inhibit the complement terminal C5b-9 complex, known as the membrane attack complex (MAC) (Singh et al., 2010). This highlights the potential interaction between haemostatic and inflammatory mediators, which is crucial for immune response and tissue homeostasis (Luo et al., 2020).

Considering the aforementioned findings, it was hypothesised that a 40-day dietary supplementation with two different levels of native chicory inulin (1% or 3%) could affect proteins associated with both innate immune response and haemostasis. The primary objective of this study was to investigate the impact of varying inulin doses on the expression of proteins associated with haemostasis and the complement system in healthy nursery pigs. The study employed a nano ultra-high performance liquid chromatography coupled with electrospray ionisation quadrupole time-of-flight mass spectrometry (nanoUHPLC-ESI-QTOF MS).

Material and methods

Experimental design and animal housing

The piglets included in the experiment were a crossbreed of PIC (Pig Improvement Company) and Penarlan. Twenty-four castrated barrows were randomly assigned into three groups (n = 8) and were offered cereal-based pre-starter diets (Table 1) from the 10th day of life: control (C) or enriched with 1% (T1) or 3% native chicory inulin (T2) (Inulin Orafti® GR, BENEO GmbH, Mannheim, Germany). Detailed information regarding the remaining diet components, nutrient and energy content, as well as the chemical composition of inulin are detailed in Tables 1 and 2, respectively.

Table 1. Ingredient and chemical composition of pig diets: control diet (C) and diets supplemented with 1% (T1) or 3% (T2) native chicory inulin

Item	C	T1	T2
Ingredients, %			
wheat	46.84	46.84	46.84
barley	20	20	20
maize starch	3	2	–
full-fat soybean	5.90	5.90	5.90
whey	9.70	9.70	9.70
fish meal	4.00	4.00	4.00
spray-dried blood plasma	4.00	4.00	4.00
soybean oil	3.40	3.40	3.40
calcium formate	0.30	0.30	0.30
limestone	0.50	0.50	0.50
dicalcium phosphate	0.60	0.60	0.60
sodium chloride	0.07	0.07	0.07
L-lysine	0.61	0.61	0.61
DL-methionine	0.23	0.23	0.23
L-threonine	0.26	0.26	0.26
L-tryptophan	0.09	0.09	0.09
mineral-vitamin premix[1]	0.40	0.40	0.40
aroma	0.10	0.10	0.10
native chicory inulin[2]	–	1	3
Chemical composition, %			
dry matter	90.03	90.15	90.13
crude ash	4.54	4.54	4.53
crude protein	20.05	20.04	20.03
ether extract	6.04	6.04	6.03
crude fibre	1.52	1.52	1.51
fructan	1.00	1.52	3.11
ME, MJ/kg[3]	14.30	14.30	14.30

[1] contained per kg: IU: vit. A 600000, vit. D_3 60000; mg: vit. E 3000, vit. K_3 120, vit. B_1 120, vit. B2 240, vit. B6 240, nicotinic acid 1600, pantothenic acid 800, folic acid 160, biotin 10, vitamin B_{12} 1.6, I 40, Se 16, Co 16; g: choline chloride 12, Mg 3.2, Fe 6, Zn 5.6, Mn 2.4, Cu 6.4; [2] inulin (Inulin Orafti®GR, BENEO GmbH, Mannheim, Germany) containing approximately 92% inulin with DP ≥ 10 and 8% of other carbohydrates (glucose, fructose and sucrose); [3] calculated values; ME – metabolizable energy

Table 2. Chemical composition (%) of inulin used as a feed supplement

Item, %	IN[1]
Dry matter	92.59
Crude protein	0.16
Crude ash	0.02
Ether extract	0.05
Crude fibre	0.18
Fructans	92.00

[1] Inulin Orafti® GR, BENEO GmbH, Mannheim, Germany (92% inulin with an average degree of polymerisation of 10, 8% simple sugars)

The chemical composition of the inulin extract contained approximately 92% inulin with DP ≥ 10 and 8% other carbohydrates (glucose, fructose, and sucrose). Animal housing conditions and diet compositions were previously described by Herosimczyk et al. (2020). Briefly, piglets were weaned after a four-week suckling period, but on day 10 of life, they were also offered a solid diet in the form of a mash placed in a feeder. On day 28, two piglets from each litter (4 litters/group) were selected and assigned to separate pens based on their dietary treatment (2 pens/group, 4 animals/pen). The average body weight of the selected piglets was consistent across all groups (7.9 ± 1.4 kg). The animals were maintained under controlled conditions of 25 °C, with a 12-hour dark-light cycle, and free access to feed and water. All diets were given in the form of pellets with a diameter of 4 mm. Feed intake and health status were monitored daily throughout the experiment. Animals were weighed at weaning and at the end of the experiment. After 40 days of feeding, the pigs were euthanised by electrical stunning followed by exsanguination (at 50 days of age, and approximate body weight of 18 kg). Directly after euthanasia, mixed blood samples were collected. Plasma for proteome analysis was obtained by centrifugation at 3000 *g* for 10 min at 4 °C and stored at −80 °C. The experimental procedures were approved by the Local Ethics Committee for the Care and Use of Laboratory Animals (No. 13/2012 of 23.05.2012).

Plasma sample preparation, LC-MS proteome analysis and method validation

All measurements were performed in triplicate. Total plasma protein concentration was determined using a modified Bradford assay (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Samples containing 10 µg of proteins were precipitated with cold (−20 °C) acetonitrile at 1:4 ratio (v/v) and incubated at −20 °C for 2 h. After incubation, the precipitated samples were centrifuged at 18000 *g* for 30 min at −9 °C, and the resulting pellet was dissolved in freshly prepared 40 mM ammonium bicarbonate. Subsequently, plasma proteins were reduced using dithiothreitol (final concentration 20 mM) and then alkylated with iodoacetamide (final concentration 40 mM). Protein digestion was carried out using Trypsin Gold (Promega, Madison, WI, USA) with a protease-to-protein ratio of 1:25. Thus prepared samples were shaken overnight at 37 °C. The resulting peptides at a final concentration of 0.1 µg/µl were diluted with 0.1% formic acid, centrifuged (15 min, 14000 *g*, 4 °C) and subjected to LC-MS/MS analysis.

Liquid chromatographic (LC) analysis was conducted using nano-UHPLC (nano-Elute, Bruker, Billerica, MA, USA). A two-column separation method was applied, comprising pre-column (300 µm × 5 mm, C18 PepMap 100, 5 µm, 100 Å, Thermo Scientific, Waltham, MA, USA) and an IonOptics Aurora-C18 column (75 µm × 250 mm, C18 1.6 µm) with a flow rate of 300 nl/min. The mobile phase consisted of 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (solvent B) with 0.1% (v/v) formic acid addition. The linear gradient elution program was as follows: 10 min 2% B, 10–100 min 2–35% B, 100–105 min 35–95% B, and 105–115 min 95% B. The injection volume was set at 14 µl. Mass spectrometric (MS) analysis was performed using an ESI-Q-TOF MS system (Compact, Bruker Daltonics, Karlsruhe, Germany) equipped with an electrospray CapticeSpray ionisation (ESI) interface. The MS acquisition was operated in positive ion mode and the mass range was set at m/z 150–2200 with a spectrum acquisition frequency of 4 Hz, and the auto MS/MS system. Sample ionisation was carried out at a nitrogen gas flow of 3.0 l/min, temperature of 150 °C and a capillary voltage of 1600 V. The quadrupole energy was set to 5.0 eV, and the collision chamber at 7.0 eV, with an ion transfer time of 90 µs.

Data acquisition was carried out using HyStar and otoControl, and processing was performed using DataAnalysis software (Bruker Daltonics, Karlsruhe, Germany). Proteins were identified using online mammalian databases (SWISS-PROT; http://us.expasy.org/uniprot/ and NCBI; http://www.ncbi.nlm.nih.gov/) using the MASCOT search engine (http://www.matrixscience.com/) implemented in ProteinScape 4.2 software (Bruker Daltonics, Karlsruhe, Germany). The search criteria included trypsin as an enzyme, carbamidomethylation as a fixed modification, and oxidation (M), deamidation (NQ),

and Gln→pyro-Glu (N- term Q) as variable modifications. Peptide mass tolerance was set to 50 ppm and a maximum of two missed cleavage sites. Results were accepted when the protein score achieved statistical significance ($P < 0.05$).

To ensure optimal quality and accuracy of measurements, the MS underwent daily external calibration using 0.5 mM sodium formate dissolved in a solution containing 90% 2-propanol and 10% water. This calibration process utilised reference m/z values ranging from 90.976644 to 1518.712539, specifically: 90.976644, 158.964068, 226.951491, 294.938915, 362.926338, 430.913762, 498.901186, 566.888609, 634.876033, 702.863456, 770.850880, 838.838303, 906.825727, 974.813150, 1042.800574, 1110.787998, 1178.775421, 1246.762845, 1314.750268, 1382.737692, 1450.725115, and 1518.712539. The calibration procedure was conducted in enhanced quadratic mode to ensure the accuracy and precision of the instrument. We considered the instrument to be correctly calibrated when the standard deviation of the measurements fell within the range of 0.0–0.5 ppm and the percentage score value ranged from 99.5 to 100% during calibration. To further refine measurement accuracy, a lock mass calibrant (C24H19F36N3O6P3) was employed, appearing as a peak at 1221.990638 m/z in every collected mass spectrum. Each individual mass spectrum was calibrated to this peak to enhance the accuracy and stability of the measurements. The order of sample analysis was randomised to minimise the risk of 'batch effect', which could introduce systematic errors in the analysis. Additionally, the entire chromatographic separation pathway between samples was thoroughly washed. This washing sequence comprised solutions of 95% ACN, 70% ACN, 2% ACN, and 98% ACN, each administered for 3 min at a constant flow rate of 300 nl/min.

Statistical analysis

All statistical analyses of the proteomic data were conducted using GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA). The mean values and standard error of the mean (SEM) were calculated. Proteomic data was first normalised to total ion count (TIC) for each sample according to the following formula:

$$xTIC = a / \Sigma pi,$$

where: xTIC – intensity of a single protein normalised to TIC, a – raw protein intensity, and Σpi – sum of intensities of all proteins within a given sample.

The data were then transformed by global median normalisation. For each sample, the median of the $\log_2$-transformed distribution was subtracted from all values. The fold change (FC) in protein abundance was calculated after normalisation and imputation of the data using the formula:

$$FC = XTICEx / XTICC,$$

where: FC – calculated fold change value, XTICEx – intensity of the protein normalised to TIC from the experimental group, XTICC – intensity of the protein normalised to TIC from the control group.

Subsequently, a logarithmic transformation of the fold change values was performed according to the formula:

$$y = \log_2 FC,$$

where: y – logarithmic value of the fold change.

Statistical analysis was performed using a two-tailed unpaired Student's t-test to compare the differences between the experimental and control groups as per the following formula:

$$T = (\bar{x}\_1 - \bar{x}\_2) / s\_(x\_1 - x\_2),$$
$$s\_(x\_1 - x\_2) = \sqrt{((n\_1 - 1) \cdot s\_1^2 + (n\_2 - 1) \cdot s\_2^2) / (n\_1 + n\_2 - 2) \cdot (1/n\_1 + 1/n\_2)},$$

where: T – Student t-test, $\bar{x}\_1$ – average value for the first group, $\bar{x}\_2$ – average value for the second group, s_(x_1-x_2) – calculated variance, s_1^2 – variance of the first group, s_2^2 – variance of the second group, and n_1 – and n_2 – indicate the sample sizes of the first and second groups, respectively.

Next, a logarithmic transformation of the *P*-value obtained from Student's t-test was performed using the following formula:

$$v = -\log_{10}(P\text{-value}),$$

where: v – logarithmic *P*-value, and $-\log_{10}$ (*P*-value) represents the negative decimal logarithm of the *P*-value.

Proteins with a $\log_2$ fold change > 0.3 and an adjusted *P*-value < 0.05 were considered statistically significant.

Bioinformatic analysis

The processing and analysis of proteomic data was conducted using GraphPad Prism Software 9.0 (San Diego, CA, USA), including volcano plots. Gene Ontology (GO) term enrichment analysis and protein-protein interactions (PPI) involving differentially expressed proteins (DEPs) were conducted to compare pathways affected by two levels of dietary inulin supplementation, utilising STRING v 11.5 (Szklarczyk et al., 2019). A medium confidence score (0.400) was applied, and significant results

were identified based on the Benjamini-Hochberg false discovery rate (FDR) procedure (Benjamini and Hochberg, 1995). An FDR threshold of < 0.05 was considered statistically significant.

Results

Animal performance

All animals were healthy and exhibited no signs of diarrhoea during the experiment. The addition of 1% or 3% native chicory inulin to the pigs' diets did not have a significant impact on their body weight (BW) at any interim point or on the final BW. These findings were previously documented in a study by Herosimczyk et al. (2020) and are outlined in Table 3. Feed intake was not subjected to statistical analysis since it was estimated only on a per-pen basis.

Table 3. Mean body weight (BW) and BW gain in pigs fed a control (C) and diets supplemented with 1% (T1) or 3% native chicory inulin (T2)

	Diet			SEM	*P*-value
Parameters	C	T	T2		
BW at weaning, kg	8.00	7.30	7.50	0.29	0.6576
Final BW, kg	17.90	16.70	17.50	0.50	0.6222
BW gain, kg	9.90	9.40	10.00	0.35	0.7339

SEM – standard error of the mean; $P > 0.05$ indicates that the data are not significantly different

Blood plasma proteome changes

To evaluate the porcine plasma profile in response to the administration of both experimental diets, we employed the LC-MS technique, identifying 1523 proteins in total, of which 316 were selected for further analysis. Only proteins present in at least 51% of the samples classified into each group (control, T1 or T2) were subjected to statistical analysis. In the group of pigs fed the T1 diet, 3 proteins were found to be significantly up-regulated, whereas 11 were down-regulated compared to the control group (Figure 1, Table 4).

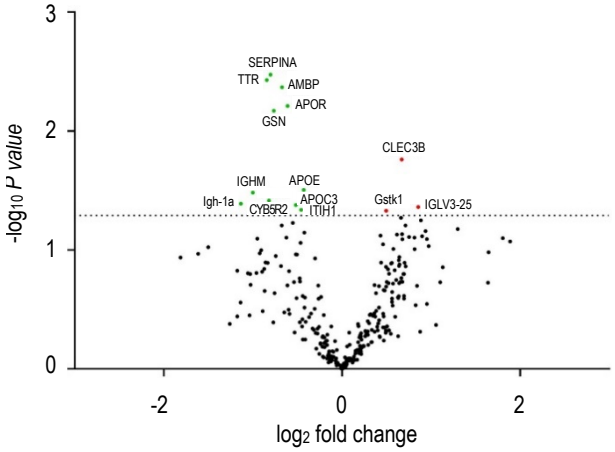

Figure 1. Volcano plot showing relative plasma protein abundance (log_2 fold change) plotted against their significance level (negative log_{10} *P*-value) in pigs fed the T1 diet normalised to the control group. Statistically signifiantly altered proteins ($P \leq 0.05$) are separated by a dashed line, which sets a cut-off point at 1.3 on the y-axis. Proteins with increased expression are marked in red, while those with decreased expression are marked in green

Gstk1 – glutathione S-transferase kappa 1, CLEC3B – tetranectin, IGLV3-25 – immunoglobulin lambda variable 3–25, Igh-1a – Ig gamma-2B chain C region, IGHM – immunoglobulin heavy constant mu, TTR – transthyretin, CYB5R2 – NADH-cytochrome b5 reductase 2, SERPINA1 – alpha-1-antitrypsin, GSN – gelsolin (fragment), APOR – apolipoprotein R, AMBP – protein AMBP (fragment), APOC3 – apolipoprotein C-III, ITIH1 – inter-alpha-trypsin inhibitor heavy chain H1, APOE – apolipoprotein E

Table 4. Statistically significantly altered proteins identified by nanoUHPLC-ESI-QTOF MS in the blood plasma of pigs fed a diet supplemented with 1% inulin (T1) compared to the control (C) group

Gene name	Protein name	C group	SEM	T1 group	SEM	Fold change	*P*-value	log_{10} *P*-value
Gstk1	Glutathione S-transferase kappa 1	0.00045	0.00011	0.00064	0.00021	1.41	0.04675	1.33
CLEC3B	Tetranectin	0.00013	0.00003	0.00022	0.00007	1.59	0.01736	1.76
IGLV3-25	Immunoglobulin lambda variable 3-25	0.00032	0.00017	0.00058	0.00028	1.81	0.04351	1.36
Igh-1a	Ig gamma-2B chain C region	0.00036	0.00012	0.00016	0.00017	0.45	0.04078	1.39
IGHM	Immunoglobulin heavy constant mu	0.00035	0.00016	0.00017	0.00010	0.50	0.03290	1.48
TTR	Transthyretin	0.00155	0.00047	0.00086	0.00030	0.56	0.00372	2.43
CYB5R2	NADH-cytochrome b5 reductase 2	0.00046	0.00021	0.00025	0.00010	0.57	0.03839	1.41
SERPINA1	Alpha-1-antitrypsin	0.00193	0.00045	0.00110	0.00048	0.57	0.00336	2.47
GSN	Gelsolin (Fragment)	0.00141	0.00042	0.00083	0.00029	0.59	0.00675	2.17
APOR	Apolipoprotein R	0.00043	0.00008	0.00027	0.00008	0.63	0.00428	2.37
AMBP	Protein AMBP (Fragment)	0.00097	0.00021	0.00064	0.00020	0.65	0.00615	2.21
APOC3	Apolipoprotein C-III	0.00067	0.00018	0.00047	0.00018	0.70	0.04195	1.38
ITIH1	Inter-alpha-trypsin inhibitor heavy chain H1	0.00148	0.00041	0.00107	0.00030	0.73	0.04591	1.34
APOE	Apolipoprotein E	0.00098	0.00018	0.00073	0.00024	0.74	0.03124	1.50

SEM – standard error of the mean; proteins with a log_2 fold change > 0.3 and an adjusted *P* – value < 0.05 were considered statistically signifi*cant*

On the other hand, the T2 diet induced slightly more pronounced changes in porcine blood plasma, as proteomic analysis identified 19 proteins with statistically significant alterations. Of these, 7 proteins displayed increased and 12 decreased expression in comparison to the control group (Figure 2, Table 5).

We also performed a functional enrichment analysis using STRING software v. 11.5 (Szklarczyk et al., 2019) to obtain additional information concerning a possible interaction between DEPs and identify core proteins within these interactions. When inputting the list of DEPs that displayed statistically significant changes in response to both experimental diets, we found significant interactions involving proteins associated with the complement and coagulation cascades, lipoprotein particle pathway (Local network cluster – STRING, $P < 0.0061$) and haemostasis pathway (Reactome Pathways, $P = 3.01$ x 10–9). These findings were used to construct the interaction map (Figure 3A). The PPI network of DEPs included 16 nodes that represented porcine plasma proteins and 86 edges, with varying thickness to reflect the strength of interactions between these proteins (Figure 3B). Figure 3C shows the average $\log_2$ fold change in expression of these 16 proteins (nodes) in the blood plasma of pigs fed two different dietary levels of inulin.

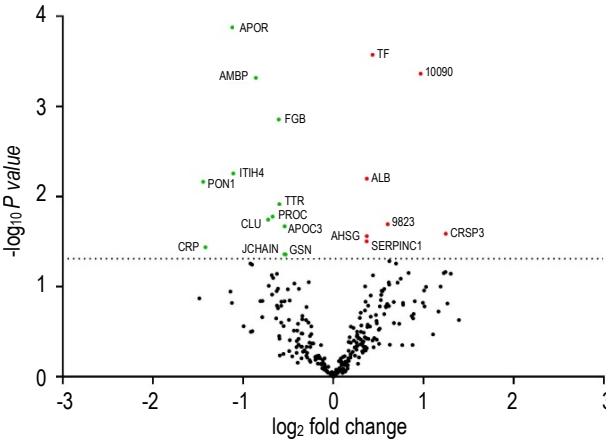

Figure 2. Volcano plot showing relative plasma protein abundance ($\log_2$ fold change) plotted against their significance level (negative $\log_{10}$ *P*-value) in pigs fed the T2 diet normalised to the control group. Statistically significantly altered proteins ($P \leq 0.05$) are separated by a dashed line, which set a cut-off point at 1.3 on the y-axis. Proteins with increased expression are marked in red, while those with decreased expression are marked in green

CRSP3 – calcitonin receptor-stimulating peptide 3, 10090 – Ig heavy chain V region MOPC 47A, 9823 – trypsin, TF – serotransferrin, ALB – serum albumin, AHSG – alpha-2-HS-glycoprotein (fragment), SERPINC1 – antithrombin-III, PON1 – serum paraoxonase/arylesterase 1, CRP – C-reactive protein, APOR – apolipoprotein R, ITIH4 – inter-alpha-trypsin inhibitor heavy chain H4, AMBP – protein AMBP (fragment), APOC3 – apolipoprotein C-III, PROC – vitamin K-dependent protein C, FGB – fibrinogen beta chain, TTR – transthyretin, JCHAIN – immunoglobulin J chain, CLU – clusterin, GSN – gelsolin (fragment)

Table 5. Statistically significantly altered proteins identified by nanoUHPLC-ESI-QTOF MS in the blood plasma of pigs fed a diet supplemented with 3% inulin (T2) compared to the control (C) group

Gene name	Protein name	C group	SEM	T2 group	SEM	Fold change	$\log_2$ fold change	*P*-value	$\log_{10}$ *P*-value
CRSP3	Calcitonin receptor-stimulating peptide 3	0.00014	0.00011	0.00035	0.00015	2.37	1.25	0.02593	1.59
10090	Ig heavy chain V region MOPC 47A	0.00251	0.00105	0.00492	0.00105	1.95	0.97	0.00043	3.36
9823	Trypsin	0.00612	0.00145	0.00931	0.00311	1.52	0.60	0.02027	1.69
TF	Serotransferrin	0.05365	0.00533	0.07252	0.00967	1.35	0.43	0.00026	3.57
ALB	Serum albumin	0.08860	0.01459	0.11460	0.01767	1.29	0.37	0.00632	2.19
AHSG	Alpha-2-HS-glycoprotein (Fragment)	0.00697	0.00140	0.00901	0.00188	1.29	0.37	0.02750	1.56
SERPINC1	Antithrombin-III	0.00069	0.00016	0.00089	0.00017	1.29	0.37	0.03141	1.50
PON1	Serum paraoxonase/arylesterase 1	0.00038	0.00020	0.00014	0.00006	0.37	−1.44	0.00686	2.16
CRP	C-reactive protein	0.00032	0.00018	0.00012	0.00008	0.37	−1.42	0.03651	1.43
APOR	Apolipoprotein R	0.00042	0.00006	0.00019	0.00005	0.46	−1.12	0.00013	3.88
ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4	0.00387	0.00170	0.00180	0.00057	0.46	−1.11	0.00555	2.25
AMBP	Protein AMBP (Fragment)	0.00097	0.00021	0.00054	0.00017	0.55	−0.86	0.00048	3.32
APOC3	Apolipoprotein C-III	0.00067	0.00018	0.00041	0.00021	0.60	−0.72	0.01805	1.74
PROC	Vitamin K-dependent protein C	0.00028	0.00006	0.00018	0.00007	0.63	−0.67	0.01672	1.78
FGB	Fibrinogen beta chain	0.00565	0.00109	0.00371	0.00083	0.66	−0.60	0.00139	2.85
TTR	Transthyretin	0.00155	0.00047	0.00103	0.00020	0.66	−0,60	0.01219	1.91
JCHAIN	Immunoglobulin J chain	0.00015	0.00005	0.00010	0.00003	0.69	−0.54	0.04369	1.36
CLU	Clusterin	0.00139	0.00042	0.00095	0.00021	0.69	−0.54	0.02140	1.67
GSN	Gelsolin (Fragment)	0.00140	0.00043	0.00097	0.00029	0.69	−0.53	0.04398	1.36

SEM – standard error of the mean; proteins with a $\log_2$ fold change > 0.3 and an adjusted *P* – value < 0.05 were considered statistically significant

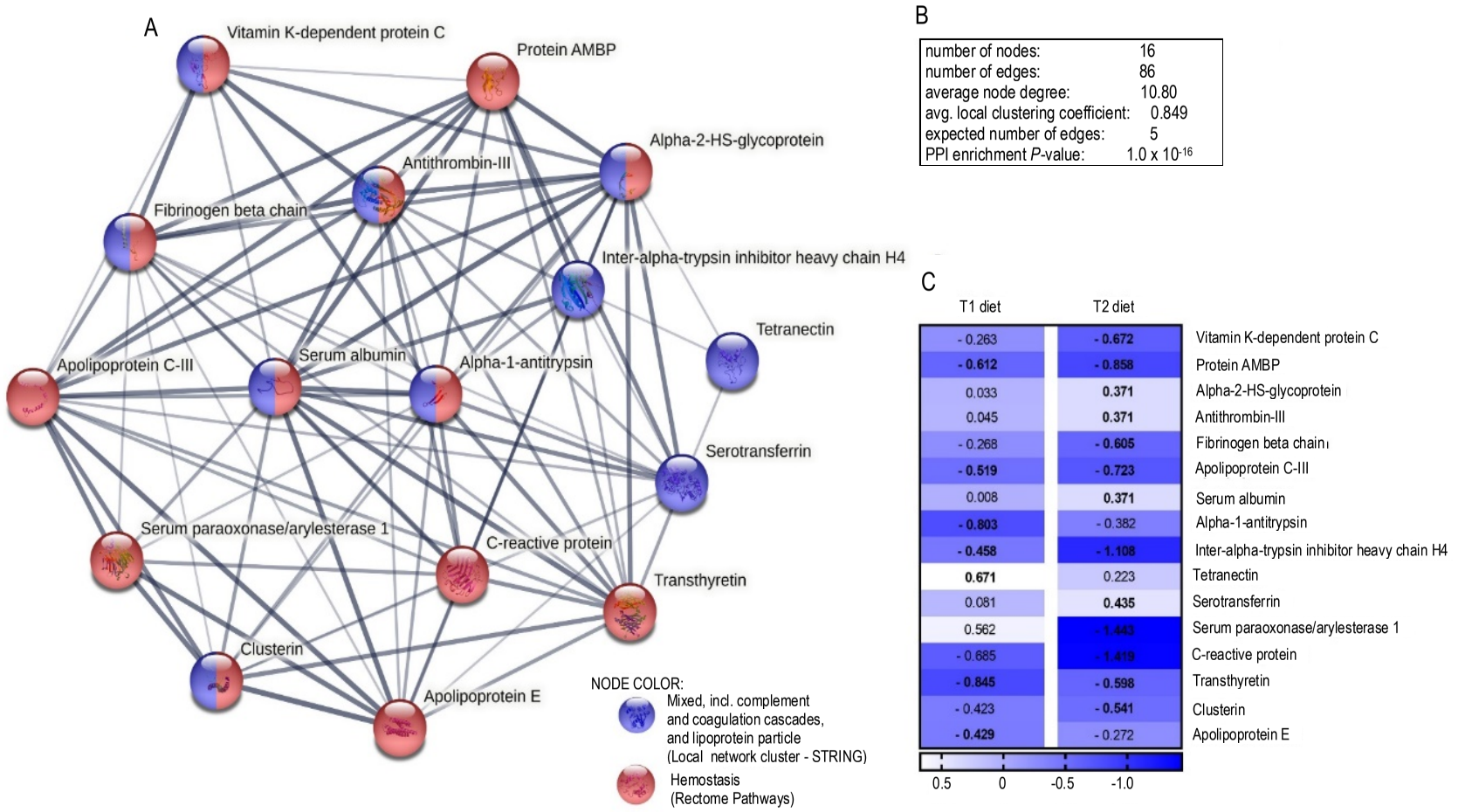

Figure 3. Analysis of apolipoproteins, complement and coagulation-related plasma proteins common to both experimental groups (T1 and T2). Panel (A): protein-protein interaction (PPI) network image displaying nodes and edges between apolipoproteins, complement and coagulation-related proteins found in the blood plasma of nursery pigs fed diets enriched with 1% (T1) or 3% native chicory inulin (T2). Proteins are shown as nodes, with the thickness of the edges indicating the strength of interactions between these proteins. Red nodes correspond to gene ontology (GO) Reactome Pathways whereas blue nodes show GO local network cluster (STRING). Panel (B): PPI network statistics, including the number of nodes and edges, average node degree, average local clustering coefficient, expected number of edges and *P*-value. Panel (C): mean normalised heat map displaying the abundance of complement and coagulation-related plasma proteins in the T1 and T2 groups. Colours correspond to $\log_2$ fold change of protein expression. Bolded values indicate statistically significant differences

Discussion

Our previous 2-DE-based studies have already demonstrated that 40-day feeding with either 2% native chicory inulin (Herosimczyk et al., 2015) or 4% dried chicory root (Lepczynski et al., 2015) led to alterations in the expression of several serum proteins involved in blood coagulation, fibrinolysis and the complement system. However, it should be noted that 2-DE electrophoresis has some major shortcomings, particularly in its capacity to analyse proteins of medium to low abundance. Therefore, in the present study, a high-throughput proteomic approach was employed to increase the accuracy and depth of porcine plasma proteome coverage, and thus to increase our knowledge of the possible impact of feeding inulin-enriched diets (1% or 3%) on changes in haemostatic and immune-related proteins. As expected, selected protein components involved in the haemostatic mechanism were differentially regulated in response to both inulin-enriched diets. These included proteins participating in biochemical reactions of blood coagulation, finally leading to clot formation (fibrinogen beta chain, serotransferrin), anticoagulant pathways (vitamin K-dependent protein C, antithrombin-III) and fibrinolysis (tetranectin). In the current study, we demonstrated that the T2 diet caused a significant down-expression of fibrinogen beta chain. This finding is consistent with our earlier observations (Herosimczyk et al., 2017), which showed that 40-day dietary supplementation with 3% inulin reduced plasma fibrinogen concentrations. Fibrinogen, the final substrate in clot formation, is also known as a positive acute-phase protein, and its plasma levels were shown to increase 2-3-fold in response to inflammation, directly inducing vasoconstriction and raising blood viscosity (Weisel and Litvinov, 2017). This confirms the findings of Abhari et al. (2016), who also observed a decrease in pro-inflammatory blood markers, such as fibrinogen and TNF-α, in rats with induced arthritis fed a diet enriched with 5% long-chain inulin. Interestingly, our analysis also revealed that the T2 diet caused a significant up-regulation of serotransferrin (TF), an iron transport protein. Recent evidence suggests that TF may promote hypercoagulability by enhancing thrombin and factor XIIa activity, while simultaneously inactivating antithrombin III (ATIII) in the apolipoprotein E-deficient mice (apoE−/−) (Tang et al., 2020). Thus, it can be postulated that the increased plasma TF expression observed in the current study may activate a potential anticoagulant compensatory mechanism, as indicated by the up-regulation of antithrombin III (ATIII), a key inhibitor of the coagulation cascade, and tetranectin (TN), a protein known to stimulate plasminogen activation (Mehic et al., 2021). This is consistent with our earlier observations, which showed that ATIII displayed an almost 2-fold increase in serum expression of pigs fed a diet supplemented with 4% dried chicory root (Lepczyński et al., 2015). On the other hand, the T2 diet was also found to have an inverse effect on the expression of vitamin K-dependent protein C (PC), i.e. the second major endogenous anticoagulant known to inhibit clotting factors Va and VIIIa, thereby limiting prothrombin activation (Mehic et al., 2021). The results of Mohammed et al. (2020) provided a potential theoretical explanation for the observed phenomenon. Since vitamin K2 (VK2, menaquinone) is a key cofactor in the synthesis of both clotting factors and anticoagulant proteins, including PC, and depleted VK2 stores, mediated by altered density of menaquinone-producing gut microbiota, could explain the observed down-regulation of PC observed in the current study. While the exact physiological mechanisms and functional consequences of these haemostatic alterations remain largely unknown, the gut microbiota has been shown to promote arterial thrombus formation in mice through TLR2-dependent regulation of von Willebrand factor synthesis in hepatic endothelium (Jäckel et al., 2017). This may suggest that the microbiota itself and/or microbiota-derived metabolites mediate an increased synthesis of several liver proteins associated with thrombosis, subsequently secreted into the main circulation. Our findings also indicated that both experimental diets induced a reduction in the levels of several proteins belonging to the superfamily of serine protease inhibitors (SERPIN), which are known to effectively counteract thrombin generation, and thus fibrin clot formation (Yaron et al., 2021). These proteins include: α-1-antitrypsin (SERPINA1), inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) and protein AMBP (α-1-microglobulin/bikunin precursor). Although it is difficult to draw definite conclusions, the observed direction in haemostatic-related protein changes, suggest that inulin supplementation may shift the balance of the coagulation system towards a more prothrombotic state. To validate this hypothesis, further large-scale studies should be conducted to obtain fully reliable results.

Moreover, consistently with our previous studies (Herosimczyk et al., 2015; Lepczynski et al., 2015), and the work of other researchers (Abhari et al., 2016), we also recorded beneficial effects of

inulin supplementation on several markers of the innate immune response, components of adaptive humoral immunity, and oxidative stress. The T2 diet significantly reduced plasma expression of C-reactive protein (CRP) considered a positive acute phase reactant (APR). Concurrently, it up-regulated serum albumin and alpha-2-HS-glycoprotein (α2HSG), which are collectively known as negative APRs, thus confirming the well-established anti-inflammatory effects of inulin (Heegaard et al., 2011). Given that APRs, especially CRP, can bind directly to various pathogens and activate the complement system via classical pathway (Singh et al., 2020), lower expression of CRP, found in the current study, was expected to induce a concomitant decrease in complement system protein levels. In contrast to our earlier findings (Herosimczyk et al., 2015; Lepczynski et al., 2015), the present study did not detect any apparent clustering of complement constituents. Moreover, administration of both experimental diets also triggered a significant down-regulation of transthyretin (TTR), a protein recently implicated in the induction of several different signalling cascades leading to increased formation of reactive oxygen species (ROS) or reactive nitrogen species (RNS), contributing to oxidative stress through redox-mediated oxidation (Sharma et al., 2019). This antioxidant effect of inulin was further supported by a significant increase in glutathione S-transferase kappa 1 levels in the T1 group, a protein involved in ROS detoxification.

Feeding pigs with both levels of dietary inulin has proven effective in reducing the expression of a protein involved in desaturation and elongation of fatty acids and cholesterol biosynthesis (NADH-cytochrome b5 reductase), as well as several apolipoproteins (Apo), protein components of plasma lipoproteins mainly responsible for lipid transport in the bloodstream (Apo C-III, Apo E, Apo J, Apo R). Many of these apolipoproteins have been identified as high-density lipoprotein (HDL), low-density lipoproteins (LDL) and very-low density lipoproteins (VLDL). Since apolipoprotein C-III is a major regulator of VLDL particles and triglyceride (TG) metabolism, its high plasma level has been associated with hypertriglyceridemia, atherogenesis and vascular inflammation (Sacks et al., 2011). In our previous study, 4% dried chicory root was shown to reduce serum apo C-II expression levels while decreasing plasma TG concentrations (Lepczynski et al., 2015). Although the reduction in plasma TG levels did not reach statistical significance, its value decreased in the plasma of pigs fed 1% or 3% native inulin (Herosimczyk et al., 2017). These results are consistent with previous studies showing that 2% inulin supplementation in the western diet reduced serum TG levels, decreased mRNA expression of fatty acid receptor involved in lipid uptake and apo C-III in the jejunum, as well as increased faecal lipid excretion in mice (Hiel et al., 2018). Moreover, our proteomic analysis also demonstrated reduced expression of Apo J (clusterin) in the T2 group, a multifunctional protein whose increased expression has recently been linked to enhanced production of pro-inflammatory mediators and oxygen-derived reactive species, contributing to the pathogenesis of atherosclerosis. In addition, a significant reduction in Apo J expression, which interacts with several inflammatory proteins, proposed as putative mediators of atherosclerosis, such as CRP and serum paraoxonase (PON1), was observed in the T2 group (Yang and Qin, 2015). These findings suggest that dietary inulin may play a role in the coordinated regulation of the interplay between lipid metabolism and the inflammatory cascade.

Conclusions

Dietary inulin, particularly when supplemented at the level of 3%, elicited profound alterations in plasma protein expression patterns. A considerable proportion of these proteins were related to haemostasis and immunity, and the direction of expression changes in proteins related to coagulation and fibrinolysis likely reflected a shift in haemostatic balance towards a more prothrombotic state. However, these findings should be interpreted in light of certain limitations that needs to be addressed in future research. The main shortcoming of this study was the lack of coagulation system tests, which prevented a comprehensive assessment of how inulin feeding might affect individual coagulation factors and their inhibitors. Nonetheless, our emerging evidence supports the notion of a close relationship between the gut microbiota and the haemostatic system, and these results may serve as a focal point for identifying remaining gaps in knowledge. Moreover, our results further confirm the well-established anti-inflammatory, immunomodulatory and lipid lowering effects of inulin. Interestingly, we also showed that inulin could coordinate the crosstalk between lipid metabolism and the inflammatory cascade, as evidenced by the observed changes in clusterin (Apo J), C-reactive protein and paraoxonase/arylesterase 1 expression levels.

Conflicts of interest

The Authors declare that there is no conflict of interest.

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