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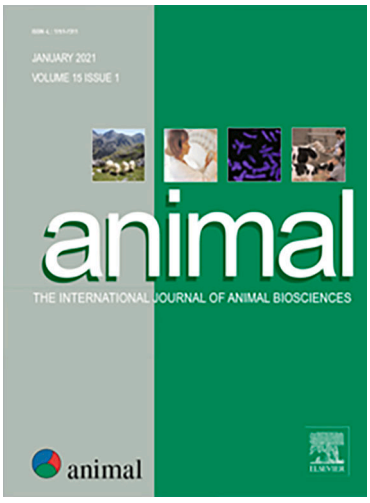

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Adipose tissue transcriptome profiles of local Krškopolje pig and modern hybrids receiving reduced protein diets

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Highlights

- Breed and low protein diet effects on adipose tissue transcriptome were studied
- Adipogenesis-related genes were increased in Krškopolje pigs compared to hybrids
- Collagen proteins, calcium signaling and inflammation varied between breeds
- Low protein diet caused minimal transcriptomic shifts in both pig breeds
- These results will help to adapt feeding and rearing strategies of Krškopolje pig

Abstract

Compared to lean pig genotypes, the local Krškopolje pig is characterized by a greater capacity for lipid and lower capacity for protein deposition, along with lower dietary protein requirements. The aim of the present study was to unravel the transcriptomic differences in subcutaneous adipose tissue between the local Krškopolje pig and modern hybrids, as well as to study the impact of the reduced protein diet in both genotypes. Differential gene expression analysis between local and modern pigs revealed 375 differentially expressed genes, with 189 upregulated and 186 downregulated in Krškopolje pig. Among the upregulated genes, several suggested more pronounced adipogenesis (*SLC7A10*, *ADIRF*, *INHBB*, and *SFRP2*) in the Krškopolje pig. In addition, genes encoding collagen proteins and extracellular matrix remodeling (e.g., *COL6A5*, *COL4A5*, *COL2A1*), calcium signalling (*TRPV4*, *CAMK2A*, *CALML5*), pro-inflammatory cytokines (e.g., *IL1A*, *TNFSF9*, *CXCL8*, *PTGS2* etc.) and cholesterol metabolism (*CYP1A1*, *CYP2A19*, *CES1* etc.) were differentially expressed. The reduced protein diet induced only minor changes in gene expression at the individual gene level. However, gene set enrichment analysis revealed that, in

Krškopolje pigs, dietary effects were associated with biological processes related to immune response, intracellular protein transport, and extracellular matrix organization. In the case of modern hybrids, reduced protein diet resulted in enrichment of gene sets related to oxidative phosphorylation, mitochondrial function, and energy metabolism. In conclusion, this study demonstrated that the adipose tissue of Krškopolje pigs compared with modern hybrids exhibited greater adipogenic potential, being also supported by higher expression of genes involved in extracellular matrix remodelling and inflammation. The impact of reduced protein diet on adipose-tissue gene expression was relatively small in both breeds, particularly in Krškopolje pig, suggesting a metabolic adaptability of this breed.

Keywords: Gene expression, breed comparison, low protein diet, adipogenesis, immune response

Implications

This study examined transcriptomic differences in subcutaneous adipose tissue between the local Krškopolje pig and modern hybrids, as well as the breed-specific response to reduced protein diets, for which a limited information is available. Relative to modern hybrids, identified gene expression networks in Krškopolje pigs suggested increased adipose tissue development and remodeling, inflammation, calcium signalling, and cholesterol metabolism. The reduced protein diet induced only minor transcriptomic changes but triggered genotype-specific biological process enrichments. These findings provide a new insight in the specific metabolic characteristics of Krškopolje pig that will help to improve rearing and feeding strategies.

Introduction

In recent years, local pig breeds have gained increased attention due to their distinctive characteristics, including superior meat quality (higher intramuscular fat content and more intensive meat colour), enhanced adaptability to local environmental conditions and greater resilience to feed shortages (Čandek-Potokar and Nieto, 2019). In addition to these traits, local pig breeds generally show lower potential for protein deposition (and thus lower protein or lysine requirements) and a greater tendency for lipid accumulation (Brossard et al., 2019) compared to modern lean breeds. The Krškopolje pig, a Slovenian local pig breed, exemplifies these characteristics, showing slower growth rates, reduced muscle development and larger fat deposition across various adipose tissue depots. It also features larger adipocytes and saturated and monounsaturated fatty acid content (Poklukar et al., 2023; Škrlep et al., 2024). Despite this, the underlying mechanisms driving the increased fat accumulation remain unexplored. Until now, only one study that compared Krškopolje pig and modern pig breeds on gene expression level has been conducted in the adipose tissue. This study employed a targeted gene expression approach focusing on the expression of the main genes involved in lipid metabolism. It demonstrated that genes involved in lipogenesis, adipogenesis and energy homeostasis were upregulated in Krškopolje pigs (Poklukar et al., 2023). Comparative studies involving other local pig breeds have revealed differential expressions of genes involved in lipogenesis, fatty acid desaturation, immune response, fatty acid transport, and extracellular matrix remodelling (Albuquerque et al., 2020; Poklukar et al., 2020; Zhang et al., 2021; Gong et al., 2022; Wu et al., 2024; Yan et al., 2025; Guo et al., 2025). However, these studies involved

various breeds (both local and modern) with different genetic backgrounds, ages, rearing conditions, and sexes, making it challenging to establish clear conclusions about metabolic properties.

In pig production, precision feeding is important for optimizing economic outcomes, enhancing sustainability, and decreasing reliance on intensive protein sources like soybean meals, reducing environmental impacts and promoting the use of locally available feed resources. In local pig diets, controlled protein reduction has been shown to affect meat quality by increasing intramuscular fat content and altering adipose tissue characteristics without compromising production performance (Madeira et al., 2013; Pugliese et al., 2013; Sirtori et al., 2014). Still, the broader impact of reduced protein diets on adipose tissue development in local breeds remains poorly explored. Transcriptomic analysis in Krškopolje pigs has shown enrichment of genes related to lipid deposition, fatty acid saturation, oxidative stress, and mitochondrial function in animals fed reduced protein diets outdoors, while immunity-related genes were enriched in indoor-reared pigs (Poklukar et al., 2025). A recent study (Škrlep et al., 2024) examined both Krškopolje and modern hybrids to assess the effects of genotype and protein reduction. It highlighted that Krškopolje pigs exhibited greater fat deposition along with greater activity of lipogenic enzymes, reduced muscle leanness, and a smaller growth rate compared to modern hybrids. It also showed that similar level of protein reduction negatively impacted modern hybrids leading to decreased growth performance and muscle development, but had no distinct effect on Krškopolje pigs. However, this differential response highlights the need for further investigation into both inter-breed differences and different response to protein reduction.

Therefore, we hypothesized that adipose tissue of Krškopolje pigs compared to modern hybrids would exhibit differential expression of genes associated with adipose tissue development and gene set enrichment of related biological processes. We further hypothesized that a reduced protein diet would induce a different transcriptional response in modern hybrids and Krškopolje pigs, reflecting greater metabolic adaptability to nutrient fluctuations in the latter. The main aim of the present study was to investigate gene expression differences and regulatory networks in the adipose tissue transcriptome of the fatty Krškopolje pig compared with modern hybrids when fattened to heavy weights, while also assessing the impact of a reduced protein diet on both breeds.

Material and methods

Study design

A total of 29 male castrates, including 15 Krškopolje pig and modern 14 hybrid pigs (progeny of Landrace x Large White sows and Pietrain boars) were allocated within breed and dietary treatments (i.e. standard and reduced protein) in four identical pens with indoor (17 m^2) and outdoor area (17 m^2). Due to the assumed breed-specific nutritional requirements, different CP levels were tested: modern hybrids receiving high (**HP**, n = 7) and medium protein (**MP**, n = 7), and the Krškopolje pigs receiving MP (n = 7) and low protein (**LP**, n = 8) diets. Three-phase feeding was applied using five different isoenergetic feeds that varied in CP level. The feeding phases and diets with CP level for each treatment group are shown in Figure 1. Briefly, the modern hybrid HP group received 16.7%, 14.6%, and 12.6% of CP across the first, second and third

phase, respectively. The modern hybrid and Krškopolje pig MP groups received 16.7%, 12.6%, and 10.4% of CP, while the Krškopolje pig LP group received 16.7%, 10.4%, and 9.3% of CP across the same phases. Pigs were fed *ad libitum* until week 12, afterwards feed intake was restricted. Thereafter, all animals received equal amounts of feed through automatic feeding stations (Compident MLP II PRO, Schauer Agrotonic GmbH, Prambachkirchen), allowing individual control of intake and animal weight. During the first feeding phase, the average feed intake was 1.64 kg per pig per day, increasing to 2.42 kg in the second phase and 2.81 kg in the third phase. More details on the experiment (feed composition, dietary treatments, in addition to productivity, carcass composition, meat quality and biochemical traits) are available in Škrlep et al. (2024). As indicated therein, the average daily gain was lower in Krškopolje pigs than in modern hybrids across all phases (611.5 and 720.8 g/day, respectively). Protein reduction significantly decreased average daily gain only in modern hybrids during the last phase (i.e. 767.5 and 720.8 g/day in HP and MP groups, respectively), whereas no significant differences were observed in Krškopolje pigs. The fattening experiment lasted for 178 days, when the pigs were slaughtered according to a commercial slaughter procedure. Immediately after slaughter, samples of backfat were collected from the carcass split line at the level of last rib and frozen at -80 °C for further RNA extraction.

RNA extraction

RNA was extracted from the subcutaneous adipose tissue using RNeasy Lipid Tissue Mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The purity of RNA was evaluated by measuring absorbance ratios at 260 nm and 280 nm, as well as at 260 nm and 230 nm using a Tecan Infinite 200 Pro M Nano+ spectrophotometer. Quantification was performed with a Qubit 4.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), and RNA integrity was assessed using the RNA 6000 Nano kit on an Agilent 5300 Fragment Analyzer (Agilent Technologies, Palo Alto, CA, USA). All samples had RNA integrity values greater than 6.

RNA library preparation and sequencing

RNA sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit (NEB, Ipswich, MA, USA) according to the protocol provided by the manufacturer. Quality assessment of the resulting libraries was performed using the NGS Kit on the Agilent 5300 Fragment Analyzer (Agilent Technologies, Palo Alto, CA, USA). Libraries were quantified using the Qubit 4.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), multiplexed and loaded onto a flow cell on the Illumina NovaSeq 6000 instrument (2x150 Pair-End configuration v1.5.). NovaSeq Control Software v1.7 was used for image analysis and base calling. The sequencing output was converted into FASTQ files and demultiplexed using the bcl2fastq software (v2.20), allowing one mismatch for index sequence identification.

Bioinformatics analysis of RNA sequencing data

Raw data quality was assessed with FastQC v1.0.0 software. Sequence reads were then trimmed using Trimmomatic v.0.36. The resulting high-quality reads were then aligned to the *Sus scrofa* 11.1 reference genome (Ensembl release 109) using the STAR aligner v2.5.2b. Gene-level quantification was performed with featureCounts

(from the Subread package v1.5.2), considering only uniquely mapped reads located within annotated exon regions. The resulting gene count matrix was subsequently used for differential gene expression analysis with the DESeq2 R package (Love et al., 2014). Differentially expressed genes between breeds (Krškopolje pigs vs. modern hybrids fed MP diet) and diets (Krškopolje pigs fed LP vs. control MP diet; modern hybrids fed MP vs. control HP diet) were identified. To generate *P*-values and adjustment of *P*-values, a Wald test and Benjamini Hochberg procedures were used, respectively. Genes with adjusted *P*-values < *0.05* and absolute $\log_2$ fold changes > 1 were called differentially expressed.

A more generic overview of biological processes impacted by breed and diet was generated by gene set enrichment analysis (**GSEA**) using the clusterProfiler R package (Yu et al., 2012; Korotkevich et al., 2021) on $\log_2$ fold change values to identify terms, that are enriched in highly ranked genes. Identified biological process terms were corrected with Benjamini–Hochberg method, and significant terms were filtered at an adjusted *P*-value cutoff of 0.05. The top 20 terms were selected for plotting. Additionally, gene ontology over-representation analysis was performed to determine biological processes enriched in Krškopolje pig compared to modern hybrids. The RNA sequencing dataset has been deposited in the NCBI Gene Expression Omnibus (Bethesda, MD, USA) under the accession number GSE309090.

Quantitative PCR

High-capacity cDNA Reverse Transcription Kit (Applied Biosystem, CA, USA) was used for reverse transcription of 0.8 µg of the total RNA according to the manufacturer's instructions. The complementary DNA samples were diluted 10-fold prior to the quantitative PCR reaction. Taq-man pre-design expression assays with fluorescent 6-FAM dye-labelled minor groove binder probes were obtained from Applied Biosystem (Table 1). Quantitative PCR was performed in 10 µl reactions using Taq man Universal PCR Master Mix II (Applied Biosystems, Waltham, MA, USA). Applied Biosystems 7500 Fast Real-time PCR System was used for quantitative PCR with the following cycling conditions: one cycle of 50 °C for 2 minutes, one cycle of 95 °C for 10 minutes, and 40 cycles of amplification (15 seconds at 95 °C and 1 minute at 60 °C). The PCR efficiency of each gene was defined using standard curves composed of three 10-fold complementary DNA dilutions. Peptidylprolyl isomerase A (*PPIA*), DNA Topoisomerase II Beta (*TOP2B*), Beta-2 microglobulin (*B2M*) and Tyrosine 3-monooxygenase (*YWHAZ*) were used as endogenous controls for data normalization with geometric averaging (Vandesompele et al., 2002). Normalized target transcripts were quantified according to the comparative Ct method (ΔCt = Ct geometric mean of controls − Ct target transcript). Normalized quantitative PCR data were analysed using Student t-test in R software.

Results

Sequencing yielded on average 46.7, 44.8, 44.3 and 50.2 million of total reads for modern hybrid HP and MP diets, and Krškopolje LP and MP diets, respectively (Supplementary Table S1). Out of these, 95.8% of all reads were uniquely mapped to *Sus scrofa* 11.1 reference genome.

Breed effect on gene expression

The transcriptome analysis of subcutaneous adipose tissue from Krškopolje pig and modern hybrids (both fed the same MP diet) revealed 375 differentially expressed genes, with 189 upregulated and 186 downregulated genes (Figure 2A, Supplementary Table S2). Among the annotated genes upregulated in Krškopolje pig compared to modern hybrids, *A2M*, *CDH1*, and *TCN* showed the highest $\log_2$ fold changes, all three ranging between 3.8 and 4.8. The downregulated genes with the greatest negative $\log_2$ fold changes (i.e. between -12.1 and -4.7) were *SLIT1*, *ADH1C*, and *SALL1*. The genes with the largest $\log_2$ fold change differences also ranked among the ones having the smallest *P*-adjusted values (being the most significant) (Figure 2B). Additionally, principal component analysis performed based on RNA-seq expression data revealed a clear separation between Krškopolje and modern hybrid samples, with the two groups forming distinct clusters (Supplementary Figure S1).

GSEA identified more than 200 enriched terms related to the biological process (Supplementary Table S3). Among the most enriched ones (Figure 3), terms related to RNA processing and translation (GO:0006412), immune response (GO:0006955), anatomical structure formation involved in morphogenesis (GO:0048646), and G protein-coupled receptor signalling pathway (GO:0007186) were identified (Figure 3). In addition, gene ontology over-representation analysis performed with differentially expressed significant genes highlighted enrichment of fat cell differentiation (GO:0045444), long-chain fatty acid metabolic process (GO:0001676), and calcium ion transmembrane import into cytosol (GO:0097553) (Supplementary Table S4).

Diet effect on adipose tissue gene expression in Krškopolje pig and modern hybrids

Differential gene expression analysis between two Krškopolje pig groups, i.e. LP and MP diet (Figure 4A, Supplementary Table S5) revealed only 2 differentially expressed genes (i.e. *DIRAS5* and *ANGPTL5*), both being downregulated in LP group. In modern hybrids (Figure 4B, Supplementary Table S6), 7 differentially expressed genes were detected, with 4 upregulated (e.g. *MATN4*, *AGMO*) and 3 downregulated genes in pigs fed MP compared to control HP diet groups (e.g. *GZMB* and *ATP2A1*).

In the case of diet-induced gene expression changes in Krškopolje pigs, GSEA based on $\log_2$ fold change (Figure 5A; Supplementary Table S7) revealed enrichment in terms related to immune response (GO:0006955), extracellular matrix organization (GO:0030198), protein transport (GO:0015031) and establishment of protein localization (GO:0045184). In the case of modern hybrids, GSEA for the genes detected comparing MP compared to control HP diet (Figure 5B; Supplementary Table S8) revealed enrichment of biological terms related to generation of precursor metabolites and energy (GO:0006091) and cellular respiration (GO:0045333), mitochondrion organization (GO:0007005), cell migration (GO:0016477) and nucleoside phosphate metabolic process (GO:0006753).

Quantitative PCR analysis of candidate gene expression and RNA sequencing validation

Targeted gene expression approach was used on candidate genes involved in lipid or energy metabolism to additionally characterize the metabolic differences between Krškopolje pig and modern hybrids, as well as the effect of dietary-induced changes in

both breeds. No significant differences were detected for the lipogenic candidate genes studied between all the comparisons (Figure 6).

Based on the RNA sequencing results, five genes were selected for additional validation using quantitative PCR approach that showed significant differences when comparing both genotypes (Figure 6A) ($P < 0.05$). Specifically, *COL6A5*, *NR4A3*, and *CES1* were upregulated ($P < 0.05$), while *CYP1A1* and *WNT10B* were downregulated ($P < 0.05$). In addition, comparing Krškopolje pig fed LP with control MP group, *NR4A3* gene was downregulated ($P = 0.01$), even though this gene was not detected with RNA sequencing (Figure 6B).

Discussion

Comparison of local Krškopolje pig with modern hybrids under the same feeding and rearing conditions allowed identification of the main molecular networks and pathways linked to breed differences in body fat accumulation. Krškopolje pigs compared to modern hybrids showed differential expression of genes associated with adipogenesis. In adult pigs, when adipocytes reach their maximum volume, adipocytes can stimulate adipogenesis and hyperplasia by promoting the recruitment of new preadipocytes that can differentiate and accumulate lipids (Mersmann and Smith, 2005; Testroet et al., 2017; Urrutia et al., 2018). In the present study, Krškopolje pigs exhibited larger backfat thickness and adipocyte surface area than modern hybrids, while the hyperplasia on histomorphological level could not be confirmed. Although there were no differences in the expression of lipogenic genes in the present study, the activity of fatty acid synthase enzyme was still larger in Krškopolje pig (Škrlep et al., 2024). Nevertheless, based on our RNA sequencing data, it can be speculated that the higher expression of lipogenic genes likely occurred earlier, and that this was still reflected on the elevated activity of lipogenic enzymes. Larger adipogenesis, which is supported by the larger expression of multiple adipogenic genes, may contribute to the ongoing development of adipose tissue in Krškopolje pigs. Surprisingly, the expression of *PPARγ*, the master regulator of adipogenesis, did not differ between the two breeds. A possible explanation could be that the functional state of *PPARγ* gene expression is modulated post-transcriptionally or post-translationally (Kim et al., 2013). Nevertheless, many other genes and networks which are tightly associated with adipogenesis, have been identified in this study (e.g. genes involved in calcium signalling, extracellular matrix remodelling, inflammatory response and IGF signalling axis). For example, the upregulation of *INHBB* gene (encoding pro-adipogenic gene activin) (Dani, 2013) and *ADIRF* gene (encoding adipogenesis regulatory factor regulating *PPARγ*; Ni et al., 2013) were identified in Krškopolje pig compared to modern hybrids. Among the gene networks, genes involved in Wnt signaling pathway were differentially expressed. The upregulated *SFRP2* gene (encoding secreted frizzled-related protein 2) in Krškopolje pig can modulate Wnt signaling by binding to Wnt proteins (including *WNT10B*) and inhibit their activity (Guan et al., 2021). Inhibition of *WNT10B* activity further promotes adipogenesis (Perkins et al., 2023). Consistent with this, *WNT10B* expression was downregulated in Krškopolje pigs.

Calcium signalling is another gene network implicated in the regulation of adipogenesis and energy metabolism (Zhai et al., 2020) that involves genes *TRPV4*, *CAMK2A*, *CALML5*, and *CACNA1S*. An imbalance in Ca^{2+} distribution can disrupt adipocyte function, leading to increased fat accumulation and contributing to obesity (Zou et al.,

2024). In this study, *TRPV4* (encoding a calcium channel gene that regulates intracellular Ca^{2+} levels) was downregulated in Krškopolje pigs compared to modern hybrids. Accordingly, reduced *TRPV4* expression has been previously associated with increased differentiation of preadipocytes into mature adipocytes (Kumar et al., 2024).

Supporting the enhanced adipogenic capacity, several genes involved in the extracellular matrix remodelling were differentially expressed (upregulation of *COL6A5*, *COL19A1*, and downregulation of *COL20A1*, *COL11A2*, *COL26A1*, *COL4A5*, *COL21A1*, and *COL2A1*) in Krškopolje pig compared to hybrids. The extracellular matrix plays a crucial role in the regulation of adipose tissue architecture by providing structural support and influencing adipogenesis. During adipocyte differentiation, the collagen network undergoes significant remodelling, facilitating changes in extracellular matrix composition that enable preadipocyte expansion and lipid accumulation (Divoux and Clément, 2011). Besides genes for collagen proteins, several additional genes involved in extracellular matrix remodelling and migration were differentially expressed between breeds, such as the downregulation of *LAMA1* (encoding laminin subunit alpha-1) or upregulation of *CCBE1* (collagen and calcium binding EGF domains 1), which provide structural support and promote cellular adhesion and migration (Goddi et al., 2021). Contrary to the present study, transcriptomic study comparing local Iberian compared to Duroc pig breed revealed the downregulation of genes involved in calcium signalling (calmodulins) and extracellular matrix (collagen) in Iberian pig (Benítez et al., 2019). Since the research of Benítez et al. (2019) was performed on younger animals, a possible explanation for the inconsistencies between their and present study could be different age, as well as different physiological maturity of the breeds (Mourot et al., 1996).

Adipose tissue is a metabolically active endocrine organ that secretes cytokines, chemokines, and adipokines involved in regulating energy homeostasis and immune function. With the expansion of the adipose tissue, hypertrophic adipocytes and immune cells undergo phenotype changes, leading to secretion of inflammatory mediators that contribute to low-grade inflammation (Kawai et al., 2021). This is in accordance with the enrichment of the immune response term and significantly expressed genes encoding pro-inflammatory cytokines (e.g., *IL1A*, *TNFSF9*) and chemokines (e.g., *CCL4*, *CXCL2*, *CXCL8*). An example of pro-inflammatory mediators identified in the present study are enzyme cyclooxygenase-2 (*PTGS2* gene), which contributes to the pro-inflammatory status of adipose tissue (Pan et al., 2022), and interleukin 1A (*IL-1A* gene), which is released in response to hypertrophic adipocyte death, promoting the recruitment of innate immune cells. This cytokine also modulates adipocyte differentiation and insulin signalling (Ballak et al., 2015) and polymorphisms have been linked to variations in intramuscular fat content and fatty acid profile in porcine muscle (Pothakam et al., 2021). The chemokines *CXCL2* (C-X-C motif chemokine ligand 2) and *CCL4* (encoding C-C motif chemokine ligand 4) were also upregulated in Krškopolje pigs compared to modern hybrids, which is consistent with findings in local Basque pigs relative to Large White pigs (Vincent et al., 2012).

In accordance with the adipose tissue pro-inflammatory status, extracellular matrix remodelling and adipogenesis, a gene ontology term response to transforming growth factor beta stimulus was significantly enriched when Krškopolje pigs were compared to modern hybrids. Transforming growth factor beta is a profibrotic and proinflammatory factor that can also negatively regulate adipogenesis (Lee, 2018). Differential expression of inflammation-related genes has previously been linked to an

increased susceptibility to obesity-associated conditions, such as insulin resistance and metabolic syndrome in the fatty Iberian pig breed (Óvilo et al., 2010; Palma-Granados et al., 2025), as well as in humans and rodents (Ruiz-Ojeda et al., 2019). However, since the present study did not include the assessment of physiological and biochemical parameters indicative of these metabolic alterations in the Krškopolje pig, such associations cannot be directly inferred from our results.

In the present study, two IGF binding proteins (*IGFBP6* and *IGFBP5*) were downregulated in the Krškopolje pig that are also associated with adipogenesis. IGFBPs regulate the IGF signalling axis and play a key role in growth and development processes such as proliferation, differentiation, as well as glucose metabolism (Bach, 2015; Liso et al., 2022) and immune response (Wang et al., 2024). Binding to IGFBP increases the half-life of IGF and blocks its potential binding to the insulin receptor (Allard and Duan, 2018). Several metalloproteinases (i.e. pappalysins *PAPPA* and *PAPPA2*) can cleave IGF from IGFBP complex, and this consequently increases the amount of bioavailable IGF. The IGF can further bind to its receptor and activate downstream signalling pathways, which are involved in growth, metabolism, and tissue development, as well as maintain homeostasis during metabolic stresses (Chang et al., 2016). In accordance, in the present study, both metalloproteinase genes were upregulated in Krškopolje pig.

RNA sequencing identified few genes involved in fatty acid metabolism, steroidogenesis and cholesterol synthesis that were downregulated in Krškopolje pig compared to modern hybrids (members the cytochrome P450 superfamily of monooxygenases; *CYP1A1*, *CYP2A19*, *CYP2B6B*). In addition, *CES1* gene (encoding an enzyme involved in the hydrolysis of fatty acyl and cholesterol esters) was upregulated in Krškopolje pigs. Similar upregulation has been reported in Korean native and Basque pigs compared to modern breeds (Li et al., 2010; Gondret et al., 2012).

In response to protein reduction, a limited number of differentially expressed genes were identified in adipose tissue of both Krškopolje pigs and modern hybrids, consistent with the lack of substantial differences in biochemical and cellularity characteristics reported previously (Škrlep et al., 2024). The analysis was therefore extended using GSEA approach on genes ranked by $\log_2$ fold change, aiming to capture coordinated transcriptional changes across gene sets that fall below the significance threshold at the individual gene level that would otherwise remain undetected.

In Krškopolje pig, the reduced protein diet revealed decreased expression of genes involved in immune response and biological processes associated with it (e.g. innate and adaptive immune response, regulation of immune system, etc.). This is in accordance with our previous study on adipose tissue of Krškopolje pigs reared in intensive indoor conditions (Poklukar et al., 2025), which demonstrated that the reduced-protein diet suppressed the expression of several immunity-related genes associated with macrophage phagocytic functions. Besides, the study of Muñoz et al. (2021) showed inhibition of pathways in muscle and liver of pigs fed the LP diet that are involved in immune system. Accordingly, administration of HP diets has been previously shown to modify immune status and contribute to immunologic defense in pigs (Wang et al., 2018). Another group of enriched terms detected with GSEA approach are those related to extracellular matrix and structural organization biological

processes, which is also closely associated with adipose tissue immune response (Williams et al., 2015; Ruiz-Ojeda et al., 2019). As previously mentioned, the extracellular matrix in adipose tissue is in a state of constant turnover, and the enrichment of genes involved could reflect altered tissue homeostasis (de Sousa Neto et al., 2022).

In modern hybrids, dietary protein reduction resulted in gene set enrichment of biological processes linked to the generation of precursor metabolites and energy (by oxidation of organic compounds), mitochondrion organization and oxidative phosphorylation. Consistent with these findings, our prior study identified upregulation of several genes involved in mitochondrial function and oxidative metabolism being upregulated in pigs fed LP diet (Poklukar et al., 2025). This is consistent with the fact that dietary protein reduction can influence energy expenditure (Pezeshki and Chelikani, 2021) as well as adipocyte differentiation, lipid homeostasis, and the oxidative capacity of adipose tissue (Muñoz et al., 2021; Poklukar et al., 2025; Zhao et al., 2010).

Conclusion

The present study elucidated key biological processes associated with greater fatness of Krškopolje pig compared to modern hybrids reared in the same environmental conditions with identical feeding. The main process identified was a larger adipogenic potential in Krškopolje pigs, supported by differential expression of inflammatory signalling and adipose tissue remodelling. These findings explain the larger adipose tissue growth of this local pig breed compared to modern hybrids. The reduced protein diet induced only minor breed-specific changes in individual gene expression. The limited transcriptomic response suggested that adipose tissue, especially of Krškopolje pig, is well adapted to variation in dietary protein and that more pronounced gene expression changes would require greater nutritional challenges. These findings indicated that feeding strategies can be tailored to better match breed specific metabolic requirements. Although RNA sequencing results were supported by gene-level quantitative PCR validation, additional functional validation would further strengthen the robustness of the findings. Future studies employing *in vitro* experiments on porcine adipocytes or *in vivo* approaches would help confirm the biological relevance of the identified pathways.

Ethics approval

The animal experiment was approved by the Ethics Committee of Agriculture Institute of Slovenia (number EK_KIS/02/14072022; 14 July 2022).

Data and model availability statement

RNA sequencing data are deposited in the NCBI Gene Expression Omnibus (Bethesda, MD, USA) under the accession number GSE309090 and can be found with the link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE309090 after 2026-09-01. Information can be made available from the authors upon request.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

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Table 1: Taq-man probes/primers used for targeted gene expression analysis comparing Krškopolje pigs with modern hybrids, as well as between animals of both breeds fed a reduced-protein diet versus a control diet.

Gene symbol	Gene name	Taq man assay	Expression method
ATP2A1	ATPase endoplasmic reticulum Ca2+ transporting 1	Ss04248500_m1	RNA-seq
COL6A2	Collagen type VI alpha 2 chain	Ss06826669_m1	RNA-seq
CYP1A1	Cytochrome P450 family 1 subfamily A member 1	Ss03394917_g1	RNA-seq
WNT10B	Wnt family member 10B	Ss03377073_u1	RNA-seq
CES1	Carboxylesterase 1	Ss03395585_g1	RNA-seq
FASN	Fatty acid synthase	Ss03386194_u1	Targeted
SCD	Stearoyl-CoA desaturase	Ss03392313_m1	Targeted
LIPE	Lipase E	Ss04955671_mH	Targeted
LPL	Lipoprotein lipase	Ss03394612_m1	Targeted
ACACA	Acetyl-CoA carboxylase	Ss03389963_m1	Targeted
PPARγ	Peroxisome proliferator-activated receptor gamma	Ss03394829_m1	Targeted
PPIA	Peptidylprolyl isomerase A	Ss03394782_g1	Housekeeping

TOP2B	DNA topoisomerase II beta	Ss04953704_m1	Housekeeping
YWHAZ	Tyrosine 3-monooxygenase	Ss03216374_g1	Housekeeping
B2M	Beta-2 microglobulin	Ss03391154_m	Housekeeping

Figure captions

Fig. 1. Dietary CP levels in Krškopolje pig and modern hybrids across feeding phases (source: Škrlep et al., 2024).

Fig. 2. Differential gene expression analysis of the subcutaneous adipose tissue of Krškopolje pig and modern hybrids. A) Volcano plots depicting differentially expressed genes in the subcutaneous adipose tissue of Krškopolje pigs relative to modern hybrids. The horizontal line indicates the significance threshold at an adjusted *P*-value of 0.05, and vertical lines denote the fold change threshold of $|\log_2(\text{fold change})| > 1$. Genes upregulated in Krškopolje pigs are shown in green; downregulated genes are shown in blue. Triangles represent genes with $-\log_{10}$(*P*-adjusted) values exceeding the plot boundary. B) Heatmap of the top 30 differentially expressed genes ranked by adjusted *P*-value. Gene expression values were scaled by row, and both genes and samples were clustered using hierarchical clustering. Columns represent individual samples.

Fig. 3. Visualization of the top 20 enriched biological process terms determined by gene set enrichment analysis comparing local Krškopolje pig and modern hybrids. Each node represents an enriched term, and edges connect overlapping gene sets. Shades in blue indicate gene ontology with predominantly downregulated genes, while shades in green represent those with upregulated genes.

Fig 4. Volcano plot depicting genes expressed in the subcutaneous adipose tissue of A) Krškopolje pigs receiving low protein versus control medium protein diet; and of B) modern hybrids receiving medium protein versus control high protein diet. Horizontal lines indicate the significance threshold of differentially expressed genes at adjusted *P*-value of 0.05. Vertical lines represent the threshold of $|\log_2$ (fold change)$| > 1$. Upregulated genes in Krškopolje pigs or modern hybrids fed with reduced protein diet are coloured in green, while the downregulated genes are coloured in blue.

Fig 5. Visualization of the top 20 enriched biological process terms identified by gene set enrichment analysis (GSEA). A) Comparison of local Krškopolje pigs receiving a low protein diet versus a control medium-protein diet. B) Comparison of modern hybrids receiving a medium-protein diet versus control high protein diet. Each node represents a significantly enriched gene ontology biological process term, and edges indicate gene set overlaps, reflecting functional similarity. Blue shades denote enriched terms with predominantly downregulated genes, while green shades indicate those enriched with upregulated genes.

Fig *6.* The expression of candidate genes and genes detected with RNA sequencing using quantitative PCR approach: Comparison of: A) Krškopolje pig and (control) modern hybrid pigs (the upregulated genes in Krškopolje pig compared to modern hybrids are coloured in green, while the downregulated genes are coloured in blue), B) Krškopolje pigs fed with low protein (LP) and medium-protein (MP) control diet (downregulated gene in Krškopolje pig fed with LP diet compared to MP diet is coloured in blue) and C) modern hybrid pigs fed with MP compared to high protein (HP) control diet. The x-axis indicates $\log_2$ fold change. Horizontal lines indicate *P*-value thresholds.