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Investigation of candidate genes for metabolic disorders expressed in liver and pituitary gland by comparing the RNA-seq data of Polish-HF and Polish-Red cattle

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Abstract. Background: Metabolic disorder is a major health problem in dairy cattle, particularly to high milk producing dairy cattle. It is worthily emphasized that metabolic diseases have a very complex etiology and pathogenesis, and the impact of these diseases on hepatic and pituitary gland gene expression and organism oxidative balance is not fully described. The presented study was aimed to determine and predict the hepatic and pituitary gland expression of potential candidate genes in context to maintenance of oxidative balance, negative nitrogen balance, as well as ketosis in Polish HF and Polish Red cattle.

Methods: Based on the RNA-seq experimental data, we investigated the candidate genes (*SOD1, SOD2, SOD3, GPx2, GPx3, GPx5, GPx6, GPx7, GPx8, BDH1, FN1, ACSL3, HMGCL, HMGCS2, BDH2, ACSL6, ACAT2, IDH3B, ACAT1, HMGCS1, ACSL4, ACSL1, PC, CPT1A, OXCT1* and *ACSL5* respectively) expressions in liver and pituitary gland tissues of Polish HF and Polish Red cattle. The RNA-seq experimental design comprised of young bulls aged between 6 to 12 months were investigated. For each breed, six liver and six pituitary gland tissues were sequenced using Next-seq 500 illumina platform. The RNA-seq expression data were normalized by the reads per kilobase of exon per million reads mapped (RPKM) method.

Results: By comparing the RNA-seq data of liver and pituitary gland tissues, the investigated candidate genes were highly expressed in the hepatic tissues than to pituitary gland in investigated cattle breeds. However, by comparing the Polish HF and Polish Red cattle breeds, results revealed a similar trend of gene expression profiling of all investigated candidate genes for both metabolic tissues. In case of hepatic gene expression profiling, the *SOD1, FN1, HMGCL, HMGCS2, ACAT2, ACAT1, HMGCS1, ACSL1* and *ACSL5* were highly expressed (FPKM values of >40), followed by *SOD2, GPX3, IDH3B, PC* and *BDH2* as moderately expressed (FPKM values: >10 to <40), and averagely expressed *SOD3, GPX5, GPX6, GPX7, GPX2, GPX8, BDH1, ACSL3, ACSL6, ACSL4, CPT1A* and *OXCT1* respectively, in Polish HF and Polish Red breeds. In case of pituitary gland gene expression profiling, the SOD1 and GPx3 were highly expressed (FPKM values of >40), followed by *SOD2, GPX8, IDH3B, ACAT1, ACSL4* and *PC* as moderately expressed (FPKM values: >10 to <40), and averagely expressed *SOD3, GPX3,GPX5, GPX6, GPX7, GPX2, BDH1, BDH2, ACSL3, ACSL6, CPT1A, OXCT1, FN1, HMGCL, HMGCS2, ACAT2, ACAT1, HMGCS1, ACSL1* and *ACSL5* respectively, in Polish HF and Polish Red breeds.

Conclusions: Based on this presented results on hepatic and pituitary gland gene expression, a further research plan is an essential pre-requisite to validate the identified candidate genes. Study indicated the understanding the genetic factors that predispose metabolic disorders in cattle would benefit the dairy industry as a whole by providing producers, breeding services, and veterinarians a tool to forecast a cow's susceptibility to metabolic disorders.

Keywords: RNA-seq; liver, pituitary gland; cattle; breeds; ketosis; *SOD*; *GPx*; antioxidants; bioinformatics.

Introduction

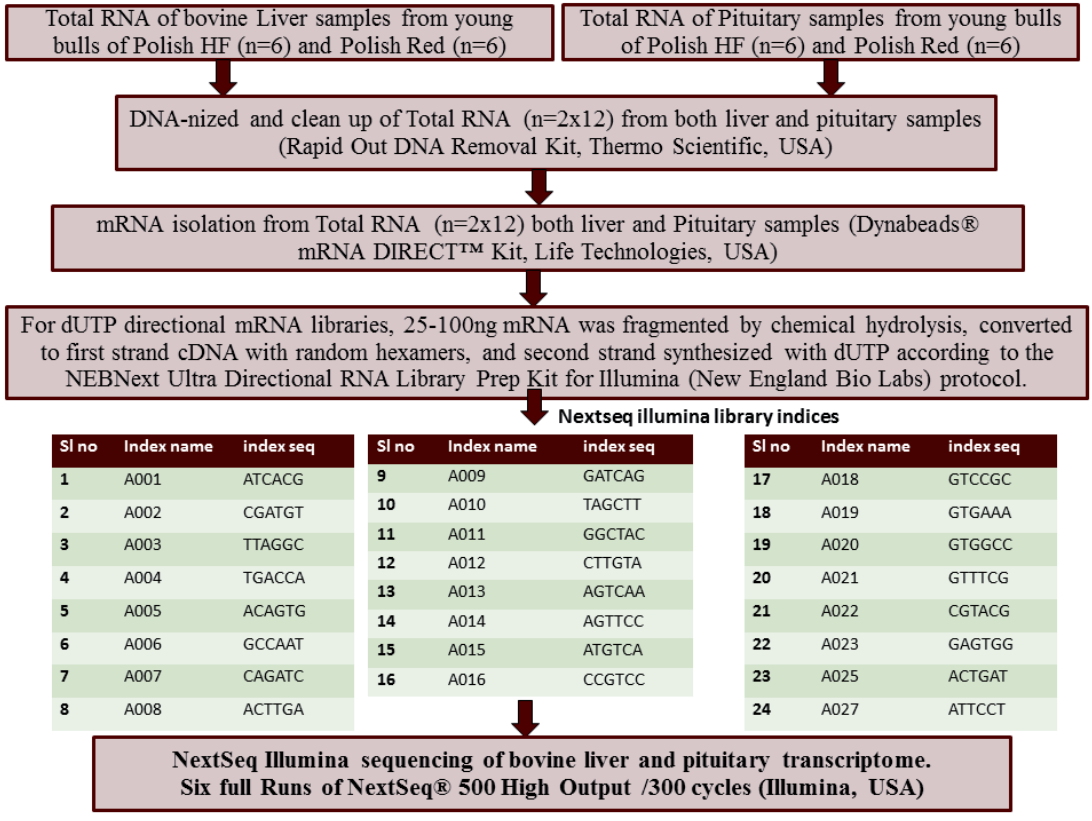
Metabolic diseases and disturbances of oxidative balance (oxidative stress) are constantly current threats to the health and welfare of dairy cows as well as the profitability of dairy farms and dairy industry all around the world [1–2]. The breeding selection and the increasing intensification of milk production made the high-yielding dairy cow an animal with a huge metabolic burden and susceptibility to metabolic diseases, such as ketosis [3–4]. High-yielding animals due to the very high rate of number of metabolic processes are also more susceptible to oxidative balance disturbances [5]. Both metabolic diseases and negative effects of oxidative stress contribute to shortening the life of animals or the need of culling them from the herd, and thus generate enormous economic losses in the dairy industry [6–7]. In recent year increased interest in ketosis and genetic studies have been observed in several manuscripts published in most reputable journals [8–13]. Furthermore there is increased interest in breed differences in transcriptome of *Bos Taurus* [14]. Ketosis in dairy cow is a multifactorial disease that is the result of a maladaptive response to negative energy balance (NEB) in early lactation. While essentially all cows undergo NEB after calving, not all cows develop ketosis. Recent study observed variable metabolic loads and differential gene expression in the liver of cows that had been pre-selected for high milk fat; despite being selected for high F:P, an indicator of ketosis, the susceptibility to ketosis was different for cows within this group [15]. Moreover, some other studies suggest that altered metabolic regulation that contributes to ketosis differs at the cow level [16–20]. Trait-association analyses that have been conducted for metabolic traits – energy balance, F:P, GPC:PC, blood BHB, BCS, milk metabolites, EBV – have identified regions of the genome, candidate genes and probable quantitative trait nucleotides (QTN) that explain some of the gene expression variation between cows [21–27]. However, further exploration of candidate genes for ketosis could contribute to our understanding of the genetic architecture of this trait. The construction of a custom designed array of candidate genes specific to the

trait of interest would allow for the investigation of many key genes simultaneously, as well as identify probable QTN with biological significance. In the paper, we investigated the gene expression variations of the GPx and SOD gene families involved in the maintenance of oxidative balance, as well as, gene expression variations among recently identified candidate genes for ketosis using the RNA-seq data of liver and pituitary gland tissues.

Materials and methods

Animals: The RNA-seq experimental design comprised of young bulls aged between 6 to 12 months were investigated. For each breed, six liver and pituitary gland tissues were sequenced using Next-seq 500 illumina platform (Figure 1). All procedures involving animals were performed in accordance with the guiding principles for the care and use of research animals. The investigated liver and pituitary gland tissues from Polish-HF and Polish Red cattle were approved by the local ethics commission of IGAB, PAS, Jastrzębiec, Poland (permission No. 3/2005).

RNA-seq laboratory work: Isolation of total RNA from liver and pituitary gland tissues were prepared from 50–60 mg of frozen tissues using the guanidinium thiocyanate method [28] (TRIzol reagent: Invitrogen, Carlsbad, CA, USA). All RNA samples were evaluated with the Agilent BioAnalyzer using the Nano RNA Kit with RNA Integrity Number (RIN) > 7.0. For the library preparation 5μg were total RNA and two biological replicates were used for each investigated tissue. The mRNA isolation was carried out by using the Dynabeads® mRNA Direct™ kit (Thermo Fisher), and followed by dUTP directional mRNA libraries preparation, according to the NEBNext Ultra Directional RNA library preparation Kit for Illumina (New England Bio Labs). The cDNA fragments were end-repaired, A-tailed, and ligated to the TruSeq y-tail single indexes from Illumina TruSeq DNA kit (Figure 1). Indexed libraries were cut with USER enzyme, and PCR amplified for 12 cycles. To achieve the highest quality data on Illumina sequencing platforms, optimum cluster



Sl no	Index name	index seq
1	A001	ATCACG
2	A002	CGATGT
3	A003	TTAGGC
4	A004	TGACCA
5	A005	ACAGTG
6	A006	GCCAAT
7	A007	CAGATC
8	A008	ACTTGA

Sl no	Index name	index seq
9	A009	GATCAG
10	A010	TAGCTT
11	A011	GGCTAC
12	A012	CTTGTA
13	A013	AGTCAA
14	A014	AGTTCC
15	A015	ATGTCA
16	A016	CCGTCC

Sl no	Index name	index seq
17	A018	GTCCGC
18	A019	GTGAAA
19	A020	GTGGCC
20	A021	GTTTCG
21	A022	CGTACG
22	A023	GAGTGG
23	A025	ACTGAT
24	A027	ATTCCT

Figure 1. Laboratory procedures of the RNA-seq experiment

deposition was made by quantitation of libraries using qPCR according to the Illumina Sequencing Library qPCR Quantification Guide (Kapa Biosciences). Finally, 156x156 bp paired-end sequence reads were generated using the Illumina NextSeq 500 platform High Output/300 cycle kits from Illumina.

Bioinformatics analysis: For all investigated liver and pituitary gland tissues (n=12), the minimum overlap length was set to 10 and error rate was set to 0.05 at cutadapt software [29]. After cutting adaptor, the low quality bases were trimmed from 3'- end. The processed short paired end reads were aligned, or mapped to the reference genome Ensembl75_UMD3-1.1 plus the Chromosome Y from Btau_4.6.1 assembly, by using BWA version 0.7.5-r404 [30]. The HT-Seq framework, version 0.5.3p9, was used to count the aligned reads in genes using the STAR BWA tools [31]. Finally, the RNA-seq expression data were normalized by the reads per kilobase of exon per million reads mapped (RPKM) method [32] to identify the gene expression variations of the candidate genes of ketosis in bovine liver and pituitary gland transcriptome.

Results and discussion

Based on the RNA-seq data, we have screened and investigated the known CGs for liver and pituitary gland transcriptome as presented in Table 1. By comparing the RNA-seq data of two metabolic tissues, study revealed that the investigated candidate genes were highly expressed in the hepatic tissues than to pituitary gland in cattle breeds (Table 2 and Table 3). However, by comparing the Polish HF and Polish Red cattle, results revealed a similar trend of gene expression profiling of all investigated candidate genes for both metabolic tissues (Table 2 and Table 3). Furthermore based on the obtained results, we have categorized gene expression profiling as: highly, moderately and averagely expressed candidate genes. In case of hepatic gene expression profiling, the *SOD1, FN1, HMGCL, HMGCS2, ACAT2, ACAT1, HMGCS1, ACSL1* and *ACSL5* were highly expressed (FPKM values of >40), followed by *SOD2, GPX3, IDH3B, PC* and *BDH2*

Table 1. lists of the candidate genes involved in ketone body metabolism of Polish HF and Polish Red cattle

Gene name	Gene symbol	Gene ensemble Id	Locus
Superoxide dismutase	*SOD1*	ENSBTAG00000018854	gnl|UMD3.1|GK000001.2:3113948-3122613
fibronectin 1	*FN1*	ENSBTAG00000008300	gnl|UMD3.1|GK000002.2:103881401-103950562
3-hydroxy-3-methylglutaryl-CoA lyase	*HMGCL*	ENSBTAG00000021832	gnl|UMD3.1|GK000002.2:129687546-129705679
3-hydroxy-3-methylglutaryl-CoA synthase 2	*HMGCS2*	ENSBTAG00000003898	gnl|UMD3.1|GK000003.2:23643771-23667741
acetyl-CoA acetyltransferase 2	*ACAT2*	ENSBTAG00000002827	gnl|UMD3.1|GK000009.2:97466404-97481898
acetyl-CoA acetyltransferase 1	*ACAT1*	ENSBTAG00000012885	gnl|UMD3.1|GK000015.2:17999931-18028984
3-hydroxy-3-methylglutaryl-CoA synthase 1	*HMGCS1*	ENSBTAG00000011839	gnl|UMD3.1|GK000020.2:31451385-31474239
long-chain-fatty-acid--CoA ligase 1	*ACSL1*	ENSBTAG00000004344	gnl|UMD3.1|GK000027.2:14223448-14288333
long-chain-fatty-acid--CoA ligase 5	*ACSL5*	ENSBTAG00000006707	gnl|UMD3.1|GK000026.2:33184652-33234956
superoxide dismutase	*SOD2*	ENSBTAG00000006523	gnl|UMD3.1|GK000009.2:97399158-97404522
glutathione peroxidase 3	*GPX3*	ENSBTAG00000043553	gnl|UMD3.1|GK000007.2:64286947-64295116
isocitrate dehydrogenase 3 (NAD(+)) beta	*IDH3B*	ENSBTAG00000018813	gnl|UMD3.1|GK000013.2:52978728-52983685
pyruvate carboxylase	*PC*	ENSBTAG00000019700	gnl|UMD3.1|GK000029.2:45508279-45611042
3-hydroxybutyrate dehydrogenase, type 2	*BDH2*	ENSBTAG00000002526	gnl|UMD3.1|GK000006.2:23047056-23077431
superoxide dismutase 3	*SOD3*	ENSBTAG00000013980	gnl|UMD3.1|GK000006.2:45927553-45930819
glutathione peroxidase 5	*GPX5*	ENSBTAG00000021987	gnl|UMD3.1|GK000001.2:95125674-95134631
glutathione peroxidase 6 precursor	*GPX6*	ENSBTAG00000012023	gnl|UMD3.1|GK000001.2:95159025-95166199
glutathione peroxidase 7	*GPX7*	ENSBTAG00000018281	gnl|UMD3.1|GK000003.2:94323975-94331673

Table 1. Lists of the candidate genes (continued)

Gene name	Gene symbol	Gene ensemble Id	Locus
glutathione peroxidase 2	*GPX2*	ENSBTAG00000048112	gnl|UMD3.1|GK000010.2:77381299-77384791
Probable glutathione peroxidase 8	*GPX8*	ENSBTAG00000021960	gnl|UMD3.1|GK000020.2:23975639-23980445
3-hydroxybutyrate dehydrogenase, type 1	*BDH1*	ENSBTAG00000000448	gnl|UMD3.1|GK000001.2:72572940-72608810
long-chain-fatty-acid--CoA ligase 3	*ACSL3*	ENSBTAG00000017258	gnl|UMD3.1|GK000002.2:111797169-111887224
long-chain-fatty-acid--CoA ligase 6	*ACSL6*	ENSBTAG00000019708	gnl|UMD3.1|GK000007.2:23779095-23882198
long-chain-fatty-acid--CoA ligase 4	*ACSL4*	ENSBTAG00000033186	gnl|UMD3.1|GK000020.2:32683995-32848723
carnitine O-palmitoyltransferase 1, liver isoform	*CPT1A*	ENSBTAG00000021999	gnl|UMD3.1|GK000029.2:46822026-46862300
3-oxoacid CoA-transferase 3	*OXCT1*	ENSBTAG00000018986	gnl|UMD3.1|GK000030.2:62476951-62508324

Table 2. Comparison of hepatic gene expression RNA-seq data of Polish HF and Polish Red breeds in context to candidate genes for metabolic disorders in cattle

Candidate genes	Hepatic gene expression FPKM values in Polish HF						Hepatic gene expression FPKM values in Polish Red					
Gene symbol	6m	6m	9m	9m	12m	12m	6m	6m	9m	9m	12m	12m
SOD1	587.806	382.506	454.909	367.82	377.889	509.811	552.273	533.482	647.915	646.17	578.4	646.66
FN1	141.253	137.056	143.107	104.746	86.4169	133.379	172.552	147.574	137.537	149.542	153.326	179.389
HMGCL	40.9679	35.2533	42.9727	33.8929	29.8469	28.5233	43.0563	33.1994	17.4588	24.3485	37.8589	33.6906
HMGCS2	133.47	318.862	266.208	242.478	133.301	141.547	299.896	177.983	77.1757	120.375	270.543	179.797
ACAT2	41.6857	45.677	40.5192	26.5138	32.892	37.0686	30.8785	23.9992	24.5161	28.0698	52.5476	50.1219
ACAT1	115.44	121.321	149.59	114.29	90.5704	111.383	170.729	154.746	178.761	166.706	168.812	170.225

Table 2. Comparison of hepatic gene expression (continued)

Candidate genes	Hepatic gene expression FPKM values in Polish HF						Hepatic gene expression FPKM values in Polish Red					
HMGCS1	59.9402	51.089	18.6792	22.7943	46.733	29.7605	6.05043	5.70433	16.1123	16.8309	67.6906	79.147
ACSL1	63.7639	81.1376	56.9695	143.965	83.8572	67.8007	76.3604	79.3234	43.9291	48.5728	82.3233	142.738
ACSL5	86.1186	82.5116	93.7492	98.0026	78.1439	89.6564	92.5392	75.3728	57.2832	65.345	95.9417	107.512
	10.6093	31.5448	26.2895	15.6248	12.91	20.5917	37.9183	26.918	13.839	33.2459	42.7187	37.7087
GPX3	33.0359	0.48964	7.94891	159.446	9.33627	5.3753	11.9042	125.113	54.0902	78.2431	14.6146	14.8297
IDH3B	31.3521	26.761	29.1937	25.505	24.9068	21.1712	32.6552	29.7186	17.3882	23.2851	28.7078	19.5314
PC	25.9998	19.2803	36.381	81.7686	11.2959	12.9058	84.8072	129.374	39.2461	59.8623	29.5949	30.6194
BDH2	20.7454	16.3494	19.2781	17.3071	14.2716	29.8659	19.443	20.5117	13.8606	17.1139	20.2104	24.6091
SOD3	0.997065	0.387089	0.478915	0.282032	0.206672	1.75654	0.60365	1.28819	0.116511	0.43481	1.04491	0.069306
GPX5	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
GPX6	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
GPX7	2.71465	2.11593	2.56983	3.13197	2.19483	2.87964	4.99105	3.75186	1.38041	2.39875	3.21558	2.00773
GPX2	0.574711	0.00000	0.061689	1.08696	0.265479	0.00000	0.3328	0.00000	0.00000	0.216759	0.578792	0.724878
GPX8	2.22946	1.97678	2.83675	1.49077	1.45654	5.04908	3.90492	4.51587	3.32298	1.85116	4.26326	3.60268
BDH1	0.072713	0.042957	0.095052	0.037434	0.00000	0.00000	0.00000	0.036415	0.00000	0.00000	0.00000	0.00000
ACSL3	0.861747	2.24732	2.22622	2.73044	1.66774	1.38317	1.58502	2.25618	1.71557	2.35547	3.19563	3.46309
ACSL6	0.178696	0.266765	0.450368	0.324652	0.169968	0.173965	0.624238	0.399946	0.193176	0.229808	0.53551	0.247538
ACSL4	0.613841	0.478862	1.01359	0.857215	0.538538	1.31658	2.33334	1.45696	1.42772	1.68123	2.48077	2.0362
CPT1A	1.8972	9.39944	10.964	14.8487	6.66172	5.91555	11.8386	15.4445	9.71804	11.2969	15.5442	17.3803
OXCT1	1.5481	0.768309	2.34889	2.29892	1.17556	1.60366	3.0995	2.57093	3.05921	3.27389	3.76858	5.21901

Table 3. Comparison of pituitary gene expression RNA-seq data of Polish HF and Polish Red breeds to in context to candidate genes for metabolic disorders in cattle

Candidate genes	**Pituitary gland gene expression FPKM values in Polish HF**						**Pituitary gland gene expression FPKM values in Polish Red**					
Gene symbol	6m	6m	9m	9m	12m	12m	6m	6m	9m	9m	12m	12m
SOD1	86.2995	53.9038	110.381	84.2797	103.59	96.3431	135.74	131.542	118.392	66.8288	113.279	90.9218
GPX3	776.417	361.776	1126.27	546.795	179.554	277.01	470.296	527.681	543.355	220.787	177.946	668.998
SOD2	19.8513	11.7984	20.3716	7.48079	14.9471	5.99109	9.95032	21.905	14.3264	5.67434	17.4066	11.238
GPX8	11.4383	7.38668	13.5565	10.4825	11.2638	9.48416	11.0325	6.97562	9.37172	6.55676	14.6727	9.63329
IDH3B	26.8771	19.8947	27.0388	24.647	24.8191	28.9725	24.4674	31.81	27.7493	15.2813	23.7175	20.923
ACAT1	36.106	20.9596	38.703	28.1692	28.4319	32.3762	37.3557	40.9214	35.2231	23.0132	51.683	23.3349
ACSL4	10.1285	9.24104	13.4465	14.0161	9.73905	7.77376	13.3709	13.8619	13.4302	7.42566	10.3305	9.59881
PC	11.2234	5.59578	6.88685	6.96704	5.96264	6.32432	6.64454	10.685	7.6261	5.72416	9.52841	6.2624
SOD3	2.62912	1.28713	1.54392	1.2261	0.641106	0.157742	1.81149	2.67144	0.826854	0.864026	2.45264	1.00815
GPX5	0.00000	0.00000	0.034628	0.091472	0.430309	0.246104	0.273666	0.215057	0.115499	0.00000	0.106467	0.194247
GPX6	0.397938	0.386262	0.18428	0.73554	0.385254	0.00000	0.318629	0.311775	0.249065	0.155349	0.141755	0.128357
GPX7	6.40528	3.9234	5.88624	6.35384	5.08026	4.75486	4.53754	5.63301	6.36146	2.76581	4.48801	5.27374

Table 3. Comparison of pituitary gene expression (continued)

Candidate genes	Pituitary gland gene expression FPKM values in Polish HF						Pituitary gland gene expression FPKM values in Polish Red					
GPX2	0.139109	0.090543	0.00000	0.344944	0.2707	0.00000	0.00000	0.073384	0.00000	0.00000	0.00000	0.00000
BDH1	2.06409	2.9456	3.37887	2.57644	4.47146	3.60652	13.5233	8.53161	9.75672	3.91156	0.588884	10.4984
FN1	8.30332	4.87045	5.07801	3.30278	1.93041	1.40863	3.60727	4.00198	0.5624	5.2591	18.7176	1.65294
ACSL3	8.19562	8.33552	13.6473	8.14815	5.87794	6.26377	11.4359	14.8789	11.1147	7.62678	7.78664	8.45531
HMGCL	9.26616	6.41665	7.35472	5.49742	8.76654	7.97851	8.58692	10.6091	7.8974	4.51019	5.95498	6.63772
HMGCS2	0.00000	0.00000	0.02730	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
BDH2	8.7833	13.105	17.3072	13.1385	15.4764	12.7814	16.6003	27.5983	16.5269	9.65395	12.8527	9.63071
ACSL6	4.66115	4.09218	5.03829	6.75911	5.61165	7.10165	4.65044	7.88833	4.95224	3.59315	2.62533	6.0886
ACAT2	7.8207	5.01241	6.99727	4.06422	5.41611	6.09655	6.60386	9.50284	6.67182	3.99656	5.37729	5.76437
HMGCS1	6.6157	6.42286	7.69707	6.15433	7.07211	7.62849	8.27939	12.0234	8.55494	5.33763	3.6239	8.90925
ACSL1	9.01769	7.82869	7.15201	9.53737	7.58949	8.37861	8.22477	13.7428	9.80515	6.37145	6.36834	8.07928
CPT1A	3.44654	2.55096	1.4916	2.62206	1.19813	1.1293	3.32617	3.90554	2.83685	2.1149	3.18305	1.01798
OXCT1	3.23663	2.99513	3.87416	2.65487	2.91203	2.1369	4.30022	5.36711	3.01314	5.30722	2.66726	4.27154
ACSL5	3.72023	1.62982	2.87451	2.33325	2.52568	1.92096	2.77289	3.93506	2.28927	2.00827	4.03437	1.71583

as moderately expressed (FPKM values: >10 to <40), and averagely expressed *SOD3, GPX5, GPX6, GPX7, GPX2, GPX8, BDH1, ACSL3, ACSL6, ACSL4, CPT1A*, and *OXCT1* respectively, in Polish HF and Polish Red breeds. In case of pituitary gland gene expression profiling, the *SOD1* and *GPx3* were highly expressed (FPKM values of >40), followed by *SOD2, GPX8, IDH3B, ACAT1, ACSL4,* and *PC* as moderately expressed (FPKM values: >10 to <40), and averagely expressed *SOD3, GPX3,GPX5, GPX6, GPX7, GPX2, BDH1, BDH2, ACSL3, ACSL6, CPT1A, OXCT1, FN1, HMGCL, HMGCS2, ACAT2, ACAT1, HMGCS1, ACSL1* and *ACSL5* respectively, in Polish HF and Polish Red breeds.

Conclusions

Understanding the genetic factors that predispose metabolic disorders in cattle would benefit the dairy industry by providing producers, breeding services, and veterinary practitioners a tool to forecast a cow's susceptibility to metabolic disorders. Considering presented results on hepatic and pituitary gland gene expression profiling study, a further research is an essential pre-requisite to validate the identified candidate genes.

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