Journal of Animal Research: v.12 n.05, p. 689-697. October 2022

DOI: 10.30954/2277-940X.05.2022.10

A Porcine Model to Study the Differential Expression of Myogenic Regulatory Factors (MRFs) in Piglets *vis-a-vis* Adult Pigs of Indigenous and Large White Yorkshire Breeds

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Received: 08 Sept., 2022 **Revised:** 26 Sept., 2022 **Accepted:** 29 Sept., 2022

ABSTRACT

The pork is considered to be highly nutritious food because it provides many essential nutrients. Pork industry is growing every year because of its high demand across worldwide. In India, pigs rearing can be adapted by weaker section of the farmers because of low investment in it. The *Longissimus dorsi* muscle is selected from the 12th to 13th rib on the back of pigs. Muscle growth is very important for the production of meat therefore *Pax7* and MRFs genes such as *MyoD, Myf5* and *Myf6* are targeted in the current study to analyse the mRNA transcript level in indigenous and Large White Yorkshire breeds of age one week old piglet and adult respectively. These genes are helpful in muscle regeneration and differentiation. *MyoD* shows significantly higher ($P<0.05$) in the indigenous pigs whereas other genes *Pax7* and other MRFs i.e., *Myf5* and *Myf6* shows significant higher ($P<0.01$) in the LWY breed of both age groups. The current study on differential expression of MRFs in one week old piglets and adult pigs of indigenous and LWY breeds served as candidate markers for muscle growth. This current study may be helpful to know the difference in expression profile in adult and piglet to unravel system biology.

HIGHLIGHTS

- We studied Differential expression of myogenic regulatory factors (MRFs) in piglets and adult pigs of indigenous and LWY breeds.
- The current study helps to understand expression profiling of genes for myogenesis.

Keywords: Indigenous, Large White Yorkshire (LWY), *MyoD, Pax7, Myf5, Myf6*

The Primary tissue that constitutes 20%-50% of total body weight dependent on growth of skeletal muscles which is used in meat production (Qazi *et al*., 2015; Wu *et al*., 2013). Several research have assessed genes during the past few decades to comprehend the fundamental mechanisms of proliferation and development of muscle tissue in pigs (Liu *et al*., 2012). A group of factors are responsible for muscle growth. First, myofibres and multinucleated fibres serve as the basic structural and functional components of skeletal muscle, and they are produced when myoblasts fuse to create multinucleated tubes (Jarvinen *et al*., 2007; Qazi *et al*., 2015). Second, satellite cells—multipotent stem cells—are thought to aid in the development of muscle fibres (Carnes and Pins, 2020; Qazi *et al*., 2015). Only 2% to 7% of the total myonuclie are satellite cells (Rudnicki *et al*., 2008). *Pax7* is expressed by satellite stem cells, also myogenic determination factor (*MyoD*)

How to cite this article: Kumar, P., Sodhi, S.S., Mukhopadhyay, C.S. and Sethi, R.S. (2022). A Porcine Model to Study the Differential Expression of Myogenic Regulatory Factors (MRFs) in Piglets vis-a-vis Adult Pigs of Indigenous and Large White Yorkshire Breeds. *J. Anim. Res.,* **12**(05): 689-697.

Source of Support: None; **Conflict of Interest:** None

and myogenic factor-5 (*Myf5*) are activated by satellite stem cells (Rudnicki *et al*., 2008). Thus, the activation of satellite cells is intimately correlated with *Pax7*, *Myf5*, and *MyoD* activity (Lepper *et al*., 2011; Murphy *et al*., 2011). Meat from pork is the cheapest source of animal protein which can be afford by cheaper section of the society Chhabra and Samantaray, 2013). It has various essential nutrients such as carbohydrates, Vitamins, Minerals and protein (Arnarson, 2015).

As compared with livestock species pigs have a strong potential to provide quick financial returns to the farmers because of high feed consumption potential, early weight gain, high proliferacy rate in short generation interval. In India, Total ten indigenous breeds have been identified (Kaur *et al*., 2020). The traits having less feed consumption but high growth rate with early maturation and maximum litter size are most reared by the famers because of less investment (Moanaro *et al*., 2011; Chauhan *et al*., 2016). Indigenous pig breed have highly nutritious meat (Pugliese and Sirtori, 2012). Large White Yorkshire which is a exotic breed originated from England found to be high growth rate compared with the indigenous pig breed however meat from indigenous breed is tender and juicy (Sodhi *et al*., 2014).

Muscle growth is highly dependent on the Muscle regulatory factors (MRFs). The genes includes myogenic determination factor (*MyoD*), Myogenic regulatory factor (*Myf5*) and myogenic regulatory factor 6 (*Myf6*) belongs to basic helix-loop-helix (bHLH) transcription factors (Te Pas *et al*., 2007). The gene *MyoD* is responsible for the differentiation of muscle cells. It has been seen that from earlier studies Paired box transcription factor (*Pax7*) and *MyoD* showed co-expression in activated satellite cells (Mesires and Doumit, 2002). *Pax7* is one of the possible genes that influence the initial stages of muscle development in the pig, and it has been reported to trigger self-regeneration of satellite cells (Patruno *et al*., 2008).

The *Myf6* contributes for coding bHLH transcription factors and also responsible to affect the muscle fibres differentiation (Wyszynska-Kokoand Kuryl, 2004). The traits which produce lean meat has shown upregulation of *Myf6* in their skeletal muscles (Te Pas *et al*., 2000). However, poor muscle growth has been observed with inactive *Myf6* level (Lin *et al*., 2012).

Limited information available in comparative transcriptomic studies related to the differential expression of genes in skeletal muscles of adult and piglets of Large White Yorkshire (LWY) and indigenous breeds of pig. Therefore, current study was designed to study the differential expression of myogenic regulatory factors (MRF) genes (*MyoD*, *Myf5, Myf6, Pax7*) in piglets and adult animals of indigenous and Large White Yorkshire (LWY) breed of pigs.

MATERIALS AND METHODS

The study was conducted at (Department of animal biotechnology, College of animal biotechnology) Guru Angad Dev Veterinary and Animal Science University, Ludhiana, Punjab.

Tissue collection

In the current study, Total 24 animals were selected having different age groups i.e. (N=12) one week old piglets and (N=12) adult pigs (1 year old) of indigenous and Large White Yorkshire breeds. The muscle tissue *Longissimus dorsi* located 12^{th} and 13^{th} rib spaces from the back of the pigs collected immediately after slaughter from the slaughter house. Tissue samples of indigenous adult and piglets were collected from the nereby slaughter house of Municipal Corporation, Ludhiana and samples of LWY adult were collected from Indo Canadian swine breeder at village Kotli, Ludhiana. The samples of LWY piglets were collected from organised farm, GADVASU. The temperature of the muscle samples were maintained by storing in ice packs and carried to the lab (COABT, GADVASU) following stored at -80°C in deep freezer for further RNA isolation. All the tips and eppendorf`s were treated with Di-ethyl pyro-carbonate (DEPC) used for RNA isolation.

Total RNA extraction

RNA isolation was done using reagent TRIzol (Qiazol) from muscle tissue (120 mg) of one week old piglets and adult pig. Tissue samples were fine chopped and treated with one ml of TRIzol. Further transported in 2 ml Eppendorf and phase separation was done using 0.2ml of chloroform (Himedia) and then incubated in ice for

15 minutes. The supernatant was treated with 0.5 ml of isopropanol (Himedia) and was incubated in -20° C for the precipitation of RNA. The RNA pellet was washed with 1 ml of 75% ethanol. Then RNA was purified using RNase-free DNase set (QIAGEN, Hilden, Germany) and quantified with thermos-scientific Nanodrop one, where the RNA integrity lied between 8.0 and 10.0 and purity ratio for 28S/18S was 1.8 to 2.0.

Qualitative analysis

The agarose gel electrophoresis (Agilent Technologies Ireland, Dublin, Ireland) was used for the qualitative analysis. The conventional PCR system was used under the conditions 95°C for 5 min (initial denaturation), 40 cycles of 95°C for 30 sec (denaturation), 60°C for 30 sec (annealing temperature), 72°C for 1 minute (extension) and 5 minutes for final extension at 72°C. The amplified PCR product was run in agarose gel (1.5%) at 100 volt/cm. The bands were visualized under UV- illumination (Bio-Rad, USA) and the final images were recorded using gel doc (G:Box Syngene).

Quantitative analysis

The primers were designed using Online Primer-3 (Table 1) (Rozen and Skaletsky, 2000) for the quantitative real time-PCR system. Quantitative levels of mRNA expression of genes *MyoD, Pax7, Myf5* and *Myf6*; in indigenous and Large White Yorkshire breeds of both respective age groups was analysed using Real-time qRT-PCR (BIO RAD model CFX96TM Optics Module real time PCR). Using SYBR Green chemistry (GoTaq® qPCR) of Promega to check the transcript levels of the target genes. The samples of each gene were conducted in duplets for the PCR experiment under following conditions 95°C for 10 min (initial denaturation), 40 cycles of 95°C for 30 sec (denaturation), 60°C for 30sec (annealing temperature), 72°C for 1 minute (extension) and then melt curve was added. The qRT-PCR efficiency was checked using standard curve method and the gene *β-actin* as endogenous control was used to defined the transcript expression of target genes (Wang *et al*., 2006). The expression levels of target genes were normalized with endogenous control i.e *β*-actin (Reboucas *et al*., 2013). The Two delta C_T method was used to quantified the transcript levels. The threshold cycle (C_T) value of *β-actin* was used to standardized the C_T value of each gene (Erkens *et al*., 2006; Van Poucke *et al*., 2001).

Statistical analysis of the differential expression patterns of the genes

The RT-PCR data were analysed using $\Delta\Delta C_T$ method where the details of average mean and standard error were given as mean ± SEM. The level of significance $P<0.05$ and $P<0.01$ were calculated using Two-Way ANOVA.

RESULTS AND DISCUSSION

The amplified band of each gene obtained from gel electrophoresis displays their significant expression and the product size as in Table 1 with reference to 1kb DNA ladder (Fig. 1, 2 and 3).

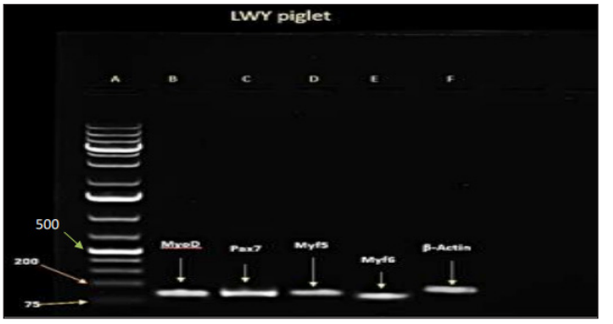


Fig. 1: The bands in gel-doc image showing ligation of primers in PCR product. Well "A" the 1kb ladder, Well "B" shows *MyoD* gene, Well "C" shows *Pax7* gene, Well "D" shows *Myf5* gene, Well "E" shows *Myf6* gene and Well "F" shows *β-actin* in LWY piglet

Fig. 2: The bands in gel-doc image showing ligation of primers in PCR product. Well "A" the 1kb ladder, well "B" shows *MyoD* gene, Well "C" shows *Pax7* gene, Well "D" shows *Myf5* gene, Well "E" shows *Myf6* gene and Well "F" shows *β-actin* in LWY adult

Table 1: Primer sequences of *MyoD, Pax7, Myf5, My6 and β- actin* for quantitative and qualitative analysis

Gene	Primer Sequence	Product Size	Tm	Gene bank ID
MyoD	F5'-TGCAAACGCAAGACCACTAA- 3'	127 bp	55°C	NM_001002824.1
	R 5'-GCTGATTCGGGTTGCTAGAC -3'			
Pax7	F 5'-GGCAGAGGATCTTGGAGACA- 3'	144 bp	55°C	AY653213.1
	R 5'- TGGGTGGGGTTTTCATCAAT – 3			
Myf5	F 5'– CCGACACAGCTTGTGGAATA- 3'	128 bp	55°C	XM_001924362.2
	R 5'– GCCAATCAACTGATGGCTTT- 3'			
Myf6	F 5'- ATCTTGAGGGTGCGGATTTC – 3'	108 bp	62°C	XM_003481764
	R 5'- CAATGTTTGTCCCTCCTTCCT – 3			
β- actin	F 5'- GACATCCGCAAGGACCTCTA - 3'	157bp	60°C	XM_003124280
	R 5'- ACACGGAGTACTTGCGCTCT - 3'			

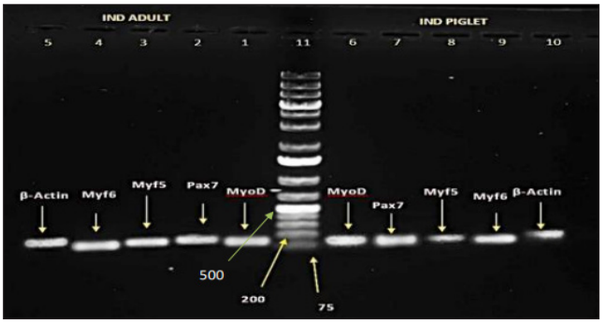
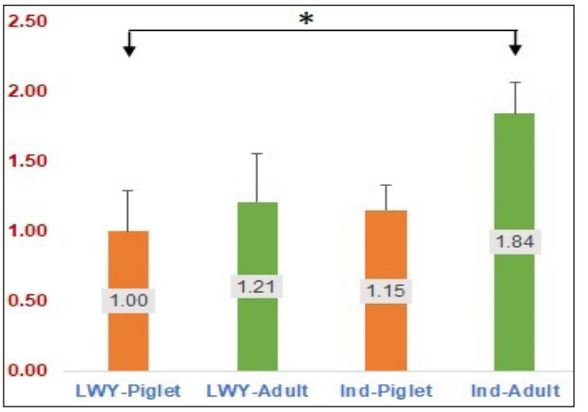


Fig. 3: The bands in gel-doc image showing ligation of primers in PCR product. Well "11" shows the 1kb ladder, well "1 and 6" is shows *MyoD* gene, Well "2 and 7" shows *Pax7* gene, Well "3 and 8" shows *Myf5* gene, Well "4 and 9" shows *Myf6* gene and Well "5 and 10" shows *β-actin* in both right side indigenous piglet and Left side indigenous adult respectively

The relative transcript mRNA expression of genes *MyoD, Myf5, Myf6* and *Pax7* obtained from real time qPCR after normalizing with *β*-actin as endogenous control. The transcript level of *MyoD* has significantly (P<0.05) upregulated in indigenous pigs of both adult (1.84) and piglets (1.15) but in LWY breed significant decreased transcript level of *MyoD* was observed in adult (1.21) (Fig. 4, Table 2). A study was conducted by Cammey *et al*. (2021) which shows resemblance with our current study. *MyoD* is involved in the differentiation of muscle cells (Li *et al.,* 2019). Their finding also suggested the role of *MyoD* by enhancing the levels of CDR1 (antisense to the cerebellar degeneration-related protein 1 transcript, also termed as ciRS-7) by joining to its promoter region in the nuclei and this results the recruitment of CDR1 in the cytoplasm. Increased expression of CDR1 results into elevation of IGF1R (insulin like growth factor 1 receptor) through binding with miR-7, Which further promotes myogenesis.



* indicates significant difference (P<0.05).

Fig. 4: Expression profiling of *MyoD* gene between different experimental groups

Moreover, the variation of fold change between the indigenous and Large White Yorkshire in comparison to one week old piglet and adult stage in respective breeds shows that *MyoD* shows minimum variation of fold change (Fig. 8). Whereas, A significant downregulation

Table 2: Relative quantification expression of *MyoD* validated by real time qPCR (SYBR Green)

Group	*MyoD* (Average Ct)	*β-actin* (Average Ct)	ΔCT (Ct *MyoD* - Ct *β -actin*)	ΔΔCT ΔCt of treated – ΔCt of untreated	Fold change
LWY piglet	22.96 ± 0.16	26.17 ± 0.18	-3.20 ± 0.28	0.0 ± 0.28	1.00
LWY adult	20.77 ± 0.26	24.24 ± 0.20	-3.48 ± 0.34	-0.28 ± 0.31	1.21
Indigenous piglet	22.26 ± 0.14	25.66 ± 0.13	-3.40 ±0.21	0.27 ± 0.40	1.15
Indigenous adult	21.91 ± 0.21	25.99 ± 0.17	-4.07 ± 0.22	-0.88 ± 0.22	1.84*

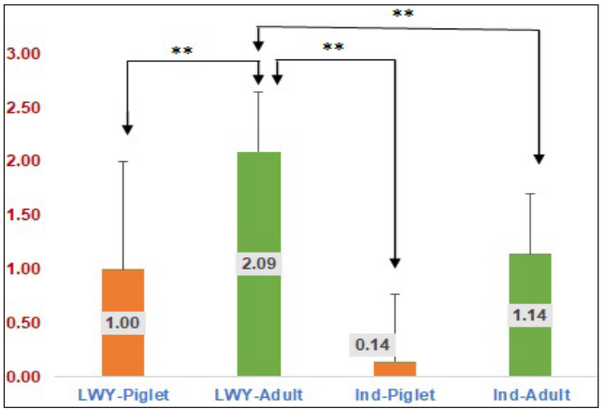
Values are mean ± SE. * indicates significant difference (P<0.05).

Table 3: Relative quantification expression of *Pax*7 validated by real time qPCR (SYBR Green)

Group	*Pax7* (Average Ct)	*β-actin* (Average Ct)	ΔCT (Ct *Pax7* - Ct *β -actin*)	ΔΔCT ΔCt of treated – ΔCt of untreated	Fold change
LWY piglet	29.29 ±0.77	26.14 ± 0.26	3.14 ± 1.44	0.0 ± 0.0	1.00
LWY adult	29.98 ± 0.39	27.89 ± 0.34	2.08 ± 0.55	-1.06 ± 0.55	2.09**
Indigenous piglet	31.10 ± 0.64	25.16 ± 0.81	5.94 ± 0.83	2.79 ± 0.63	0.14**
Indigenous adult	30.61 ± 0.26	27.69 ± 0.51	2.95 ± 0.60	0.19 ± 0.56	1.14**

Values are mean ± SE. ** indicates significant difference (P<0.01).

of *Pax7* (P<0.01) is observed in one week old piglet (0.14) and in adult (1.14) of indigenous breeds while in comparison with LWY the level of *Pax7* is significant (P<0.01) higher in adult (2.09) as compared with LWY piglet. In comparison between the breeds the fold change was significantly higher in LWY adult (Fig. 8) which is supported by previous study (Sodhi *et al*., 2021). The lower levels of *Pax7* indicates lesser expression of muscle stem cells affecting self-regeneration activity in indigenous breed (Fig. 5, Table 3.). However, previous study reported that transcript level of *Pax7* depends on type of breed (Ropka-Molik *et al*., 2011).

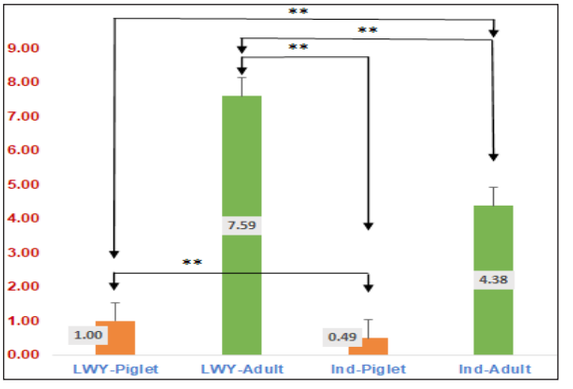


** indicates significant difference (P<0.01).

Fig. 5: Expression profiling of *Pax7* gene between different experimental groups

These results also showed similarity with earlier study done by (Kim *et al*., 2021) in which downregulation of *Pax7* was observed in the one week old piglet and adult pigs of indigenous breed. Knockdown of *Pax7* gene has resulted in decreased size of body and early death after birth.

The transcript expression of *Myf5* shows significant (P<0.01) downregulated in indigenous adult (4.38) and in one week old piglet (0.49) which is comparative less from piglet to adult stage while in LWY the level of *Myf5* is significant higher in adult (7.59). In comparison between the two breeds from one week old piglet to adult stage it is significantly higher in LWY breeds (Fig. 6, Table 4).



** indicates significant difference (P<0.01)

Fig. 6: Expression profiling of *Myf5* gene between different experimental groups

Table 4: Relative quantification expression of *Myf5* validated by real time qPCR (SYBR Green)

Group	*Myf5* (Average Ct)	*β-actin* (Average Ct)	ΔCT (Ct *Myf5* - Ct *β -actin*)	ΔΔCT ΔCt of treated – ΔCt of untreated	Fold change
LWY piglet	32.38 ± 0.32	23.065±0.25	8.82±0.42	0.0 ± 0.41	1.00
LWY adult	30.28 ± 0.71	24.39 ± 0.12	5.89 ± 0.35	-2.92 ±0.77	7.59**
Indigenous piglet	33.43 ± 0.35	23.60 ± 0.13	9.83 ± 0.37	1.01 ± 0.38	0.49**
Indigenous adult	31.85 ± 0.57	25.16 ± 0.18	6.69 ± 0.53	-2.13 ± 0.54	4.38**

Values are mean ± SE. ** indicates significant difference (P<0.01).

Table 5: Relative quantification expression of *Myf6* validated by real time qPCR (SYBR Green)

Group	*Myf6* (Average Ct)	*β-actin* (Average Ct)	ΔCT (Ct *Myf6* - Ct *β -actin*)	ΔΔCT ΔCt of treated – ΔCt of untreated	Fold change
LWY piglet	26.74 ± 0.44	25.35 ± 0.2	1.39 ± 0.61	0.0 ± 0.31	1.00
LWY adult	28.62 ± 0.48	27.48 ± 0.31	1.14 ± 0.66	-0.25 ± 0.32	1.19**
Indigenous piglet	31.42 ± 0.68	26.36 ± 0.27	506 ± 0.90	3.67± 0.15	0.08**
Indigenous adult	28.64 ± 0.78	25.03 ± 0.17	3.65 ± 0.69	2.22 ± 0.12	0.21*

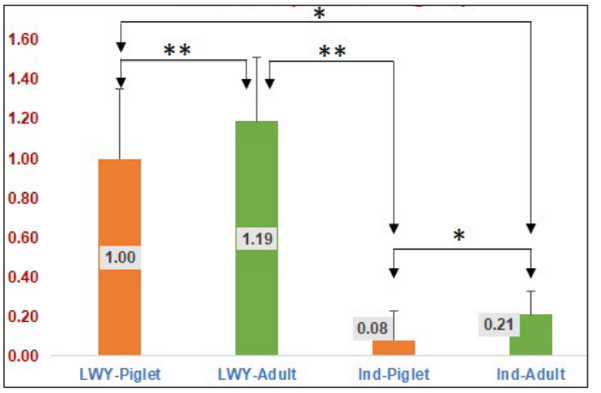
Values are mean ± SE. * indicates significant difference (P<0.05), ** indicates significant difference (P<0.01).

Co-expression of *MyoD* with *Myf5* genes observed in muscle stem cells differentiation and muscle growth rate (Zammit, 2017). *Myf5* shows maximum variation among other MRFs genes from one week old piglet to adult stage which results *Myf5* may be a important candidate for gene assisted selection of indigenous pigs to increased muscle growth

Earlier studies by Muroya *et al*. (2002) resulted that myosin heavy chain (MyHc) expression is affected by *Myf5* gene, Moreover the expression of muscle fibre types also affected by *Myf5* gene. Significant lesser expression of *Myf5* mRNA has been seen in slow muscle fibres. However, variations in intramuscular fat and meat moisture content were substantially associated with a *Myf5*/Hsp92II polymorphism that resulted in an amino acid substitution (Liu *et al*., 2008).

Hou *et al*. (2015) observed that *Myf5, MyoD*, and *Myf6* genes are induced by the myogenic regulatory factors (MRF) family in the back muscular tissue of Wuzhishan pigs. They also observed the expression of *Myf5* and *MyoD* mRNA in the tissues of the heart, liver, lung, kidney, muscle, and intestine.

Similar trend was observed for gene *Myf6* where the transcript level was significantly (P<0.01) downregulated in the indigenous adult (0.21) and in indigenous piglet (0.08) while in LWY the transcript level of *Myf6* is significantly higher (1.19) (Fig. 7, Table 5).



* indicates significant difference (P<0.05), ** indicates significant difference (P<0.01).

Fig. 7: Expression profiling of *Myf6* gene between different experimental groups

Myf5 shows maximum variation followed by *Myf6* among the *Pax7* and other MRFs (Fig. 8) which results this *Myf6* might be a important gene for gene assisted selection (GAS). *Myf6* is associated with daily weight gain and increased muscle fibre area (Wyszynska-Koko and Kuryl, 2004). *Myf6* is responsible for maintenance of the muscle

Fold change				
Breed	*MyoD*	*Pax7*	*Myf5*	*Myf6*
LWY piglet	1	1	1	1
Indigenous piglet	1.15	0.14	0.49	0.08
LWY adult	1.21	2.09	7.59	1.19
Indigenous adult	1.84	1.14	4.38	0.21

Fig. 8: Fold Change expression of *MyoD, Pax7, Myf5, Myf6 mRNA* using real time PCR (SYBR Green); No common superscript between levels of effects

tissues and meat quality (Maak *et al*., 2006). Our results shows similarity with the earlier study done by Sodhi *et al*. (2021) in which the level of *Myf5* and *Myf6* are down regulated in the indigenous breeds of one week old piglet and adult pigs.

Previous studies reported Xu *et al*. (2018) that *MyoD* showed co-expression with *Pax7* other MRFs i.e *Myf5 and Myf6* (Zammit, 2017) hence, inspite of high expression of *MyoD* in the indigenous breed but due to the downregulation of *Pax7* and other MRFs, *MyoD* is not able to contribute in the differentiation of muscle cell and myotube formation, hence muscle growth is poor in the indigenous pigs. The mRNA transcript levels except *MyoD* shows downregulation of the *Pax7* and MRFs genes So, reference to this data obtained from current study indicates retard muscle growth in the indigenous breed.

CONCLUSION

Improvements in body growth rate and quality of pork are top concerns of the breeding programmes**.** Indigenous breeds have poor muscle growth rate but meat from this breed is tender and juicy as compared with commercial breeds. Continuous decrease in quality and carcass characteristics have influence the breeders to upgrade these parameters. LWY breed is known to produce lean meat and high muscle growth as compared to indigenous breeds. Our study on differential expression of MRFs genes in piglets and adult of indigenous and LWY breeds marked them as candidate genes which are linked with muscle growth and body weight of pigs. Limited information available in comparative transcriptomic studies related to the differential expression of genes in skeletal muscles of adult and piglets of Large White Yorkshire (LWY) and indigenous breeds of pig. *Myf5* and *Myf6* genes indicated significant variation between indigenous and LWY breeds for two age groups. Therefore, these genes can act as important candidate for gene assisted selection of indigenous pigs (increased muscle growth). *Myf5* has exhibited maximum variability and *MyoD* has exhibited minimum variability between the experimental groups. The findings of the current study may be helpful in understanding the difference in expression profile in adult and piglet to unravel system biology and use of proposed markers of current study for Marker Assisted Selection (MAS) in indigenous pigs to have higher muscle mass and lean meat in indigenous pigs.

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