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COMPARATIVE PROTEOMIC STUDY OF MUSCLE TISSUE OF WILD BOARS AND DOMESTIC PIG BREEDS

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Abstract
In the modern conditions, there is a growing consumer interest in products from local (autochthonous) breeds of animals raised in extensive animal husbandry systems. Meat of such animals is often associated with high quality characteristics; however, its molecular foundations remain to be studied insufficiently. Comparative analysis of proteomic profiles of such breeds and their wild ancestor — wild boar — is of particular interest for understanding the fundamental consequences of domestication and selection as well as for revealing key marker proteins determining meat product properties. A comparative proteomic analysis of muscle tissue (M. longissimus dorsi) of wild boar and four pig breeds (Livny breed, Altai meat-type breed, Landrace, Mangalitsa) was carried out to reveal breed-specific molecular patterns associated with key meat quality characteristics. Proteomic profile was studied by two-dimensional electrophoresis (2-DE) and mass spectrometry (MALDI-TOF/TOF). Functional analysis of protein-protein interactions and gene ontology (GO) enrichment were carried out using the STRING database. Twenty one proteins forming a functionally linked network were identified. Significant breed related differences in the composition and modifications of proteins of the contractile apparatus (products of MYL1, MYL2, MYL3, MYL6B, TNNT3, TNNI2 genes), energy metabolism (products of ENO3, ALDOA, CKM, AK1, ATP5F1A genes) and stress response (products of CRYAB, HSPB6 genes) were revealed. The highest degree of proteome transformation was noticed in the Livny breed, which demonstrated a significant similarity with wild boar in terms of several parameters including appearance of atypical myosin light chain MYL6B and a decrease in the level of muscle enolase (products of ENO3 gene). For Mangalitsa, a unique modification of the pattern of expression of myosin light chains and a significant increase in the level of small chaperons were characteristic, which correlates with the conditions of its free-range keeping. Bioinformatics analysis in STRING corroborated statistically significant formation of functional clusters responsible for muscle contraction, metabolism and maintenance of proteostasis. The data obtained suggest that both gene pool (breed) and environmental factors (keeping conditions) exert a complex effect on the proteomic landscape. The revealed protein signatures and their network interactions not only deepen the understanding of the biological foundations of meat quality but also open new prospects for the development of molecular markers in breeding and meat industry aimed at production of products with target properties.

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Introduction

Pork is considered the most consumed meat type in the world. In pork production, the most economically significant characteristics are the growth rate and meat quality. It is these requirements that determined domination of large-scale, highly productive pig husbandry, which led to the fact that four commercial breeds (Landrace, Duroc, Large White (Yorkshire), Pietrain) account for more than 95 % of pork production [1]. However, due to the high concern of the society about intensive methods of pig husbandry, consumers more and more often prefer meat from animals that were raised using the extensive method of animal husbandry, which is linked with the possibility of free range for animals, more diverse diets with possibilities of foraging. Extensive pig husbandry is more applicable to local breeds, which differ significantly from highly productive breeds of the traditional production by both productivity and quality indicators of carcasses and meat [2].

In response to this consumer demand together with risks of disappearance of autochthonous breeds, more and more studies are aimed at investigation of a relationship between meat quality characteristics and genetic structure.

Particularly many publications are aimed at the comparison of commercial and local breeds, methods of raising and fattening of pigs. At the first stages, investigations dealt with comparison of physico-chemical properties of meat raw materials. The study by Estévez et al. [3], can serve as a prominent example. It presents the results of the investigation of *M. longissimus dorsi* from free-range Iberian pigs slaughtered at a live weight of 90 kg compared to muscles from commercial pig breeds. The authors present data about a higher content of fat upon the low content of phospholipids, higher content of heme iron and distinct darker (red) color (a^* value) of muscles compared to those of the commercial pigs. *M. longissimus dorsi* from pigs of commercial breeds contained higher proportions of polyunsaturated fatty acids and higher ratio of omega-3 to omega-6. Also, a recent study [4] demonstrated higher moisture content, cooking losses, high a^* and b^* values and content of linoleic acid, linolenic acid and arachidonic acid upon a lower content of crude fat and ash, and L^* value in *M. longissimus dorsi* from Korean native pigs compared to hybrid pigs (Lanrace × Yorkshire x Duroc).

The obtained extensive data about differences in the physico-chemical properties of meat from native and commercial pig breeds formed the basis for conducting deeper molecular investigations that became possible with the development of the modern methods of proteomics and genomics and enable discovering a mechanism of manifestation of meat quality characteristics.

Proteomic differences between native and commercial pig breeds were noticed in a significant number of publications. For example, in [5] significant differences were revealed in expression of protein, synthesis of fatty acids, content of catalase and glutathione peroxidase when comparing the Nero d'Abruzzo with commercial hybrid pigs. Wang et al. [6] presented the results of the proteomic analysis of M. *Longissimus dorsi* from six-month-old pigs of two indigenous Chinese breeds (Tibetan pig and Diannan Small-Ear pig) and two introduced Western pig breeds (Yorkshire and Landrace) using isobaric tag for relative and absolute quantitation (iTRAQ). The authors revealed 288 differentially expressed proteins. Among them, 169 proteins were upregulated and 119 proteins were downregulated. The authors linked differences in muscle growth and formation of muscle fibers between the studied groups with such proteins as aldolase C, enolase 3, phosphoglycerate kinase 1 and 2, troponins (TNNT1, TNNC2, TNNT3), tropomyosins (TPM1, TPM2, TPM3), myosin light (MYL3) and heavy (MYH4) chains. Differences in the ability to deposit lipids are regulated by expression of lipoprotein lipase (LPL); apolipoproteins (APOA1 and APOC3); acyl-CoA dehydrogenase; fatty acid binding protein; acyl-CoA dehydrogenase, C-4 to C-12 straight chain; acetyl-CoA acyltransferase 2; acetyl-CoA acetyltransferase 1; hydroxyacyl-CoA-dehydrogenase and peroxisomal 3,2-trans-enoyl-CoA isomerase.

When comparing tenderloins from Tibetan pigs and Yorkshire pigs, 171 proteins were identified as differentially abundant proteins (DAPs). In tenderloin from Tibetan pigs, upregulated proteins took part mainly in biological pathways of energy production, muscle contraction, immunity and defense, while downregulated proteins in glutathione metabolism [7].

Pan et al. [8] present the results of the transcriptome sequencing of muscle *Longissimus dorsi* from Duroc and Luchuan pigs, which showed differential expression of 3,682 genes. Special attention was paid to MYL2 gene expression in Luchuan pigs, which was significantly higher than that in Duroc pigs at 2 and 8 months of age. The authors link this to the different times at which the maximum growth rate of different pigs appears. With that, 40 genes were associated with biological pathways of growth of skeletal muscles, metabolism of fatty acids and deposition of intramuscular fat.

Kim et al. [9] reported on the results of the complex study of the meat quality characteristics and differences in transcriptome of *M. longissimus dorsi* between the Landrace breed and Jeju native pigs. Phenotypical analysis of meat quality traits revealed that meat from the local breed was characterized by higher content of intramuscular fat and redness. RNA sequencing of muscle samples revealed 427 differentially expressed genes upregulated in Jeju pigs, while 821 genes were upregulated in Landrace pigs. Among candidate genes being indicators of growth and meat quality and also facilitating improvement of indicators of growth and development of pigs, the following key genes were determined: encoding myosins (MYH2, MYH6, MYH7B, MYO5B), growth factors (IGF1, IGFBP5, EGFL6, LINGO1), glycoproteins (SFRP2), matrilin-3, hyaluronan and proteoglycan link protein 1, fibulin-7.

Therefore, data of transcriptomic and proteomic investigations clearly demonstrate molecular differences between pig breeds and prove that genetics and targeted selection exert a profound influence on pork quality traits. Nevertheless, current scientific context has a significant gap: there are no comparative studies of pigs and their common ancestor — wild boar. Proteomic landscape of wild boar (*Sus scrofa*) is to a large extent understudied. Inclusion of the wild phenotype into comparative analysis is a necessary link for understanding the initial muscle proteome not modified by selection, which allows for revealing evolutionary conserved pathways and molecular adaptations that were acquired in the process of domestication and targeted selection.

In this respect, the aim of this work was the comparative proteomic analysis of *M. longissimus dorsi* samples from wild boars, pigs of the commercial breed (Landrace), two local breeds (Altai and Livny) and the introduced breed (Mangalitsa). This study is aimed at revealing universal and unique breed-specific protein patterns associated with growth rate of muscles, transformation of muscle fiber type and metabolic status, which will enable disclosure of molecular mechanisms of meat quality formation that form the base of adaptation to different conditions of keeping and genetic heritage.

Objects and methods

Objects of research were muscle tissue samples of *M. longissimus dorsi* from pigs of two local breeds — Livny ($n = 6$; LV) and Altai meat-type ($n = 5$; AL), commercial pigs (Landrace) ($n = 5$; LD), Mangalitsa breed ($n = 5$; MG) as an introduced breed, as well as wild boar ($n = 8$, WB).

The Livny breed (Livny, Orlov region) is a local Russian meat-fat breed of pigs registered in 1949. Boars of the White Long-Eared, Yorkshire, Large White, and Berkshire breeds were used in its production. At present, only a small population of Livny pigs is kept in one enterprise in the Orlov region. Pigs of the Livny breed achieve slaughter weight for 155–160 days.

The Altai meat-type breed (Altai Republic) is a local Russian meat-type breed registered in 2017. White Large and Landrace pigs and boars of the MAXGRO™ Terminal Line were used in its development. An average yield of lean meat is 58–59 %. Altai pigs grow very fast and achieve body weight of 100 kg for 145–150 days.

The Mangalitsa breed (Krasnoyarsk region) was imported from Hungary to Russia in 2000, raised in free-range conditions with access to pastures.

Wild boars are represented by individuals from different populations (Central European boar *S. scrofa scrofa*, Moscow region; Ussuri boar *S. scrofa ussuricus*, Amur River region).

Livny, Altai and Landrace pigs were kept in conditions of the commercial pig husbandry farm and fed with complete combined feed. After the average live weight of pigs reached 110 ± 10 kg, pigs were transported to the slaughterhouse, were allowed to rest for 12 hours and then slaughtered. Carcasses were chilled at a temperature of 0 °C for 24 hours. Cold carcasses were assessed 24 hours after slaughter. Pieces of *M. longissimus dorsi* with a weight of 500 g (± 10 g) were cut off from each carcass. Samples were obtained, as a minimum, from three pigs at slaughterhouses after conventional processing of carcasses or after shooting in field conditions.

To carry out the experiment, the following reagents were used: acrylamide, methylenebisacrylamide, agarose, Tris, glycine, sodium dodecyl sulfate, ammonium persulfate, Triton X-100, 2-mercaptoethanol, BSA, ampholines pH 3–10 and 5–8, Coomassie Brilliant blue R-250, trypsin (Sigma, USA), amberlite IRA-150L (Amersham Biosciences, Sweden), PageRuler™ Prestained Protein Ladder (Fermentas, USA), urea (Across Organics, Belgium), silver nitrate (Dia-M, Russia).

To perform the proteomic analysis, a minced specimen (100 mg) of muscle tissue was homogenized in 2 ml in the Teflon-glass system in a ysis solution of the following composition: 9M urea, 5 % mercaptoethanol, 2 % Triton X-100, 2 % ampholytes. The homogenate was centrifuged at 800 g for 5 min.

Two-dimensional electrophoresis (2-DE) by O'Farrell was carried out using a chamber Bio-Rad (Bio-Rad, USA) by isoelectric focusing in ampholine-polyacrylamide gel (IEF-PAGE, equilibrium variant), in glass tubes in the first direction and SDS-PAGE in the second direction with slight modifications [11,12,13]: IEF in the first direction was performed in 2.4 mm × 180 mm cylindrical gels until reaching 3650 Volt-hours. Non-equilibrium variant of IEF (with an increase to 2500 Volt/hour) was also used to reveal atypical fractions of troponin. For visualization of proteins, 2DE gels were stained with Coomassie blue R-250 and then with silver nitrate according to [14]. Stained gels were analyzed by scanning (300 points per inch, 48 bit, color,.TIFF) using a scanner Epson Expression 1680 (Epson, Suwa, Nagano, Japan) [15]. Densitometry was carried out using the software ImageMaster 2D Platinum, version 7 (GE Healthcare, Switzerland). No less than three 2-DE were tested for each breed/cross.

For protein identification, individual fractions were cut out from 2-DE gels, cut-out fragments were ground and their trypsinolysis was performed as described earlier [16]. Then, the corresponding sets of peptides were studied by MALDI-TOF MS and MS/MS spectrometry using a MALDI / time-of-flight mass spectrometer Ultraflex (Bruker, Bremen, Germany) with UV laser (336 nm) in a positive ion mode in a range of 500–500–8000 Da with their calibration by known trypsin autolysis peaks. Analysis of the obtained mass spectra (peptide fingerprints) was carried out using the Mascot software (FlexControl 3.3, FlexAnalisis 3.3 and Biotools 3.2), options Peptide Fingerprint (Matrix Science, Boston, Massachusetts, USA), with accuracy of mass measurement MH+ 0.01 %, and was accompanied by the search in the NCBI databases "Proteins of porcine skeletal muscles (*Sus scrofa*)" [17].

Analysis of protein-protein interactions (PPI) and functional enrichment was performed using the STRING database [18,19]. Analysis included all identified proteins (n = 19). A PPI-network was constructed with an average confidence level (score ≥ 0.5). Biological interpretation of the network was carried out based on the gene ontology (GO) enrichment analysis by three directions: biological processes (Biological Process), cellular components (Cellular Component) and molecular functions (Molecular Function). Statistical significance of enrichment was determined with adjustment for false discovery (False Discovery Rate, FDR) with a value of < 0.05.

Statistical processing was carried out using a software package Statistica 10.0. Results are presented as means and standard deviations (Mean ± SD). Statistical significance was calculated with the use of non-parametric Mann–Whitney U test. A probability of 0.05 was chosen as a significant level.

Results

Upon visual clustering using the STRING database, the statistically significant (score > 0.9, PPI enrichment *p*-value: 1.89e-15, local clustering coefficient: 0.692) protein-protein interaction network was distributed into three main clusters: two interlinked heat shock proteins CRYAB and HSPB6, a triangle of interactions of ubiquitin system RPS27A-UBB-KXD1, and a wide network from 14 protein nodes linked by 32 edges (Figure 1).

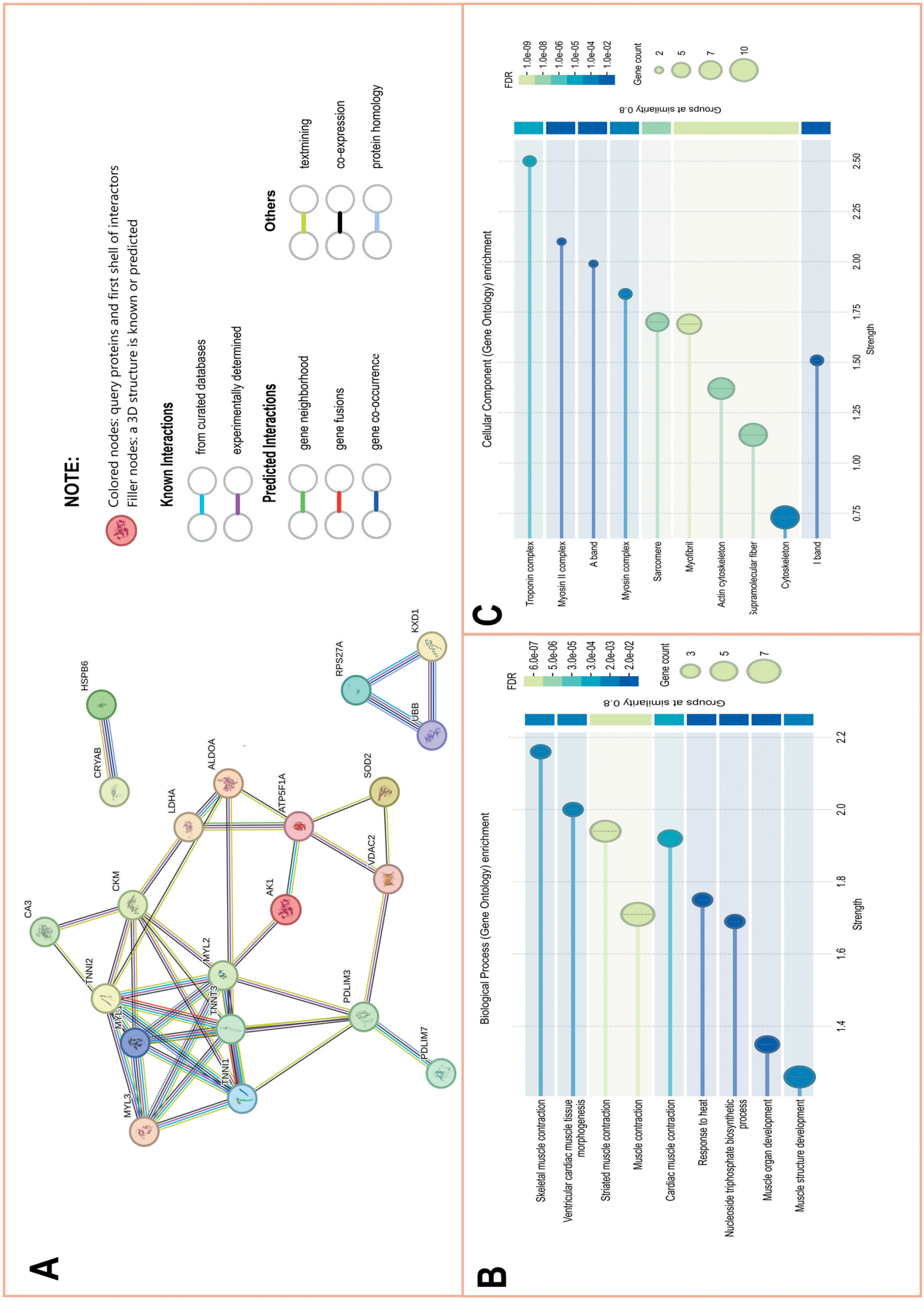

Figure 1. Visualization of protein-protein interactions (A) of identified proteins and distribution by related biological processes (B) and cellular components (C)

The largest cluster of 14 molecules represents a network with a clear functional distribution into key modules reflecting the main biological processes in muscle tissue: contractile apparatus (GO:0006936, GO:0006941, GO:0060048, GO:0003009), energy metabolism (GO:0009142) and mitochondrial functions, including defense against the oxidative stress. The most dense and highly significant module (score > 0.95) is formed by structural and regulatory proteins of myofibrils (GO:0030016, Myofibril), which indicate their close functional and physical relationship in the composition of sarcomeres (GO:0030017, Sarcomere), for example, interaction MYL1-TNNT3 (score 0.967), MYL1-TNNI2 (score 0.951) and TNNI2-TNNT3 (score 0.986). The second key module of the network unites enzymes of glycolysis and the system of generation and buffering of ATP: creatine kinase M-type (CKM) demonstrates multiple links both with contractile proteins (MYL1, score 0.868; TNNT3, score 0.625), and with enzymes of glycolysis (LDHA, score 0.520), which underlines its central role in buffering of ATP and rapid rephosphorylation of ADP near the places of its consumption — myofibrils. In addition, we visualized the interaction between aldolase A (ALDOA), a key enzyme of glycolysis, and lactate dehydrogenase A (LDHA) (score 0.915), which corroborates their cooperation in the anaerobic degradation of glucose. Adenylate kinase 1 (AK1) interacts with mitochondrial ATP syntase (ATP5F1A) (score 0.618) and myosin regulatory chain MYL2 (score 0.509), integrating mitochondrial ATP production with its intracellular turnover and consumption.

PDZ and LIM domain protein 3 (PDLIM3) acts as a link interacting both with contractile elements (MYL1, MYL2, TNNI1, TNNT3), and mitochondrial protein VDAC2. This determines it possible role in the spatial organization of sarcomere (GO:0030017) and location of mitochondria near myofibrils (GO:0030016) for effective ATP supply, which corresponds to its annotation to the component of Z-disc (GO:0030018, Z disc) and actin cytoskeleton (GO:0015629) [25].

Cluster of small chaperons (CRYAB and HSPB6, score 0.603) is involved into the function of the defense system against proteotoxic stress preventing protein denaturation and aggregation (GO:0009408, Response to heat) [26].

Proteins RPS27A (endogenous ribosomal protein), UBB (polyubiquitin, central for proteasome degradation) and KXD1 (interacts with ubiquitin ligases) form a stable functional complex, which central link is the ubiquitin system (score>0.9). RPS27A (GO:0042254, ribosome biogenesis) and UBB (GO:0000209, protein polyubiquitination) are components of this system [27], and KXD1 (GO:0140356, ubiquitin binding) acts as a regulator and adapter linking it with other cellular processes, in particular, with intracellular transport (GO:0006886, intracellular protein transport) through interaction with regulators Rab GTPases (RabGDI), which apparently ensures the directed transfer of ubiquitin substrates [28] and indicates the active processes of targeted degradation of damaged proteins (GO:0006508, proteolysis) and other non-membranous organelles (GO:0043232), regulation of cellular functions. This complex is mostly located in cytosol (GO:0005829) and other non-membranous organelles (GO:0043232), which is a prerequisite for constant renewal of protein pool in the dynamic muscle tissue.

Figure 2 presents two-dimensional electropherograms of *M. longissimus dorsi* from breeds under study, which were stained with Coomassie. First of all, we assessed protein fractions that corresponded to the myosin essential light chains (MLC). It is known that myosin light chains are in the composition of the heads of the myosin molecule as two types of molecules — essential (MYL1) and regulatory (phosphorylated), which ensure the contractile activity of muscle fiber due to changes in a degree of phosphorylation of the regulatory isoform (MYL2). Products of MYL1 gene in vertebrates are presented in a form of two isoforms — long and short (fast type). Each of two heads in the myosin molecule from fast skeletal muscles contains only one MLC isoform (either long or short); therefore, myosin in muscle fibers can exist either as homodimer containing the same MLC in both heads, or as a heterodimer containing different MLC isoforms [20]. Traditionally, essential light chains in domestic pigs are presented as two isoforms - products of MYL1 (fast type, 93 %) and MYL3 (slow type, 7 %) genes.

Two MYL1 isoforms were revealed for *M. longissimus dorsi*: major, fast type (quantitative content 90 %) and minor (10 ± 5 %). Among the breeds under study, the ratios of isoforms had insignificant quantitative differences. It was found that the proportion of the myosin long chain (MLC) changes quantitatively depending on the breed. MLC1 (MYL1, fast type, No. 1–3 in Table 1) was the main component, but MYL3 (slow type, No. 7, 8 in Table 1) was also found. An amount of MYL3 for the studied breeds was not higher than 6.5 % of the total amount of essential MLC. With that, for *L. Dorsi* of the Landrace breed the slow isoform accounted for 13.9 %, while in the Livny and Altai breeds it reached 25 %. In the samples from wild boar, two isoforms of the products of the MYL1 gene were detected: major, fast type, as well as fragments of the zone of regulatory myosin light chains and short product of the MYL1 gene.

For *L. Dorsi* of the Mangalitsa breed, part of MYL1/MYL3 molecules (no more than 1 %) acquired an unusual property — proteins in the norm have pI 4.8–5.0; with that, the detected fractions showed a shift in pI to a level of 9.50. Apparently, part of molecules of these proteins underwent atypical post-translational modification.

In five out of eight samples of *L. Dorsi* from wild boar, the presence of the additional fraction of myosin essential light chain, MYL6B (No. 9–11 in Table 1), was revealed. It belongs to the smooth muscle or non-muscle type and as a rule, is not detected in skeletal muscle of domestic pigs. This protein for porcine muscle tissue was denoted for now as not characterized, although it is without doubt one of the variants of essential myosin light chains.

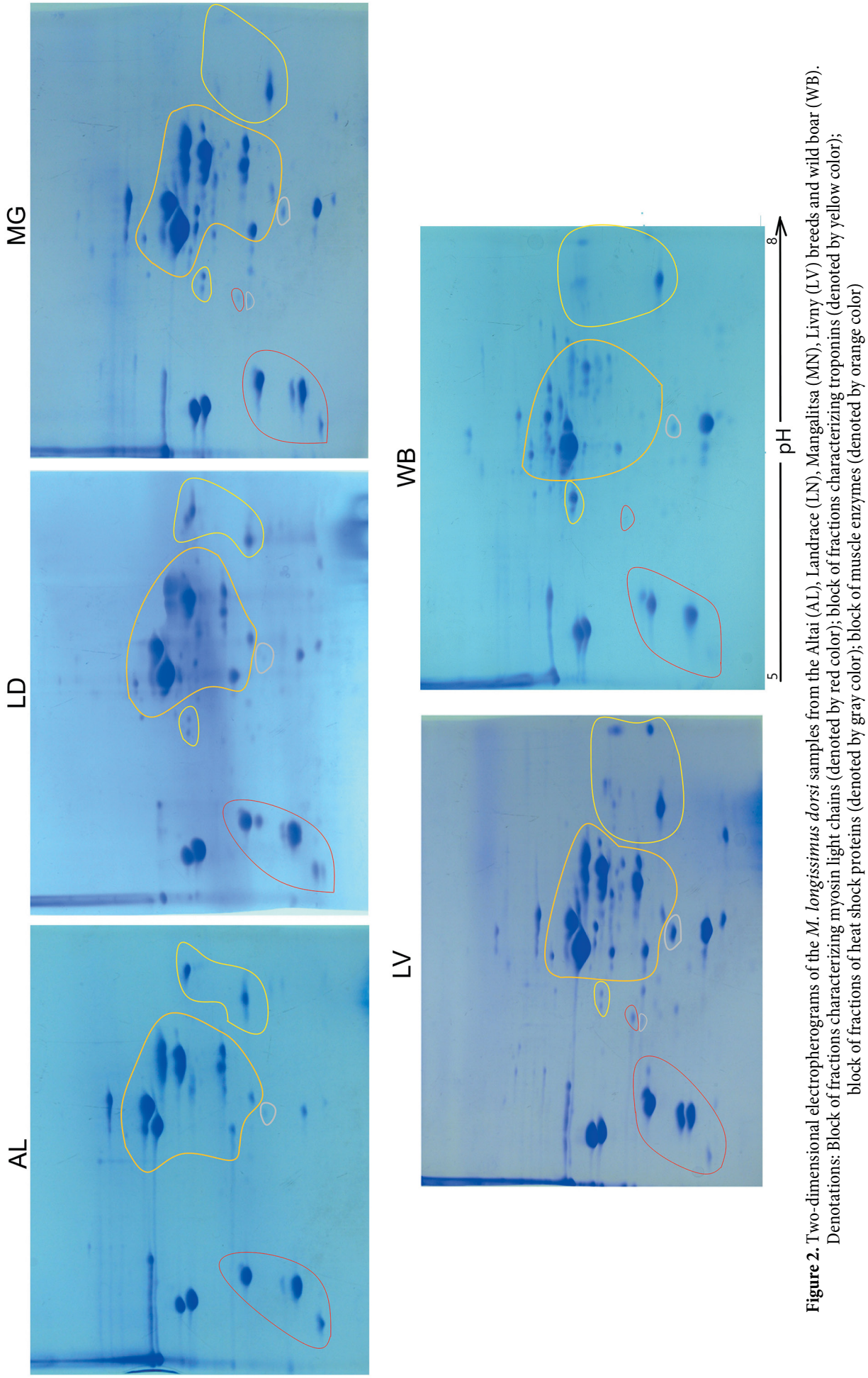

Figure 2. Two-dimensional electropherograms of the *M. longissimus dorsi* samples from the Altai (AL), Landrace (LN), Mangalitsa (MN), Livny (LV) breeds and wild boar (WB). Denotations: Block of fractions characterizing myosin light chains (denoted by red color); block of fractions characterizing troponins (denoted by yellow color); block of fractions of heat shock proteins (denoted by gray color); block of muscle enzymes (denoted by orange color)

With that, the main protein material in the analyzed samples was in the high-molecular-weight fraction, where peptides of the amino acid sequence from 6 to 224 positions out of 224 were determined. However, a minor fraction of the fragment 29–219 was additionally revealed, which was detected in four out of eight samples. In addition, in the major fraction, there is an additional spot shifted by pI to the acid side, apparently due to post-translational modification that has not been identified yet. The presence of the stable fragment indicates that the lifetime of muscle fibers containing MYL6B is less than that of the standard muscle fibers. An increase in the quantity of MYL6B is always linked with a decrease in the quantity of MYL3. It is worth noting that we identified the full-size MYL6B fraction in four out of eight samples, and in one case also its fragment, absolutely similar to what was revealed in the *L. Dorsi* samples from the Livny breed — slow MYL6B isoform accounted for 8.1% on average, which in total accounted for 1/3 of the quantity of MLC in muscle tissue. Therefore, the two-fold decrease in the quantity of shorter (out of two) forms of MYL1 in the Livny breed correlated with replacement of part of long MYL1 isoform with slow type of essential chains MYL3 and MYL6B.

With regard to the regulatory myosin light chain (MYL2, No. 4–6 in Table 1), significant changes in the samples of the analyzed breeds were also noted. According to the results of the computer densitometry, the quantity of MYL2 in *M. L. Dorsi* from Livny and Mangalitsa pigs increased by 5.5 times compared to other breeds. This calcium regulated contractile protein in muscle tissue is presented in a form of two closely located fractions. For *L. Dorsi* from Landrace, phosphorylation at the amino acid position 15S was found in one of the fractions, which determines the mechanism of contraction. For domestic pig, this modification was confirmed for the first time, although in humans this variant was revealed long before with experimental support [21,22].

Among identified proteins, there were also other representatives of slow and fast isoforms of proteins, in particular, a complex of fractions of troponin T of slow skeletal muscles, which in domestic pigs is represented as a set from a range of fractions differing by pI and weight. With that, the main quantity of these fractions belongs to higher-molecular-weight ones. Due to various types of post-translational modifications, this polymorphic protein is represented in a form of three fractions differing by pH, which ratio in the skeletal muscle of domestic pigs is 10/70/20% according to the results of computer densitometry.

Two representatives of the troponin family, troponin T (TNNT3, No. 12 in Table 1) and troponin I (TNNI2, No. 13–15 in Table 1), which belong to the fast type, had quantitative differences. Troponin I in the Altai and Landrace breeds was found in lower quantities than in other breeds. These genes express a whole family of various isoforms, which quantity is quite stable in muscle tissues. To reveal the presence of isoforms, we used the non-equilibrium variant of IEF with an increase to 2500 Volt/hour and not 3600 as in the equilibrium variant. As a result, the presence of additional fractions was found for *L. Dorsi* of the Livny breed: a fragment of 4.5 LIM domain-containing protein and troponin I (No. 20, 21 in Table 1), which belongs to the slow type and appeared as a fragment in a large quantity and without N-terminal part.

For the samples of wild boar and Mangalitsa, a clear link with a decrease in the quantity of slow isoforms was not revealed; the disappearance of the alkaline isoform was mainly noted. With that, it is possible to note that in the samples containing maximum quantity of *MYL6B* the redistribution of isoform by quantity took place. They became equal, which possibly was determined by changes in the ratio of muscle fibers of fast and slow types. Also, the fraction of cytoplasmic malate dehydrogenase was identified. It was found in trace quantities in all samples from wild boar.

Changes in the composition and distribution of heat shock proteins were noticed. αB crystallin (No. 23 in Table 1) is actively expressed in tissues with a high level of oxidative metabolism, such as skeletal and cardiac muscles [23], and performs a broad range of important functions. Its increased expression was found in red fibers (slow type) and is induced by various stress factors (temperature jumps and other extreme conditions), the presence of deep oxidative stress caused by disorder of mitochondrial metabolism. The importance of αβ-crystallin for a network of intermediate filaments is well known. αβ-crystallin can inhibit the assembly of intermediate filaments in cytoskeletal fractions, linked with intermediate filaments, thereby regulating interactions of intermediate filaments with other cellular proteins. It was assumed that down-regulation of αβ- crystalline facilitates proteolytic degradation of actin and myosin, which leads to more tender meat. For *L. Dorsi* from the Livny breed, Mangalitsa and wild boar, its quantity increased up to three times compared to that from Landrace and Altai breeds of pigs. The ratio of the crystalline and MYL6B levels allows for assuming a correlation of these indicators. In addition, an increase in the content of heat shock protein HSPB6 (No. 24 in Table 1) was revealed, which was acetylated by the N-terminal part in *L. Dorsi* from the Livny breed and Mangalitsa. Also in three samples of wild boar, the quantity of HSPB6 was higher compared to that in the Livny and Landrace breeds. This protein is similar in structure and function with αβ-crystallin and, apparently, an increased level is also related to changes in the proportion of fast and slow muscle fibers.

Variability in the content of muscle enzymes was observed. In *L. Dorsi* from Mangalitsa, Landrace and four samples from wild boar, the ratios of ATP synthase subunit alpha (ATP5F1A, No. 25 in Table 1) were at a close level. With that, its content was increased in three samples from wild boar and the samples from the Livny breed, which can be linked with an increase in the quantity of mitochondria in muscle cells. For the Livny breed, a fluctuation in the quantity of aldolase A (No. 35–36 in Table 1) was also observed, which was more dense in *L. Dorsi* from the Altai breed (by more than 1.35 times ($p < 0.05$) compared to other pigs).

Table 1. Identified protein fractions from domestic pig and wild boar characterizing breed differences

No.	Name of protein (symbol of gene)	Number in database	S/M/C*	MM/ pI (exp.)**	MM/ pI (calc.)**
	Myosin light chains				
1	Myosin light chain 1 (MYL1)***(1)	A0A5G2QVS9	154/23/70	23,0/9,50	20,9/4,86
2	Myosin light chain 1 (MYL1)***(2) + Acetyl (N-term)	A0A287BJF1	116/27/75	17,0/4,75	20,6/4,94
3	Myosin light chain 1 (MYL1)***(2) + Acetyl (N-term)	A0A287BJF1	198/20/72	17,0/4,75	20,6/4,94
4	MYL2 (MYL2)***(3)	Q8MHY0	288/49/93	19,0/4,85	18,9/4,8
5	MYL2 (MYL2)***(3) + Phospho (15S)	Q8MHY0	286/45/95	19,0/4,85	18,9/4,86
6	MYL2 (MYL2)***(2)	Q8MHY0	389/44/93	19,0/4,85	18,9/4,86
7	Myosin light chain 3 (MYL3) + Acetyl (Protein N-term	F1SNW4	200/29/80	22,0/5,00	21,7/5,00
8	Myosin light chain 3 (MYL3) ***(1)	F1SNW4	164/33/69	22,0/5,00	21,7/5,00
9	Fragment Uncharacterized protein (MYL6B)***(1)	F1SM01	207/23/82	22,0/5,20	24,0/5,53
10	Mixture of Uncharacterized protein (MYL6B)***(1) and Carbonic anhydrase 3 (CA3)***(1)	F1SM01 Q5S1S4	326/30/92 75/10/50	24,0/5,80	24,0/5,53 29,4/7,72
11	Mixture of Uncharacterized protein (MYL6B) and Carbonic anhydrase 3 (CA3)***(1)	F1SM01 Q5S1S4	374/33/92 106/6/32	24,0/5,80	24,0/5,53 29,4/7,72
	Troponins				
12	Troponin T, fast skeletal muscle (TNNT3)***(2)	Q75NG7	231/57/79	30,0/9,50	30,7/8,69
13	Troponin I (TNNI2) + Acetyl (Protein N-term)	Q4JH15	254/24/64	21.0/9,30	21,3/9,02
14	Troponin I (TNNI2) + Acetyl (Protein N-term)	Q4JH15	398/44/86	21.0/9,30	21,3/9,02
15	Troponin I (TNNI2)+ Acetyl (Protein N-term) with fragment PDZ and LIM domain 7 (PDLIM7)	Q4JH15 A0A287AZ96	285/34/73 48/7/33	21.0/9,30	21,3/9,02 25,2/9,65
16	Mixture of Troponin I1, slow skeletal type (TNNI1) and Myosin light chain 3 (MYL3)	A0A287BG25 F1SNW4	67/9/32 41/7/28	23,0/9,50	24,8/9,77 21.7/5.00
17	Mixture of Four and a half LIM domains (FHL1)***(1) and Troponin T, fast skeletal muscle (TNNT3)***(1)	A0A5K1U1D3 Q75NG7	69/4/7 197/4/26	30,0/9,50	40,7/9,24 30,7/8,69
18	Mixture of Isoform 2 of Troponin T, slow skeletal muscle (TNNT1); L-lactate dehydrogenase A chain (LDHA) and Voltage-dependent anion-selective channel protein 2 (VDAC2)	Q75ZZ6-2 P00339 F1S2F6	201/33/73 88/15/55 70/12/57	30,0/7,40	30,0/6,41 36,6/8,18 31,6/7,48
19	Mixture of Isoform 2 of Troponin T, slow skeletal muscle (TNNT1) + Acetyl (Protein N-term); L-lactate dehydrogenase A chain (LDHA) and Voltage-dependent anion-selective channel protein 2 (VDAC2) + Acetyl (Protein N-term); fragment Creatine kinase (CKM)	Q75ZZ6-2 P00339 F1S2F6 A0A287AMP3	267/37/79 49/9/35 56/10/53 41/10/36	30,0/7,40	30,0/6,41 36,6/8,18 31,6/7,48
20	Troponin T, fast skeletal muscle (TNNT3)***(2) with C-term fragment Four and a half LIM domains 1 (FHL1)***(1)	Q75NG7 A0A5K1UHX7	189/30/55 108/20/48	30,0/8,40	30,7/8,69 40,1/8,82
21	Four and a half LIM domains 1 (FHL1)***(1)	A0A5K1UHX7	309/58/74	29,0/8,70	40,1/8,82
22	Troponin I (TNNI1)	B3VCE8	399/52/92	22,0/9,00	21,6/9,61
	Heat shock proteins				
23	Alpha-crystallin B chain (CRYAB) + Acetyl (Protein N-term)	Q7M2W6	322/27/99	20,0/7,60	20,1/6,76
24	Heat shock protein family B (small) member (HSPB6) + Acetyl (Protein N-term)	A0A287AQR8	254/15	17,5/6,00	17,4/5,95
	Muscle enzymes				
25	ATP synthase subunit alpha (ATP5F1A)	F1RPS8	399/44/66	52,0/7,40	58,2/8,95
26	Mixture of Adenylate kinase isoenzyme 1 (AK1) and C-term fragment of Superoxide dismutase [Mn], mitochondrial dismutase (SOD2)	P00571 A0A287A8A8	375/36/86 315/8/68	20,5/7,90	21,6/8,38 48,5/10,68
27	Mixture of Adenylate kinase isoenzyme 1 (AK1) and C-term fragment of Superoxide dismutase [Mn], mitochondrial dismutase (SOD2)***(2)	P00571 A0A287A8A8	22/144/70 359/17/39	20,5/7,90	21,6/8,38 48,5/10,68
28	Mixture of Adenylate kinase isoenzyme 1 (AK1) and fragment PDZ and LIM domain protein 3 (PDLIM3)***(1)	P00571 Q6QGC0	268/31/82 119/05/21	21,5/7,95	21,6/8,38 39,5/6,79
29	Mixture of Adenylate kinase isoenzyme 1 (AK1) and Superoxide dismutase (SOD2)***(1)	P00571 A0A287A4Z2	304/36/86 164/19/76	20,5/7,90	21,6/8,38 27,1/8,52
30	Adenylate kinase isoenzyme 1 (AK1)	P00571	509/44/92	21,5/7,95	21,6/8,38
31	Creatine kinase M-type (CKM)***(1)	Q5XLD3	389/42/84	43,0/7,40	43,0/6,61
32	Creatine kinase M-type (CKM)***(1)	Q5XLD3	467/47/88	43,0/7,40	43,0/6,61
33	2-phospho-D-glycerate hydro-lyase (ENO3)***(1)	A0A5Г2P7P6	806/54/85	45,0/7,70	46,9/8,05
34	2-phospho-D-glycerate hydro-lyase (ENO3)***(3)	A0A5Г2P7P6	817/57/84	45,0/7,70	46,9/8,05
35	Fructose-bisphosphate aldolase (ALDOA)***(1)	A0A286ZYX8	551/44/85	40,0/8,60	39,8/8,49
36	Fructose-bisphosphate aldolase (ALDOA)	A0A286ZYX8	522/44/84	40,0/8,60	39,8/8,49
37	Mixture of Acyl-CoA-binding protein (DBI), Cardiac phospholamban (PLN)***(1), C-term fragment of Adenylate kinase isoenzyme 1 (AK1)***(1)	A0A5S6I0G9 P61013 A0A286ZQ79	82/8/62 134/6/42 92/3/13	10,0/6,80	9,9/8,07 6,1/9,15 29.1/9.08
38	Ubiquitin B (UBB)***(2), Ubiquitin-60S ribosomal protein L40 (UBA52)***(2) Ubiquitin carboxyl extension protein 80 (RPS27A)***(2)	A7U5U2 P63053 A0A287AZA7	325/31/62 288/31/73 277/31/36	8,0/6,50	25,7/6,86 14,7/9,87 22,8/9,69
39	Ubiquitin B (UBB)***(2)	A7U5U2	354/16	8,0/6,50	25,7/6,86

* S/M/C — the Mascot Score is an indicator of conformity or "scorecard"; match peptides are the number of matched peptides; coverage is the percentage of the complete amino acid sequence of the protein using the identified peptides.

** MM/pI (exp.) are the obtained estimates according to electrophoretic mobility in the DE, MM/pI (calc.) are the estimates made using the data on the amino acid sequence, taking into account signal peptide removal, but with no consideration of other post-synthetic modifications using the ExPASy Compute pI/Mwtool software.

*** msms — indication of confirmatory identification by tandem mass spectrometry, the number of sequenced tryptic peptides is indicated in parentheses.

For *L. Dorsi* from the Altai, Livny, Landrace and Mangalitsa breeds, a mixture of adenylate kinase 1 and C-terminal fragment of mitochondrial superoxide dismutase (No. 26 in Table 1) was found and its quantity was 2–3 times higher than that in wild boar. Presumably, the fraction of adenylate kinase 1 has two isoforms, one of which is modified and its quantity is changed depending on the breed. Adenylate kinase catalyzes the interconversion of various adenosine phosphates (ATP, ADP and AMP) and plays an important role in cellular energy homeostasis.

The fraction of muscle enolase (ENO3) in muscle tissue is one of the major in all analyzed samples. In domestic pigs it is comparable in quantity with muscle creatine phosphokinase (Figure 1, No. 31–34 in Table 1), except for the Livny breed and wild boar, where the quantity reduced practically three times (by 16 % when norming by the quantity of muscle creatine phosphokinase), which can be linked to reduced glycogen synthesis. In the Altai, Mangalitsa and Livny breeds, modified fraction of ENO3 was 21.5 % of the major fraction. In the samples of *L. Dorsi* from wild boar, the quantity of ENO3 varied — in five samples the quantity reduced up to two times, while in three samples it reduced even more.

Upon identification of the ENO3 fraction in the Livny breed, the amino acid replacement 298Q/K — glutamine with lysine — was revealed. In the samples of the Altai breed, 298Q (glutamine) was present in this position. The sequence corresponded to Landrace. In the Uniprot database, the information about amino acid replacements in protein is absent, and it is denoted as a canonical form 298Q like in the database Sus_scrofa scrofa_20210319 (47259 sequences; 25814658 residues). In one sample from wild boar, the results of identification (No. 48) showed that it is muscle enolase ENO3; however, the spectrum of tryptic peptides contained a mass peak corresponding to the variant 298Q, and tandem mass spectrometry of the peak with m/z 1050.5 showed (like in the Livny breed) that the variant 298K is also present. Therefore, this animal was heterozygous and possibly the occurrence of these variants in wild animals is different from domestic breeds/ hybrids, which are more homogeneous in terms of genome.

In the Livny breed, Mangalitsa breed and wild boar, the presence of the ubiquitin fraction (No. 38,39 in Table 1) was revealed. This protein was discovered in 1975 [24]. Mammals (including humans) have four different genes encoding ubiquitin — UBB, UBC, UBA52 and RPS27A. Each of these genes codes a single copy of ubiquitin in the composition of polyprotein (polypeptide consisting of the precursors of several proteins that subsequently separate as a result of restricted proteolysis). The product of the UBA52 gene is initially synthesized as ubiquitin "sewn" to ribosomal protein L40, and the product of the RPS27A gene as ubiquitin "sewn" to S27A. Ubiquitin contains 74 amino acid residues, the calculated parameters of mass/pI is 8.45/6.56. All revealed peptides were exactly within its amino acid sequence. One of the functions of ubiquitin is lipid binding and formation of fat layers in muscle tissue. Apparently, an increased quantity of ubiquitin can be realized through the enhanced formation of fat layers.

Discussion

Table 2 presents qualitative differences in changes in proteins between studied domestic pig breeds and wild boar.

When performing investigations of the muscle tissue samples from breeds/hybrids of domestic pigs, it was found that they clearly differ by the composition of different variants of fast and slow types of muscle tissue. Myosin light chains are traditionally linked with the growth of muscles. In pigs, muscle growth and differences in muscle fibers can be regulated by MYL3 [29]; with that, MYL3 transcripts were found in abundance in Pietrain and Polish Landrace [30]. We revealed in our work an increase in expression of MYL3 in the samples of tissues from the Altai and Livny breeds as well as in Landrace.

Puig-Oliveras et al. [31] showed differential expression of MYL6B in muscles when comparing groups with high and low level of backcrossing of Iberian x Landrace by the peculiarities of the fatty acid composition. High expression of myosin light chains (MYL2, MYL3 and MYL6B) was revealed in normally stained part of m. *biceps femoris*, in cardiac muscle [32]. A high level of expression of MYL2 gene

Table 2. Summary table of the obtained results on changes in protein fractions

Fractions	Altai breed	Landrace breed	Mangalitsa breed	Livny breed	Wild boar
Myosin light chains	↑↑↑ MYL3;	↑↑ MYL3	MYL1/MYL3 pI shift to 9.5	↓↓↓ MYL1; ↑↑MYL2; ↑↑↑ MYL3; appearance of MYL6B	Replacement of MYL3 with MYL6B isoform;
Troponins	↓↓ TNNT3, TNNI2	↓↓ TNNT3, TNNI2		Formation of fragment FHL1; appearance of TNNI1	
Heat shock proteins			↑↑↑ CRYAB, HSPB6	↑↑↑ CRYAB, HSPB6	↑↑↑ CRYAB
Muscle enzymes	↑↑ATP5F1A ↑↑AK1+SOD2	↑↑ATP5F1A ↑↑AK1+SOD2	↑↑ATP5F1A ↑↑AK1+SOD2 ↑↑UBB	↑↑↑ ATP5F1A ↑↑AK1+SOD2 ↓↓ ENO3+a.з. 298Q/K ↑↑↑ UBB +a.з. 68M/H	↓↓↓ ENO3, +a.з. 298Q/K ↑ UBB

was established in skeletal muscles of Duroc and Large White pigs compared to the local breed Piau. The authors indicated the relation of the gene to the growth of mammalian skeletal muscles [8]. We revealed appearance of the MYL6B fractions in *M. Dorsi* from pigs of the Livny breed and replacement of a part of MYL3 fractions with MYL6B isoform in the samples from wild boar.

A decrease in the MYL1 expression upon an increase in MYL2 in pigs of the Livny breed as well as the shift of the isoelectric point of MYL1/MYL3 fractions in Mangalitsa can be associated with the increased formation of intermuscular and intramuscular fat, which classifies them as fat-type breeds. This is confirmed by the results of [33]. Based on the results of the revealed different patterns of MYL1 expression, the authors characterized the local breed Lantang as fatty and commercial breed Landrace as lean.

The quantity of troponin T is regarded as a good indicator of meat tenderness [34]. TNNI1 plays an important role in muscle development and determines quality characteristics of meat, which was demonstrated in [35] on meat samples from pigs of the Mongcai breed. Appearance of slow troponin I in the muscle tissue samples from Livny pigs can also be linked with an increased content of intramuscular fat [36]. TNNI1 is assigned to the sex-specific biomarker upon low level of intramuscular fat [37] and is linked with type I fiber in Large White pigs [38]. It can be assumed that in the Livny breed there are certain differences in the FHL1 gene, which encodes 4.5 LIM domain-containing protein responsible for the development of skeletal and cardiac muscles [33,39]. These differences lead to the accelerated decomposition of this protein and changes in the development of muscle fibers as defects in this gene result in the development of a range of myopathies [40].

The content of HSPB6 can decrease during rigor mortis and is usually associated with beef tenderness [41,42]. Its increased content in Mangalitsa and Livny pigs can be an indicator of higher toughness of meat, which is balanced by increased fatness.

It is interesting that the ATP5F1A gene is linked with increased female and male fertility in Swallow-bellied Mangalitsa [43] and, correspondingly, its expression was increased in all analyzed pig breeds except for wild boar.

An increase in the expression of the mixture of adenylate kinase 1 (AK1) and C-terminal fragment of mitochondrial superoxide dismutase (SOD2) was revealed in muscle tissue of the Altai, Livny, Landrace and Mangalitsa breeds despite data about their reduced content in lean commercial breeds [44] and high expression in combination with an increased content of ENO3 in fat-type local breeds [33].

The presence of polymorphisms in the ENO3 gene can be linked with a percentage of fat, average thickness of backfat, meat marbling and amount of intramuscular fat in two different populations of domestic pigs [45]. For Mangalitsa and wild boar, the revealed combination of the reduced level of ENO3 with the amino acid replacement 298Q/K can facilitate better survival of animals, in particular, due to specific features of the development of fat layer upon temperature fluctuations [46].

An increased expression of protein ubiquitin (UBB) in our study was shown for Livny pigs, Mangalitsa and wild boar, which can be related to the characteristics of semen [47] and is linked with the regulation of the transcriptional network of the innate immunity [48]. In addition, the ubiquitin — proteasome system influences muscle homeostasis and development of fat layers [49].

Predictions of the protein interaction network demonstrated that differences in the growth of muscle and muscle fibers between Chinese local breeds and introduced Western breeds are regulated by genes ENO3, TNNT3 and MYL3. Gene TNNT3 also was described as a candidate gene for meat quality in Tunchang pigs [50].

Conclusion

Identified proteins represent the coherent functional system and are included in the functional clusters of muscle work: from the contractile apparatus (troponin, myosin) to the systems of its energy supply (glycolysis, creatine kinase system, oxidative phosphorylation) and defense against the accompanying metabolic stress (antioxidant system), including for maintenance of the long-term integrity of muscle fibers in conditions of high functional load.

Compared to the Altai and Landrace breeds, pigs of the Livny and Mangalitsa breeds are more similar to wild boar by a broad range of traits. For pigs of the Livny breed changes touch all analyzed blocks of protein fractions. Of particular interest is the appearance of the myosin fractions MYL6B, hydrolase ENO3 with the amino acid replacement 298Q/K upon an increase in the content of alpha-crystallin CRYAB, which is typical of wild boar.

The revealed similarity of the proteomic profile between pigs of the Livny breed and wild boar, apparently, is a consequence of their genetic closeness, determined by crossbreeding of local long-eared pigs, which theoretically could crossbreed with wild boar. Keeping the Mangalitsa pigs in the free-range conditions upon lowered environmental temperatures determine the characteristic changes in the protein profile of muscle tissue, namely, modification of the expression of myosin light chains and an increase in the level of alpha-crystallin B-chain (CRYAB), which is approximated to the living conditions of wild boar.

The revealed systemic interrelations form a significant basis for the following deepened comparative analysis of proteomic landscapes and understanding of molecular evolution between different pig breeds and wild boar. Atypical proteins (MYL6B, variants ENO3 and UBB) are promising targets for deep molecular genetic analysis aimed at establishing their contribution to quality and functional-technological properties of meat raw materials.

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