*Original article*

# Differentially expressed proteins in the blood serum of piglets in response to a diet supplemented with inulin

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## Abstract

In the present study we introduced a two-dimensional electrophoresis and matrix-assisted laser desorption/ionisation time of flight mass spectrometry-based proteomic workflow to identify proteins that show altered expression as a result of the addition of 2% of water extract of inulin-type fructans to the diet of growing piglets. This analysis allowed us to detect an average of 240 spots per gel with a mass range from 10 to 250 kDa and a pH ranging from 3 to 10. Twenty protein spots were found to show statistically significant differences in their expression. Of these, 7 protein spots were up-regulated, whereas 13 showed down-regulation in response to the experimental diet. In total, 13 spots were identified, representing 8 distinct gene products. The experimental diet caused a significant change in proteins directly or indirectly involved in hemostasis and the innate immune response. Increased levels of fibrinogen along with decreased plasminogen expression may indicate that a fructan-rich diet favours the deposits of fibrin and promotes blood clotting. We also found increased expression of vitronectin and the alpha subunit of the complement component C8 which may protect the host organism against excessive cytolitic activity of the activated complement. The piglets from the experimental group had slightly increased values of IgG and IgA, whereas the IgM level tended to be decreased. The fructan-rich diet did not have any influence on plasma total cholesterol, HDL and LDL cholesterol and triglyceride levels.

**Key words**: piglets, blood serum, inulin, proteome, two-dimensional electrophoresis, mass spectrometry

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## Introduction

Fructans such as oligofructose (OF) and inulin are defined as components of the diet that are not digested in the upper gastrointestinal tract of monogastric animals and are able to selectively stimulate the growth and activity of beneficial intestinal microflora, mainly *Bifidobacteria* and *Lactobacilli* (Robefroid 2007). These functional food ingredients are commonly extracted from chicory roots (*Cichorium intybus*), which contains approximately 15-20 g/100 g (fresh weight) of inulin with a different degree of polymerisation (DP). The ingestion of a fructan-rich diet leads to increased bacterial production of short-chain fatty acids (SCFA), mainly acetic, propionic and butyric acids, which create an acidic environment in the large intestine that limits colonisation of pathogenic microorganisms (Robefroid 2007). SCFA stimulate epithelial cell proliferation and differentiation in both the large and small intestine, due to the trophic effect, which may have fundamental importance for animals during intensive growth and maturation. Moreover, SCFA are largely absorbed into the bloodstream and are metabolized by peripheral tissues, where they are involved in many processes.

Over the past decade, tremendous progress has been made in characterising structural and functional genomics as well as proteomics in pigs. Currently, proteomics has emerged as a valuable tool that can considerably extend our knowledge and complement transcriptome analysis. Proteomics is aimed at analysis of the proteome, i.e. the entire set of proteins expressed in cells, tissue samples and body fluids, at a defined time and under specific conditions (Bendixen et al. 2010). As proteome changes dynamically in response to environmental alternations it can be assumed that any modifications in the diet composition will affect the functions of complex networks of proteins expressed in various tissues, including blood.

The above-mentioned findings prompted us to conduct a study to determine the effect of feeding a diet supplemented with 2% inulin on the blood serum proteome of piglets.

## Materials and Methods

### Animals and sample collection

The experiment was carried out on a total of 12 castrated male piglets (PIC x Penarlan P76), randomly divided into two groups (n = 6). From 10 days of age the animals were offered an unsupplemented cereal-based diet (control group, C) or a diet supplemented with 2% water extract of inulin from chicory roots (treatment group, T). The remaining components of the diet were as follows: wheat (46.84%), barley (20%), corn starch (3% in C vs. 1% in T), full-fat soybean meal (5.9%), whey (9.7%), fish meal (4%), spray-dried blood plasma (4%), soybean oil (3.39%), calcium formate (0.3%), limestone (0.5%), dicalcium phosphate (0.6%), sodium chloride (0.07%), L-lysine (0.614%), DL-methionine (0.232%), L-threonine (0.264%), L-tryptophan (0.089%), mineral-vitamin premix (0.4%) and aroma (0.1%). The chemical composition of the inulin extract contained approximately 92% of inulin with DP≥10 and 8% of other carbohydrates (glucose, fructose and sucrose). Piglets were kept with sows until weaning at 28 days of age, and then in groups of three animals per pen with free access to experimental feed and water. Blood samples were collected after 40 days of feeding of both C and T diets (50 days of age). Serum for proteome analysis was obtained by blood centrifugation (1500 x g for 15 min at 4°C) after clotting. Blood samples were also collected into heparinized tubes to obtain plasma for biochemical assays by centrifugation at 3000 rpm for 10 min at 4°C. Serum and plasma samples were stored at -80°C until further analyses. The experimental procedures were approved by the Local Commission of Ethics for the Care and Use of Laboratory Animals (No. 13/2012 of 23.05.2012).

### Two-dimensional electrophoresis (2-DE)

Serum samples were processed in duplicate with a ProteoMiner™ Protein enrichment Large-Capacity kit (Bio-Rad) to decrease the concentration of high-abundance proteins and to increase the concentration of low-abundance proteins. Protein samples, each containing 800 µg of total protein, were mixed with lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v DTT, 0.2% w/v 3-10 ampholytes, 2 mM TBP) to adjust to a final volume of 600 µl per sample. The mixtures were then applied to 3-10, 24 cm NL (nonlinear) ReadyStrip™ IPG Strips (Bio-Rad). Isoelectrofocusing (IEF) was run in a total of 90 000 Vh using a Protean® IEF Cell (Bio-Rad). After IEF, the IPG strips were reduced for 15 minutes in equilibration buffer (6 M urea, 0.5 M Tris/HCl, pH 6.8, 2% w/v SDS, 30% v/v glycerol) containing 1% DTT (w/v), and then alkylated for 20 minutes with equilibration buffer containing iodoacetamide (2.5% w/v). The second dimension was run in 12% SDS polyacrylamide gels (20 x 25 cm) at 40 V for 2.5 hours, and then at 100 V for 16 hours at 10°C using a Protean Plus™ Dodeca Cell™ electrophoretic chamber

(Bio-Rad). After 2-DE separation, the gels were stained for 72 hours with colloidal Coomassie Brilliant Blue G-250.

## Image analysis

After 2-DE, patterns of the gels were scanned using a GS-800™ Calibrated Densitometer (Bio-Rad) and analysed using PDQuest Advanced 8.0.1 (Bio-Rad). The coefficient of variation (CV) was calculated for each replicate group to measure the variability within the group. Qualitative and quantitative comparisons were performed to find the differences in the protein spot patterns and examine changes in the protein expression level. Molecular mass (kDa) was computed for each protein spot identified based on the molecular range standard.

## Protein identification by MALDI-TOF MS

The protein spots showing statistically significant differences were manually excised from the gels and decolorized by washing in a buffer containing 25 mM $NH_4HCO_3$ in 5% v/v acetonitrile (ACN), followed by two washes in a solution of 25 mM $NH_4HCO_3$ in 50% v/v ACN. The gel pieces were dehydrated (100% ACN), vacuum dried and incubated overnight with trypsin (8 µl/spot of 12.5 µg trypsin/ml in 25 mM $NH_4HCO_3$; Promega, Madison, USA) at 37ºC. The resulting peptides were extracted with 100% ACN, combined with an equal volume of matrix solution (2.5 mg/ml CHCA, 0.1% v/v TFA, 50% v/v ACN) and loaded onto a MALDI-MSP AnchorChip™ 600/96 plate (Bruker Daltonics, Germany). Peptide Mass Standard II was used (mass range 700-3200 Da, Bruker Daltonics) to calibrate the mass scale. Mass spectra were acquired in the positive-ion reflector mode using a Microflex™ MALDI TOF MS (matrix-assisted laser desorption/ionization time of flight mass spectrometer) (Bruker Daltonics, Germany). Peptide mass fingerprinting (PMF) data were compared with mammalian databases (SWISS-PROT; http://us.expasy.org/uniprot/ and NCBI; http://www.ncbi.nlm.nih.gov/) with the aid of the MASCOT search engine (http://www.matrixscience.com/). Search parameters applied included: trypsin as an enzyme, carbamidomethylation of cysteine as a fixed modification, methionine oxidation as a variable modification, mass tolerance to 150 ppm and a maximum of one missed cleavage site. The results were further validated by the MASCOT score and sequence coverage.

## Measurement of plasma biochemical parameters

The levels of blood plasma Igs, i.e.: immunoglobulin A (IgA), immunoglobulin G (IgG) and immunoglobulin M (IgM), were determined by the immuno-turbidimetry method using ready-to-use reagents according to the manufacturer's instructions (APTEC Diagnostics nv, Sint-Niklaas, Belgium). Turbidity was measured at 340 nm and Ig concentrations were calculated using four-parameter logistic curves.

Fibrinogen concentration was analyzed chronometrically, according to the method of Clauss (1957), using reagents from DIAGON Ltd. (Budapest, Hungary). Plasma samples were diluted in imidazole buffer mixed with thrombin, and the clotting time was measured using photometers supplied with 470 nm LEDs.

The levels of total cholesterol, high-density lipoprotein (HDL) and triglycerides were determined spectrophotometrically using ELITech ready-to-use reagents (ELITech Group, Puteaux, France). The level of low-density lipoprotein (LDL) was calculated using Friedewald's formula (LDL = TC – HDL – [TG/2.2]).

All biochemical analyses were performed on a MAXMAT PL multidisciplinary diagnostic platform (Erba Diagnostics France SARL, Montpellier, France).

## Statistical analysis

The mean values and standard error of the mean (SEM) were calculated. Significance of the differences in protein expression pattern was confirmed by image analysis using Student's t-test in PDQuest Analysis software. Biochemical blood parameters were also analysed by Student's t-test using STATGRAPHICS Centurion XVI ver. 16.1.03.

## Results

On average, 240 protein spots per gel were detected with a mass range from 10 to 250 kDa and a pH range from 3 to 10. The expression of 20 protein spots differed significantly between groups. Seven of these spots were up-regulated, whereas 13 showed down-regulation in response to the experimental diet (Fig. 1). The analysis of intragroup variation showed that CV for replicate groups amounted to 58.86% and 62.47% for the C and T groups, respectively. In total, 13 spots, representing 8 distinct gene products were identified. Detailed information concerning the average abundance, fold change and significance of differences of the spots analyzed are summarized in
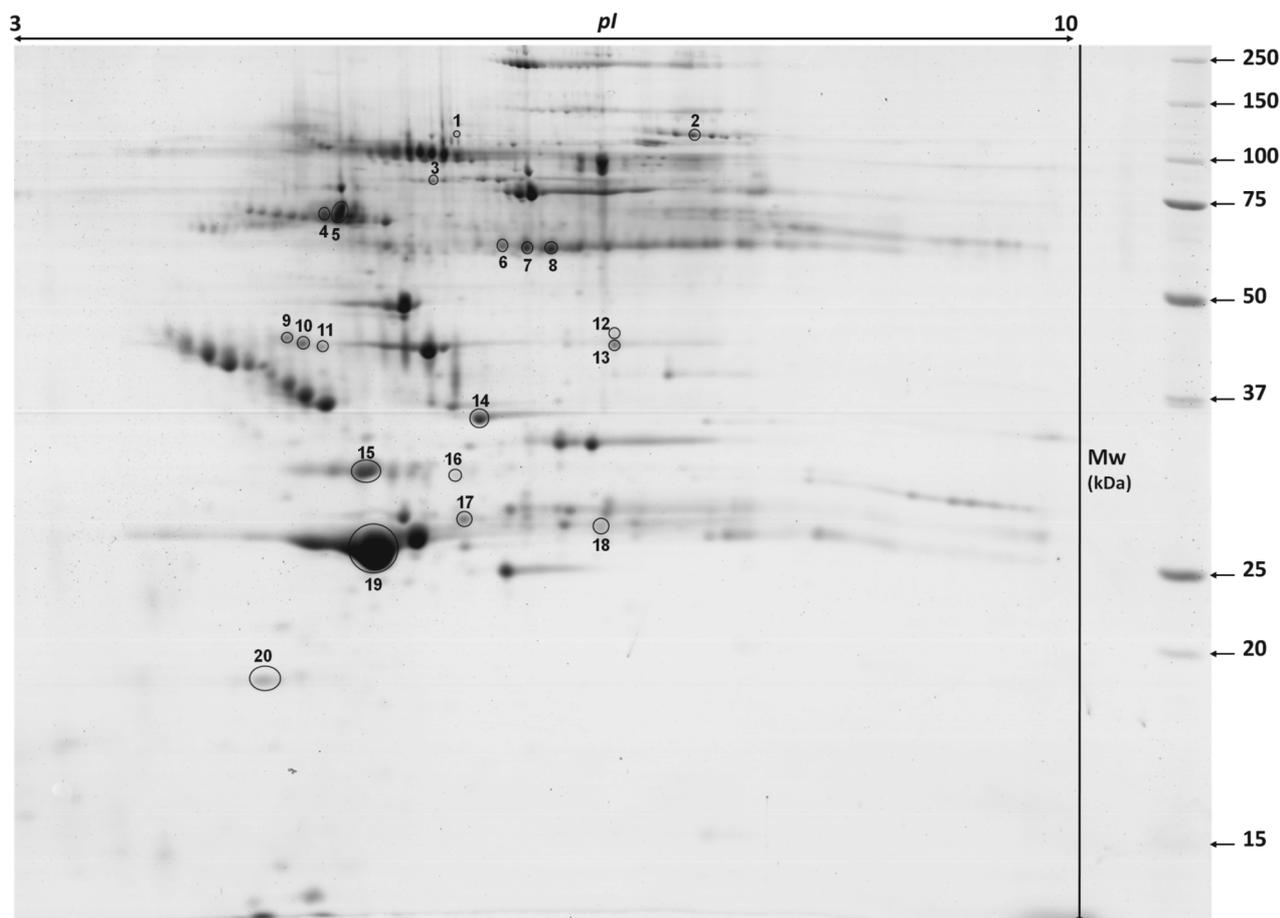
Fig. 1. 2-DE map of differentially expressed protein spots identified in 50-day-old piglets fed a diet supplemented with 2% inulin compared to the control group. Proteins (800 μg) were applied on the IPG strip (24 cm, pH 3-10) for the first dimension, and the second dimension was performed on 12% SDS-PAGE gels. The gels were stained with Coomassie brilliant blue G-250. Spot numbers correspond to those in Table 1.

Table 1. Differences in abundance and changes in spot intensities for the identified proteins are presented in Fig. 2.

Piglets fed the T diet did not differ significantly in IgA, IgG and IgM concentrations compared to the C diet. There was also no difference in the level of fibrinogen and lipid profile between groups (Table 2).

## Discussion

In the present study, feeding a diet with 2% of inulin resulted in a different expression of 8 proteins identified in the blood serum of pigs. Two of these proteins are directly involved in haemostasis, i.e. vitronectin (VT) and plasminogen. Ingestion of the experimental diet increased expression of VT, whereas plasminogen was found to be down-regulated. Vitronectin is a known regulatory protein engaged in numerous biological processes, including blood coagulation and fibrinolysis (Goswami et al. 2013). Previous

studies have shown that VT has binding sites for plasminogen activator inhibitor type-1 (PAI-1), which indicates that VT plays a key role in the regulation of plasminogen activation (Xu et al. 2001, Zhou et al. 2003). Vitronectin stabilizes PAI-1 in its active conformation and enables the inhibitor to bind to fibrin clots. PAI-1 is the main physiological inhibitor of both tissue plasminogen activator and urokinase plasminogen activator. These proteins are responsible for the conversion of plasminogen to plasmin, which forms fibrin degradation products (Sharp et al. 1999). Moreover, it was demonstrated that VT can bind plasminogen in a concentration-dependent manner (Xu et al. 2001, Preissner 1990), hence the decreased expression of plasminogen may be the result of VT up-regulation caused by inulin. Furthermore, the decreased plasminogen level is thought to favour fibrin deposition and contribute to atherothrombosis (Lima et al. 2012).

Vitronectin is also involved in the control of innate immune response. It regulates the terminal

Table 1. Relative expression, fold changes, significance of differences and MALDI-TOF-based identification of differentially expressed protein spots in piglet sera.

| | Protein name | Accession number | MALDI-TOF MS | | | Mean abundance | | | Fold | Significance |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Peptides matched | Sequence coverage (%) | pI/ Mr theoretical [pH/kDa] | Observed Mr [kDa] | C group | T group | | |
| 1 | Not identified | | | | | 119.90 | 144.40 | 257.10 | + 1.78 | p≤0.05 |
| 2 | Plasminogen | NP._001038055 | 39 | 55 | 7.00/93.29 | 119.00 | 962.80 | 503.30 | - 1.91 | p≤0.01 |
| 3 | Complement component C8 alpha chain | NP_001090919 | 17 | 35 | 5.61/67.72 | 88.60 | 420.50 | 821.90 | + 1.95 | p≤0.05 |
| 4 | Vitronectin | BAA09616 | 14 | 39 | 5.48/44.61 | 71.70 | 2059.10 | 6699.20 | + 3.25 | p≤0.05 |
| 5 | | | 14 | 39 | | 72.20 | 6131.60 | 17305.20 | + 2.82 | p≤0.05 |
| 6 | IgG heavy chain | BAM75569 | 17 | 56 | 5.91/52.20 | 62.50 | 3348.70 | 1325.30 | - 2.53 | p≤0.01 |
| 7 | | | 17 | 56 | | 62.90 | 3975.10 | 2774.00 | - 1.43 | p≤0.05 |
| 8 | | | 15 | 43 | | 63.50 | 3800.40 | 2311.60 | - 1.64 | p≤0.05 |
| 9 | C4b-binding protein beta chain | XP-003130503 | 8 | 31 | 5.23/29.88 | 44.50 | 1131.30 | 856.30 | - 1.32 | p≤0.05 |
| 10 | | | 8 | 31 | | 43.80 | 1506.10 | 1184.80 | - 1.27 | p≤0.05 |
| 11 | | | 8 | 31 | | 43.40 | 1011.30 | 731.60 | - 1.38 | p≤0.05 |
| 12 | Not identified | | | | | 41.30 | 415.00 | 224.90 | - 1.84 | p≤0.05 |
| 13 | Not identified | | | | | 39.90 | 1883.70 | 850.70 | - 2.21 | p≤0.05 |
| 14 | Apolipo-protein E | NP_999473 | 23 | 52 | 5.62/36.63 | 35.40 | 2019.40 | 4497.40 | + 2.23 | p≤0.05 |
| 15 | Ig lambda chain C region | P01846 | 4 | 68 | 6.75/11.17 | 31.50 | 3522.20 | 4410.30 | + 1.25 | p≤0.05 |
| 16 | Not identified | | | | | 31.30 | 1489.40 | 458.90 | - 3.24 | p≤0.05 |
| 17 | Not identified | | | | | 28.30 | 1305.90 | 751.80 | - 1.74 | p≤0.05 |
| 18 | Not identified | | | | | 27.80 | 1192.20 | 901.30 | - 1.32 | p≤0.05 |
| 19 | Not identified | | | | | 18.70 | 1845.60 | 1109.70 | - 1.66 | p≤0.05 |
| 20 | Apolipo-protein A-I | AAA30992 | 24 | 66 | 5.38/30.31 | 26.30 | 10124.00 | 19740.30 | + 1.95 | p≤0.01 |

Table 2. Blood plasma biochemical parameters in piglets fed a control diet (C) or a diet supplemented with 2% inulin (T).

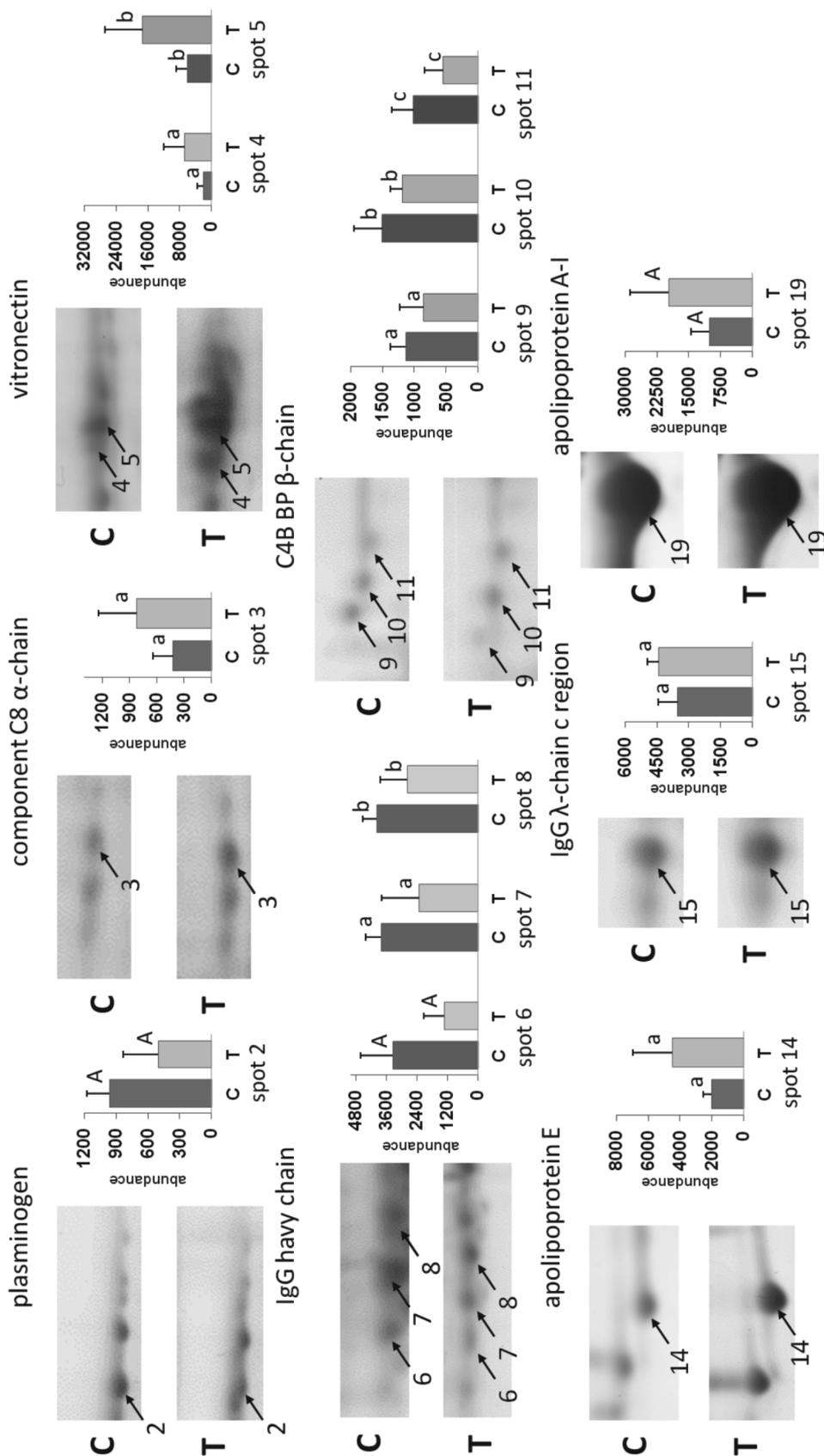| Biochemical parameters | Groups | | SEM | P-value |
|---|---|---|---|---|
| | C | T | | |
| IgG (mg/dl) | 717.07 ± 8.06 | 723.16 ± 11.43 | 0.6638 | 717.07 ± 8.06 |
| IgM (mg/dl) | 138.75 ± 1.68 | 131.43 ± 5.74 | 0.2161 | 138.75 ± 1.68 |
| Ig A (mg/dl) | 150.37 ± 10.12 | 169.95 ± 12.32 | 0.2473 | 150.37 ± 10.12 |
| Fibrinogen (g/l) | 1.67 ± 0.17 | 1.96 ± 0.25 | 0.3469 | 1.67 ± 0.17 |
| Total cholesterol (mmol/l) | 2.14 ± 0.14 | 2.15 ± 0.26 | 0.9731 | 2.14 ± 0.14 |
| HDL cholesterol (mmol/l) | 0.80 ± 0.05 | 0.81 ± 0.12 | 0.9602 | 0.80 ± 0.05 |
| LDL cholesterol (mmol/l) | 1.04 ± 0.10 | 1.03 ± 0.12 | 0.9774 | 1.04 ± 0.10 |
| Triglycerides (mmol/l) | 0.65 ± 0.05 | 0.67 ± 0.08 | 0.8661 | 0.65 ± 0.05 |

Fig. 2. Differentially expressed serum proteins of piglets fed a control diet (C) or a diet supplemented with 2% inulin (T). Names of the proteins identified, spot numbers, graph depicting the differences in abundance and changes in spot intensities are presented in the expanded view of the gel. Capital letters show the level of statistical significance at p≤0.01, while lowercase letters correspond to the level of statistical significance at p≤0.05.

pathway of complement activation, the formation of the membrane attack complex (MAC), consisting of five components from C5b to C9 (Milis et al. 1993, Su 1996, Singh et al. 2010). It has been postulated that VT interacts with C5b-7 and forms a complex (VT-C5b-7) that cannot be introduced into the cell membrane. The second mode of action involves binding to the C9 component that prevents lytic pore formation (Su 1996, Singh et al. 2010). Taken together, these findings may indicate that the increased expression of VT observed in the present study, may protect the piglet organism against excessive cytolytic action of activated complement. The studies of Cooper (1995) and Cooper and Petrovsky (2011) clearly indicate that γ and δ inulin have the ability to activate the alternative complement pathway via activation of the complement C3 receptors localised on the surface of macrophages.

The results of the present study also demonstrate that feeding a diet supplemented with inulin increased expression of the α subunit of complement component 8 (C8α), which is one of the MAC members. Complement component 8 is a serum glycoprotein consisting of three subunits (C8α, C8β and C8γ) encoded by three separate genes (Do et al. 2012). It should be emphasised that C8 plays a dual role in the complement-mediated cytolysis manifested by promoting or inhibiting its activity (Nemerow et al. 1979). Increased C8α expression, observed in the current work, may be potentially associated with the inhibition of the formation of C5b-9 channels, thereby preventing excessive disruption of cell membranes. However, this is only a hypothesis, as we have not measured the levels of the complement components and its activation products in the blood plasma of piglets.

C4b binding protein (C4b-BP) is not only involved in the regulation of classical complement pathway, but it also inhibits the blood coagulation process. This protein is composed of α- and β-chains linked together at their terminal parts. The β-chain, found to be down-regulated in the serum of piglets fed a diet containing inulin, interacts with the vitamin K-dependent protein S (PS), whereas α-chains have binding sites for the complement protein C4b and heparin (Esmon 2005). Protein S is an anticoagulant protein circulating in two forms, bound to C4b-BPβ and free form. Only the free form of PS can exert an anticoagulant effect (Taylor et al. 1991). Previous studies by Zoller et al. (1995) and Morb2uf et al. (1998) have shown that the level of PS regulates the concentration of the β-chain of C4b-BP. Therefore, we postulate that the decreased expression of C4b-BPβ caused by inulin may be associated with the elevated plasma concentration of the free form of PS.

Feeding a diet supplemented with 2% inulin decreased the expression of IgG heavy chains. This is quite surprising, as the total plasma IgG did not differ significantly between groups. The mechanisms underlying this phenomenon are difficult to explain, and remain unclear. In addition, we found no the effect of inulin on the level of IgA and IgM in growing piglets, which is consistent with the results of Taranu et al. (2012). Similar results of these authors were obtained with respect to the total plasma IgG concentration (Taranu et al. 2012).

Expressions of apo E and apo A-I were increased in the serum of piglets fed an inulin-containing diet. These proteins are involved in the transport of lipids and lipoprotein metabolism. They are known as the main protein components of high-density lipoproteins (HDL) and act as cofactors for the lecithin-cholesterol acyltransferase (LCAT). LCAT is an enzyme responsible for the production of cholesteryl esters, which are transported into the core of HDL particles, and this in turn causes their functional maturation (Birchbauer et al. 1993). In contrast, supplementation of the human diet with short-chain FOS for 4 weeks did not affect the concentration of serum apo A-I (Luo et al. 1996). Giacco et al. (2004) also observed no significant differences in plasma apo A-I levels in human subjects with mild hyperlipidemia when short-chain FOS were added to the diet for 2 months. The up-regulation of apo A-I and apo E observed in the present study did not correspond to the lipid profile of blood plasma, as the total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides remained essentially unchanged in comparison to the control group. This is consistent with previous studies where inulin-type fructans have been shown to have no effect on blood lipid levels in humans with normal initial concentrations of these lipids (Luo et al. 1996, van Dokkum et al. 1999).

## Conclusion

In conclusion, feeding a diet supplemented with 2% inulin leads to a significant change in the expression of serum proteins directly or indirectly involved in hemostasis and the innate immune response. Inulin favours the deposition of fibrin, promotes blood clotting and protects the piglet organism against excessive cytolitic activity of activated complement. However, further research is required to assess the precise mechanism of inulin effect on the blood serum proteome of piglets.

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## Acknowledgements

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