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Comparative Study Targeting Differentially Expressed Genes Related to Metabolic Response, Skeletal System and Body Growth in Postnatal Fat Tissue of Adult Large White Yorkshire and Non-Descript Pig Breed of Punjab

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ABSTRACT

Fat is a significant factor that influences the quality of pork. In carcass, subcutaneous fat accounts for the maximum percentage of fat. Since subcutaneous fat is negatively correlated with the quality of pork as increased level of subcutaneous fat decreases the Intramuscular fat (IMF) content. Thus, reducing the fat percentage has become a major goal. Current study was designed with an objective to investigate the comparative expression analysis of differentially expressed genes (*COL2A1, COL9A1, PNPLA3, PDK4* and *FASN*) in fat tissue of Large White Yorkshire (LWY) and non-descript pig breed. Real-time qPCR was utilized for identifying the differential expression of genes. Results from current study revealed a significant lower expression of *COL2A1* ($p<0.01$) and *PDK4* ($p<0.05$) in non-descript pigs, indicating toward poor growth and lesser active metabolic response. However, a higher expression of *COL9A1* ($p<0.05$), *PNPLA3* ($p<0.05$), and *FASN* ($p<0.01$) is observed in non-descript pigs indicating high fat development in non-descript pigs. Therefore, current results suggests higher fat deposition in non-descript breed which may be because of lesser active metabolic response. Current study is the first report to differentiate between LWY and non-descript breed on the basis of differential expression of mRNA transcript in fat tissue. Results generated by current study can further be used for targeting other candidate genes for fat development in pigs. Furthermore, genes used in current study can be subjected to use as biomarker for selection, development and promoting lean meat production in non-descript pigs.

HIGHLIGHTS

- Focused to understand key genes involved in higher fat development in non-descript pigs.
- Genes under study are key DEGs related to metabolic response, skeletal system and body growth.

Keywords: Large White Yorkshire (LWY), non-descript pigs, *PNPLA3*, *PDK4* and *FASN*

The pig is an important livestock animal, as pork is considered as one of the best source of animal protein. Apart from protein, pork contains a wide range of essential nutrients (Sodhi *et al*., 2014). In India, pigs account for only 1.69% of the total livestock population. The total population of pigs is 9.06million, of which 76% of the population accounts for indigenous pigs whereas, 24% of the population is of exotic/crossbreed (Sahu and Gupta, 2022). Pigs are exclusively reared for the production of pork by economically weaker sections of society as it is a profitable source of income (Haldar *et al*., 2017). Pork of indigenous breed is having a better taste, better marbling

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quality, and tenderness. However, the problem associated with non-descript breeds is that they exhibit poor growth rate, low lean meat production, and high-fat deposition (Yang *et al*., 2003). On the other hand, exotic breeds are known for their higher lean meat percentage, higher growth rate, and low-fat deposition (Dai *et al*., 2009). In pigs, the percentage of subcutaneous fat is measured by backfat thickness. Since subcutaneous fat is negatively associated with the quality of pork as its abundance reduces the intramuscular fat content (IMF) which ultimately affects the quality of meat (Zhang *et al.,* 2022; Zambonelli *et al*., 2016). Thus, promoting intramuscular fat content and decreasing the accumulation of subcutaneous fat has become a major goal of the pork industry. Therefore, to overcome this situation, approaches like cross-breeding is being employed for the improvement of the non-descript breed to promote reproductive health, production of leaner meat, and reducing fat content (Čandek-Potokar *et al*., 2012; Zhang *et al*., 2019).

Skeletal development, metabolism, and body growth affect the growth of an animal drastically. In skeletal development and body growth, Collagen type II alpha 1 chain (*COL2A1*) and Collagen Type IX alpha 1 chain (*COL9A1*) play an essential role. *COL2A1* encodes for the α1 chain of type 2 collagen which is found in cartilage. Moreover, it is essential for the normal growth and development of the skeleton. Mutation in *COL2A1* results in a wide range of diseases collectively known as Type II collagenopathies (Zhang *et al*., 2020). Collagen Type IX alpha 1 (*COL9A1*), encodes for one of the three chains of Type IX collagen (Diab *et al*., 1996). *COL9A1* is present at SSC1 which is recognized as QTL for front leg and rear view of pigs. Moreover, it is also associated with the body conformation trait (length, width, and depth) (Fan *et al*., 2009). Genes *PNPLA3*, *PDK4*, and *FASN* are associated with metabolism as well as body growth. *PNPLA3* is found in the lipid droplet where the function is to hydrolyze the triglycerides (Huang *et al*., 2011). *PNPLA3* encodes for a protein called adiponutrin, it is an adipocyte which is found in both adipose tissues as well as in hepatocytes where it participates in the production and breakdown of fat (Johansson *et al*., 2008; Lake *et al*., 2005) *PNPLA3* regulates fat deposition and is involved in the metabolic process (Chen *et al*., 2011). Pyruvate Dehydrogenase Kinase4 (*PDK4*), is a mitochondrial gene that resides in the mitochondrial matrix. *PDK4* participates in aerobic oxidation of carbohydrate fuels like fatty acid oxidase and glucose (Majer *et al*., 1998; Boulatnikov and Popov, 2003). *PDK4* inhibits the PDC complex by phosphorylating one of its subunits as a result of which consumption of glucose decreases and fat metabolism increases (Sugden and Holness, 2006; Manio *et al*., 2016). Fatty acid synthase (*FASN*), catalyzes the synthesis of palmitate by utilizing acetyl CoA and malonyl-CoA, which is further utilized for the production of the long chain of saturated fatty acid with the help of other fat related genes i.e. *ELOVL6* and *ME1* (Fig.1) (Xing *et al*., 2014).

Therefore, the current study was designed with the objective to investigate the differential expression of genes related to metabolic response, skeletal system, and body growth in fat tissue of Large White Yorkshire (LWY) and non-descript pigs.

MATERIALS AND METHODS

Animal and sample preparation

Animal used in current study were non-descript pigs and Large White Yorkshire pig. Targeted sample for current study was subcutaneous fat from 5th lumbar vertebra. Total of 12 fat samples, 6 from each breeds were collected. Samples for non-descript pigs were collected from local slaughter house and for exotic breed; samples were collected from exotic breeder which exclusively rare Large White Yorkshire breed. After slaughtering, fat samples were kept in dry ice and immediately brought to lab (College of Animal Biotechnology, Guru Angad Dev Veterinary Science and Animal University, Ludhiana, Punjab). Samples were stored at -80˚C till further use for RNA extraction.

Extraction and qualitative and quantitative analysis of RNA

All the plastic ware used for RNA extraction was treated with 1% DEPC prior extraction. 500mg of fat tissue was used for extraction of RNA. RNA was isolated using TRIZOL reagent (Qiazol). A paste was made out of fat sample by using pestle and mortar, into the paste 2 ml of trizol was added. After centrifugation trizol phase was transferred in a new tube and into it 200µl chloroform (Himedia) was added for phase separation. Followed

by this, 1µl of isopropanol (Himedia) was added for precipitation of RNA pallet. RNA pallet was washed using 75% ethanol. For making RNA sample free from contamination RNAse-free DNase kit (QIAGEN, Hilden, Germany) was used. Quantification of isolated RNA was measured by Thermo Scientific Nanodrop-one. Integrity of extracted RNA samples were in range of 1.8-2.0 (A260/280).

Qualitative Analysis

Agarose gel electrophoresis (Agilent Technologies Ireland, Dublin, Ireland) was used for qualitative analysis. Conventional PCR was employed for amplification of sample at standardized conditions. Followed by PCR amplification, samples were run at 1% Agarose gel at 80 volt/cm. UV-illumination (Bio-Rad, USA) and gel doc (G:BoxSyngene) were used for visualization and capturing images of gel.

Quantitative Analysis

Quantitative analysis of mRNA expression of *COL2A1*, *COL9A1, PNPLA3, PDK4 and FASN* genes was done by Real-Time qRT-PCR (Bio-Rad D model CFX96TM Optics Module real time PCR). SYBR® Green chemistry (GoTaq® qPCR, Promega) was used for quantifying the transcripts of genes. Primers were selected from literature (Ghosh *et al*., 2015; Sodhi *et al*., 2014) (Table 1). The samples were run in duplicates and standard curve method was used to assess the real-time PCR efficiency. Amplified genes were compared to *β–actin*, an endogenous control (Rebouças *et al*., 2013). The comparative C_T approach was used to quantify the transcript levels. The outcomes are shown as relative expression normalized using the transcript level of the endogenous reference (*β–actin*) (Erkens *et al*., 2006).

Statistical Analysis

Differential expression of genes were analyzed using ΔΔCt method (Livak and Schmittgen 2001) where mean and standard error were mentioned as mean ± SEM. Paired t-test was employed for calculating the level of significance ($p<0.05$, $p<0.01$).

RESULTS AND DISCUSSION

Qualitative analyses of genes were determined using 1% Agarose gel electrophoresis (Fig. 2). Primer efficiency was determined using the regression curve method, primers used in current study showed an efficiency range 94-99%. The differential expression profiling of genes was quantified using the real-time qPCR.

Table 1: Primers used for quantitative PCR to analyze differential expression of genes in fat tissue of non-descript and LWY pig breeds

Gene	Primer Sequence	Product Size	Annealing temperature	GenBank ID
COL2A1	F- GATGTTGGTGAGAAAGGCCC R-GTCCAGTCTCTCCACGTTCA	181	60˚	NC_010447
COL9A1	F-TGTGCTCGGAAAACTTGTGG R-TCACCGTCAATACCGTCGAT	228	60˚	NC_010443
PNPLA3	F-CAGAACAAAGCACTGTCCCC R-CGCAGATCTAGCGTGTAGGA	247	60˚	NW_003538349
PDK4	F-CCTCATTCCTCCACCAAGAA R-GAGAAATGCTCGACCTCTCG	244	60˚	NC_010451
FASN	F-ATGACTACGGGCCCTTCTTC R-GATGCGGATGGAGGTGAAAC	176	58˚	NC_010454
β–actin	F-GACATCCGCAAGGACCTCTA R-ACACGGAGTACTTGCGCTCT	157	60˚	XM_0031242

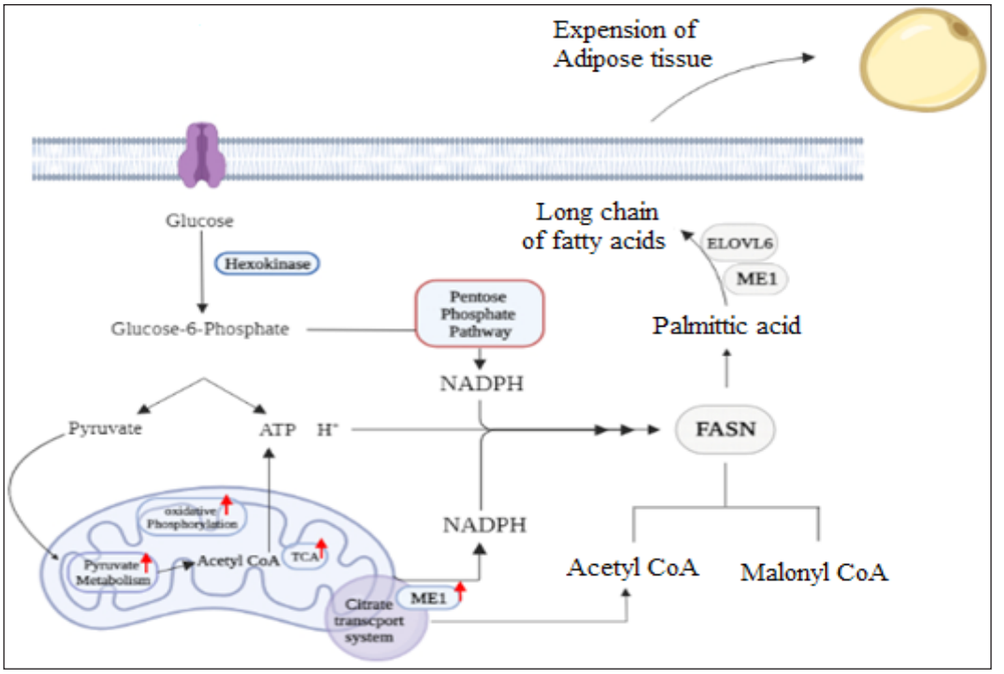


Fig. 1: Role of FASN in *de novo* fatty acid synthesis

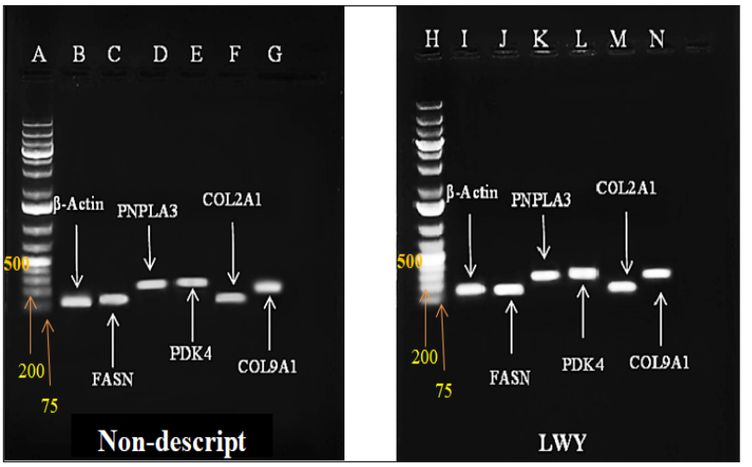


Fig. 2: Agarose Gel Electrophoresis- The bands in gel doc image showing ligation of primer in PCR products. Well A and H contain 1Kb DNA ladder, well B and I represents β-Actin, well C and J contain *FASN* gene, well D and K –*PNPLA3* gene, well E and L - *PDK4* gene, well F and M - *COL2A1* gene and well G and N contains *COL9A1* gene

Differential expression of genes involved in body growth and skeletal system development

Current study targeted two genes *COL2A1* and *COL9A1* which are related to skeleton system development, current study depicted a significant lower expression of *COL2A1* ($p<0.01$) in fat tissue of non-descript pigs. Moreover, in non-descript pigs *COL2A1* expression was 0.45 times lesser expressed as compared to LWY (Table 2) (Fig. 3). *COL2A1* is essentially required for the growth and development of skeletal system and current study is

Table 2: Validation of *COL2A1* mRNA expression using real time qPCR (SYBR green)

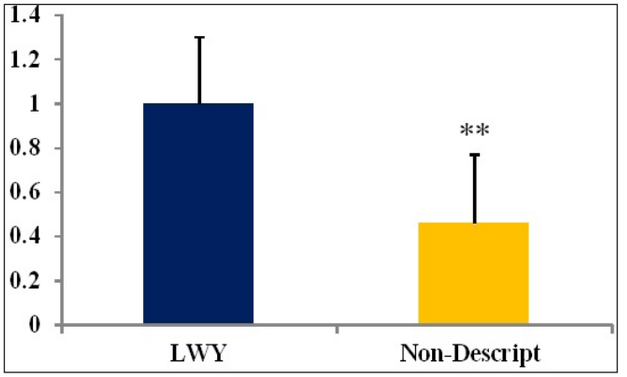
Group	*COL2A1* (Average C_t)	*β-Actin* (Average C_t)	ΔCT (*COL2A1* C_t - *β-Actin* C_t)	ΔΔCT (ΔC_t ND - ΔC_t LWY)	Fold Change
LWY	26.35 ± 0.33	27.59 ± 0.29	-1.24 ± 0.29	0.0 ± 0.30	1
Non-Descript	26.23 ± 0.15	26.33 ± 0.30	-0.11 ± 0.30	1.13 ± 0.31	0.46**

Values are Mean ± SE. ** shows significant difference at p <0.01.

Table 3: Validation of *COL9A1* mRNA expression using real time qPCR (SYBR green)

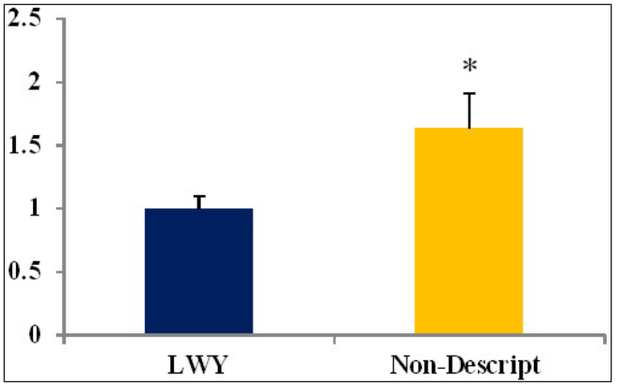
Group	*COL9A1* (Average C_t)	*β-Actin* (Average C_t)	ΔCT (*COL9A1* C_t - *β-Actin* C_t)	ΔΔCT (ΔC_t ND - ΔC_t LWY)	Fold Change
LWY	21.49 ± 0.10	22.86 ± 0.10	-1.34 ± 0.13	0.0 ± 10	1
Non-Descript	21.82 ± 0.33	23.90 ± 0.21	-2.08 ± 0.30	-0.72 ± 0.27	1.64*

Values are Mean ± SE. * shows significant difference at p <0.05.



** indicates significance difference (p<0.01)

Fig. 3: *COL2A1* gene expression profiling in fat tissue of LWY and non-descript pigs



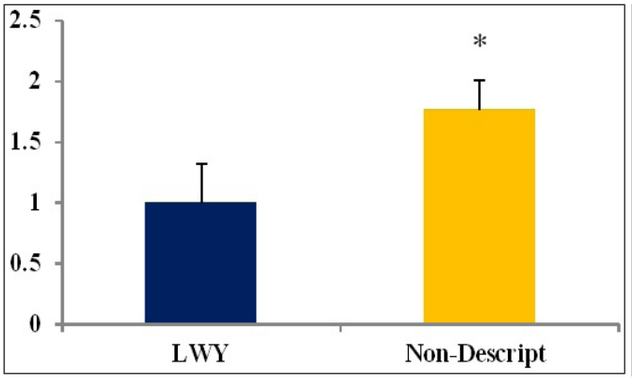
* indicates significance difference (p<0.05)

Fig. 4: *COL9A1* gene expression profiling in fat tissue of LWY and non-descript pigs

providing a significant evidence against non-descript pigs. Ghosh *et al*. (2015) reported significant down-regulated expression of *COL2A1* in Jeju native pigs indicating their poor growth and development. However, commercial breeds like Berkshire showed higher expression of *COL2A1* suggesting better growth. On the other hand, significantly higher expression of *COL9A1* was found in non-descript pigs (p<0.05) (Fig. 4). Non-descript pigs showed an expression 1.64 times higher in comparison to LWY (Table 3). An up-regulated expression of Collagen Type IX was recorded in Jeju native and large white pigs (Ghosh *et al*., 2015; Fan *et al*., 2009). Therefore, the lower expression of key genes involved in growth and development of skeletal system indicates towards the poor growth of non-descript pigs.

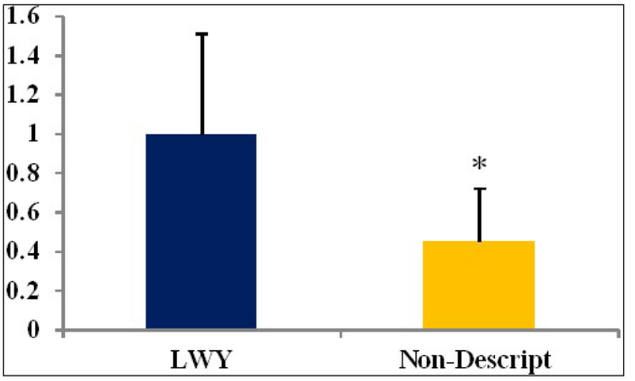
Differential expression of genes involved in metabolic response

PNPLA3, *PDK4* and *FASN* are the genes involved in metabolic processes. The expression of metabolic gene; *PDK4* was found to be significantly down-regulated in non-descript pigs which signifies poor metabolic response of non-descript pigs (Fig. 6). *PDK4* is responsible for aerobic oxidation of carbohydrate fuels i.e. glucose and fatty acids (Majer *et al*., 1998; Boulatnikov *et al*., 2003).



* indicates significance difference (p<0.05)

Fig. 5: *PNPLA3* gene expression profiling in fat tissue of LWY and non-descript pigs



* indicates significance difference (p<0.05)

Fig. 6: *PDK4* gene expression profiling in fat tissue of LWY and non-descript pigs

Table 4: Validation of *PNPLA3* mRNA expression using real time qPCR (SYBR green)

Group	***PNPLA3*** **(Average C_t)**	***β-Actin*** **(Average C_t)**	**ΔCT (*PNPLA3* C_t - *β-Actin* C_t)**	**ΔΔCT (ΔC_t ND - ΔC_t LWY)**	**Fold Change**
LWY	29.48 ± 0.27	30.56 ± 0.20	-1.08 ± 0.51	0.0 ± 32	1
Non-Descript	28.08 ± 0.29	29.98 ± 0.21	-1.90 ± 0.28	-0.82 ± 0.24	1.77*

Values are Mean ± SE. * shows significant difference at p <0.05.

Table 5: Validation of *PDK4* mRNA expression using real time qPCR (SYBR green)

Group	***PDK4*** **(Average C_t)**	***β-Actin*** **(Average C_t)**	**ΔCT (*PDK4* C_t - *β-Actin* C_t)**	**ΔΔCT (ΔC_t ND - ΔC_t LWY)**	**Fold Change**
LWY	26.29 ± 0.28	27.49 ± 0.13	-1.20 ± 0.51	0.0 ± 51	1
Non-Descript	27.26 ± 0.26	27.32 ± 0.19	-0.06 ± 0.45	1.14 ± 0.27	0.46*

Values are Mean ± SE. * shows significant difference at p <0.05.

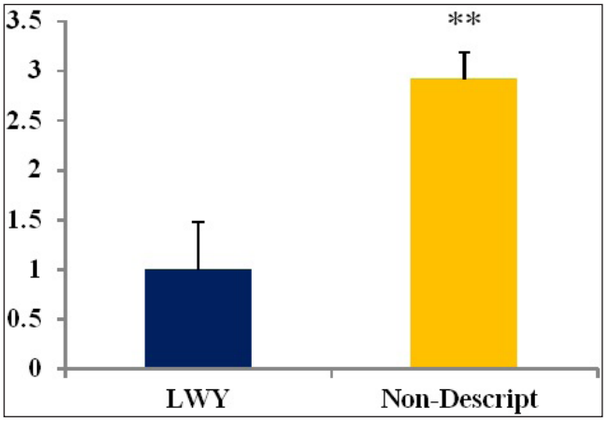
PDK4 regulates cellular metabolism via inhibition of PDC or Pyruvate Dehydrogenase Complex (Yang *et al*., 2019). In current study, significant lower (p<0.05) expression of *PDK4* was observed. Non-descript pigs showed 0.46 times fold lower than that of LWY (Table 5). Similarly, Sodhi *et al*. (2014) reported down-regulation of *PDK4* in Jeju native pigs. Moreover in active metabolic state, *PDK4* overexpression is recorded as a result of which the fat metabolism increases and similar pattern was reported in mouse, signifying a strong relation between *PDK4* And fatty acid oxidation (Pettersen *et al*., 2019; Manio *et al*., 2016). Since the expression of gene related to metabolic response is expressed poorly, the gene responsible for fat deposition expressed dominantly in non-descript pigs. *PNPLA3* gene is a candidate gene promoting fat development in pigs as it is found in lipid droplet where its major function is to hydrolyze triglycerides (Pingitore and Romeo, 2019). In present study, the expression of *PNPLA3* was found to be significantly up-regulated (p<0.05) in non-descript pigs (Fig. 5). *PNPLA3* expressed 1.77 times more in non-descript pigs as compared to LWY (Table 4). PNPAL3 is reported to express predominantly in adipose tissue of Meishan pigs, suggesting its role in deposition of fat and obesity (Chen *et al*., 2011). Higher expression of *PNPLA3*

Table 6: Validation of *FASN* mRNA expression using real time qPCR (SYBR green)

Group	*FASN* (Average C_t)	*β-Actin* (Average C_t)	ΔCT (*FASN* C_t - *β-Actin* C_t)	ΔΔCT (ΔC_t ND - ΔC_t LWY)	Fold Change
LWY	29.48 ± 0.56	30.12 ± 0.39	-0.55 ± 0.48	0.0 ± 0.48	1
Non-Descript	27.74 ± 0.74	29.84 ± 0.36	-2.10 ± 0.27	-1.55 ± 0.27	2.92**

Values are Mean ± SE. ** shows significant difference at p <0.01.

gene increased the size of lipid droplet via accumulation of abnormal intracellular lipids (Chamoun *et al*., 2013). *FASN* is an essential gene responsible for fat deposition as it produces long chain of fatty acid by catalyzing synthesis of palmitic acid (Zappaterra *et al*., 2019). In pigs, *FASN* is associated with the backfat thickness (Renaville *et al*., 2015). In present study, the expression of *FASN* gene was significantly higher (p<0.01) in non-descript pigs (Fig. 7).



** indicates significance difference (p<0.01)

Fig. 7: *FASN* gene expression profiling in fat tissue of LWY and non-descript pigs

FASN was found to be 2.92 times fold higher expression as compared to LWY (Table 6). Previous studies reported higher expression of *FASN* in obese animals, confirming its significant association with fat development (Shan *et al*., 2022; Piórkowska *et al*., 2020). Therefore, the Results from current study clearly depicts the lower expression of metabolic gene suggesting poor metabolic response in non-descript pigs due to which the efficiency to metabolize fat is compromised and thus, the expression of gene promoting fat deposition expressed dominantly

CONCLUSION

To best of available resources, this study is the first report to address the difference between LWY and non-descript pigs on the basis of DEGs in fat tissue, supported by statistical data. Pork of non-descript pigs is tenderness and juicy, but fat has become a significant factor that influences the quality of pork (Sodhi *et al*., 2014), thus reducing it is essential. Current study investigated expression of key genes involved in fat development, growth and metabolic response. Results of current study showed optimal expression of *COL9A1*; indicating optimal body conformation of non-descript pigs. However, significant down-regulation of *COL2A1* (p<0.0.1) and *PDK4* (p<0.05) gene responsible for skeletal growth and metabolic response; indicates towards the poor growth and lesser active metabolism of non-descript pigs due to which the expression of genes related to fat development *PNPLA3* (p<0.05), and *FASN* (p<0.0.1), expressed dominantly in non-descript pigs explaining presence of higher fat content in respective breed. Furthermore, the genes proposed in current study can be used for early selection in approaches like marker assisted selection (MAS) for promoting lean meat production and growth improvement of non-descript pigs. Moreover, current study provides a base for target candidate genes related fat development and cutting the higher fat percentage in pork industry.

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