Modification of ileal proteome in growing pigs by dietary supplementation with inulin or dried chicory root

A. Lepczyński[1,3], A. Herosimczyk[1], M. Ożgo[1], M. Barszcz[2], M. Taciak[2] and J. Skomiał[2]

[1] West Pomeranian University of Technology, Department of Physiology, Cytobiology and Proteomics,
Klemensa Janickiego 29, 71-270 Szczecin, Poland
[2] The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Department of Animal Nutrition,
Instytucka 3, 05-110 Jabłonna, Poland

[3] Corresponding author:
e-mail: adam.lepczynski@zut.edu.pl

ABSTRACT. Recently, numerous plant-based preparations have been used for health promotion or disease prevention in animals. Chicory is one of the plants that contain various nutraceutics, mainly inulin type fructans (ITFs) showing prebiotic character. In pigs, a significant proportion of ITFs is fermented in the distal part of the small intestine to lactate and short-chain fatty acids (SCFAs). Previous studies have shown that SCFAs are involved in structural rearrangement of the intestinal epithelial mucosa and stimulate intestinal barrier assembly. These changes should be accompanied by a modification of enterocyte protein composition and abundance. Thus, we hypothesized that ITFs, due to their direct or indirect effect, can modify ileal proteome. The experiment was performed on 24 castrated male pigs (PIC × Penarlan P76) assigned to 3 groups (n = 8) fed cereal-based diets: control or experimental: supplemented with 4% of dried chicory root or with 2% of inulin. Mucosa proteins were separated using two-dimensional electrophoresis, followed by the identification of statistically valid proteins with the aid of Matrix Associated Laser Desorption Ionization – Time of Flight mass spectrometry (MALDI-TOF MS). Experimental diets significantly altered expression of proteins involved in: glycolysis/gluconeogenesis, biosynthesis of amino acids, cytoskeleton rearrangement, protein synthesis and processing, cell proliferation and differentiation, and iron absorption. Changes in the expression of proteins associated with energetic metabolism, cell proliferation and cytoskeleton rearrangement may suggest an impact of dried chicory root on the functional maturation of the ileal mucosa. Additionally, changes in transferrin abundance suggest the significance of chicory root and inulin supplementation for intestinal iron absorption.

Introduction

Recently, numerous plant-based preparations have been used for health promotion or disease prevention in humans and animals. Chicory (*Cichorium intybus* L.) is one of the attractive plants that contain various nutraceutics. Inulin-type fructans (ITFs) are the main group of health-improving substances of this plant and constitute approximately 63% of dried chicory root powder (Gałązka, 2002). Purified ITFs are classified as soluble fibre fraction with prebiotic properties, which means that they are carbohydrates resistant to hydrolysis by mammalian enzymes, but are degraded by the symbiotic microbiota and exert positive effects on the host (Gibson et al., 2017).

In pigs, a significant proportion of ITFs (20–50%) is fermented in the distal part of the small intestine (Yasuda et al., 2009). The remaining pool, the size of which differs depending on fructose chain length and dietary level (Barszcz et al., 2018), is fermented by the microbiota in the large intestine to short-chain fatty acids (SCFAs) that may directly or indirectly exert health-improving effects. Previous studies have shown that SCFAs are involved in structural rearrangement of the intestinal epithelial mucosa. Supplementation of a diet with butyrate results in morphological changes in the small intestine epithelium and this effect is positively correlated with the dose (Lu et al., 2008). Additionally, SCFAs stimulate intestinal barrier function by increasing mRNA expression of tight junction-related genes in pigs (Diao et al., 2017). These changes should be accompanied by a modification of enterocyte protein composition and abundance. Since the form of inulin (extract or dried chicory root) may influence pig metabolism (Lepczyński et al., 2016; 2017), we have decided to analyse their influence on ileal mucosa proteome. We hypothesized that ITFs, due to their direct or indirect effect, modify proteome of the distal part of the small intestine.

The present study was aimed to compare the influence of inulin, supplemented as chicory root or water extract of chicory inulin, on the proteome expression pattern of the ileal mucosa in growing pigs.

Material and methods

Animals and sample collection

The experiment was performed on 24 castrated male piglets (PIC × Penarlan P76). Animals were assigned to 3 groups (n = 8) and fed cereal-based isoenergetic diets: control (C) or experimental – supplemented with 4% of dried chicory root (CR) or with 2% of inulin extracted from chicory root (IN). In diets for both experimental groups inulin content was similar.

Animal housing and feeding conditions were the same as described by Lepczyński et al. (2017). Detailed composition of the diets is given in Table 1. Briefly, animals were offered experimental diets from the 10th day of life. At weaning, in 28th day of life, male piglets were distributed among group pens (n = 4) with free access to feed and water. Feed intake and health status were monitored daily during the whole experimental period. Animals were weighted in two time points: at weaning and at the end of the experiment. At the 50th day of life, at approximately 18 kg of body weight, animals were euthanized, and

Table 1. Composition of control diet (C) and diets supplemented with 2% of chicory inulin (IN) or 4% of dried chicory root (CR)

Indices	Diet		
	C	IN	CR
Composition, g/kg			
wheat	468.4	468.4	458.4
barley	200.0	200.0	200.0
maize starch	30.0	10.0	0.0
inulin	0.0	20.0	0.0
dried chicory root	0.0	0.0	40.0
full fat soybean	59.0	59.0	59.0
whey	97.0	97.0	97.0
fish meal	40.0	40.0	40.0
spray-dried plasma	40.0	40.0	40.0
soybean oil	33.9	33.9	33.9
calcium formate	3.0	3.0	3.0
calcium carbonate	5.0	5.0	5.0
calcium monophosphate	6.0	6.0	6.0
sodium chloride	0.7	0.7	0.7
L-lysine HCL (78.5%)	6.1	6.1	6.1
DL-methionine (99%)	2.3	2.3	2.3
L-threonine (98%)	2.6	2.6	2.6
L-tryptophan (98%)	0.9	0.9	0.9
mineral-vitamin mix[1]	4.0	4.0	4.0
aroma	1.0	1.0	1.0
Nutrients, % dry matter			
dry matter, %	90.03	90.14	90.15
crude ash	4.54	4.54	4.55
crude protein	20.05	20.04	20.05
ether extract	6.04	6.03	6.04
crude fibre	1.52	1.51	1.53
fructans	1.00	2.21	2.74
EM[2], MJ/kg	14.3	14.3	14.2

[1] supplied per kg of diet: IU: vit. A 2400, vit. D_3 240; mg: vit. E 12, nicotinic acid 6.4, pantothenic acid 3.2, choline chloride 48, Mg 3.2, Fe 24, Zn 22.4, Mn 9.6, Cu 25.6, I 0.16; µg: vit. K_3 480, vit. B_1 480, vit. B_2 960, vit. B_6 960, folic acid 640, biotin 40, vit. B_{12} 6.4, Se 64, Co 64; [2] calculated values; EM – metabolic energy

last 5 cm of distal ileal part were collected as samples for further analysis. The obtained tissue fragments were washed twice with 0.9% NaCl (4 °C), and then with 20 mM Krebs-HEPES buffer (NaCl, KCl, $CaCl_2$, $MgSO_4$, K_2HPO_4, $NaHCO_3$, pH 7.4; 4 °C; Noxygene, Elzach, Germany). Next, the samples were frozen in liquid nitrogen and subsequently stored at −80 °C.

All 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, West Pomeranian University of Technology, Szczecin, Poland).

Chemical composition of diets

Diets were analyzed for dry matter, crude ash, crude protein, ether extract, crude fiber, and fructans according to AOAC International (2011) procedures.

Protein sample preparation

After thawing the ileal fragments in the 0.9% NaCl, the intestinal mucosa was immediately scraped with the back of a surgery knife. A 100 mg of a mucosal sample was homogenized with steel beads using Tissue Lyser (QIAGEN, Hilden, Germany) at a frequency of 22 000 Hz for 5 min. Subsequently, samples were homogenized in 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. Extant cell debris were sedimented by centrifugation (20 800 g for 3 min at 4 °C), and next the supernatant containing solubilized mucosal proteins was collected.

2-DE (two-dimensional electrophoresis)

After the measurement of the total protein concentration using Bradford assay (Bio-Rad Protein Assay, Bio-Rad, Hercules, California, USA), linear 3–10, 24 cm ReadyStrip™ IPG Strips (Bio-Rad, Hercules, California, USA) were rehydrated with 650 µl of sample containing 1000 µg of total ileal mucosa protein (in two replicates). The isoelectrofocusing (IEF) was conducted in total of 90 000 Vh using a Protean i12® IEF Cell (Bio-Rad, Hercules, California, USA). The IPG strips were incubated for 15 min in basal equilibration buffer (6 M urea, 0.5 M Tris/HCl, pH 6.8, 2% w/v SDS, 30% w/v glycerol) with 1% DTT followed by 20 min in the same basal buffer with 2.5% iodoacetamide. Second dimension run was performed at 40 V for 2.5 h, and then at 100 V for 16 h at 10 °C with 12% polyacrylamide gels in Tris-glycine buffer (25 mM Tris–HCl, 192 mM glycine, 0.1% SDS) in a Protean Plus™ Dodeca Cell™ electrophoretic chamber (Bio-Rad, Hercules, California, USA). After 2-DE separation, proteins were visualized with colloidal Coomassie Brilliant Blue G-250.

Image analysis

Gels digitalization was performed using GS-800™ Calibrated Densitometer (Bio-Rad, Hercules, California, USA). For analysis of protein spots expression pattern the PDQuest Advanced v. 8.0.1 (Bio-Rad, Hercules, California, USA) 2D-Gel analysis software was used. Experiment normalization was performed according to local regression model (LOESS). For the statistical analysis of the differences in relative abundance of protein spots Student's t-test was used as integrated in the PDQuest software. Significance of the differences was set at the level of $P \leq 0.05$. To analyze the intragroup variability coefficients of variation (CV) for each group were calculat-

ed. The experimental molecular masses of identified protein spots were determined using Precision Plus Protein™ Kaleidoscope™ Standard for SDS-PAGE (Bio-Rad, Hercules, California, USA) as reference.

Mass spectrometry

For the identification of protein spots of statistically different expression MALDI-TOF (Matrix Associated Laser Desorption Ionization – Time of Flight) mass spectrometric identification was performed as previously described by Ozgo et al. (2015). For the identified gene products Gene Ontology analysis was performed based on the on-line tool STRING 11.0 (https://string-db.org/).

Histological analysis

Ileal tissue samples were taken, rinsed with physiological saline and fixed in Bouin's solution for 4 days. After dehydration in graded concentration of ethanol, tissues were cleared in xylene and embedded in paraffin blocks. The samples were then sliced into 5-µm sections and stained with haematoxylin and eosin. For each sample two slides were prepared with a minimum of 4 sections. Villous height, crypt depth, mucosa thickness and myenteron thickness were determined using a Zeiss Axio Star Plus (Carl Zeiss, Göttingen, Germany) bright field microscope and Axio Vision LE Rel. 4.5 image analysis software (Carl Zeiss, Göttingen, Germany). At least 30 measurements of each parameter were performed per one sample and individual means were calculated. Data were analysed by one-way analysis of variance, followed by post hoc Tukey's HSD test using STATGRAPHICS® Centurion XVI ver. 16.1.03 statistical package (StatPoint Technologies, Inc., Warrenton, VA, USA).

Results

Productive traits

Feeding diets supplemented with IN or CR neither significantly affected feed intake and average daily gain nor final body weight of piglets (Lepczyński et al., 2017).
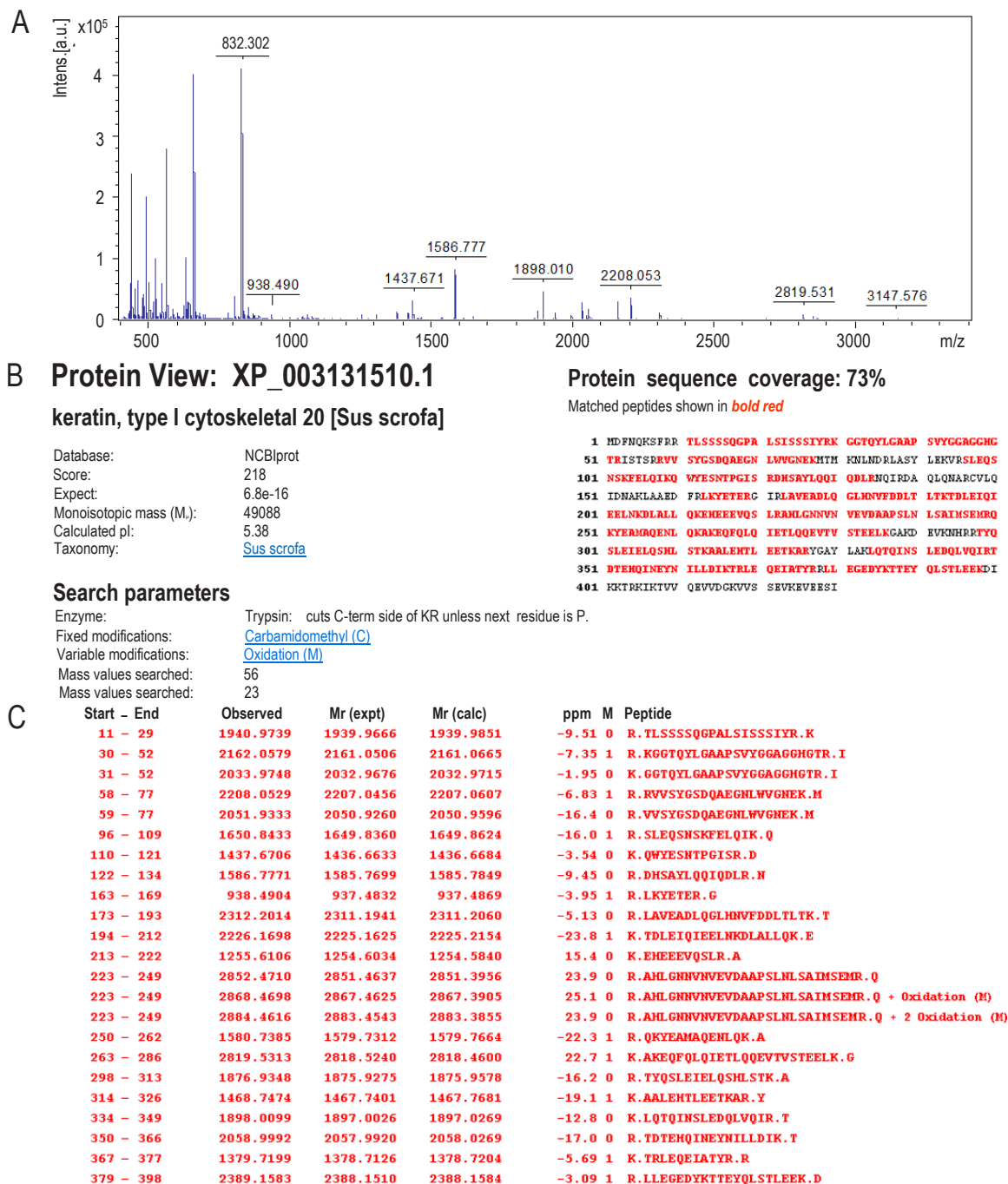
Analysis of ileal proteome differences

Bioinformatic analysis showed a similar number of protein spots on each analysed 2-DE gel representing ileal mucosa proteome (ranging from 457 to 524). Of these, 408 spots were included in the quantitative analysis aimed at comparing the expression pattern between control and experimental groups. The CV was estimated at the level of 49.20,

45.99 and 51.70% for the C, IN and CR group, respectively.

Most of the significantly altered protein spots between gel images representing the C group and the IN or CR groups demonstrated similar trends in expression patterns; however, the differences were more pronounced in animals fed the CR diet.

Feeding the IN diet significantly altered the expression of 8 protein spots, 5 of which were down-regulated and the remaining 3 were up-regulated

in comparison to the C group. The CR diet was shown to induce significant expression changes of 35 protein spots, 18 of which were up-regulated and 17 were down-regulated in comparison to the C group. Detailed data concerning fold changes in protein expression and detailed parameters of protein spots identification are presented in Table 2. Additionally, example mass spectrum with original MASCOT search engine identification details are presented on Figure 1. Differentially expressed

A

B Protein View: XP_003131510.1

Protein sequence coverage: 73%

keratin, type I cytoskeletal 20 [Sus scrofa]

Matched peptides shown in *bold red*

Database:	NCBIprot
Score:	218
Expect:	6.8e-16
Monoisotopic mass (M$_r$):	49088
Calculated pI:	5.38
Taxonomy:	Sus scrofa

```
  1 MDFNQKSFRR TLSSSSQGPA LSISSSIYRK GGTQYLGAAP SVYGGAGGHG
 51 TRISTSRRVV SYGSDQAEGN LWVGNEKMTM KNLNDRLASY LEKVRSLEQS
101 NSKFELQIKQ WYESNTPGIS RDHSAYLQQI QDLRNQIRDA QLQNARCVLQ
151 IDNAKLAAED FRLKYETERG IRLAVEADLQ GLHNVFDDLT LTKTDLEIQI
201 EELNKDLALL QKEHEEEVQS LRAHLGNNVN VEVDAAPSLN LSAIMSEMRQ
251 KYEAMAQENL QKAKEQFQLQ IETLQQEVTV STEELKGAKD EVKNHRRTYQ
301 SLEIELQSHL STKRALEHTL EETKARYGAY LAKLQTQINS LEDQLVQIRT
351 DTEHQINEYN ILLDIKTRLE QEIATYRRLL EGEDYKTTEY QLSTLEEKDI
401 KKTRKIKTVV QEVVDGKVVS SEVKEVEESI
```

Search parameters

Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M)
Mass values searched:	56
Mass values searched:	23

C

| Start – End	Observed	Mr (expt)	Mr (calc)	ppm	M	Peptide
11 – 29	1940.9739	1939.9666	1939.9851	-9.51	0	R.TLSSSSQGPALSISSSIYR.K
30 – 52	2162.0579	2161.0506	2161.0665	-7.35	1	R.KGGTQYLGAAPSVYGGAGGHGTR.I
31 – 52	2033.9748	2032.9676	2032.9715	-1.95	0	K.GGTQYLGAAPSVYGGAGGHGTR.I
58 – 77	2208.0529	2207.0456	2207.0607	-6.83	1	R.RVVSYGSDQAEGNLWVGNEK.M
59 – 77	2051.9333	2050.9260	2050.9596	-16.4	0	R.VVSYGSDQAEGNLWVGNEK.M
96 – 109	1650.8433	1649.8360	1649.8624	-16.0	1	R.SLEQSNSKFELQIK.Q
110 – 121	1437.6706	1436.6633	1436.6684	-3.54	0	K.QWYESNTPGISR.D
122 – 134	1586.7771	1585.7699	1585.7849	-9.45	0	R.DHSAYLQQIQDLR.N
163 – 169	938.4904	937.4832	937.4869	-3.95	1	R.LKYETER.G
173 – 193	2312.2014	2311.1941	2311.2060	-5.13	0	R.LAVEADLQGLHNVFDDLTLTK.T
194 – 212	2226.1698	2225.1625	2225.2154	-23.8	1	K.TDLEIQIEELNKDLALLQK.E
213 – 222	1255.6106	1254.6034	1254.5840	15.4	0	K.EHEEEVQSLR.A
223 – 249	2852.4710	2851.4637	2851.3956	23.9	0	R.AHLGNNVNVEVDAAPSLNLSAIMSEMR.Q
223 – 249	2868.4698	2867.4625	2867.3905	25.1	0	R.AHLGNNVNVEVDAAPSLNLSAIMSEMR.Q + Oxidation (M)
223 – 249	2884.4616	2883.4543	2883.3855	23.9	0	R.AHLGNNVNVEVDAAPSLNLSAIMSEMR.Q + 2 Oxidation (M)
250 – 262	1580.7385	1579.7312	1579.7664	-22.3	1	R.QKYEAMAQENLQK.A
263 – 286	2819.5313	2818.5240	2818.4600	22.7	1	K.AKEQFQLQIETLQQEVTVSTEELK.G
298 – 313	1876.9348	1875.9275	1875.9578	-16.2	0	R.TYQSLEIELQSHLSTK.A
314 – 326	1468.7474	1467.7401	1467.7681	-19.1	1	K.AALEHTLEETKAR.Y
334 – 349	1898.0099	1897.0026	1897.0269	-12.8	0	K.LQTQINSLEDQLVQIR.T
350 – 366	2058.9992	2057.9920	2058.0269	-17.0	0	R.TDTEHQINEYNILLDIK.T
367 – 377	1379.7199	1378.7126	1378.7204	-5.69	1	K.TRLEQEIATYR.R
379 – 398	2389.1583	2388.1510	2388.1584	-3.09	1	R.LLEGEDYKTTEYQLSTLEEK.D

Figure 1. Protein identification data on the example of keratin type I cytoskeletal 20
A – mass spectrum obtained using MALDI-TOF mass spectrometer (Microflex, Bruker, Bremen, Germany); B – protein identification parameters using the Mascot Search Engine: protein name, searched database, MASCOT score, expectation rate, number of matched peptides and amino acid sequence coverage; C – list of matched peptides; presented data are corresponding to those for spot number 6 in Table 2

Table 2. Differentially expressed protein spots between the control (C) and treatment groups (diets supplemented with 2% of chicory inulin (IN) or 4% of dried chicory root (CR))

Spot No.	Accession No. UniProt/NCBI	Protein name	Gene name	Ratio IN/C	Ratio CR/C	Sequence coverage (%) / MASCOT score	MP	SL	Theo. pI / MW (kDa)	Exp. pI / MW (kDa)
Glycolysis/gluconeogenesis[a] and biosynthesis of amino acids[b]										
1	BAI48105	triosephosphate isomerase 1[ab]	TPI1	1.25	**2.66**	53/115	9	CP	6.54/26.88	6.9/27.8
2	A0A287AJQ2	phosphoglycerate mutase 1[ab]	PGAM1	1.16	**2.72**	46/94	9	CP	6.67/28.90	6.8/29.0
3	XP_020949154	alcohol dehydrogenase [NADP+] isoform X2[a]	AKR1A1	0.97	**2.33**	32/96	8	CP	6.51/36.84	6.9/36.6
4	P00355	glyceraldehyde-3-phosphate dehydrogenase[ab]	GAPDH	0.83	**0.63**	25/61	7	N, CP, CS	8.51/36.04	4.9/37.8
5	XP_005672193	isocitrate dehydrogenase [NADP] cytoplasmic isoform X2[b]	IDH1	1.01	**1.87**	27/82	8	CP, PX	6.48/47.15	6.6/46.1
Cytoskeletal and related proteins										
6[†]	XP_003131510	keratin type I cytoskeletal 20	KRT20	**1.43**	**3.06**	73/218	23	CS	5.38/49.09	5.5/51.5
7				0.98	**1.55**	71/218	25			5.4/54.2
8				0.85	**1.57**	36/110	10			5.5/54.4
9	XP_001929280	keratin type II cytoskeletal 8	KRT8	**1.46**	**1.74**	54/246	33	CS	5.70/54.39	5.8/58.3
10	P02543	vimentin	VIM	1.19	**0.50**	36/151	17	CS	5.06/53.69	4.6/51.3
11	P67937	tropomyosin alpha-4 chain	TPM4	0.91	**0.78**	40/98	12	CS	4.67/28.62	4.3/33.1
12	NP._001123419.1	tropomyosin beta chain	TPM2	1.09	**0.59**	22/89	8	CS	4.62/33.38	4.2/40.4
13	Q6QAQ1	actin cytoplasmic 1	ACTB	1.09	**0.56**	37/80	11	CS	5.29/42.05	5.3/38.8
14	NP_001127815	actin related protein 3	ACTR3	0.96	**3.03**	31/109	12	CS	5.61/47.85	5.6/51.5
15	XP_005659594	annexin A2	ANXA2	1.04	**3.03**	43/119	12	CP, N, CM	6.92/38.84	7.0/39.6
16	Q99MZ8	LIM and SH3 domain protein 1	LASP1	1.20	**2.17**	40/95	13	CS	6.61/30.35	6.6/38.7
17	P02554	tubulin beta chain	TUBB	0.93	**0.65**	52/197	22	CS	4.78/50.29	4.8/37.8
18				**0.67**	**0.43**	31/86	10			4.9/69.7
19				0.73	**0.09**	29/77	13			4.4/57.9
20	XP_003358947	T-complex protein 1 subunit theta	CCT8	0.89	**0.69**	20/79	8	CS, CP	5.40/60.15	5.3/67.6
Protein synthesis and processing										
21	P85834*	elongation factor TU, mitochondrial	TUFM	0.9	**1.28**	15/62	6	MT	7.23/49.89	6.5/48.2
22	XP_020939748	elongation factor 2 isoform X2	EEF2	1.17	**1.53**	38/133	19	CP, N	6.41/94.87	6.8/78.9
23	P68102*	eukariotic translation initiation factor 2 subunit 1	EIF2S1	0.57	**0.24**	39/77	8	N, CP	5.02/36.37	5.1/40.0
24	XP_003133652	elongation factor 1-beta	EEF1B2	**0.79**	**0.69**	29/91	7	CP	4.51/24.96	4.3/33.1
25	Q3T165*	prohibitin[d]	PHB	0.86	**0.19**	51/92	8	MT, EX	5.57/29.84	5.4/31.0
26	NP_001182041	protein disulfide isomerase A3 precursor	PDIA3	1.76	**1.52**	26/86	10	ER	5.93/57.28	5.9/61.8
27	XP_020946788	endoplasmin	HSP90B1	1.11	**1.50**	24/90	13	ER	4.73/92.56	4.6/111.4
Cell proliferation and differentiation										
28	XP_003359931	proliferating cell nuclear antigen	PCNA	**0.58**	**0.75**	54/139	13	N	4.57/29.10	4.3/36.1
29	P61288	translationally-controled tumor protein	TCTP	**0.77**	**0.79**	51/89	9	CP	4.84/19.70	4.7/25.6
30	XP_020954120	chloride intracellular channel protein 1	CLIC1	**0.6**	**0.52**	45/91	9	N, CM	5.17/27.30	5.2/36.4
Other proteins										
31	CAA30943	transferrin, partial	TF	1.34	**1.74**	22/86	12	ES	6.73/78.95	6.6/83.4
32	XP_003129266	annexin A5	ANXA5	1.06	**0.53**	66/220	22	CP, CM	4.94/36.17	4.8/34.1
33	CAA30970	albumin	ALB	1.19	**2.30**	47/192	20	EX	5.92/71.36	6.0/72.3
34				**1.98**	**2.07**	46/221	21			5.9/71.0
35	Q99028	catechol o-methyltransferase	COMT	1.02	**0.52**	50/61	5	CP, CM	6.10/20.89	5.0/26.9

Spot number indicates the number labeling the spots in Figure 1; for each protein, accession number and protein name are shown as in Uniprot/NCBI database; the fold change (ratio IN/C; ratio CR/C), the predicted and estimated molecular weight (MW) and isoelectric point (pI), the percentage of the sequence coverage, the Mascot score and the number of peptides matched per protein (MP) are also indicated; spots significantly differentially expressed at level $P \leq 0.05$ are bolded; proteins are grouped according to their function; subcellular localization (SL): CM – cell membrane, CP – cytoplasm, CS – cytoskeleton, ER – endoplasmic reticulum, ES – endosome, EX – extracellular, MT – mitochondrion, N – nucleus, PX – peroxisome; [a] – proteins involved in glycolysis/gluconeogenesis; [b] – proteins involved in biosynthesis of amino acids; [ab] – proteins involved in both glycolysis/gluconeogenesis and biosynthesis of amino acids; * – proteins identified as other organisms homologues; [†] – the protein spot which Maldi-TOF MS identification profile is presented on Figure 2
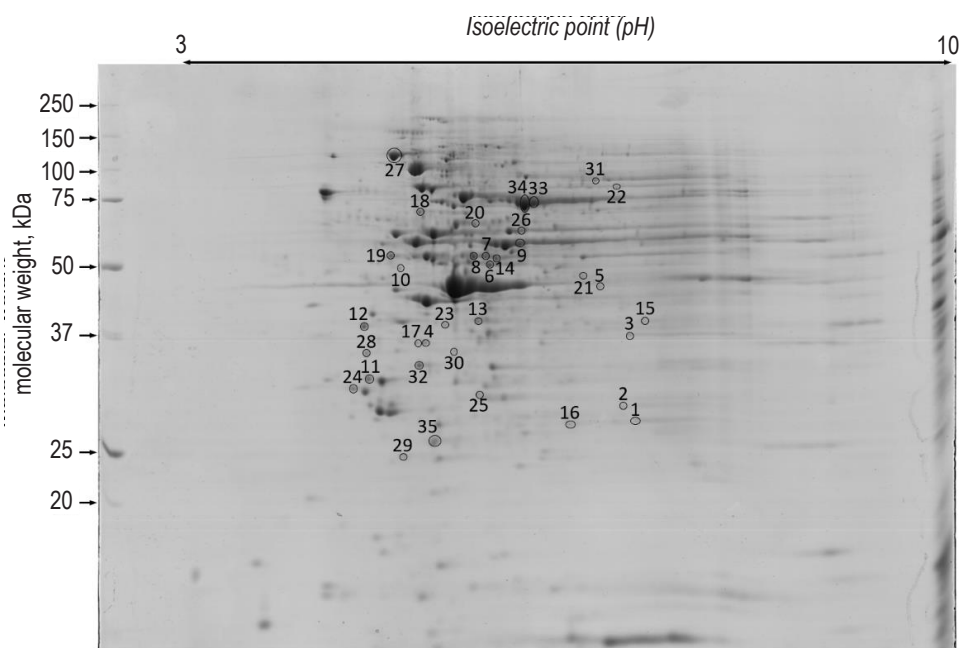
Figure 2. 2-DE map of differentially expressed protein spots found in the ileal mucosa of 50-day-old pigs fed both supplemented diets. Spot numbers correspond to those in Table 2

protein spots are marked on the representative gel image of the ileal mucosa protein profile (Figure 2).

Significantly altered proteins were categorized according to their biological function and known metabolic pathways based on the on-line Gene Ontology enrichment tool STRING 11.0 (https://string-db.org/). On that basis the identified proteins were manually assigned to the following protein groups: glycolysis/gluconeogenesis and biosynthesis of amino acids, cytoskeletal and related proteins, protein synthesis and processing, cell proliferation and differentiation, and other proteins (Table 2). Significantly altered proteins were also grouped accordingly to their subcellular localisation (Table 2). The highest number of proteins in this group was classified as cytoplasmic and cytoskeletal proteins.

Histological analysis

Histological analysis including villous height, crypt depth, villus:crypt ratio, mucosa thickness and myenteron thickness did not show any statistical differences (Table 3).

Table 3. Morphological parameters of the ileum of pigs, µm

Group	Villous height (VH), µm	Crypt depth (CD), µm	VH:CD	Mucosa thickness, µm	Myenteron thickness, µm
Control	341	246	1.42	586	619
Inulin (2%)	324	235	1.41	565	652
Dried chicory root (4%)	339	227	1.52	541	578
SEM	7.3	4.4	0.047	10.0	31.2
P-value	0.6332	0.1677	0.6055	0.1799	0.6499

Discussion

In the present study, a significant influence of native inulin and inulin rich-chicory root was observed on the expression level of several proteins involved in glycolysis/gluconeogenesis and metabolism of amino acids. The influence of ITFs on the protein and transcriptome expression pattern of the small intestine cells has not been analysed yet, but several studies evidenced the effect of inulin and SCFAs (butyrate, propionate and acetate) on Caco-2 cells (Lamers et al., 2003; Chang et al., 2008) that may partially reflect the metabolic properties of ileal enterocytes. Lamers et al. (2003) noticed that inulin and SCFAs cause changes in the level of metabolites associated with glucose metabolism and glutamate synthesis in this cell line. An increase in glucose, glutamate and alanine concentrations was observed after incubating the cells in a medium containing native inulin. On the other hand, an increase in the concentration of metabolites related to tricarboxylic acid cycle activity (i.e. alanine, succinate, lactate and 2-oxoglutarate), proline and its precursors (nicotinamide, nicotinate) was observed as a result of incubation of Caco-2 cells with inulin fermentation products, which could indicate increased glycolytic activity of these cells. The effect of butyrate on the expression profile of glycolytic enzymes (i.e. PGAM1) at the transcript level in Caco-2 cells was also reported by Chang et al. (2008). Additionally, Wang et al. (2012) demonstrated an increased expression of isocitrate dehydrogenase (IDH)

protein isoforms in the jejunum of weanling piglets as a result of dietary supplementation with the *Lactobacillus fermentum* I5007 probiotic strain. Thus, it can be concluded that changes in the expression of glycolytic enzymes: triose phosphate isomerase (TPI1), phosphoglycerate mutase 1 (PGAM1), alcohol dehydrogenase [NADP+] (AKR1A1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and isocitrate dehydrogenase 1 (IDH1), in our study may result from an increased number of probiotic bacteria colonizing the small intestine and/or subsequent inulin fermentation in this segment of the small intestine. This hypothesis is strengthened by the fact that ITF fermentation in the small intestine of growing pigs was confirmed by Yasuda et al. (2009). Our findings also showed that ITFs affected cell proliferation and differentiation by down-regulating proliferating cell nuclear antigen (PCNA) and translationally-controled tumor protein (TCTP) in animals fed diets supplemented with inulin and chicory root. PCNA expression is strictly corelated with proliferative cells at the final stage of the G1 phase and the initial stage of the S phase typically occurs in intestinal crypt cells (Paunesku et al., 2001). It has been also confirmed that the administration of prebiotics and probiotics in pigs cause a decrease in the number of PCNA-positive crypt cells in the ileal mucosa, that exhibited progression towards reduced epithelial proliferation (Rekiel et al., 2010). TCTP is a protein involved inter alia in cell growth, cell cycle progression and stress resistance as a molecular chaperone (Bommer, 2012). TCTP shows strong polarisation along the crypt-villus axis. Its expression is relatively high in the crypt and low in the villi (Sheverdin et al., 2013). In our study we have not observed significantly important differences in morphometric measurements of ileal mucosa, but the fact that animals fed diet supplemented with dried chicory root had the relatively shorter crypt depth, the lowest expression of PCNA and TCTP. Taken all together it may evidence the relatively highest maturity of intestinal mucosa in this animal group. The crypt depth is a marker of intestinal mucosa maturity. Increased crypt cell proliferation counteracts the rate of reduction in villous height and eventually equalled the rate of cell loss from the villi in post-weaning intestine rearrangement (Hampson, 1986; Masri et al., 2015). It may suggest that decreased crypt depth in the animals fed both experimental diets may be the effect of less intense rearrangement of villi in the postweaning period. Additionally, Masri et al. (2015) stated that morphometric indices (villous height, crypt depth, villous:crypt ratio)

should not be considered individually, but should be treated as an entity, forming an overall picture of mucosa status. In our opinion, this statement should replenish molecular markers of functional mucosa maturity e.g., protein expression pattern.

In our study, significant differences in the expression of ileal cytoskeletal proteins were observed in animals fed diet supplemented with chicory root. These changes were observed for both intermediate filament and thin filament constituents as well as microfilaments and cytoskeleton-associated proteins. It clearly shows that inulin affects mucosal maintenance and integrity. It has been confirmed that ITFs and SCFAs, mainly butyrate, appear to play an important role in regulating epithelial barrier integrity by regulating the expression and distribution of tight junction proteins *in vitro* (Wu et al., 2017) and *in vivo* in the pig ileum (Diao et al., 2017). In our study, we did not observe differences in the expression of main tight junction-forming proteins (e.g., zona ocludens-1, claudins, ocludins), but we found a 3-fold up-regulation of annexin A2 (ANXA2) in the CR group. ANXA2 is proposed as a member of tight junction-related proteins responsible for its proper function and assembly (Lee et al., 2004). ANXA2 was also up-regulated in the ileum of broilers treated with *Enterococcus fecium* probiotic (Luo et al., 2013). Moreover, ANXA2 may protect against oxidative stress. According to Madureira et al. (2011), ANXA2 was responsible for protecting lipids, proteins and nucleic acids from oxidative damage by participating in H_2O_2 reduction. The proposed mechanism included oxidation of the ANXA2 Cys-8 residue by H_2O_2 and its subsequent reduction by the thioredoxin system. It is known that ITFs (Pasqualetti et al., 2014) and their metabolites (Hamer et al., 2010) exhibit antioxidant effects in intestinal epithelial cells. Recently, such an effect of ITFs has also been found in the liver of piglets (Lepczyński et al., 2017). The antioxidant activity of inulin can be enhanced by polyphenol compounds present in chicory root (Montefusco et al., 2015).

In our study, we also observed a marked up-regulation of actin-related protein 3 (ACTR3) in the mucosa of animals fed diet supplemented with chicory root. This protein is crucial for the formation and stabilisation of intestinal epithelium adherence junctions (Bernadskaya et al., 2011). Additionally, Johnson-Henry et al. (2014) observed an intense rearrangement of actin cytoskeleton in the Caco-2BBe subclones epithelial cell layer after its co-incubation with inulin and fructooligosaccharides. It can be assumed that up-regulation of these proteins

may reflect the improved integrity of the intestinal mucosa. Inulin itself and its fermentation products may influence the development of intestinal mucosa (Lu et al., 2008; Awad et al., 2013). In our study, we observed an increased expression of keratin type I cytoskeletal 20 (KRT20) and keratin type II cytoskeletal 8 (KRT8) in animals fed both supplemented diets in comparison to control animals; these proteins are the most abundant cytokeratins in ileal epithelial cells (Zhou et al., 2003). It has been recently described that incubation of Caco-2 cells in the medium with β-hydroxybutyrate can stimulate its differentiation into absorptive enterocyte phenotype exhibiting strong expression of KRT20, which is a known marker of absorptive epithelial cells (Wang et al., 2017). Higher expression of KRT20 in the absorptive part of the intestine was also confirmed in mouse (Chang et al., 2008). Moreover, larger ileal villi and higher KRT20 expression were observed in broiler chickens fed diet supplemented with probiotic *Enterococcus faecium* (Luo et al., 2013). According to these findings, the increased KRT20 expression, accompanied by ANXA2 up-regulation, may facilitate rapid turnover of cellular proteins. Changes in KRT8 expression may be the effect of SCFA production by inulin-fermenting bacteria, as KRT8 may be involved in butyrate metabolism in colonocytes (Helenius et al., 2015). Additionally, its increased abundance was observed in mucosal cells of probiotic-supplemented piglets (Wang et al., 2012).

Transferrin attracted our great attention from the group of other differentially expressed proteins. This protein is not expressed in the ileum (Idzerda et al., 1986), but it can be transferred to the enterocyte *via* the receptor-mediated endocytosis pathway (Morgan and Oates, 2002). Transferrin receptor (TfR) expression and transferrin abundance in the small intestine mucosa strictly reflect iron status and are inversely correlated with iron level in the enterocyte under physiological conditions (Kolachala et al., 2007). Tako et al. (2008) described the impact of ITFs on duodenal and caecal expression of proteins involved in iron absorption and metabolism at both transcript and protein levels in growing pigs. These authors observed increased TfR expression in the intestine and higher blood haemoglobin concentration in pigs fed for 6 weeks diet supplemented with ITFs in comparison to animals fed standard diet. Furthermore, the up-regulation of genes coding for iron enzymes, receptors and binding protein in the duodenum suggests that inulin itself may directly affect gene expression in enterocytes

(Tako et al., 2008). Additionally, in our previous paper, the analysis performed on the same group of animals showed that pigs fed a diet supplemented with dried chicory root had significantly higher blood plasma iron concentration (30.5 μmol/l) in comparison to animals fed a control diet (15.6 μmol/l) and a diet supplemented with 2% of native chicory inulin (19.8 μmol/l) (Lepczyński et al., 2016). Thus, the increased ileal accumulation of transferrin, found in the present study, may be caused by direct effect of chicory root bioactive compounds leading to the improvement of iron status in growing pigs.

Conclusions

Changes in the expression of proteins associated with energetic metabolism, cell proliferation and cytoskeletal proteins may suggest an impact of dried inulin type fructans-rich chicory root on the functional maturation of the ileal mucosa. Additionally, changes in transferrin abundance suggest the significance of chicory root and native inulin supplementation for iron absorption in the small intestine of growing pigs. Nevertheless, further studies are necessary to better understand the mechanism underlying inulin effect on the ileum of growing pigs.

Acknowledgment

References

AOAC International, 2011. Official Methods of Analysis of AOAC International. Current Through Revision 4. 18th Edition. Gaithersburg, MD (USA)

Awad W.A., Ghareeb K., Paßlack N., Zentek J., 2013. Dietary inulin alters the intestinal absorptive and barrier function of piglet intestine after weaning. Res. Vet. Sci. 95, 249–254, https://doi.org/10.1016/j.rvsc.2013.02.009

Barszcz M., Taciak M., Skomiał J., 2018. Influence of different inclusion levels and chain length of inulin on microbial ecology and the state of mucosal protective barrier in the large intestine of young pigs. Anim. Prod. Sci. 58, 1109–1118, https://doi.org/10.1071/AN16014

Bernadskaya Y.Y., Patel F.B., Hsu H.-T., Soto M.C., 2011. Arp2/3 promotes junction formation and maintenance in the *Caenorhabditis elegans in*testine by regulating membrane association of apical proteins. Mol. Biol. Cell 22, 2886–2899, https://doi.org/10.1091/mbc.e10-10-0862

Bommer U.-A., 2012. Cellular function and regulation of the translationally controlled tumour protein TCTP. Open Allergy J. 5, 19–32, http://doi.org/10.2174/1874838401205010019

Chang J., Chance M.R., Nicholas C. et al., 2008. Proteomic changes during intestinal cell maturation *in vivo*. J. Proteomics 71, 530–546, https://doi.org/10.1016/j.jprot.2008.08.003

Diao H., Jiao A.R., Yu B. et al., 2017. Stimulation of intestinal growth with distal ileal infusion of short-chain fatty acid: a reevaluation in a pig model. RSC Adv. 7, 30792–30806, https://doi.org/10.1039/C7RA03730A

Gałązka I., 2002. The composition of chicory flour of selected chicory cultivars Polanowicka and Fredonia in relation to root sizes and the date of harvest. Zywn.-Nauk Technol. Jakosc 3(32), Suppl., 37–45

Gibson G.R., Hutkins R., Sanders M.E. et al., 2017. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat. Rev. Gastroenterol. Hepatol. 14, 491–502, https://doi.org/10.1038/nrgastro.2017.75

Hamer H.M., Jonkers D.M., Vanhoutvin S.A., Troost F.J., Rijkers G., de Bruine A., Bast A., Venema K., Brummer R.J., 2010. Effect of butyrate enemas on inflammation and antioxidant status in the colonic mucosa of patients with ulcerative colitis in remission. Clin. Nutr. 29, 738–744, https://doi.org/10.1016/j.clnu.2010.04.002.

Hampson D.J., 1986. Alterations in piglet small intestinal structure at weaning. Res. Vet. Sci. 40, 32–40, https://doi.org/10.1016/S0034-5288(18)30482-X

Helenius T.O., Misiorek J.O., Nyström J. et al., 2015. Keratin 8 absence down-regulates colonocyte HMGCS2 and modulates colonic ketogenesis and energy metabolism. Mol. Biol. Cell 26, 2298–2310, https://doi.org/10.1091/mbc.E14-02-0736

Idzerda R.L., Huebers H., Finch C.A., McKnight G.S., 1986. Rat transferrin gene expression: tissue-specific regulation by iron deficiency. Proc. Natl. Acad. Sci. U. S. A. 83, 3723–3727, https://doi.org/10.1073/pnas.83.11.3723

Johnson-Henry K.C., Pinnell L.J., Waskow A.M., Irrazabal T., Martin A., Hausner M., Sherman P.M., 2014. Short-chain fructo-oligosaccharide and inulin modulate inflammatory responses and microbial communities in Caco2-bbe cells and in a mouse model of intestinal injury. J. Nutr. 144, 1725–1733, https://doi.org/10.3945/jn.114.195081

Kolachala V.L., Sesikeran B., and Nair K.M., 2007. Evidence for a sequential transfer of iron amongst ferritin, transferrin and transferrin receptor during duodenal absorption of iron in rat and human. World J. Gastroenterol. 13, 1042–1052, https://doi.org/10.3748/wjg.v13.i7.1042

Lamers R.-J.A.N., Wessels E.C.H.H., van de Sandt J.J.M., Venema K., Schaafsma G., van der Greef J., van Nesselrooij J.H.J., 2003. A pilot study to investigate effects of inulin on Caco-2 cells through in vitro metabolic fingerprinting. J. Nutr. 133, 3080–3084, https://doi.org/10.1093/jn/133.10.3080

Lee D.B., Jamgotchian N., Allen S.G., Kan F.W.K., Hale I.L., 2004. Annexin A2 heterotetramer: role in tight junction assembly. Am. J. Physiol. Renal Physiol. 287, F481–F491, https://doi.org/10.1152/ajprenal.00175.2003

Lepczyński A., Herosimczyk A., Barszcz M., Ożgo M., Taciak M., Skomiał J., 2016. Inulin-type fructans trigger changes in iron concentration and activity of bone metabolism biomarkers in blood plasma of growing pigs. J. Anim. Feed Sci. 25, 343–347, https://doi.org/10.22358/jafs/67471/2016

Lepczyński A., Herosimczyk A., Ożgo M., Marynowska M., Pawlikowska M., Barszcz M., Taciak M., Skomiał J., 2017. Dietary chicory root and chicory inulin trigger changes in energetic metabolism, stress prevention and cytoskeletal proteins in the liver of growing pigs – a proteomic study. J. Anim. Physiol. Anim. Nutr. 101, e225–e236, https://doi.org/10.1111/jpn.12595

Lu J.J., Zou X.T., Wang Y.M., 2008. Effects of sodium butyrate on the growth performance, intestinal microflora and morphology of weanling pigs. J. Anim. Feed Sci. 17, 568–578, https://doi.org/10.22358/jafs/66685/2008

Luo J., Zheng A., Meng K., Chang W., Bai Y., Li K., Cai H., Liu G., Yao B., 2013. Proteome changes in the intestinal mucosa of broiler (*Gallus gallus*) activated by probiotic *Enterococcus faecium*. J. Proteomics 91, 226–241, https://doi.org/10.1016/j.jprot.2013.07.017

Madureira P.A., Hill R., Miller V.A., Giacomantonio C., Lee P.W.K., Waisman D.M., 2011. Annexin A2 is a novel cellular redox regulatory protein involved in tumorigenesis. Oncotarget 2, 1075–1093, https://doi.org/10.18632/oncotarget.375

Masri Al S., Hünigen H., Al Aiyan A., Rieger J., Zentek J., 2015. Influence of age at weaning and feeding regimes on the postnatal morpholo y of the porcine small intestine. J. Swine Health Prod. 23, 186–203

Montefusco A., Semitaio G., Marrese P.P., Iurlaro A., De Caroli M., Piro G., Dalessandro G., Lenucci M.S., 2015. Antioxidants in varieties of chicory (*Cichorium intybus* L.) and wild poppy (*Papaver rhoeas* L.) of Southern Italy. J. Chem. 2015, 923142, http://doi.org/10.1155/2015/923142

Morgan E.H., Oates P.S., 2002. Mechanisms and regulation of intestinal iron absorption. Blood Cells Mol. Dis. 29, 384–399, https://doi.org/10.1006/bcmd.2002.0578

Ozgo M., Lepczynski A., Herosimczyk A., 2015. Two-dimensional gel-based serum protein profile of growing piglets. Turk. J. Biol. 39, 320–327, https://doi.org/10.3906/biy-1408-45

Pasqualetti V., Altomare A., Guarino MP. Et al., 2014. Antioxidant activity of inulin and its role in the prevention of human colonic muscle cell impairment induced by lipopolysaccharide mucosal exposure. PLoS One. 9, e98031. https://doi.org/10.1371/journal.pone.0098031

Paunesku T., Mittal S., Proctić M., Oryhon J., Korolev S.V., Joachimiak A., Woloschak G.E., 2001. Proliferating cell nuclear antigen (PCNA): ringmaster of the genome. Int. J. Radiat. Biol. 77, 1007–1021, https://doi.org/10.1080/09553000110069335

Rekiel A., Bielecki W., Więcek J., Kulisiewicz J., 2010. Histological changes in the small intestinal epithelium in fattening pigs fed selected feed additives. Acta Vet. Brno 79, 67–71, https://doi.org/10.2754/avb201079010067

Sheverdin V., Jung J., Lee K., 2013. Immunohistochemical localization of translationally controlled tumor protein in the mouse digestive system. J. Anat. 223, 278–288, https://doi.org/10.1111/joa.12077

Tako E., Glahn R.P., Welch R.M., Lei X., Yasuda K., Miller D.D., 2008. Dietary inulin affects the expression of intestinal enterocyte iron transporters, receptors and storage protein and alters the microbiota in the pig intestine. Br. J. Nutr. 99, 472–480, https://doi.org/10.1017/S0007114507825128

Wang Q., Zhou Y., Rychahou P., Fan T.W.-M., Lane A.N., Weiss H.L., Evers B.M., 2017. Ketogenesis contributes to intestinal cell differentiation. Cell Death Differ. 24, 458–468, https://doi.org/10.1038/cdd.2016.142

Wang X., Yang F., Liu C., Zhou H., Wu G., Qiao S., Li D., Wang J., 2012. Dietary supplementation with the probiotic *Lactobacillus fermentum* I5007 and the antibiotic aureomycin differentially affects the small intestinal proteomes of weanling piglets. J. Nutr. 142, 7–13, https://doi.org/10.3945/jn.111.147074

Wu R.Y., Abdullah M., Määttänen P., Pilar A.V., Scruten E., Johnson-Henry K.C., Napper S., O'Brien C., Jones N.L., Sherman P.M., 2017. Protein kinase C δ signaling is required for dietary prebiotic-induced strengthening of intestinal epithelial barrier function. Sci. Rep. 7, 40820, https://doi.org/10.1038/srep40820

Yasuda K., Dawson H.D., Wasmuth E.V., Roneker C.A., Chen C., Urban J.F., Welch R.M., Miller D.D., Lei X.G., 2009. Supplemental dietary inulin influences expression of iron and inflammation related genes in young pigs. J. Nutr. 139, 2018–2023, https://doi.org/10.3945/jn.109.110528

Zhou Q., Toivola D.M., Feng N., Greenberg H.B., Franke W.W., Omary M.B., 2003. Keratin 20 helps maintain intermediate filament organization in intestinal epithelia. Mol. Biol. Cell 14, 2959–2971, https://doi.org/10.1091/mbc.E03-02-0059