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EFFECTS OF DIFFERENT SOURCES OF FAT IN THE DIET OF PIGS ON THE LIVER TRANSCRIPTOME ESTIMATED BY RNA-SEQ*

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Abstract

In this study, we have attempted to analyse the impact of dietary fats on the liver transcriptome in pigs. Four nutritional groups were created. The animals' diets differed among groups in terms of the presence of corn dried distillers' grains with solubles (DDGS) (group I – no DDGS, groups II, III, IV– 20% DDGS) as well as the type of fat (rapeseed oil – groups I and II, beef tallow – group III, coconut oil – group IV) used. Using the RNA-Seq method we identified 39 differentially expressed genes (DEGs) as a result of Cuffdiff analysis of the differences among all groups. Analysis of these genes with Panther Gene Classification System revealed that among identified DEGs, genes responsible for lipid and fatty acids metabolism are overrepresented as well as the genes engaged in oxidoreductase and catalytic activity. The article presents for the first time the RNA-seq analysis of the liver transcriptome in pigs fed with different sources of fats. The results may be useful for the elaboration of new therapies for cardiovascular diseases in humans as well as for the preparation of new nutrition strategies in animals.

Key words: nutrigenomics, RNA-seq, transcriptome, fatty acids, pigs

Diet is one of the major environmental factors that affect obesity and health status in humans and carcass performance in farm animals. A growing amount of evidence suggests that substances present in food affect gene expression; however, little is known about how nutrients act at the molecular level. It is well proven that several transcription factors (PPARs, SREBPs) are nutrient sensors and influence the expression of other genes engaged in metabolism (Müller and Kersten, 2003). Moreover, many experiments have demonstrated the role of nutrition in epigenetic modifica-

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tion (Jimenez-Chillaron et al., 2012). Nonetheless, nutrigenomics is still in its early stages, and understanding the relation between genomes and nutrition is one of the major goals of bioscience.

The impact of dietary fats on human and animal health has been under investigation for many years. At present the positive correlation between dietary saturated fatty acid (SFA) intake and plasma cholesterol and mortality from cardiovascular disease (CVD) has been proven. However, dietary SFAs are often replaced by carbohydrates, which are not effective in CVD prevention (Michas et al., 2014). Therefore, strong evidence for the CVD-reducing effects of particular sources of fats is necessary in order to prepare appropriate dietary recommendations.

Another issue is the potential for modification of an animal product's features through feeding. Consumers demand healthy, high-quality products. Unfortunately, sometimes it is difficult to achieve these two attributes simultaneously. A high content of unsaturated fatty acids (UFA) is beneficial in terms of CVD risk, yet it lowers the quality parameters of animal fat, which becomes more susceptible to oxidation. Animal nutrition specialists elaborate feeding mixtures so as to achieve a compromise between the costs of these mixtures and the quality parameters and beneficial impacts on human health of animal products. Modern molecular biology offers a wide range of tools to help us to understand the mechanisms involved in modulating these traits (Li et al., 2015; Tan et al., 2011).

Dried distillers' grains with solubles (DDGS) is a by-product obtained during grain fermentation for ethanol production, widely used in animal feeding due to its reasonable price, high protein content and good quality (Agyekum et al., 2014). DDGS contains high amounts of unsaturated fatty acids. This is beneficial from the point of view of human nutrition, because animals fed with DDGS show a high content of UFA in their tissue. However, the fat of these animals is of low technological quality; therefore, feeding with DDGS should be supplemented with additives that reduce the oxidation of lipids. The addition of fats containing high amounts of SFA to pigs' diets improves pork fat technological parameters by balancing the UFA/SFA ratio. Beef tallow, chosen for the present experiment, is the most popular animal fat, being cheap and easily available. It contains about 58% SFA, mostly palmitic (36%) and stearic (18%) acids, but also large amounts of cholesterol, in contrast to coconut or rapeseed oils. Coconut oil is very rich in SFA (95%) compared to other vegetable oils. Its popularity on the European market is growing, since it features protective action against CVD. It increases serum HDL, reduces total cholesterol, triglyceride, and phospholipid levels in blood serum and heart, liver and kidney tissue (Nevin and Rajamohan, 2004), and improves antioxidant enzyme activities in various tissues (Nevin and Rajamohan, 2006). Coconut oil contains high amounts of medium long-chain fatty acids, which are easily digested and immediately used as an energy source. It is composed of ~50% lauric acid, which, after conversion into monolaurin, possesses some health-promoting antibacterial and antiviral properties. In contrast, rapeseed oil contains a low level (7%) of SFAs but substantial amounts of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) (61% oleic, 21% linoleic and 11% alpha-linolenic acid) known to be cardioprotective substances.

RNA-seq is a new cutting-edge technology which enables quantitative analysis of a whole transcriptome. This technique has been used several times in exploring porcine transcriptomes from different tissues (Esteve-Codina et al., 2011; Jung et al., 2012; Ramayo-Caldas et al., 2012; Samborski et al., 2013; Gunawan et al., 2013). The results mostly confirm the strong correlation between RNA-seq and microarray or quantitative PCR (qPCR) and prove the great potential of this method in genome-wide studies. To date, RNA-seq has not been utilised for screening transcriptome changes as a response to different diets in pigs. Nonetheless, several microarray experiments have described the transcriptome changes induced by particular forms of nutrition (Jun et al., 2010; Keller et al., 2011; Döring et al., 2013). Jun et al. (2010) identified 648 DEGs in pigs fed with different dietary amylose and amylopectin ratios, while Keller et al. (2011) observed altered gene expression in the skeletal muscle of pigs fed with a carnitine-supplemented diet.

Recently, analysis of the liver transcriptome of pigs with extreme phenotypes for intramuscular fatty acid composition showed potential gene networks which affect fatty acid metabolism in the liver (Ramayo-Caldas et al., 2012). These results indicate strong interactions between gene expression in the liver and meat quality.

The aim of our experiment was to determine the effect on the liver transcriptome of various types of fat added to feedstuff in pigs given a mixture with or without corn DDGS, as the liver is a crucial organ in lipid metabolism. We hoped to provide new information on the potential for inducing gene expression changes through nutrition. Moreover, determination of the effects of different sources of dietary fat on the liver transcriptome will help us to understand the molecular processes involved in modulating lipid metabolism in animals and humans.

Material and methods

Animals

Twenty-four fatteners (12 gilts and 12 barrows) originating from (Polish Landrace × Polish Large White) sows mated with (Duroc × Pietrain) boars were divided into 4 groups, with 6 pigs in each (3 gilts and 3 barrows). Animals were kept in individual straw-bedded pens with free access to water. The fattening experiment continued from about 60 kg to 118 kg of body weight (BW). During this period the body weights of all animals were checked every two weeks. All procedures relating to the use of live animals included in the experiment were in agreement with the local Ethics Committee for Experiments with Animals.

Diets and treatment

All groups obtained diets with similar energy and protein concentrations covering the nutritional requirements of pigs (~13.4 MJ ME, ~165 g crude protein, ~9.1 g Lys, ~5.9 g Met+Cys, ~5.8 g Tre, ~1.9 g Trp, crude fat ~49 g/Kg). Mash diets included barley, wheat, corn, wheat bran, soybean meal, vitamin-mineral additives and crystalline amino acids, but differed among groups in terms of the presence of

corn DDGS (groups II, III, IV – 20%) as well as the type of fat used (rapeseed oil – groups I and II, beef tallow – group III, coconut oil – group IV). Feed mixtures differed significantly in their profile of fatty acids and SFA/UFA ratios (Table 1). In feed mixtures for groups III and IV, a higher amount of saturated fatty acids (SFA) and lower iodine value of fat was noted when compared to groups I and II. All pigs were fed restricted feed amounts twice a day, according to body weight: from 2.8 kg d^{-1} at 61–70 kg BW and 3.0 kg $^{d-1}$ at 71–80 kg BW to 3.2 kg $^{d-1}$ at 80+ kg of BW. Dietary intervention lasted 59–61 days.

Table 1. Fatty-acid profile (g/100 g of estimated fatty acids) of feed mixtures used in the experiment

	Group I feed mixture with rapeseed oil –DDGS	Group II feed mixture with rapeseed oil + DDGS	Group III feed mixture with beef tallow + DDGS	Group IV feed mixture with coconut oil + DDGS
SFA[1]	17.35	15.5	31.6	53.9
UFA[1]	80.9	83.4	67.5	45.6
MUFA[1]	44.4	36.4	32.4	15.7
PUFA *n-6*[1]	30.4	41.1	32.1	27.7
PUFA *n-3*[1]	6.04	5.80	2.98	2.16
PUFA[1]	36.4	46.9	35.1	29.9
SFA/UFA ratio	0.21	0.19	0.47	1.18
IV (g/100 g[2])	104.5	116.2	90.5	66.6

[1]Sum of saturated (SFA), unsaturated (UFA), monounsaturated (MUFA), polyunsaturated (PUFA) fatty acids.
[2]IV – iodine value of fat.

Collection of samples and chemical analysis

At the end of the fattening experiment, all pigs were slaughtered. Immediately after slaughter, samples of livers for gene expression analysis were collected from 6 animals in each group; immediately after separation, samples were frozen in liquid nitrogen and kept in a freezer (–85ºC). Carcass cuts were evaluated according to standard methods used at the Pig Performance Testing Stations. The fatty acids in the feed mixtures were determined as methyl esters in hexane using gas chromatography (ISO, 2011). Percentages of individual fatty acids were used to calculate the iodine value (IV) of fat according to the following equation (AOCS, 1998): $IV\ (g/100\ g) = (\%C_{16:1}) \times 0.95 + (\%C_{18:1}) \times 0.86 + (\%C_{18:2}) \times 1.732 + (\%C_{18:3}) \times \times\ 2.616 + (\%C_{20:1}) \times 0.785 + (\%C_{22:1}) \times 0.723$.

RNA isolation

RNA was isolated with Trireagent (Life Technologies, USA) according to Chomczyński and Sacchi (1987) and additionally purified using lithium chloride precipitation (Life Technologies). RNA concentration was estimated with the use of NanoDrop (Thermoscientific, Wilmington, USA) and RNA integrity was evaluated with the use of 2% agarose gel electrophoresis.

NGS library preparation

Indexed cDNA libraries were constructed using 300 ng of total RNA from 14 samples (three or four per group) and the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA) as decribed in Ropka-Molik et al. (2014). From 24 samples, we chose only those with the best RNA integrity. The following steps were undertaken in accordance with the manufacturer's protocol: RNA purification and fragmentation (poly-A selection, denaturation), synthesis of first and second strand of cDNA, end-repair and A-tailing of cDNA, ligation of adaptors. The libraries were amplified in 15 cycles of PCR. The size of the obtained libraries' fragments was approximately 260 bp.

Whole transcriptome sequencing

Whole transcriptome sequencing was performed in two separate runs, each with different parameters. HiSeq Version 3 flow cell clustering was performed using TruSeq Cluster Kit v3-cBot-HS (Illumina). Samples (after pooling) were diluted to a final concentration of 10 pM according to the manufacturer's protocol and loaded into two separate flow cells (two and three samples per lane). Sequencing by synthesis of the libraries was performed on a HiScanSQ System with 50 single-end cycles and 200 paired-end cycles using a TruSeq SBS Kit v3-HS (Illumina). The raw sequences and demultiplexing of samples were obtained using CASAVA software (Illumina).

Aligning raw reads to the pig transcriptome

The Illumina raw sequences were controlled for quality using FastQC software. Flexbar software (Dodt et al., 2012) was used for trimming low quality sequences and polyA stretches. Reads shorter than 32 base pair (bp) and reads with qualities lower than 10 were removed from the dataset. Trimmed sequences were aligned to *Sus scrofa* genome assembly (Sscrofa 10.2.70) with a transcriptome reference file containing 26,126 gene transcripts annotated in an Ensembl database (Flicek et al., 2012).

Alignment to reference sequences was conducted using Bowtie2 software (Langmead and Salzberg, 2012) implemented in a TopHat splice junction mapper (Trapnell et al., 2009). Following this step, mapping quality statistics were generated in RNA-SeQC (Deluca et al., 2012).

Analysis of differentially expressed genes

Differential expression analysis (DE) was performed using Cufflinks software (Trapnell et al., 2010). The DE model fitted sex (gilts and barrows), library type (single-end and paired-end) and treatment effect. Differentially expressed genes and their corresponding P-values were determined using tests based on negative binomial distribution, which can reflect these properties. All obtained p-values were adjusted for false discovery rate with multiple testing procedures used to control for type I errors (Benjamini and Hochberg, 1995). The level of statistical significance for differently expressed genes between groups was set for the adjusted P-value (false discovery rate – FDR control) <0.05.

Reverse transcription and qPCR

Reverse transcription and qPCR has been described previously (Świątkiewicz et al., 2016). In brief, 24 samples (6 for each group) were reverse-transcribed using a cDNA Archive Kit (Life Technologies) and qPCR on an Eco instrument (Illumina) was performed using the GoTaq Probe qPCR mastermix (Promega) and TaqMan assays (Life Technologies) in triplicate for each sample. The relative quantity of mRNA was calculated according to Pfaffl (2001) and the statistical analyses were performed with SAS software (GLM procedure) with feed and sex as the fixed factors.

Statistical analysis of carcass backfat quality, IMF content and liver weight

Analyses of the treatment effect on carcass backfat quality, IMF content and liver weight were conducted by one-way analysis of variance with comparison of means using Duncan's multiple range test and Statistica 12.0.

Results

RNA-seq statistics

After RNA-seq, 14 liver samples collected from animals fed with different diets were analysed; between 6.5 and 41.5 million raw reads were obtained per sample. The percentage of alignment to the reference pig genome assembly Sscrofa 10.2 amounted to 90.93–92.68% and 78.29–79.60% for single-end and paired-end sequencing, respectively (Table 2). The exonic rate of mapped reads was high when compared to previous RNA-seq experiments in pigs (0.712–0.833) (Ramayo-Caldas et al., 2012) (Figure 1).

Table 2. Samples used in the experiment. RNA-seq statistics

Sample number	Pooling	Sex	Type of library	Number of raw reads	Number of reads after filtration	Number of mapped reads	Percentage of aligned reads
+DDGS+Beef tallow1	1	boar	pe	29240298	28152218	22107052	78.53%
+DDGS+Beef tallow2	1	boar	pe	27462794	26464106	20825348	78.69%
+DDGS+Beef tallow3	2	boar	se	10233066	10144025	9326122	91.94%
+DDGS+Beef tallow4	2	sow	se	6539747	6032004	5506216	91.28%
+DDGS+coconut oil1	1	sow	pe	27849242	26800682	21332548	79.60%
+DDGS+coconut oil2	1	boar	pe	30936782	29866142	23510782	78.72%
+DDGS+coconut oil3	2	boar	se	7874454	6789180	6173287	90.93%
+DDGS+rapseed oil1	1	boar	pe	34335890	33102680	26036008	78.65%
+DDGS+rapseed oil2	1	sow	pe	41577520	40209447	31974770	79.52%
+DDGS+rapseed oil3	2	boar	se	10307396	10182879	9437546	92.68%
–DDGS+rapseed oil1	1	boar	pe	29991326	28974628	22685577	78.29%
–DDGS+rapseed oil2	1	sow	pe	28495336	27484941	21594889	78.57%
–DDGS+rapseed oil3	2	boar	se	7306659	6501289	5925507	91.14%
–DDGS+rapseed oil4	2	sow	se	12097712	12005725	11109872	92.54%

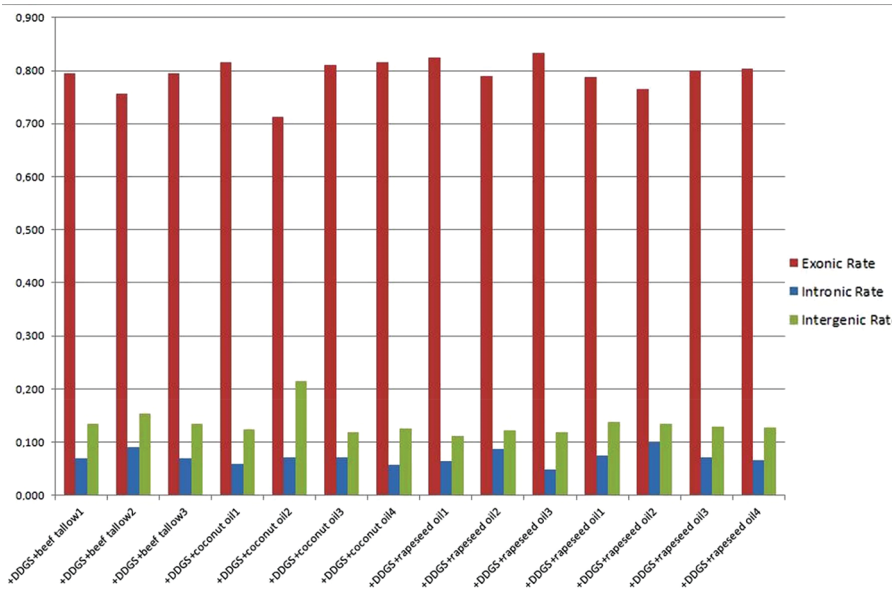

Figure 1. Proportion of exonic, intronic and intergenic regions aligned to the reference genome Sus scrofa 10.2

Differentially expressed gene (DEG) analysis by Cuffdiff software and Panther Classification System

Cuffdiff analysis revealed 39 DEGs (Table 3) when all analysed groups were compared to each other (P adjusted <0.05). We performed a functional classification of identified 39 DEGs using the Panther Classification System (http://www.pantherdb.org/). For 33 genes, we found mapped IDs in the database. The highest number of genes were involved in metabolism (21), cellular processes (15), localisation (9) and development (8) (data not shown). We also performed a statistical overrepresentation test (http://www.pantherdb.org/) with Bonferroni corrections and found that among PANTHER GO-Slim Biological Process two processes were overepresented: fatty acid metabolic process (GO:0006631) (P<0.00019) and lipid metabolic process (GO:0006629) (P<0.000126) (Table 4). Genes engaged in these processes were: *ENSSSCG00000021940*, *ENSSSCG00000024537*, *CYP7A1*, *HMGCS1*, *APOA4*, *CYP2C49*, *ACSL5*, *PLIN2*, *CYP2B22* and *ABCD3*. Moreover, a significant Panther pathway – a nicotinic acetylcholine receptor signaling pathway with three genes (ACTA1, ACTA2, MYH1) of different expression was found (P<0.048) (Table 4).

In addition, among molecular functions, oxidoreductase activity (GO:0016491) (genes: *ENSSSCG00000021940, CYP7A1, ENSSSCG00000011148, ENSSSCG00000024537, GSTO1, CYP2C49, CYP2B22)* and catalytic activity (GO:0003824) (genes: *ENSSSCG00000021940, CYP7A1, ENSSSCG00000011148, ENSSSCG00000024537, GSTO1, CYP2C49, CYP2B22, HMGCS1, APDA4, ENSSSCG00000022724, CKM, PMM1, KIF1A, ACSL5, MYH1, SERPINA1, CCRN4L, AMPD3)* were overrepresented (P<0.008, P<0.027 respectively) (Table 4).

Table 3. Results of the differential expression gene analysis using Cuffdiff software. Q-value – P-value after Bonferroni correction. FPKM (fragments per kilobase of exon per million fragments mapped)

Gene	Group I	Group II	FPKM	FPKM	Log2 (fold-change)	P-value	Q-value
1	2	3	4	5	6	7	8
CYP7A1	–DDGS+rapeseed oil	+DDGS+beef tallow	29.28	4.18	–2.81	0.00	0.00
MYH1	–DDGS+rapeseed oil	+DDGS+beef tallow	4.84	0.87	–2.47	0.00	0.04
CH242-185F9.2	–DDGS+rapeseed oil	+DDGS+beef tallow	33.28	6.99	–2.25	0.00	0.05
CHST7	–DDGS+rapeseed oil	+DDGS+beef tallow	0.66	4.99	2.93	0.00	0.05
CYP2C49	–DDGS+rapeseed oil	+DDGS+beef tallow	47.91	366.41	2.94	0.00	0.01
ZFAND2A	–DDGS+rapeseed oil	+DDGS+beef tallow	6.15	49.54	3.01	0.00	0.00
FST	–DDGS+rapeseed oil	+DDGS+beef tallow	2.35	19.65	3.06	0.00	0.01
APOA4	–DDGS+rapeseed oil	+DDGS+beef tallow	219.53	2000.82	3.19	0.00	0.04
ENSSSCG00000024537	–DDGS+rapeseed oil	+DDGS+beef tallow	0.43	6.09	3.82	0.00	0.01
ACSL5	–DDGS+rapeseed oil	+DDGS+beef tallow	0.14	2.99	4.37	0.00	0.03
GSTO1	–DDGS+rapeseed oil	+DDGS+beef tallow	0.65	69.07	6.73	0.00	0.00
SAA4	–DDGS+rapeseed oil	+DDGS+coconut oil	642.51	5.33	–6.91	0.00	0.00
KLHL8	–DDGS+rapeseed oil	+DDGS+coconut oil	5.71	0.80	–2.84	0.00	0.04
AMPD3	–DDGS+rapeseed oil	+DDGS+coconut oil	8.51	1.80	–2.24	0.00	0.04
AHNAK	–DDGS+rapeseed oil	+DDGS+coconut oil	4.40	1.02	–2.11	0.00	0.05
PLIN2	–DDGS+rapeseed oil	+DDGS+coconut oil	4.55	20.59	2.18	0.00	0.02
CYP2B22	–DDGS+rapeseed oil	+DDGS+coconut oil	5.08	23.75	2.22	0.00	0.00
PMM1	–DDGS+rapeseed oil	+DDGS+coconut oil	8.48	39.71	2.23	0.00	0.00
ABCD3	–DDGS+rapeseed oil	+DDGS+coconut oil	8.30	39.87	2.26	0.00	0.00
CYP2C49	–DDGS+rapeseed oil	+DDGS+coconut oil	47.91	237.82	2.31	0.00	0.03
TMP-CH242-74M17.6	–DDGS+rapeseed oil	+DDGS+coconut oil	20.27	108.53	2.42	0.00	0.03
ENSSSCG00000027008	–DDGS+rapeseed oil	+DDGS+coconut oil	22.82	132.21	2.53	0.00	0.01

HBA	–DDGS+rapeseed oil	+DDGS+coconut oil	233.50	1498.23	2.68	0.00	0.04
FGF13	–DDGS+rapeseed oil	+DDGS+coconut oil	0.88	6.63	2.91	0.00	0.00
G0S2	–DDGS+rapeseed oil	+DDGS+coconut oil	13.14	100.18	2.93	0.00	0.00
APOA4	–DDGS+rapeseed oil	+DDGS+coconut oil	219.53	2754.34	3.65	0.00	0.00
KIF1A	–DDGS+rapeseed oil	+DDGS+rapeseed oil	1.24	0.02	–5.68	0.00	0.05
TNNC2	–DDGS+rapeseed oil	+DDGS+rapeseed oil	21.63	2.66	–3.02	0.00	0.04
FOS	–DDGS+rapeseed oil	+DDGS+rapeseed oil	20.43	2.88	–2.83	0.00	0.00
CKM	–DDGS+rapeseed oil	+DDGS+rapeseed oil	11.27	1.65	–2.77	0.00	0.05
MYH1	–DDGS+rapeseed oil	+DDGS+rapeseed oil	4.84	0.78	–2.64	0.00	0.03
ACTA1	–DDGS+rapeseed oil	+DDGS+rapeseed oil	26.93	5.07	–2.41	0.00	0.04
ENSSSCG00000011148	–DDGS+rapeseed oil	+DDGS+rapeseed oil	4.64	32.62	2.81	0.00	0.02
ENSSSCG00000027008	–DDGS+rapeseed oil	+DDGS+rapeseed oil	22.82	193.86	3.09	0.00	0.03
CYP2B22	–DDGS+rapeseed oil	+DDGS+rapeseed oil	5.08	53.67	3.40	0.00	0.00
ENSSSCG00000022724	–DDGS+rapeseed oil	+DDGS+rapeseed oil	0.08	16.24	7.64	0.00	0.00
FGF13	+DDGS+coconut oil	+DDGS+beef tallow	6.63	0.26	–4.65	0.00	0.00
ENSSSCG00000027008	+DDGS+coconut oil	+DDGS+beef tallow	132.21	15.74	–3.07	0.00	0.00
HMGCS1	+DDGS+coconut oil	+DDGS+beef tallow	20.65	4.66	–2.15	0.00	0.02
ZFAND2A	+DDGS+coconut oil	+DDGS+beef tallow	10.61	49.54	2.22	0.00	0.03
ENSSSCG00000004170	+DDGS+coconut oil	+DDGS+beef tallow	3.94	29.68	2.91	0.00	0.01
FST	+DDGS+coconut oil	+DDGS+beef tallow	2.56	19.65	2.94	0.00	0.02
CH242-51E15.1	+DDGS+coconut oil	+DDGS+beef tallow	0.48	6.33	3.72	0.00	0.03
ACSL5	+DDGS+coconut oil	+DDGS+beef tallow	0.06	2.99	5.69	0.00	0.05
SAA4	+DDGS+coconut oil	+DDGS+beef tallow	5.33	330.25	5.95	0.00	0.00
GSTO1	+DDGS+coconut oil	+DDGS+beef tallow	0.49	69.07	7.14	0.00	0.00

Table 3 – contd.

1	2	3	4	5	6	7	8
ENSSSCG00000022724	+DDGS+rapeseed oil	+DDGS+beef tallow	16.24	0.17	–6.60	0.00	0.00
FGF13	+DDGS+rapeseed oil	+DDGS+beef tallow	5.18	0.26	–4.29	0.00	0.01
ENSSSCG00000027008	+DDGS+rapeseed oil	+DDGS+beef tallow	193.86	15.74	–3.62	0.00	0.00
CYP7A1	+DDGS+rapeseed oil	+DDGS+beef tallow	30.47	4.18	–2.87	0.00	0.00
C5ORF13	+DDGS+rapeseed oil	+DDGS+beef tallow	12.58	1.81	–2.80	0.00	0.02
TMP-CH242-74M17.5	+DDGS+rapeseed oil	+DDGS+beef tallow	57.08	9.96	–2.52	0.00	0.03
HMGCS1	+DDGS+rapeseed oil	+DDGS+beef tallow	26.70	4.66	–2.52	0.00	0.04
ZFAND2A	+DDGS+rapeseed oil	+DDGS+beef tallow	8.24	49.54	2.59	0.00	0.03
CCRN4L	+DDGS+rapeseed oil	+DDGS+beef tallow	6.09	41.66	2.77	0.00	0.04
ACTA2	+DDGS+rapeseed oil	+DDGS+beef tallow	7.82	56.35	2.85	0.00	0.03
ENSSSCG00000024537	+DDGS+rapeseed oil	+DDGS+beef tallow	0.71	6.09	3.10	0.00	0.05
FST	+DDGS+rapeseed oil	+DDGS+beef tallow	0.78	19.65	4.65	0.00	0.00
ACSL5	+DDGS+rapeseed oil	+DDGS+beef tallow	0.07	2.99	5.52	0.00	0.01
GSTO1	+DDGS+rapeseed oil	+DDGS+beef tallow	0.30	69.07	7.86	0.00	0.00
SAA4	+DDGS+rapeseed oil	+DDGS+coconut oil	317.50	5.33	–5.90	0.00	0.00
ENSSSCG00000022724	+DDGS+rapeseed oil	+DDGS+coconut oil	16.24	0.40	–5.34	0.00	0.00
ENSSSCG00000021940	+DDGS+rapeseed oil	+DDGS+coconut oil	9.67	0.55	–4.15	0.00	0.00
ACTA1	+DDGS+rapeseed oil	+DDGS+coconut oil	5.07	29.29	2.53	0.00	0.02
SERPINA11	+DDGS+rapeseed oil	+DDGS+coconut oil	17.13	99.57	2.54	0.00	0.03
CKM	+DDGS+rapeseed oil	+DDGS+coconut oil	1.65	11.72	2.83	0.00	0.04

Table 4. Results of the Panther Classification System statistical overrepresentation test of Cuffdiff results

Type of analysis		Sus scrofa REFLIST (21483)	Observed	Expected	Over/ under	Fold Enrichment	P-value
PANTHER GO-Slim Biological Process	fatty acid metabolic process (GO:0006631)	214	6	0.33	+	> 5	1.91E-04
	lipid metabolic process (GO:0006629)	889	10	1.37	+	> 5	1.26E-04
PANTHER GO-Slim Molecular Function	oxidoreductase activity (GO:0016491)	650	7	1	+	> 5	8.38E-03
	catalytic activity (GO:0003824)	5167	18	7.94	+	2.27	2.71E-02
PANTHER Pathways	nicotinic acetylcholine receptor signaling pathway (P00044)	87	3	0.13	+	> 5	4.86E-02

Effect of dietary lipid composition on gene expression in relation to backfat tissue and meat quality

We observed no significant effects of the experimental treatment on animals' growth performance. During the experimental period, in all groups, average weight gains amounted to 958–991 g per day and feed utilisation equalled 3.0–3.2 kg/kg. On the other hand, a significant effect of dietary fat composition on backfat tissue quality was found and described in detail elsewhere (Świątkiewicz et al., 2016). In brief, the relationship between fatty-acid content in the diet and in fat tissue was confirmed using a Pearson correlation. The correlation coefficients (R) between dietary SFA, UFA, MUFA, PUFA, and iodine value and the same parameters in pig backfat were positive, ranging from 0.77 to 0.92 ($P<0.01$). In the backfat of pigs fed diets with corn DDGS, significantly higher (by 8%) iodine and (by 20%) TBA-RS values, indicating high susceptibility to the lipid oxidative process, were observed when compared with group I. The addition of beef tallow or coconut oil to feed mixtures containing DDGS led to the accumulation of more SFA in backfat tissue in comparison to pigs obtaining the same feed mixture with rapeseed oil. The SFA/UFA ratio, iodine values and TBA-RS in the group receiving beef tallow were similar to the results found in the control group I (–DDGS + rapeseed oil). The backfat of pigs obtaining coconut oil in their feed mixture had the highest content of SFA ($P<0.001$), the lowest iodine value ($P<0.001$) and the highest resistance to lipid oxidation (TBA-RS lower by 28–40% in comparison to the remaining groups) (Świątkiewicz et al., 2016).

Dietary treatment had no significant influence on carcass backfat thickness (Table 5); however, an insignificant tendency for lower backfat thickness in pigs receiving DDGS (group II) in comparison to animals fed the same mixture but without DDGS (group I) was noticed.

Table 5. Parameters of carcass backfat quality, IMF content and liver weight in pigs fed with different diets

	Group I (control) feed mixture with rapeseed oil	Group II feed mixture with rapeseed oil + DDGS	Group III feed mixture with beef tallow + DDGS	Group IV feed mixture with coconut oil + DDGS	P-level	SEM
Average backfat thickness from 5 measurements (cm)	2.36	2.04	2.08	2.21	ns*	0.065
Backfat thickness at point "C" (cm)	1.13	0.93	0.90	1.04	ns	0.039
Neck backfat with skin (kg)	0.98	0.89	0.97	1.02	ns	0.088
Ham backfat with skin (kg)	1.44	1.31	1.28	1.40	ns	0.057
Loin backfat with skin (kg)	2.19	1.96	1.95	2.37	ns	0.027
Inner-carcass fat (kg)	0.76	0.66	0.63	0.62	ns	0.036
M. Longissimus fat content (%)	0.92 A	1.12 AB	1.59 B	1.56 B	0.019	0.096
Liver (kg)	1.84	1.83	1.81	1.65	ns	0.036

A, B – means with different letters are significantly different.

A higher content of intramuscular fat was observed in *longissimus* muscles in experimental pigs receiving beef tallow or coconut oil (Table 5). This finding is especially valuable, because modern selection for high meat content results in pork with a low IMF content, i.e. low-quality pork with insufficient tenderness and juiciness. Proper IMF content significantly determines the meat quality trait mentioned above and improves taste properties as well as positively affecting the technological processes related to heat treatment of the meat (Tyra and Żak, 2012).

Validation of RNA-seq by results of qPCR

Six DEGs (*APOA4*, *ACSL5*, *CYP7A1*, *CYP2BC9*, *CYP2B22*, *GSTO1*) were selected in order to compare their expression in the feeding groups by qPCR. All 24 samples were analysed, in contrast to the RNA-seq experiment in which only 14 samples were examined. Pearson correlation analysis, performed by comparing mean Relative Quantity (RQ) and mean FPKM (fragments per kilobase of exon per million fragments mapped) for groups, showed that the RNA-seq results were highly correlated with qPCR data ($r=0.8$; $P<0.001$) despite the different number of analysed samples. The detailed description of the changes in the expression levels of the analysed genes is included elsewhere (Świątkiewicz et al., 2016). In brief, statistical analysis (GLM) with sex as a fixed factor revealed that the expression of none of the genes (*APOA4*, *ACSL5*, *CYP7A1*, *CYP2BC9*, *CYP2B22*, *GSTO1*) was influenced by sex. We could not confirm expression differences for *CYP7A1*, while all other genes, i.e. *APOA4*, *ACSL5*, *CYP2C499*, *CYP2B22*, and *GSTO1*, showed significant differences between groups (Figure 2). Animals obtaining coconut oil in the feedstuff showed the highest expression of *APOA4, CYP2C49, CYP2B22* genes, while *GSTO1* gene was the most expressed in the animals receiving beef tallow (Figure 2).

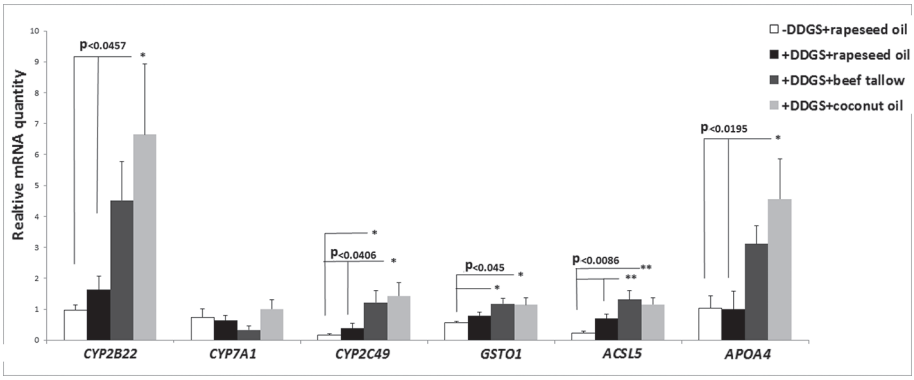

Figure 2. Relative expression of mRNA of selected genes after different diet treatments

Discussion

Panther Classification System analysis of DEGs identified in this study revealed overrepresentation of genes engaged in fatty acids and lipid metabolic processes (Table 4). This observation is in a great agreement with the assumption of the study and shows that nutrition may induce changes in the expression of genes associated with metabolic processes. Among the DEGs identified by Cuffdiff software, there were several genes from the cytochrome P450 family (*CYP2C49*, *CYP2B22*), which are known to be responsible for lipid homeostasis (Ramayo-Caldas et al., 2012). Several previous studies identified genes from the cytochrome P450 family as DEGs in RNA-seq or microarray experiments. One of the experiments compared the liver transcriptome profile with extreme phenotypes of intramuscular fatty acid (IMF) composition and found that *CYP7A1*, *CYP2C49*, *CYP4A11*, and *CYP2C19* are differentially expressed between high and low IMF groups (Ramayo-Caldas et al., 2012). IMF content is one of the major factors determining meat quality in pigs. Our results, being in agreement with previous results, show that genes from the CYP family may be important regulators of the taste of meat.

Moreover, Gunawan et al. (2013) observed that *CYP2B22*, *CYP2C33*, *CYP4A11* and *CYP4B1* are differentially expressed in the testes of boars with high and low androstenone levels, while *CYP7A1* is differentially expressed in the liver of the animals analysed. Similarly, *CYP7A1*, *CYP11A1*, *CYP26B1* and *CYP4F11* were differentially expressed in the livers of lactating dairy cows divergent in negative energy balance (McCabe et al., 2012), while the level of expression of the genes *Cyp2b10, Cyp3a11* and *Cyp4a10* in mouse livers was changed by non-alcoholic fatty liver disease induced by a high-fat diet (Kirpich et al., 2011). Finally, hepatic gene expression profiling of obesity induced by a long-term high-fat diet in a mouse model revealed changes in *Cyp3a11* and *Cyp4a10* genes (Kim et al., 2004). Taken together, these data strongly support the important function of *CYP* genes in lipid metabolism.

In addition, genes involved in lipid metabolic processes or their homologs have been identified in several previous studies concerning liver transcriptome changes as a result of different diet conditions. It has been shown that the *APOA2* and *APOB* genes are overexpressed in the livers of pigs with high contents of IMF in muscle (Ramayo-Caldas et al., 2012). Moreover, a study by Kirpich et al. (2011) demonstrated that expression of *Apoa4* mRNA is upregulated, while *HMGCS1* is downregulated, in the livers of mice on a high-fat diet. Upregulation was also observed in protein levels for *ApoE*. Differential expressions of *APOA4* and *ABCD1* were also noticed in a very similar experiment performed on mice fed with low- and high-fat diets (Partridge et al., 2014).

On the other hand, among molecular functions, oxidoreductase activity and catalytic activity were overrepresented (Table 4). This is in concordance with the observations that genes engaged in metabolic processes are over or under expressed depending on the diet. Genes assigned to oxidoreductase and catalytic activity are mainly catalytic enzymes responsible for metabolic processes.

The only affected metabolic pathway in the present study was the nicotinic acetylcholine receptor signaling (P00044). This result is difficult to interpret since

nicotinic acetylcholine receptor is mainly expressed in nervous system. Although it might suggest that different diet affects autonomic nervous system.

Although little is known about the associations between the identified genes and meat quality traits in pigs, we can speculate that some of the genes involved in lipid metabolism modulate lipid content in meat and thus affect meat quality. It would be interesting to investigate these genes further in order to correlate their expression with meat quality traits. Furthermore, identification of new polymorphisms within these genes would afford an opportunity to investigate the interactions between genotypes and environmental factors (diet) in the development of certain phenotypes. Recently, regions of SSC12 containing actin cytoskeleton genes (*MYH1*, *MYH2*, *MYH4*) have been identified in a GWAS (Genome-Wide Association Study) of meat quality traits in a porcine Large White × Minzhu inter-cross population (Luo et al., 2012). Significant associations were identified for IMF, marbling, moisture and colour of meat. These results, in combination with ours, suggest that *MYH* genes should be explored further with regard to meat quality traits.

Coconut oil has become very popular recently, because of its beneficial effect on many aspects of health in humans. It was shown among others that coconut oil is associated with a beneficial lipid profile in pre-menopausal women in the Philippines (Feranil et al., 2011). Our results are in concordance with these suggestions, since in our study, animals obtaining coconut oil exhibited higher expression of *APOA4* gene – which codes for apolipoprotein A-IV, known for antiatherogenic properties (Culnan et al., 2009)

Our results show that the addition of beef tallow or coconut oil to feedstuffs does not change backfat thickness or liver weight; however, it improves IMF content in the *longissimus dorsi* muscle. At the same time, RNA-seq and qPCR analysis revealed that these fats increase the expression of several genes involved in lipid metabolism in the liver. Interestingly, Ramayo-Caldas (2012) identified differential expressions of some of these genes (*CYP2C49*, *GSTO1*, *FOS*) in the livers of pigs with extreme phenotypes of intramuscular fatty-acid composition. These results, taken together, indicate that meat quality traits may be modulated by both genetic and nutritional factors. It would be very interesting to continue these investigations by comparing the phenotypes of the animals with different genotypes in the identified genes and their response to different diet treatments.

Conclusions

In the present paper, we have demonstrated for the first time the effect of different lipids in the diet on porcine liver transcriptome by RNA-seq. We have shown that the expression of genes involved in lipid and fatty acids metabolism is changed following dietary stimulation. The RNA-seq results for five genes were confirmed by qPCR analysis. The most interesting seems to be the differences in the expression of genes from the *CYP* family, as well as the genes engaged in lipid and fatty acids metabolism e.g. *APOA4, ACSL5*. These results may be useful in the better understanding of the effect of usage of different fats in human and animal nutrition.

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