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Proteome changes in ileal mucosa of young pigs resulting from different levels of native chicory inulin in the diet

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ABSTRACT. The analysis of mucosa proteomes was performed using a two-dimensional electrophoresis combined with mass spectrometry to determine the effect of dietary level of inulin on protein expression patterns in the ileum. The experiment was carried out on 24 castrated male piglets, allocated to three groups, fed from the day 10 of life an unsupplemented diet (C) or diet supplemented with 1% (T1) or 3% (T2) of native chicory inulin. Samples of ileal tissue and blood were collected after 40 days of feeding. Comparative proteomic analysis of the T1 group showed 10 protein spots with a decreased expression, whereas the T2 diet caused overexpression of 24 spots in comparison to the C diet. Inulin levels differed in their effects on the expression of ileal proteins involved in intracellular molecular mechanisms controlling cell division and growth. The T1 diet down-regulated, whereas the T2 diet induced substantial up-regulation of proteins engaged in transcriptional and translational activities, folding and posttranslational modifications, which may indicate stimulation of epithelial cell proliferation. Inulin did not affect plasma levels of phosphorus, magnesium, calcium and iron in piglets but improved plasma prooxidant-antioxidant balance in animals fed the T2 diet. The results of this study might be considered as preliminary since further confirmation using more sophisticated quantitative proteomic tools is required to better understand and answer the unresolved issues concerning the mechanism underlying the inulin effect on the ileum in growing pigs. Nevertheless, a general insight into how inulin molecules or their fermentation end-products may induce changes in protein expression patterns in the ileum was presented.

Introduction

In recent years, a wide variety of prebiotics, including inulin, has gained researchers attention as possible feed additives due to their possible positive effects on intestinal health, and thus young animal performance. The root of *Cichorium intybus* L. is considered one of the most important sources of inulin, consisting of 15–20% of this prebiotic (Niness, 1999). Chicory inulin is a mixture of linear oligomers and polymers composed of β-D-fructosyl units linked together by (2→1) glycosidic bonds.

Its degree of polimerisation (DP) ranges from 2 to 60 fructose monomers with an average 10–12 DP

(Mensink et al., 2015). Previous studies on microbial profiles and activities in the gastrointestinal tract of pigs demonstrated that the effects of inulin were depended on dietary level, DP and intestinal segment (Yasuda et al., 2007; Patterson et al., 2010; Barszcz et al., 2018). The results of these studies also indicated that inulin (1–4% addition to diets of growing pigs, average DP ≥ 23) was predominantly fermented in the large intestine, mainly in the caecum (Yasuda et al., 2007; Patterson et al., 2010; Barszcz et al., 2018). On the other hand, in the previous study by Loh et al. (2006) was shown that a significant amount of chicory inulin with an average DP of 12 (20–50%) was fermented in the porcine jejunum by the resident microflora. Similar observations were reported by Böhmer et al. (2005), who found 57% precaecal digestibility of inulin in pigs fed diet supplemented with 2% of this carbohydrate. Considering the above results, it was hypothesized that inulin implementation with an average DP of 10 into the diet of growing pigs would induce proteome changes in the ileum resulting from its earlier degradation. The results of this study might help to gain additional insight into how inulin may transmit signals changing protein expression patterns in the ileum, and thereby provide an additional evidence demonstrating its prebiotic effect on the distal part of the pig small intestine. Therefore, the influence of feeding young pigs a diet supplemented with 1% and 3% of native chicory inulin with an average DP of 10 on the expression of ileal mucosa proteins was evaluated.

Material and methods

Animals and sample collection

In total 24 castrated male piglets (PIC × Penarlan P76) were used in the study. Animals were allocated to three experimental groups (n = 8). At the beginning of the experiment piglets were kept with their sows (4 sows and litters per group) in farrowing pens, on a commercial farm. Sows and their litters were randomly allotted to the different treatments. The sows were in the third or fourth parity. During the first 9 days of life piglets received only colostrum and milk of their mothers, while from the day 10 of life they were offered a milk-based diet with the addition of a solid-feed diet. The control group (C group) received an unsupplemented cereal-based diet and the experimental groups – a diet supplemented with 1% (treatment group 1, T1) or 3% of native chicory inulin (treatment group 2, T2). Inulin (Inulin Orafti®GR, BENEO GmbH, Mannheim, Germany) containing approximately 92% of inulin with DP ≥ 10 and 8% of other carbohydrates (glucose, fructose and sucrose) was included into the diets instead of maize starch. The remaining components of the diet were, %: wheat 46.84, barley 20, maize starch 3 in C, 2 in T1 and 0 in T2, full-fat soybean 5.90, whey 9.70, fish meal 4, spray-dried blood plasma 4, soybean oil 3.40, calcium formate 0.30, limestone 0.50, dicalcium phosphate 0.60, sodium chloride 0.07, L-lysine 0.61, DL-methionine 0.23, L-threonine 0.26, L-tryptophan 0.09, mineral-vitamin premix 0.40 and aroma 0.10. All diets contained, %: crude protein 20, crude ash 4.54, crude fibre 1.52 and 14.3 MJ/kg of metabolizable energy. The composition of the diets was previously described by Barszcz et al. (2018). At day 28 of life piglets were weaned and then 2 males from each litter were chosen and transported to the experimental facility. The solid based diets were offered *ad libitum* and its composition has not been changed through the whole experiment. Animals had free access to water. Feed intake and health status was monitored every day during the experimental period. At the age of 50 days animals were stunned by electric shock and killed by exsanguination. The samples of mixed blood were collected into heparinized tubes and plasma for biochemical analyses was obtained by centrifugation at 3000 *g* for 10 min at 4 °C and stored at –40 °C until further analyses. Ileal tissue samples were taken immediately and washed twice with 0.9% NaCl and thereafter twice with 20 mM Krebs-HEPES buffer (99 mM NaCl, 4.69 mM KCl, 2.50 mM $CaCl_2$, 1.20 mM $MgSO_4$, 1.03 mM K_2HPO_4, 25 mM $NaHCO_3$, 5.60 mM D-(+)-glucose, pH 7.4). Tissue samples were snap frozen in liquid nitrogen and then stored at –80 °C until further analysis. The experimental procedures were approved by the Local Commission of Ethics for the Care and Use of Laboratory Animals (No. 13/2012, Poland).

Protein extract preparation

Ileal mucosa was removed by scrubbing with a glass slide on plastic plate and then homogenized in 1000 µl of lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v DTT, 2% v/v Biolyte, 1% v/v protease inhibitor cocktail, 0.1% v/v nuclease) at a frequency of 20 000 Hz for 60 min using a mechanical homogenizer (Tissue Lyser, Qiagen, Hilden, Germany). Insoluble tissue debris were removed by centrifugation (4 °C, 15 000 *g*, 15 min) and the supernatant was used for two-dimensional electrophoresis.

Two-dimensional electrophoresis (2-DE)

Analyses were performed in duplicate. A modified Bradford assay (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA) was used to estimate total protein concentration. Samples containing 1000 µg of proteins were mixed with lysis buffer to the total volume of 650 µl and applied to 3–10, 24 cm ReadyStrip™ IPG Strips (Bio-Rad, Hercules, CA, USA). Isoelectrofocusing was run using Protean® IEF Cell (Bio-Rad, Hercules, CA, USA) by ramping the voltage to a maximum of 5000 V and terminated when a total of 90 000 Vh was reached. The strips were reduced with DTT in equilibration buffer (6 M urea, 0.5 M Tris/HCl, pH 6.8, 2% w/v SDS, 30% w/v glycerol and 1% w/v DTT) for 15 min and then alkylated with iodoacetamide (2.5% w/v) for 20 min. The second dimension was run on 12% SDS polyacrylamide gels (20 × 25 cm) at 100 V for 17 h at 15 °C using a Protean Plus™ Dodeca Cell™ electrophoretic chamber (Bio-Rad, Hercules, CA, USA). After electrophoretic separation, the gels were stained with colloidal Coomassie Brilliant Blue G-250 (Sigma-Aldrich, St. Louis, MO, USA).

Image acquisition and data analysis

Gel image acquisition was performed using a GS-800™ Calibrated Densitometer (Bio-Rad, Hercules, CA, USA). 2-D images were analysed using PDQuest Analysis software version 8.0.1 Advanced (Bio-Rad, Hercules, CA, USA). The spots present on at least four gels from each group were designated as expressed protein spots and were further analysed. The gel that displayed the highest number of spots was marked as the master gel for matching the remaining gels. The spot volume was used as the parameter for quantifying protein expression after normalisation based on the local regression model. After normalisation, the volume of each spot was averaged for two replicates of each biological sample. To measure the variability within the group, the coefficients of variation (CV) were calculated and additionally for each protein spot the mean spot quantity value was calculated. The level of statistical significance ($P < 0.01$ or $P < 0.05$) was first used to select differential protein spots that then were ranked by a fold-change with a cut-off of 1.5. The degree of difference between protein groups was expressed as an average ratio. The ratio values are presented in Table 1 for the T1 group (C vs T1) and in Table 2 for the T2 group (C vs T2). Based on the standard 2-D markers, the experimental isoelectric point (pI) and molecular weight (M_r) values were computed for each identified protein spot.

Mass spectrometry and bioinformatic data analysis

Protein spots were excised from the gels and washed in a solution of 25 mM NH_4HCO_3 in 5% v/v acetonitrile (ACN), followed by two washes with a buffer containing 25 mM NH_4HCO_3 in 50% v/v ACN. The gel pieces were dehydrated with 100% ACN, dried by centrifugation under vacuum and further digested overnight with trypsin (20 µl/spot of 12.5 µg trypsin/ml in 25 mM NH_4HCO_3; Promega, Madison, WI, USA) at 37 °C. After digestion, the peptides were extracted with 100% ACN, combined with the matrix solution (5 mg/ml CHCA, 0.1% v/v TFA, 50% v/v ACN) and placed onto a MALDI-MSP AnchorChip™ 600/96 plate (Bruker Daltonics, Hamburg, Germany) in a final volume of 1 µl. Droplets were allowed to dry at room temperature. A Microflex™ MALDI-TOF (matrix-assisted laser desorption/ionization time of flight) mass spectrometer (Bruker Daltonics, Hamburg, Germany) was operated in positive ion reflector mode. External calibration was performed using a Peptide Mass Standard II (Bruker Daltonics, Hamburg, Germany) based on monoisotopic values of the following peptides: bradykinin 1-7 (757.3992 Da), angiotensin II (1046.5418 Da), angiotensin I (1296.6848 Da), substance P (1347.7354 Da), bombesin (1619.8223 Da), ACTH clip 1-17 (2093.0862 Da), ACTH clip 18-39 (2465.1983 Da) and somatostatin 28 (3147.4710 Da). The mass spectra were acquired with 150 shots of a nitrogen laser operating at 20 Hz and were internally calibrated using porcine tryptic autolytic products (842.51 and 2211.10 m/z). Peptide mass fingerprinting (PMF) data were compared to mammalian databases (SWISS-PROT; http://us.expasy.org/uniprot/ and NCBI; http://www.ncbi.nlm.nih.gov/) with the aid of MASCOT search engine (http://www.matrixscience.com/). Search criteria 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. Contaminating peaks of keratin and trypsin were removed from the peptide mass list prior to database search. The results of PMF-based identification were accepted when the protein score was significant ($P < 0.05$) with at least five matching peptides and 25% peptide coverage.

Analysis of biochemical parameters of blood plasma

Biochemical blood parameters were determined spectrophotometrically using ready-to-use reagents

Table 1. Summary of the differentially expressed protein spots identified by MALDI-TOF MS analysis in the ileum of pigs fed a diet supplemented with 1% of inulin (T1) compared to the control (C) group. Spot number correspond to those at Figure 1A

Spot No.	Accession No. UniProt/NCBI	Protein name	Cellular component[1]	Ratio T1/C[2]	Level of significance	SC[3] (%)/ MS[4]	PM[5]	Theo. pI/M_r[6]	Exp. pI/M_r[7]
Down-regulated protein spots									
Structural proteins									
3	F1RKM0	Lamin B1	N	0.45	$P < 0.05$	33/131	22	5.08/66.67	4.80/64.50
6	XP_005668474	Plastin-2	CP, CS	0.52	$P < 0.05$	52/321	29	5.25/70.79	5.10/73.00
Proteins involved in metabolism									
5	P49720	Proteasome subunit beta type-3	CP, N	0.35	$P < 0.05$	42/95	10	6.15/23.23	5.60/27.30
7	XP_001925115	Inorganic pyrophosphatase	CP	0.39	$P < 0.01$	49/141	13	5.44/33.22	5.30/38.80
Oxidative-stress related proteins									
4	P52552	Peroxiredoxin-2	CP	0.66	$P < 0.05$	43/95	8	4.70/14.27	5.00/25.30
9	P80276	Aldose reductase	CP	0.48	$P < 0.05$	48/108	9	5.89/36.19	6.10/38.20
Cell cycle and apoptosis									
2	K9IVR0	Histone-binding protein RBBP4	N	0.64	$P < 0.05$	26/100	10	4.74/47.65	4.60/58.40
10	XP_003132938	Phosphatidylethanolamine-binding protein 1	CP	0.42	$P < 0.05$	50/82	6	6.96/21.07	7.70/22.40
Protein biosynthesis									
8	Q29387	Elongation factor 1-gamma	CP	0.59	$P < 0.05$	46/123	20	6.15/50.67	6.20/50.00
Other									
1	P10775	Ribonuclease inhibitor	CP	0.58	$P < 0.05$	52/168	15	4.76/50.70	4.40/53.40

[1] cellular components: N – nucleus, CP – cytoplasm, CS – cytoskeleton; [2] ratio of expression for protein spots for the ileum of pigs fed a diet supplemented with 1% (T1) of chicory inulin and the control (C) group; [3] the percentage of sequence coverage; [4] the MASCOT score; [5] the number of peptides matched; [6] theoretical pI and M_r values based on the Uniprot/NCBI databases; [7] experimental pI and M_r values computed for each identified protein spot based on the standard 2-D markers

Table 2. Summary of the differentially expressed protein spots identified by MALDI-TOF MS analysis in the ileum of pigs fed a diet supplemented with 3% of inulin (T2) compared to the control (C) group. Spot number correspond to those at Figure 1B.

Spot no.	Accession no. UniProt/NCBI	Protein name	Cellular component[1]	Ratio T2/C[2]	Level of significance	SC[3] (%)/ MS[4]	PM[5]	Theo. pI/M_r[6]	Exp. pI/M_r[7]
Up-regulated protein spots									
Structural proteins									
6	Q2XVP4	Tubulin alpha-1B chain	CP, CS	1.83	$P < 0.05$	32/78	9	4.94/50.80	6.00/58.50
12	XP_005659576	Tropomyosin alpha-1 chain isoform X3	CP, CS	3.31	$P < 0.01$	41/170	17	4.67/32.62	4.30/42.60
14	XP_005659583	Tropomyosin alpha-1 chain isoform X9	CP, CS	2.98	$P < 0.05$	38/115	17	4.71/32.54	4.40/38.90
13	NP_001123419	Tropomyosin beta chain	CP, CS	3.18	$P < 0.01$	55/186	23	4.62/33.38	4.30/42.60
24	P29269	Myosin regulatory light polypeptide 9	CP	4.47	$P < 0.01$	40/74	8	4.80/19.87	4.70/20.00
23	P19105	Myosin regulatory light chain 12A	CP	2.25	$P < 0.05$	40/72	6	4.67/19.84	4.40/19.90
9	XP_005668164	Vimentin isoform X2	CP, CS	2.17	$P < 0.05$	33/98	13	5.09/49.25	4.40/57.90
18	EDL81026	EF hand domain containing 2, isoform CRA_a	CT	2.40	$P < 0.01$	35/79	6	6.23/20.46	4.90/33.70
Proteins involved in metabolism									
1	NP_999445	Transitional endoplasmic reticulum ATPase	CP, ER, N	3.15	$P < 0.05$	28/148	22	5.13/89.92	5.00/104.40
2	NP_999445	Transitional endoplasmic reticulum ATPase	CP, ER, N	1.72	$P < 0.05$	33/150	18	5.13/89.92	5.10/105.80
19	NP_001138373	Proteasome subunit alpha type-5	CP, N	2.10	$P < 0.01$	56/97	10	4.74/26.41	4.40/29.10
20	XP_005662207	Phosphomannomutase 2	CS	1.75	$P < 0.01$	32/95	9	5.19/28.39	4.90/29.90
Cell cycle and apoptosis									
3	XP_003360037	Protein-glutamine gamma-glutamyltransferase 2	CT, ER	3.36	$P < 0.01$	32/163	15	5.19/78.20	5.10/95.00
10	XP_003360037	Protein-glutamine gamma-glutamyltransferase 2	CT, ER	1.80	$P < 0.05$	56/271	34	5.19/78.20	4.80/55.60
5	P54612	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	CP, N	2.17	$P < 0.05$	41/137	18	5.00/66.10	5.10/71.50
Protein biosynthesis									
15	XP_005653148	Elongation factor 1-delta isoform X1	N, CP	1.83	$P < 0.01$	42/99	11	4.94/31.13	4.80/37.80
16	XP_005653148	Elongation factor 1-delta isoform X1	N, CP	1.56	$P < 0.05$	46/130	14	4.94/31.13	4.90/37.80
11	NP_001032223	40S ribosomal protein SA	CM, CP, N	1.63	$P < 0.01$	52/139	12	4.80/33.02	4.60/46.60
Chaperone proteins									
4	P28491	Calreticulin	CP, ER	2.40	$P < 0.01$	50/183	16	4.32/48.43	3.80/54.00
7	G9F6X8	Protein disulfide isomerase	ER	1.67	$P < 0.05$	40/144	15	4.78/56.76	4.60/61.90
Calcium-binding proteins									
17	XP_003129266	Annexin A5	CS	2.74	$P < 0.01$	41/123	11	4.94/36.17	4.90/34.30
22	F1S340	Sorcin	CP	2.14	$P < 0.01$	43/82	9	5.32/21.73	5.10/20.40
Other									
8	P10775	Ribunuclease inhibitor	CP	2.13	$P < 0.05$	29/85	9	4.76/50.70	4.40/53.40
21		Not identified		2.02	$P < 0.01$				5.10/27.00

[1] cellular components: CP – cytoplasm, CS – cytoskeleton, CT – cytosol, ER – endoplasmic reticulum, N – nucleus, CM – cell membrane; [2] ratio of expression for protein spots for the ileum of pigs fed a diet supplemented with 3% (T2) of chicory inulin and the control (C) group; [3] the percentage of sequence coverage; [4] the MASCOT score; [5] the number of peptides matched; [6] theoretical pI and M_r values based on the Uniprot/NCBI databases; [7] experimental pI and M_r values computed for each identified protein spot based on the standard 2-D markers

(ELITech Group, Puteaux, France). The prooxidant-antioxidant balance (PAB) in blood plasma was determined according to the method of Koliakos and Hamidi Alamdari (2009). All 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. Biochemical blood parameters were analysed by one-way analysis of variancefollowed by Tukey's test using STATGRAPHICS® Centurion XVI ver. 16.1.03 statistical package (StatPoint Technologies, Inc., Warrenton, VA, USA).

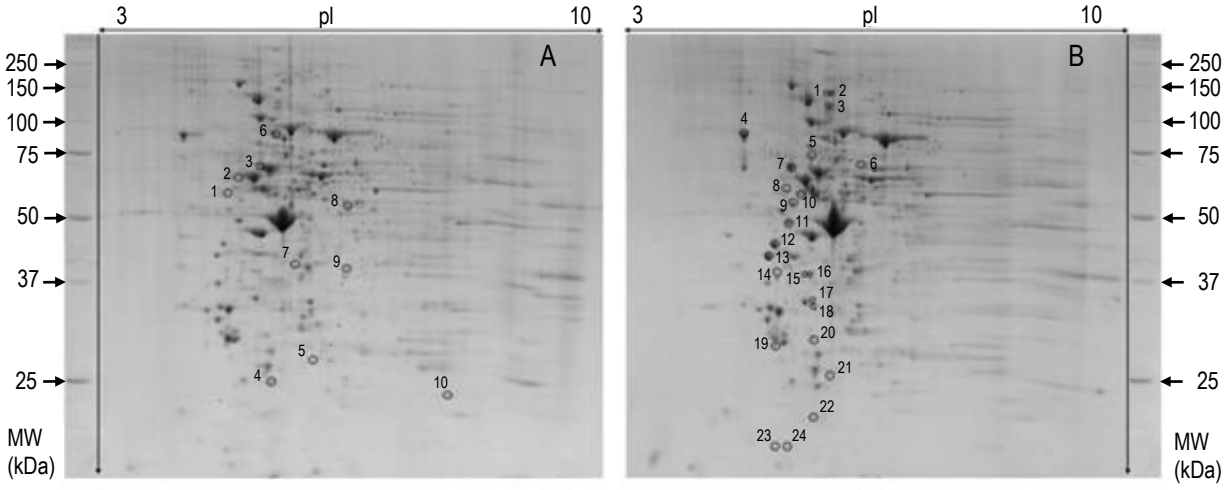

Figure 1. Representative two-dimensional (2-D) gel images presenting differentially expressed ileal protein spots found in growing piglets fed diets supplemented with 1% (A) or 3% (B) of native chicory inulin. 1000 µg of proteins were applied on the IPG strips (24 cm, pH 3–10) for the first dimension, the second dimension was performed on a 12% SDS-PAGE gels that were stained with Coomassie Brilliant Blue G-250. Spot numbers correspond to those in Tables 1 and 2

The significance level was set at $P < 0.05$. For the statistical analysis of the differences in protein expression pattern, Student's t-test was used, which was integrated in the PDQuest Analysis software version 8.0.1 Advanced (Bio-Rad, Hercules, CA, USA) and $P < 0.05$ was considered as significant.

Results

Differentially expressed ileal proteins

Comparative proteomic analysis of the T1 group versus the C group revealed 10 spots with a significantly decreased expression. Spots No. 5 and No. 7, identified as proteasome subunit beta type-3 and inorganic pyrophosphatase, showed the highest decrease in the T1 group (0.35 and 0.39-fold decrease, respectively, Figure 1A). The T2 diet caused a significant overexpression of 24 ileal protein spots when compared to the C diet. Among these proteins, the most pronounced changes were observed in the expressions of myosin regulatory light polypeptide 9 (spot No. 24) and protein-glutamine gamma-glutamyltransferase 2 (spot No. 3) (4.47 and 3.36-fold increase, respectively, Figure 1B). The analysis of intragroup variation showed that CV amounted to 51.40%, 50.95% and 50.10% for group C, T1 and T2, respectively.

Among 34 protein spots with altered expression, 33 were successfully identified and the majority of these proteins (85%) were recognized based on *Sus scrofa* identities. Protein identification was approved when matches had a MASCOT score greater than 70. Proteins were identified with peptide matches ranging from 6 to 34.

The experimental M_r and pI value of the identified proteins ranged from 19.90 to 105.80 kDa and from 3.80 to 7.70, respectively. It should be noted that the high-molecular weight proteins (> 30 kDa) were in the majority among the identified proteins (70%). Small shifts between experimental and theoretical M_r and pI values were observed in all analysed spots. Lists of proteins containing detailed information concerning 2-DE and MALDI-TOF coordinates of each individual protein spots are provided in Tables 2 and 3. All identified ileal proteins were categorized according to their subcellular localization as well as biological functions on the basis of the UniProtKB database (Tables 1 and 2).

Blood plasma biochemical parameters

There were no effects of inulin level on phosphorus, magnesium, calcium and iron concentrations, whereas chloride concentration was higher in piglets fed T2 than T1 diet. Feeding the T2 diet decreased PAB values ($P < 0.05$) in comparison to the C and T1 groups (Table 3).

Table 3. Biochemical blood parameters in pigs fed control diet and diets supplemented with 1% (T1) and 3% (T2) of native chicory inulin

Biochemical parameters	Group			SEM	*P*-value
	C	T1	T2		
Chloride, mmol/l	97.06^{ab}	93.75^{a}	101.56^{b}	1.118	0.009
Phosphorus, mmol/l	2.60	2.79	2.54	0.065	0.266
Magnesium, mmol/l	0.47	0.48	0.51	0.018	0.733
Calcium, mmol/l	2.97	3.01	2.82	0.062	0.463
Iron, µmol/l	18.27	23.89	19.64	1.778	0.4122
PAB values, HK	303^{b}	297^{b}	201^{a}	15.30	0.002

PAB – prooxidant-antioxidant balance; ^{ab} – mean values with different superscripts within the same row are significantly different at $P < 0.05$

Discussion

Proper gut functioning depends mainly on four components (mechanical, chemical, immunological and biological) forming the intestinal mucosal barrier that provides the anterior defensive line separating the internal tissue and the surrounding environment. The biological barrier is a mutually dependent microecosystem composed of intestinal microbiota, which mainly consists of commensal bacteria living on mucosal surfaces (Ribet and Cossart, 2015; Wang et al., 2016). The distal ileum of pigs contains significantly higher amount of bacteria (10^9 CFU/g), with a clear dominance of Gram-positive lactic acid bacteria, especially *Lactobacillus* spp. in comparison to humans and other monogastric animals (Konstantinov et al., 2006). As a consequence, more rapid inulin fermentation may occur, which is accompanied by an elevated production and absorption of short-chain fatty acids (SCFAs) (Böhmer et al., 2005; Loh et al., 2006).

The results of our previous study on growing pigs (Barszcz et al., 2018) demonstrated that both levels of native inulin increased fructan concentrations in the caecum, proximal, middle and distal colon, indicating that inulin had reached the large intestine. However, these results also showed that both dietary inulin levels did not affect relative amounts of *E. coli*, *Lactobacillus*, *Bifidobacterium* and *Clostridium* spp. in the digesta samples from different colon segments. These findings are consistent with the results of other studies performed in pigs (Loh et al., 2006; Paßlack et al., 2012).

Based on the results of our previous research (Herosimczyk et al., 2017; Lepczyński et al., 2017) and the results of the present study, it is clear that the dietary level of inulin has a direct impact on the total amount of expressed proteins as well as the direction of their changes. The expression of some ileal proteins, such as the ribonuclease inhibitor, elongation factor and proteasome subunit beta showed an opposite direction of changes in response to the experimental diets. The above-mentioned proteins were found to be down-regulated when pigs were fed the diet supplemented with 1% of inulin, whereas the diet with 3% inulin addition caused their up-regulation. Therefore, changes in protein expression caused by the T1 and T2 diets will be addressed separately in the discussion.

Ileal protein changes in response to the T1 diet. Lower expression levels were observed for 10 proteins in pigs fed the T1 diet in comparison to the C group. Two of these proteins, i.e. aldose reductase (AR) and peroxiredoxin-2 (PRDX2) were closely associated with oxidative stress. AR has been shown to efficiently metabolize reactive aldehydes yielding products that activate and perpetuate inflammatory process (Ramana et al., 2006; Srivastava et al., 2011). Studies performed on cellular and animal models revealed that AR was able to mediate the activation of NF-κB, an oxidative stress inducing factor, as well as transcription induction of cytokines and chemokines (Ramana et al., 2006; Reddy et al., 2009; Srivastava et al., 2011). AR inhibitors are known as anti-inflammatory agents, therefore, it seems that the down-regulation of ileal AR, observed in the present study, may reflect enhanced defence against oxidative stress to counteract endogenous reactive oxygen species (ROS) accumulation. Moreover, PRDX2 expression level was also decreased in response to 1% inulin addition. This protein is predominantly known as a cellular antioxidant exerting a critical role in oxidative stress prevention caused by a sudden increase in ROS generation. However, further characterization has revealed that PRDX2 may also induce inflammatory cytokine production, and thus be involved in the redox-dependent signalling mechanism during inflammation (Mullen et al., 2015). This finding may support a general hypothesis concerning putative anti-inflammatory and antioxidant effects of inulin on the intestinal mucosa layer (Pasqualetti et al., 2014).

Previously, it was found that both experimental diets reduced the expression of structural proteins and those related to cell mobility and proliferation in the liver of piglets (Herosimczyk et al., 2017). Similarly, this study demonstrated decreased expression of two structural proteins (lamin-B1, plastin-2), proteins directly or indirectly involved in DNA, RNA, protein and polysaccharide synthesis (inorganic pyrophosphatase, elongation factor 1-gamma), as well as those associated with the regulation of cell proliferation and apoptosis (histone-binding protein RBBP4, phosphatidylethanolamine-binding protein 1) in response to the T1 diet. Down-regulation of these proteins suggests that the T1 diet might have limited the turnover rate of ileal epithelial cells, which is in line with the findings of previous experiments conducted on growing piglets fed a formula supplemented with 0.3% of inulin (Kien et al., 2006, 2007).

Ileal protein changes in response to the T2 diet. Previously, in weaned pigs fed diet supplemented with 10% of dietary fructooligosaccharides (FOS) for 10 days an increase in the number of cecal epithelial, mitotic and mucin-containing cells was demonstrated (Tsukahara et al., 2003).

These authors postulated that the observed effect was mainly due to the increased luminal concentration of not only butyrate, but also valerate. This is consistent with a previous study of Barszcz et al. (2018). Although the concentrations of butyric and valeric acids did not reach statistical significance, they were elevated in different segments of the large intestine in pigs fed diet with 3% of native inulin in comparison to the control group (Barszcz et al., 2018). The results of the current study seem to support the above-mentioned findings, as the up-regulation of several proteins involved in intracellular molecular mechanisms controlling cell division and growth was revealed.

An increase in the expression of ileal cytoskeletal proteins, proteins engaged in the regulation of mRNA transcription, RNA binding and translation activity (40 S ribosomal protein SA, ribonuclease inhibitor, elongation factor 1-delta isoform X1), proteins involved in protein folding (transitional endoplasmic reticulum ATPase, protein disulfide isomerase) as well as those catalysing protein posttranslational modifications, mainly glycosylation (phosphomannomutase 2, protein-glutamine gamma-glutamyltransferase 2) was shown in pigs fed the T2 diet. It should be also noted that transitional endoplasmic reticulum ATPase is implicated in the regulation of the G2-M transition stage, thus the increased expression of this protein found in the T2 group seemed to drive cell cycle progression from one stage to another (Ye et al., 2017). Similar results were reported in other studies that showed higher protein synthesis rates in the distal part of the small intestine as well as in the caecum and colon of rats fed diet containing 8% of pectin for 14 days (Pirman et al., 2007). The observed trophic effect was associated with enhanced total SCFA production, mainly butyrate which is known for its role as a major mediator stimulating intestinal mucosal cell proliferation. Apart from its function as an energy substrate for colonocytes, butyrate has also been shown to directly stimulate the release of glucagon-like peptide-2 (GLP-2) from the enteroendocrine L cells, located in the terminal ileum and colon (Burrin et al., 2001). GLP-2 acts *via* binding to a G protein-coupled membrane receptor (GLP-2R) localized along the gastrointestinal tract, including ileum (Tappenden et al., 2003). This protein exerts a trophic effects on the intestinal mucosa, both in small and large intestines, where it stimulates cell proliferation and inhibits apoptosis. In this regard, secretion of GLP-2 *via* increased butyrate concentration in the ileum might be the one of a possible explanation for the enhanced expression of proteins involved in controlling cell division and growth, observed in the group of pigs fed the T2 diet. It might be anticipated that the main cause for diametrically opposite effect of the T1 on the proteome changes might be attributed to decreased values of the butyric acid observed in the large intestine of pigs fed diet with 1% of inulin in comparison to the control group (Barszcz et al., 2018).

Furthermore, an up-regulation of two gene products, i.e. annexin A5 (AnxA5) and sorcin (CP22), which belong to the family of calcium-binding proteins was demonstrated in this study. AnxA5 and CP22 are primarily involved in the regulation of calcium homeostasis by binding Ca^{2+} as well as the regulation of calcium channel activity, increasing, in consequence, the concentration of this ion in the endoplasmic reticulum and mitochondria (Ilari et al., 2015). Elevated expression of calcium-binding proteins is considered as a possible mechanism of increasing intestinal calcium absorption and, as a result, its plasma levels. However, we did not find any effect of inulin level on plasma calcium concentration, which is consistent with our previous study (Lepczyński et al., 2016).

Conclusions

Several new ileal proteins affected by dietary level of native chicory inulin in growing pigs were revealed in the study. Based on the biological role of those protein, it is most likely that supplementation with 1% inulin caused down-regulation, whereas 3% inulin addition induced substantial up-regulation of proteins engaged in transcriptional and translational activity, folding and posttranslational modification, which may indicate a stimulating effect of this diet on the intestinal cell proliferation. The results of this study might be considered as preliminary since further confirmation using more sophisticated quantitative proteomic tools is required to better understand and answer the unresolved issues concerning the mechanism underlying inulin effect on the ileum in growing pigs. Nevertheless, a general insight into how inulin molecules or their fermentation end-products may induce changes in protein expression patterns in the ileum was presented.

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