COMPARATIVE EXPRESSION ANALYSIS OF LIVER ASSOCIATED METABOLIC AND IMMUNE RESPONSE GENES OF LARGE WHITE YORKSHIRE (LWY) AND INDIGENOUS PIGS

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

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(Minor Subject: Veterinary Microbiology)

By

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(L-2019-BT-02-M)

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CERTIFICATE I

This is to certify that the thesis entitled, **"COMPARATIVE EXPRESSION ANALYSIS OF LIVER ASSOCIATED METABOLIC AND IMMUNE RESPONSE GENES OF LARGE WHITE YORKSHIRE (LWY) AND INDIGENOUS PIGS"** submitted for the degree of **M.Sc.** in the subject of **Biotechnology** (Minor subject: **Veterinary Microbiology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Amanpreet Kaur** Registration No. **(L-2019-BT-02-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE – II

This is to certify that the thesis entitled, **"COMPARATIVE EXPRESSION ANALYSIS OF LIVER ASSOCIATED METABOLIC AND IMMUNE RESPONSE GENES OF LARGE WHITE YORKSHIRE (LWY) AND INDIGENOUS PIGS"** submitted by **Amanpreet Kaur** Registration No. **(L-2019-BT-02-M)** to the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in partial fulfillment of the requirements for the degree of M.Sc., in the subject of **Biotechnology** (Minor subject: **Veterinary Microbiology**) has been approved by the Student's Advisory Committee along with the Dean of the College after an oral examination on the same, in collaboration with an external examiner.

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ABSTRACT

Pigs are an important part of livestock sector. The pig rearing economically supports the lower section of society. The pork is highly preferred due to its high nutritional value. The current study is based on the differential expression of genes (*ACAT2*, *ACAA2*, *APOA4* & *SOCS2*) involved in metabolism and immune response in Large White Yorkshire (LWY) and indigenous pig where liver is taken as tissue of choice. The qualitative and quantitative analysis were done by gel-elctrophoresis and real-time qPCR. *ACAT2* has shown significantly (P<0.05) higher mRNA expression in indigenous pig in comparison to LWY. Whereas, *ACAA2* shows no significant difference (P<0.05) between both breeds with lower quantitative transcript levels in indigenous pig. *APOA4* shows significantly (P<0.05) negligible mRNA expression with only 1% expression of LWY. *SOCS2* shows significantly (P<0.05) lower mRNA expression in indigenous pigs than in LWY. Therefore, the current study on expression profile of genes under study can be one of the platforms to understand the differences of genetic mechanisms between indigenous breed and LWY.

Keywords: Indigenous, Large White Yorkshire (LWY), *ACAT2*, *ACAA2*, *APOA4*, *SOCS2*.

Signature of Major Advisor **Signature of the student**

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LIST OF ABBREVIATIONS

@	:	At the rate of
ACAA2	:	Acetyl Coenzyme A Acyl Transferase
ACAT2	:	Acetyl Coenzyme A Acetyl Transferase
APAP	:	Acetaminophen
APOA4	:	Apolipo Protein A 4
bp	:	Base pair
C	:	Degree Celsius
cDNA	:	Complementary Deoxyribo-nucleic acid
CNS	:	Central Nervous System
DNA	:	Deoxyribo-nucleic acid
dNTP	:	deoxyribonucleotide triphosphate
EDTA	:	Ethylene-diamine-tetraacetic acid
et al	:	*et alia* (L. means “and others”)
Fig.	:	Figure
GH	:	Growth Hormone
HCC	:	Hepatocellular Carcinoma
Hr	:	Hour(s)
IL	:	Interleukin
IMF	:	Intramuscular Fat
IND	:	Indigenous
JAK 2	:	Janus Kinase 2
LWY	:	Large White Yorkshire
mRNA	:	Messenger Ribonucleic acid
NAFLD	:	Non Alcoholic Fatty Liver Disease
ng	:	Nanogram
PBMCs	:	Peripheral Blood Mononuclear Cells
RA	:	Rheumatoid Arthritis
rhGH	:	Recombinant Human Growth Hormone
RNA	:	Ribo-nucleic acid
ROS	:	Reactive Oxygen Species
sec	:	second (s)
SLE	:	Systemic Lupus Erythematosus

SNP	:	Single Nucleotide Polymorphism
SOCS2	:	Suppressor of Cytokine Signalling 2
TG	:	Triglyceride
Th	:	T-Helper cells
µg	:	Microgram
µl	:	Microliter
µM	:	Micromole

CHAPTER I

INTRODUCTION

The pig farming had been source of income for lower economic class of the society but with industrialization of livestock rearing, pig farming is growing at very fast pace. Early sexual maturity, less feed consumption with high fat and short generation interval are the economic traits due to which pig farming is being adopted by large number of people. The pigs can also be reared in low-input production system, fed on vegetables and kitchen waste (Moanaro et al., 2011 and Chauhan et al., 2016). India has fifth rank in pig rearing in the world whereas North-Eastern states are main contributer as pig producers of the country. According to 20th livestock census, Assam had the highest pig population in the country. The pig population in India is constantly declining from year 2012 to 2020 i.e., -12% (Singh, 2019). According to a report, it has been found that Indians consume least amount of meat in the world which is seven pounds per person (Tepper 2012) and cultural and religious matters are main reason behind it (Devi et al., 2014). Therefore, less consumption leads to restricted import and export of pork; only processed meat is imported. In 2018, the import and export of total meat including pork was 500 and 270 metric tons (Statista, 2021).

The large part of total pig population is reared in rural areas that becomes a great source of employment for economically weaker sections of society. Local breeds of pigs are the cheapest source of protein, providing high grade meat (Bonneau et al., 2010 and Pugliese et al., 2012). One-third part of the total indigenous pigs are nondescript with high lean meat and disease resistance property. Therefore, it is necessary to generate genomic data about the non-descript breeds (Sulabh et al., 2017).

The breeding programs were adopted to enhance the performance rate of indigenous pigs. The cross-breed pigs have better reproduction and growth potential, high lean carcass, reduced fat including intramuscular fat (IMF) content which leads to production of meat with good quality (Zhang et al., 2019 and Čandek-Potokar et al., 2012). Various breeds used for cross-breeding are Large White Yorkshire (LWY), Landrace, Middle White Yorkshire, Berkshire, Hampshire, Tamworth, Duroc, Chesterwhite and Hereford. The major risks in cross-breeding are lack of

knowledge, management methods and prevention of disease (http://gain.fas.usda.gov).

Large White Yorkshire (LWY) is an exotic breed of pig adapted to Asian continent especially India. Exotic breeds of pig are economically viable breeds because of their higher growth rate. In India, LWY is the most economically viable breed for meat production; however, pork from indigenous breed is tender and juicy, but their growth rate is poor (Sodhi et al., 2014, a). Few reports are available on comparative transcriptomic studies on indigenous breeds of pigs.

Pork is a nutritious food that is commonly consumed worldwide since 5000 BC. It provides various nutrients; potassium, riboflavin, zinc, proteins, vitamin A, B2, B6 and essential amino acids (Arnarson 2015). Liver comprises 1% - 4.5% of the total weight in pigs and it is one of the tissues that are considered edible. The moisture, fat and protein content of liver are 71.59%, 2.94% and 22.05%, respectively (Seong et al., 2014). The global production and demand of pork is increasing at a high rate due to its high nutritive value, less cost and cheap source of animal protein. It has been observed that in India, the production and utilization of pork was same with only 0.1% of imports and exports in year 2019-2020 (FAO 2021).

Liver is an important organ involved in metabolism, conversion of nutrients into fuel and then exports them to different tissues through blood. In pigs, it contains higher average weight, fat and moisture content (Florek et al., 2012; Ngapo et al., 2012). The microbiological evaluation showed that it contains high iron, zinc, protein and lipid content (Srebernich et al., 2018). The lipids regulates expression of genes at mRNA level and are involved in cellular differentiation (Uddin et al., 2011). Sodhi et al., (2014, a) suggested that increased immune cells signifies better immunity which leads to better growth.

Liver and intestine are source organs for Acetyl Coenzyme A acetyl transferase (*ACAT2*) gene present at chromosome 1 of the pig. The different domains of *ACAT2* function in metabolic processes, transfers acyl groups and performs transferase and catalytic activity (Sodhi et al., 2014 b). It plays essential role in biosynthesis of cholesterol as it catalyzes cholesterol esterification that occurs during cholesterol adsorption (Cerqueira et al., 2016). The study was conducted where two rat groups were fed with glucose or fructose rich diet and the results showed that there is high expression of *ACAT2* in fructose fed mice with high concentration of

triglycerides and cholesterol in liver indicating that gene is linked with fructose induced hypertriglyceridemia (Ichigo et al., 2019).

Acetyl Coenzyme A acyl transferase (*ACAA2*) and *ACAT2* are members of one protein family. *ACAA2* involved in metabolism; it catalyzes the end reaction of beta oxidation of fatty acids in mitochondria and facilitates catabolism of lipids (Houten et al., 2015). Further, Peng et al., (2018 b) demonstrated that *ACAA2* plays pivotal role in fat deposition in chicken embryos. Zhang et al., (2018) conducted a study on sheep to observe the impact of *ACAA2* gene expression on adipose tissue synthesis. The study revealed that gene knockdown results in inhibition of adipocyte differentiation. So, it was concluded that *ACAA2* can be used in improving meat quality. The study was conducted to find various processes involved in pig fatness, observed that miR-24-3p; miRNA sequence is dependent on *ACAA2* in regulating differentiation of fat cells (Ropka-Molik et al., 2020).

Apolipoprotein A-4 *(APOA4)* gene is found in liver and intestine. It has various physiological functions related to metabolism and immune response. It is an antioxidant, anti-inflammatory protein and protects against artherosclerosis. It also promotes reverse cholesterol transport and promotes insulin secretion (Wang et al., 2015). Tang et al., (2021) observed the downregulation of *APOA4* in sera of rats having chronic intermittent hypoxia (CIH) with obstructive sleep apnea (OSA). Thus, *APOA4* can be used as biomarker as it may contributes in the development of OSA. Schizophrenia is a psychiatric disease, characterized by hallucinations, cognitive dysfunction which results in disability to move for lifetime (Rosenberg et al., 2020). It has been found that disturbance in lipid metabolism pathway leads to development of schizophrenia. Comparative studies showed that level of *APOA4* gene is lower in diseased patients than the normal ones. Therefore, *APOA4* may be used as marker in the prognosis of disease.

Suppressor of cytokine signaling (*SOCS*2) negatively correlates with cytokine and growth factor signaling. Monti-Rocha et al., (2019) found that *SOCS2* prevents adverse immune reactions caused by acetaminophen treatment in liver. Jian et al., (2021) demonstrated that *SOCS2* gene acts as repressor of endometrial cancer, suppresses cell maturation and induces cell death. The down regulation of *SOCS2* represents the critical stage of tumor, so this gene can be used as biomarker for the presence of endometrial cancer. Also, it can be used for curing non–small-cell lung

cancer (NSCLC) regulated by circular RNA; Circ_0006677 (Yang et al., 2021). Li et al., (2021 b) observed that SOCS2 gene downregulates with NASH progression. The normal and SOCS2 knockout samples were studied and found that upregulation of gene results in inhibition of NF-κB signaling pathway. Thus, inhibits inflammation and apoptosis which plays key role in development of NASH.

Moreover, no information is available related to the differential expression of genes linked with immune response and metabolic processes in liver of LWY and indigenous pig. Therefore, the current study relies on the following objective:

1. To study the differential expression of genes (*ACAT2*, *ACAA2*, *APOA4* and *SOCS2*) involved in metabolic processes and immune response in the liver of Large White Yorkshire (LWY) and indigenous pig.

CHAPTER II

REVIEW OF LITERATURE

In India, more than 70% of people are dependent on agriculture and livestock for earning and pig farming contributes about half of the income in the scrubland of the northeast states of India (Ajala et al., 2007). There are 1.90 and 7.16 million of exotic and indigenous/ non-descript breeds of pigs in India and the trend is decreased by 12.03% in census 2019 in comparison to census 2012.The total pig population is 9.06 million. Punjab has 52452 pigs with the highest population of 6755 pigs in district Ludhiana. The district ranks first in exotic pig breed production with 5692 exotic pigs and third in indigenous breed production with 1063 pigs (Singh 2019).

2.1 Breed Characteristics and importance of Large White Yorkshire (LWY) Pigs:

The large White Yorkshire (LWY) is a well known british breed used for pork production. It was originally found in Yorkshire and adjoining nations in northern England. It is famous as 'mother breed' having largest litter size than other breeds. These pigs possess huge outlined body which is counted as significant quality for marketing. The developed body weight of a pig is 300-400 kg and sow 230-320 kg (Mathivanan 2014). The highly considered maternal traits are number of piglets conceived alive, number weaned, and 21-day litter weight. The Yorkshire sow has 7 pairs of teats. Some of the teats are accepted to be less efficient than others i.e. inverted, small, visually impaired, extra, flat or telescopic teats (Chalkias 2013). The weight of LWY piglets during weaning at twenty eighth day and fifty sixth day were 8.73 ± 1.32 and 14.77 ± 0.77, kg and there was no impact on marketing weight of 28^{th} day weaned piglets (Narayanan et al., 2008). Gopinathan et al., (2011) concluded that the LWY have better carcass traits than other pig breeds. The growth studies showed that the LWY pigs possess higher weight of body and better feed conversion ability at fifth month of their age in comparison to desi and cross-breed pigs. Reiland (1978) reported that LWY pigs get sexually matured at age of six months and till eighteen months age is counted as adolescence age. It has been seen that these pigs are sensitive to the heat stress in contrast with the native breed of pig. At high temperature, the observations showed that LWY meat had rapid glycolysis, lower

water holding limit and higher protein denaturation which indicates that increase in temperature compromises with the quality of meat (Parkunan et al., 2017).

2.2 Breed Characteristics and importance of Indigenous Pigs:

In India, the indigenous pigs contribute seventy six percent to the total pig population. The population trend of indigenous pigs is decreased by 8.66% between 2012 and 2019 (Singh 2019). The popular indigenous breeds are Ghungroo, Niang Megha, Ankamali, Agonda Goan, and Tany-Vo (NBAGR https://nbagr.icar.gov.in/en/registered-pig/) (Table 1). Ghungroo is a native pig breed originated from North Bengal. It is famous for its quality of pork. It has litter size of six to twelve piglets with one kilogram weight during birth and weaning weight is seven to ten kilograms respectively (Zaman et al., 2013).

The native piglet and matured pig weighs 40 kg and 70kg respectively. The mature female pig at age of twenty four months possess six pairs of teats. The pigs have 12-17 cm long bristles with diameters of 210-320 µm (Kumaresan et al., 2006). The important traits such as disease resistance, hardiness and adaptability to harsh climatic makes the indigenous pig more preferable in their respective regions (Govindasamy et al., 2019). The Kumar et al., (2018) stated that the muscle mass and marbling score of indigenous pigs are better than LWY pigs but lacks in growth rate. Moreover, the meat has good sensory quality with higher fat content, juiciness and tenderness.

2.3 History of domestication of pigs:

Pig is an even-toed ungulate animal belongs to Suidae family. The *Sus scrofa domesticus* is the domesticated breed of Europe, emerged from a wild boar *Sus scrofa* (Zeller et al., 2019). The two domesticated forms of pigs i.e., *Sus scrofa* and *Sus indicus* were identified by Darwin. It was believed that *Sus scrofa* was origined from European wild boar but the origin of *Sus indicus* was unknown. During early 19th centuries the Asian pigs were used to improve the European domestic pigs (Darwin 1868). Bosse et al., (2015) demonstrated that the cross breeding between Asian and European pig breeds results in increase in fertility and backfat in Large White pigs. With the help of microsatellite markers, it was estimated that the time divergence between Chinese Meishan pigs and European domestic pigs was of 2000 years (Paszek et al., 1998).

2.4 Pork and its nutritional value:

Pork is rich in minerals and vitamins i.e., B12, B6. The 100gm of pig meat provides various nutrients i.e., potassium, riboflavin and zinc respectively (Arnarson 2015). The study demonstrated that the organ having highest yield is liver i.e., 1.35 % of total yield. Whereas all organs contain moisture, fat, ash, and protein also (Seong et al., 2014). Hong et al., (2014) performed liquid chromatography-tandem mass spectrometry and found that there are twenty-two beta-agonists that are present in the pork liver. The qualitative analysis of pork has been done in industries depends upon colour, pH, water holding capacity, firmness and marbling. The reddish pink coloured meat is considered as best quality meat, where the colour score ranges from 1-6. The desirable pH after 45 minutes of post-mortem must be in range of 6.7-6.3 whereas, the pH after 24 hours can be 6.1-5.7 respectively. Similarly, firmness ranges from one to five, the desirable one must be 3-4 in range. The drip loss or water holding capacity in cooler is measured in lab that ranges between 3-6 % (Lucy 2016). Bertol et al., (2015) demonstrated that the colour intensity increases while intramuscular fat remains same in meat yielded from heavy weight pigs.

Table 1: Registered breeds of indigenous pig.

S.No.	Breed	Geographical location
1.	Purnea	Bihar and Jharkhand
2.	Mali	Tripura
3.	Ghurrah	Uttar Pradesh
4.	Tenyi Vo	Nagaland
5.	Ghoongroo	West Bengal
6.	Zovawk	Mizoram
7.	Doom	Assam
8.	Agonda Goan	Goa
9.	Niang Megha	Meghalaya
10.	Nicobari	Andaman & Nicobar

Source: NBAGR

2.5 Genes associated with metabolic process and immune response:

The metabolism is the mechanism involves various chemical reactions that empowers the cell to grow, proliferate and differentiate. It involves catabolism and anabolism. The metabolic pathways include: electron transport chain, fatty acid β-oxidation, glycolysis, gluconeogenesis, kreb cycle, phosphogluconate pathway and urea cycle. These metabolic pathways provides ATP as primary fuel; are regulated by a variety of genes and vice-versa (Carthew 2021). The literature was studied to find such genes that have effective role in lipid metabolism and energy production. The genes involved in beta-oxidation, plasma triglyceride metabolism and HDL metabolism are *ACSL1*, *HADH*, *ACAA2*, *ACADS*, *GCDH*, *ACAT2*, *CYP2E1*, *APOA4*, *CACNA2D1*, *HMGCG2*, *ACACA*, *LPL*, *FASN* and *ABCA1* (Zhong et al., 2012; Pedrelli et al., 2014; Sodhi et al., 2014 a; Sztankoova et al., 2016).

The immune system contributes equally to cellular growth by eliminating invading pathogens and preventing various tissue injuries (Chaplin 2010). *APOA4*, *APOA1*, *APOC3* are the genes that function collaborately and highly influence the inflammatory and immune response (Salviano-Silva et al., 2018 and Sodhi et al., 2014 a). The *SOCS* gene family includes eight genes i.e. *CIS*, *SOCS1* to *SOCS7* that regulates cellular growth by modulating GH secretion (Sodhi et al., 2014a and Santos et al., 2020).

The genes selected for this study are *ACAT2*, *ACAA2*, APOA4 and *SOCS2* respectively.

2.6 ACAT2:

ACAT2 gene majorly facilitates cholesterol synthesis. It functions in regulation of cholesterol stored as lipid droplets in the cell, absorbs extra cholesterol and plays role in formation of chylomicrons (Ahmed 2019). Zhang et al., (2010) found that ACAT2 protein involves in conversion of cholesterol and fatty acids into cholesterol esters.

The studies on African green monkey explained the membrane topology for *ACAT2* which is trans-membrane protein with seven domains and facilitates the cholesteryl esters into apoB-containing lipoproteins (Anderson et al., 1998). The primary function of gene is studied by its knockdown in mice which shows decreased cholesterol ester content in the liver (Zou et al., 2018). Sodhi et al., (2014, b) found

that mutations at residues 122 and 281 showed the functional inhibition of *ACAT2* due to change in charge of residue. The other study revealed that *ACAT2* found in liver can be used for selection as it is able to facilitate or inhibit the fat accumulation in abdomen (Zhu et al., 2018).

Turley et al., (2010) found that *ACAT2* knockout mice shows low absorption of intestinal cholesterol and results in unesterified fat buildup. The *ACAT* inhibitors can be used to treat hypercholesterolemia and cholesterol gallstone formation (Portincasa et al., 2017, Nagarthna et al., 2020). Further, Parini et al., (2004) conducted a study using *ACAT2* inhibitor; pyripyropene A to demonstrate the *ACAT2* activity in different samples. It was found that *ACAT2* activity was higher in liver samples while *ACAT1* gene was expressing similarly in all samples. The conclusion was made that the diseases caused by cholesterol metabolism dysregulation can be prevented and treated by regulating expression of *ACAT2* gene. The other study also provided the similar data suggesting to medicate atherosclerosis and fatty liver disease by using *ACAT2* inhibitors (Alger et al., 2010).

The gallstone formation; accumulation of excess cholesterol treatment is given by a study suggesting the use of *ACAT* inhibitors that targets both the *ACAT1* and *ACAT2* proteins in their source organs. As *ACAT2* inhibition reduces the cholesterol absorption and directly increase the bile cholesterol (Chang et al., 2000).

Hussain (2014) revealed that there is a incredible impact of *ACAT2* inactivity on the homeostasis of intestinal cholesterol. It was observed that in absence of *ACAT2* the unesterified cholesterol is absorbed through basolateral efflux pathway by denosine triphosphate–binding cassette transporter A1 (*ABCA1*).

Weng et al., (2020) conducted a study showing that pro-proliferative activity of *ACAT2* gene positively corelated with colorectal cancer (CRC). They demonstrate that the CRC cells with *ACAT2* knockdown result in cell cycle arrest. The fact was, that the gene knockdown leads to low expression of cyclin D1 and CDK2; regulators of G0/G1 phase. The study done by Wu et al., (2020 b) found that *ACAT2* is the one of the hub genes that is responsible for occurrence of immunoglobulin A nephropathy (IgAN); end stage kidney disease.

Nguyen et al., (2012) demonstrated that *ACAT2* gene is majorly involved in metabolic process. The study confirmed that in absence of *ACAT2*, inefficient sterol

absorption occurs as the unesterified sterol takes part in forming chylomicron coat which significantly lower the amount of sterol in lymph.

2.7 ACAA2:

A transcriptome analysis in pigs revealed that *ACAA2* is one of the main gene in metabolism of lipid (Zhao et al., 2019). *ACAA2* gene regulates fatty acid metabolism and encodes thiolase enzyme catalyzing the elongation and degradation of mitochondrial fatty acids (Miltiadou et al., 2017). The thiolase enzyme is the main enzyme involve in energy production from fat by conversion of acetyl-Coenzyme A into acetoacetyl-CoA and ketone bodies production (Kiema et al., 2014). The study conducted by Wang et al., (2012 b) identified *ACAA2* as one of nine protein coding genes involved in mitochondrial energy metabolism. It was observed that protein encoded by *ACAA2* lowers down due to myocardial ischaemia- reperfusion injury and intermittent hypobaric hypoxia treatment.

Sodhi et al., (2014, b) constructed the phylogenetic tree of *ACAA2* and *ACAT2* of Bos taurus (cattle breed) and Sus Scrofa (domesticated pig) which showed that both are present in same clade. It has been found that *ACAA2* and *ACAT2* are linked with various metabolic pathways but *ACAA2* shows no association with lipid metabolism pathway. Cao et al., (2008) demonstrated reduction of apoptosis after the injection of *ACAA2* in human hepatocellular carcinoma HepG2 cells with over-expression of BNIP3 (role in cell death) gene.

Paten et al., (2015) studied that *ACAA2* gene expresses both in liver as well as in mammary gland of ovine. Also, the expression of *ACAA2* regulates the beta-oxidation pathway in the late pregnancy period. It is identified that the SNP's in *ACAA2* gene are linked with protein content in milk as the alleles of gene express differentially in Chios sheep (Orford et al., 2012). Tomilov et al., (2016) studied that p46Shc negatively regulates the expression of *ACAA2* gene by binding strongly with *ACAA2* encoded thiolase enzyme. The mice lacking p46Shc has high lean, low obesity and higher oxidation of lipids.

As *ACAA2* is involved in lipid metabolism and the *ACAA2* overexpression results in inhibition of triglyceride synthesis and apoptosis of cells. It has been observed that *ACAA2* expression is inversely proportional to the expression of miR-152; tumor suppressor microRNA (Yang et al., 2018). It has been found that the key

genes involved in development of glioma; brain tumor. The comparative expression of *ACAA2* in isocitrate dehydrogenase (IDH) mutant and wild-type groups was observed which signifies that the gene is highly involved in lower grade glioma production (Wu et al., 2020 a). Xia et al., (2016) characterized the IncRNAs positively regulating the *ACAA2* gene associated with nonalcoholic steatohepatitis (NASH); energy metabolism disorder.

It has been found that *ACAA2* gene has nuclear localization sequence which is linked with a histone variant; H2A.Z. With no H2A.Z in human HAP1 cells the enzymes associated with chromatin also decrease in amount (Choi et al., 2019).

Another study suggested that *Qishen* granules (QSG) can be used to cure heart diseases in rats by targeting *ACAA2* involved in lipid metabolism (Gao et al., 2020). The HIV-1 viral protein R decreases *ACAA2* expression which slow down the β-oxidation results in hepatic steatosis (Agarwal et al., 2017). The study carried out by Saei et al., (2018) choose both the attached and detached cancer cells for applying comparative proteomics. It was observed that knocking down the *ACAA2* gene results in antiproliferative effects.

Wan et al., (2019) conducted a study in neonatal pig liver inserted with maternal fecal Bacteria (FMT) and amoxicillin (AM) solution. The metabolomic analysis revealed that early insertion of FMT and AM leads to downregulation of *ACAA2* gene, thus lower down the fatty acid oxidative catabolism.

2.8 APOA4

The gene apolipoprotein A4 (*APOA4*) expresses in small intestine and liver (Motoi et al., 2021). It enters in circulation by large triglyceride-rich lipoproteins i.e. chylomicrons, then transported to various tissues (Qu et al., 2019). Qu et al., (2021) found that gene is regulated by LRP1during fasting and facilitates glucose uptake.

Wang et al., 2019 recognized the role of *ApoA-IV* gene in liver and white adipose tissue from *ApoA-IV* knockout rats. The absence of *ApoA-IV* affects the genes linked with glucose and pyruvate synthesis and *de novo* synthesis of lipids. It has been observed that the gene is age-independent and closely associated with glucose and lipid. It controls glucose balance by insulin secretion (Wang et al., 2012 a) and decreases gluconeogenesis in liver by nuclear receptor NR1D1 (Li et al., 2014).

The study revealed that *ApoA-IV* acts as an antioxidant in-vivo and have therapeutic role in treating atherosclerosis by inhibiting oxidative damage in tissues (Peng et al., 2018 a). It reduces the cholesterol accumulation in arteries by three different ways. It affects high density lipoprotein mediated reverse cholesterol transport, decreases low-density lipoprotein oxidation and facilitates aggregation of platelets (Qu et al., 2019). It has an anti-ulcer role by which it inhibits gastric acid secretion (Okumura et al., 1995). The gene also acts as satiation factor as it reduces the food intake when it was fed to fasted rats (Qu et al., 2021).

The anti-inflammatory effect of *ApoA-IV* was observed in WT and *ApoA-IV* knockout mice fed with 3% DSS for 7 days. It has been determined that the gene reduce the P-selective mediated adhesive interactions between leukocytes and platelets. Thus, diminish the progression of experimental colitis i.e. inflammation of colon (Vowinkel et al., 2004; Li et al., 2021 c). It is linked with HDL and chylomicron remnant. It also facilitates reverse-cholesterol transport and has a vital role in protection from cardiovascular disease (Wang et al., 2015).

The overexpression of gene is linked to liver fibrosis, a chronic disease caused by accumulation of extracellular matrix proteins in the liver. Therefore, this gene can be used as selection marker for early disease diagnosis (Wang et al., 2017). The other study demonstrated that it protects liver by inhibiting fibrotic mediators, inflammatory cytokines and modulation of T lymphocytes (Wang et al., 2021). Li et al., (2021 b) explored that the gene plays protective role during liver damage. In the absence of *APOA4*, recruitment of monocytes increased whereas overexpression of gene results in less macrophages and dendritic cells.

The study carried out by VerHague et al., (2013) demonstrated that the hepatic triglyceride (TG) accumulation and *APOA4* RNA levels increase in parallel. As liver protects itself by efficient TG export from toxic accumulation of intracellular lipids, so it was concluded that increasing hepatic *APOA4* expression help in lower down the cases of fatty liver disease.

2.9 SOCS2

SOCS2 gene belongs to family of proteins that negatively regulates cytokine and growth factor signalling (Yoshimura et al., 2021). It has been studied that low level of gene prevents GH, prolactin and interleukin signalling cascades whereas

upregulation of SOCS2 accelerates the growth factors signalling (Morris et al., 2018).

The progressive nerve damage causes impairment of central nervous system (CNS) that leads to multiple sclerosis (MS). Cramer et al., 2019 used Experimental Autoimmune Encephalomyelitis (EAE) as animal model and determined that there is less cure rate of locomotor impairment in KO mice. Hence, it is clear that the gene has positive role in recovery of multiple sclerosis. It has important role in balancing the functions of the brain, liver and heart as it regulates the mechanisms of CNS and maintains calcium homeostasis (Esper et al., 2012 and Brant et al., 2016).

Cui et al., (2016) demonstrated that overexpression or absence of SOCS2 have no significant effect on cell proliferation but it inhibits metastasis by creating obstruction in migration of HCC cells. Thus, it has therapeutic function in treating hepatocellular carcinoma. Chen et al., (2018) discovered that overexpression of RNA N6-adenosine methyltransferase, METTL3 in HCC which prevents the expression of SOCS2. Hence, by silencing METTL3 mRNA expression of SOCS2 can be restored.

The progression in fatty liver leads to nonalcoholic fatty liver disease (NAFLD) that involves aggressive inflammation and liver failure. *SOCS2* inhibits Janus kinase 2 (JAK2)-signal transducer which in response inhibits the growth hormone (GH) secretion. Lowering of GH dysregulates the hepatic triglyceride (TG) metabolism and leads to NAFLD. Therefore, *SOCS2* involved in development of NAFLD (Vesterlund et al., 2011; Morris et al., 2018). The other study found that *SOCS2* deficient mice results in gigantism due to GH dysregulation (Rico-Bautista et al., 2005). Tannahill et al., (2005) studied that *SOCS2* improves signalling of cytokines by degradation of *SOCS3* that marked as mechanism leading to gigantism.

The study carried out by Monti-Rocha et al., (2019) revealed that when acetaminophen (APAP) exposure results in various inflammations then *SOCS2* plays defensive role in decreasing the liver damage. Without *SOCS2*, there can be increase in hepatic necrosis which leads to increase in immune reactions like neutrophil recruitment, expression of the neutrophil-active chemokine and ROS generation. The inhibition of GH secretion by SOCS2 have great role in liver recovery. During partial hepatectomy, it has been observed that SOCS2 limits the early hepatocyte proliferation by regulating GH pathway. Later, it improves proliferation by diminishing serum IGF-1 (Masuzaki et al., 2016).

The study conducted by Yang et al., (2013) demonstrated that diet rich in fat fed mice treated with rhGH results in increase in expression of *SOCS2* in subcutaneous fat mass. The other study identified *SOCS2* deficient mice has increased triglyceride concentration in liver when fed with diet containing extra fat, which may leads to development of fatty liver disease. Thus, concluded that *SOCS2* gene regulates the lipid metabolism (Zadjali et al., 2012).

The atopic disorders are defined as immune response against various antigens or allergens that facilitates Th2 differentiation (Monti-Rocha et al., 2019). Knosp et al., (2011) studied that *SOCS2* deficient mice are more prone to atopy as there is great surge in secretion of various interleucins

SOCS2 modulates the innate as well as acquired immune response and facilitate the production and differentian of various T helper and T regulatory cells (Esper et al., 2012 and Brant et al., 2016). Tsao et al., (2008) demonstrated that the absence of SOCS2 gene involves in progression of Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). The increased IL-1β and TNFα are observed in peripheral blood mononuclear cells (PBMCs) with low SOCS2 level. The use of TNFα blocking agent results reported that TNFα regulates the SOCS2 expression in PBMCs . Isomäki et al., (2007) reported increase of SOCS2 gene in RA affected peripheral blood T cells. Rasche et al., (2008) reported that SOCS2 also associates with type-2 diabetes.

Choi et al., (2009) determined high levels of SOCS2 in rat suffering from brain ischemia. The other studies showed that low level of SOCS2 gene reported in breast, lung, hepatocellular and ovarian cancers (Qiu et al., 2013; Zhu et al., 2013; Li et al., 2017; Liu et al., 2020; Yang et al., 2020 and Zhang et al., 2021).

CHAPTER III

MATERIALS AND METHODS

The current study was done to find the comparative expression profiling of genes associated with metabolic process and immune response in Large White Yorkshire and indigenous breeds of pigs.

3.1 Chemicals and Reagents.

Trizol (Qiazol), Chloroform (Himedia), Isopropanol (Himedia), Ethanol 75% (Omnie), DEPC (Himedia) and nuclease free water (Ambion).

3.2 Animals

The pig samples were collected from slaughter house.

3.3 Experimental design

The pigs (N = 12) were divided into two groups, Large White Yorkshire (N = 6 each) and indigenous breeds (N = 6 each).

3.4 Sample Collection

3.4.1 Collection of tissues

In this work, the samples were taken from adult animals of Large White Yorkshire and indigenous pigs. Pigs of LWY breed were reared on an organized farm at Village Kotli, district Ludhiana. The breed of animals was characterized, based on their phenotypic traits and pedigree. The pigs from non-descript (desi) breed were reared at unorganized farms. All the animals were of similar age (1 year) and were castrated animals. All the animals were vaccinated & dewormed. The non-descript animals were slaughtered at slaughter house of Municipal Corporation, Ludhiana. The median hepatic lobes of the lever were harvested from the slaughtered animals. The tissue samples were transported in RNA later to lab. After that, the samples were washed with DEPC and kept at -80^0 C in the sealed packets.

3.5 Di ethyl pyro carbonate (DEPC) treatment of tips and eppendorfs:

The vials used for RNA extraction and PCR reaction (i.e., tips, eppendorfs and PCR tubes) were treated with DEPC at 1:1000 ratio (1 ml of DEPC in 1 litre of distilled water). Vials were submerged in DEPC treated water for overnight and then

dried in hot air oven at 37^0 C for overnight and autoclaved twice at 121^0 C for 20 minutes.

3.5.1 Extraction of RNA

3.5.2 Extraction of RNA by Trizol method

The working platform, pestle & mortar were made RNase free everytime with RNase Zap™ (Invitrogen, Thermo Fisher Scientific).

1. 120 mg of liver tissue was homogenized in a pestle and mortar sanitized with RNase Zap™ (Invitrogen, Thermo Fisher Scientific). Then, it was thoroughly mixed with 1 ml of Trizol.
2. After thorough minsing, the mix was transferred into 2 ml eppendorf.
3. The homogenized paste of liver tissue was vortexed vigorously and incubated for 5 minutes on ice.
4. Afterwards, it has been centrifuged for 15 minutes at 12,000 rpm where temperature was 4^0 C and supernatant was collected for further steps.
5. Then 200 µl of chloroform was added to supernatant. After vortexing, 15 minute incubation in ice was given. Then, it was centrifuged at 13000 rpm for 15 minutes at 4° C.
6. Half of the aqueous phase was poured into 1.5 ml tube without touching the inter-phase.
7. 0.5 ml isopropyl alcohol was mixed with aqueous phase. Then the mixture was incubated at -20° C for 10 minutes.
8. Afterwards, it was centrifuged at 12000 rpm at 4° C for 10 minutes and the pellet was obtained by centrifugation.
9. The pellet was washed with one ml of 75% ethanol after discarding the supernatant. Then it was vortexed for fifteen seconds and centrifuged at 13000 rpm for fifteen minutes at 4° C.
10. The washing step can be repeated again (if needed).
11. Then the pellet was air dried and dissolved in 35 µl nuclease free water with the help of pippet.
12. The dissolved RNA had been preserved at -80^0 C for further use.

3.6 RNA Quantification

3.6.1 Spectrophotometric measurement

The isolated RNA samples were treated with RNase-free DNase kit (QIAGEN, Hilden, Germany) to get rid of genomic DNA contamination and it was purified with RNeasy mini kit according to the user guidelines (QIAGEN, Hilden, Germany). The quantity of RNA was examined by the thermo scientific Nanodrop one and quality check was done by automated capillary gel electrophoresis according to manufacturer's guidelines (Agilent Technologies Ireland, Dublin, Ireland). The mRNA samples were measured between the ratio of 1.8 to 2.0 whereas RNA integrity values were in the range of 8.0 to 10.0 (Table 2).

Table 2: Quality and concentration of extracted RNA

Breed	**LWY**		**Indigenous**	
Sr. No.	**Concentration (ng/ µl)**	**Quality (A 260/280)**	**Concentration (ng/ µl)**	**Quality (A 260/280)**
1.	1452.8	1.89	3002.4	1.90
2.	2508.6	1.81	3191.0	1.83
3.	2294.2	1.80	3067.8	1.88
4.	2984.9	1.84	2877.3	1.91
5.	1788.6	1.99	1666.9	1.85
6.	2572.7	1.86	1543.6	1.82

3.7 First Strand cDNA synthesis

Total RNA extracted was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Catalog no. 4368814) as per manufacturer's instructions. The components of kit and their specific volume are given in Table 3.

Preparation of saturated Reverse Transcription master mix (2X)

1. First of all the kit components were thawed on ice.
2. Then the volume of components required for each reaction was calculated.

Table 3: Components of 2X RT cDNA master mix per reaction.

Component	Volume
25X dNTP Mix (100 mM)	0.8 µl
MultiScribe™ Reverse Transcriptase	1.0 µl
10X RT Buffer	2.0 µl
10X RT Random Primers	2.0 µl
Nuclease-free H_2O	4.2 µl
Total per reaction	10 µl

3. Then the 2X RT master mix was kept on ice.

Preparation of the reverse transcription reactions

1. The volume for 2 µg of RNA sample was calculated and was pipette in each well with rest of nuclease free water to make 10 µl volume.
2. Then, 10 µl of 2X RT master mix was added into the wells. The whole mixture was pipetted up and down gently to mix thoroughly.
3. The tubes were sealed properly.
4. Then, tubes were spinned to mix the content and to eliminate the air bubbles.
5. The tubes were placed on ice until they were ready to be loaded in the thermal cycler.

Table 4: List of primer sequences of *ACAT2, ACAA2, APOA4* and *β- actin* for PCR.

Gene	Primers	Size of Product	Annealing temperature (Ta)	GenBank ID
ACAA2	F-TAGGCTCTGTGGCTCTGGTT R-GTAATTGCCATCGGGATTTG	225 bp	61° C	NM_001167638
ACAT2	F-ATCACCAAGGAGCGAATCC R–CCTCTTCTGCTTGTCCCAAC	245 bp	58.5° C	NM_001243427
APOA4	F-GATGAAGAAGCAGGCAGAGG R-TGTTGAAGGTCTCCCCGTAG	215 bp	61° C	NM_214388
SOCS2	F-ATTGGTTTTGTGGCTGGAAG R-AGCACAGAATCGGAACATCC	188 bp	61° C	NC_010447
β- actin	F-GACATCCGCAAGGACCTCTA R-ACACGGAGTACTTGCGCTCT	250 bp	60° C	XM_003124280

Table 5: Conditions followed for synthesis of cDNA

Conditions	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Temperature	25º C	37º C	85º C	4º C
Time (in minutes)	10 min	120 min	5 min	∞

1. The volume of reaction was set at 20 µl.
2. Then the tubes were loaded into thermal cycler after entering conditions as per mentioned in Table 5.
3. Then, the reverse transcription run was started.

Table 6: Reaction mixture composition for amplification of *ACAT2, ACAA2, APOA4* and *SOCS2* gene coding sequence

Components	**Volume/reaction (l)**
Forward primer	2 µl (0.5 picomole/ µl)
Reverse primer	2 µl (0.5 picomole/ µl)
Master mix	10 µl
CDNA Template	2 µl
Nuclease free water	4 µl
Total volume	20 µl

3.8 Optimization of cDNA for Real Time PCR reaction

Online Primer-3 software was used to design primers for the qRT-PCR (Rozen and Skaletsky, 2000) and the list of primers has been given in Table 4. Real-time qRT-PCR (BIO RAD model CFX96^{TM} Optics Module real time PCR) was used for quantitative assessment of mRNA transcript levels of *ACAT2, ACAA2, APOA4* and *SOCS2* genes; in indigenous and LWY. The EvaGreen (Biotium, USA) dye was used to quantify the transcripts of target genes.

3.9 Standardization of PCR

The reaction mixture for the amplification of coding sequences of *ACAT2, ACAA2, APOA4* and *SOCS2* genes as mentioned in Table 6 were prepared after

thorough mixing. Then PCR tubes were put in thermocycler (BIO RAD) as per conditions mentioned in Table 7.

Table 7: PCR conditions for amplifying gene coding sequences using the *ACAT2, ACAA2, APOA4* and *SOCS2* gene specific primers

Steps	Time	Temperature (ºC)	No. of cycles
Initial Denaturation	2 min	95	1
Denaturation	30 sec	95	35
Annealing	30 sec	ACAT2: 58.5	
		ACAA2: 61	
		APOA4: 61	
		SOCS2: 60	
Extension	1 min	72	
Final extension	5 min	72	1
Hold	∞	4	

3.9.1 Agarose gel electrophoresis

1. 100 ml agarose gel was prepared by adding 1.5 g agarose in 100 ml of 1X Tris Acetate EDTA. Then mixture was heated for two minutes and left for five minutes to cool down. Then 4 µl of ethidium bromide (1 µg/ ml) was added and mixed by slow shaking of beaker.
2. Gel was poured into a casting tray. After fitting the comb, gel was left to solidify.
3. After solidification of gel, the comb was removed carefully and the gel was transfered into the electrophoresis tank containing 1X TAE buffer.
4. No loading dye was used as a dye was already present in the mastermix.
5. Equal volume of PCR samples were loaded in the adjacent wells with 5 µl of 50 bp ladder in first well.
6. Electrophoresis was carried out at 90-100 volts/ cm till bromophenol blue has migrated at least 2 cm from the well. The bands were visualized under UV-illumination (Bio-Rad, USA) and gel doc (G:Box Syngene).

3.9.2 Reaction conditions

The Applied Biosystems by Life technologies SYBR® Green PCR kit was used for preparing reaction mixture. The components of reaction mixture are mentioned in Table 8.

Table 8: Master Mix composition for real time PCR

Composition	Concentration
Nuclease free water	2 µl
SYBR	5 µl
Primer	F - 0.5 µl (0.5 picomole/ µl) R -0.5 µl (0.5 picomole/ µl)
cDNA template	2 µl (120 ng/ µl)
Total	10 µl

The reactions were run in duplicates and were carried out in Real Time PCR using the following cycling parameters (Table 9) that have been found optimum for amplification of genes fragment with β- actin as an endogenous control.

Table 9: Conditions of *ACAT2, ACAA2, APOA4* and *SOCS2* for Real Time PCR (SYBR Green chemistry)

Step		Temperature (°C)	Time
I	Initial denaturation	94	5 min
II	Denaturation	94	30 sec
III	Annealing	60	30 sec
IV	Extension	72	1 min
V		Melt curve added	

The efficiency of real-time PCR was assessed by standard curve method. The different delusions of cDNA were used for generation of standard regression curve. The slope of the curve was used to determine the efficiency of PCR. The *β-actin* gene

was used as endogenous control to compare with the amplified target genes (Wang et al., 2003). The comparative C_T method was used for quantification of mRNA transcript levels (Livak and Schmittgen., 2001). The results were obtained by calculating relative expressions of target genes normalized with transcript levels of the endogenous gene (Van Poucke et al., 2001; Erkens et al., 2006).

Statistical analysis of the differential expression patterns of the genes:

ANOVA was used for the statistical analysis of the differential expression patterns of the genes. The values have been written as mean ± SEM. $P<0.05$ has been set as statistical level of significance and subsequently Tukey's b- test was performed for analyzing the level of significance.

CHAPTER IV

RESULTS AND DISCUSSION

The present study envisaged the comparative expression profiling of genes associated with metabolic process and immune response between Large White Yorkshire (LWY) and indigenous pigs.

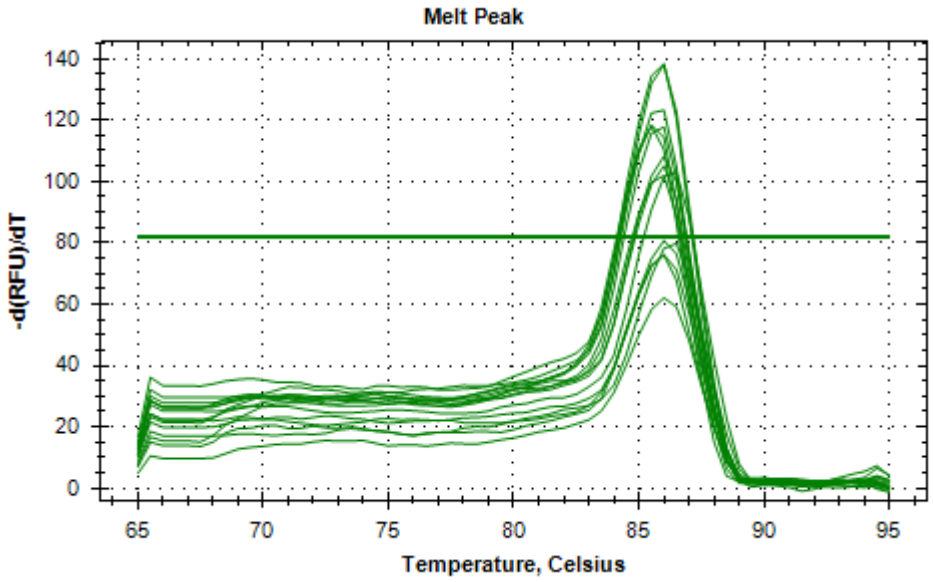
4.1 mRNA expression of *ACAT2*

In the present study the mRNA expression of *ACAT2* was evaluated by using real-time PCR in liver tissue of LWY and indigenous pigs. The fold change expression in indigenous breeds is 3.78 times more than LWY demonstrating higher expression of *ACAT2* in indigenous pigs (Table 10). *ACAT2* is reported as involved in lipid metabolism, tissue growth and differentiation (Pedrelli et al., 2014). This gene regulates the formation of chylomicrons and their transport from liver to intestines through blood stream (Hussain et al., 2014). The upregulation of *ACAT2* leads to development of atherosclerosis, hypercholesterolemia and gallstone formation due to dysregulation of cholesterol absorption, biosynthesis and transport (Guo et al., 2018). Similarly, the gene is involved in progression of IgA neuropathy, end stage kidney disease by increasing the cholesterol level in blood (Wu et al., 2020 b). Further, Yuan et al., (2013) demonstrated that the cellular growth is highly influenced by interactions between metabolites and the genes linked with metabolic pathways. The Human Protein Atlas (2021) demonstrated the higher expression of *ACAT2* in liver tissue than all other tissues and organs in humans. Li et al., (2020) stated that the expression of gene at neonatal stage is higher in Large White than Erhualian (EHL) piglets (at birth and 25-day-old). Findings of Sodhi et al., (2014 a) showing significant upregulation of *ACAT2* gene in adult JNP in comparison to the Berkshire breed of pigs goes parallel to the finding of current study. In the present study, *ACAT2* has shown significantly higher transcript levels in indigenous pig as compared to LWY (Fig.1 and Fig.2). The findings of current study are in accordance with the earlier reports and it can be said that the higher expression of gene depicts better cellular growth in indigenous pigs than LWY but the upregulation of gene may cause diseases due to high cholesterol.

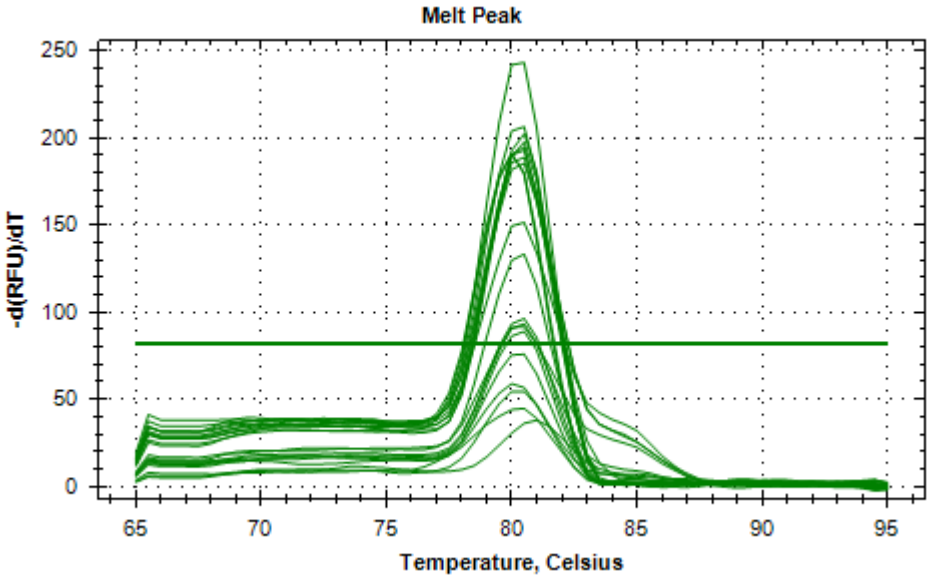
Table 10: Validation of expression of *ACAT2* mRNA using real time PCR (SYBR green)

Group	*ACAT2* (Average C_t)	β-actin (Average C_t)	ΔCT (C_t *ACAT2* - C_t β-actin)	ΔΔCT ΔCt of treated – ΔCt of untreated	Fold change
LWY	27.03 ± 0.17	23.80 ± 0.26	3.22 ± 0.26	0.00 ± 0.26	1.00
Indigenous	22.79 ± 0.13	21.47 ± 0.16	1.32 ± 0.18	-1.92 ± 0.36	3.78*

Values are Mean ± SE. Mean's having superscript * shows significant difference at $p < 0.05$.



Beta-Actin



ACAT2

Fig. 1: Melt curve analysis of ACAT2

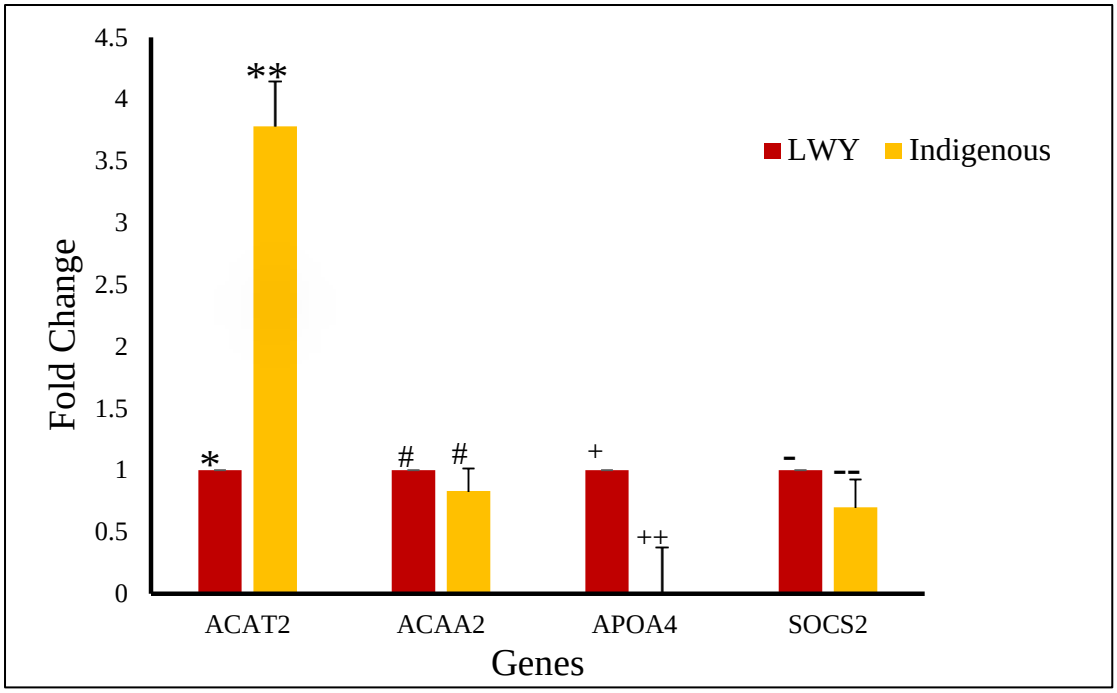


Figure 2: Relative transcript level (RQ) of *ACAT2*, *ACAA2*, *APOA4* and SOCS2 in liver of Large White Yorkshire and Indigenous pigs. RQ of genes in indigenous samples has been normalized with mRNA expression of endogenous reference gene β-actin where LWY was taken as control. The bars having different superscripts indicates significant differences in the transcript levels between the two breeds ($p < 0.05$).

Indigenous LWY

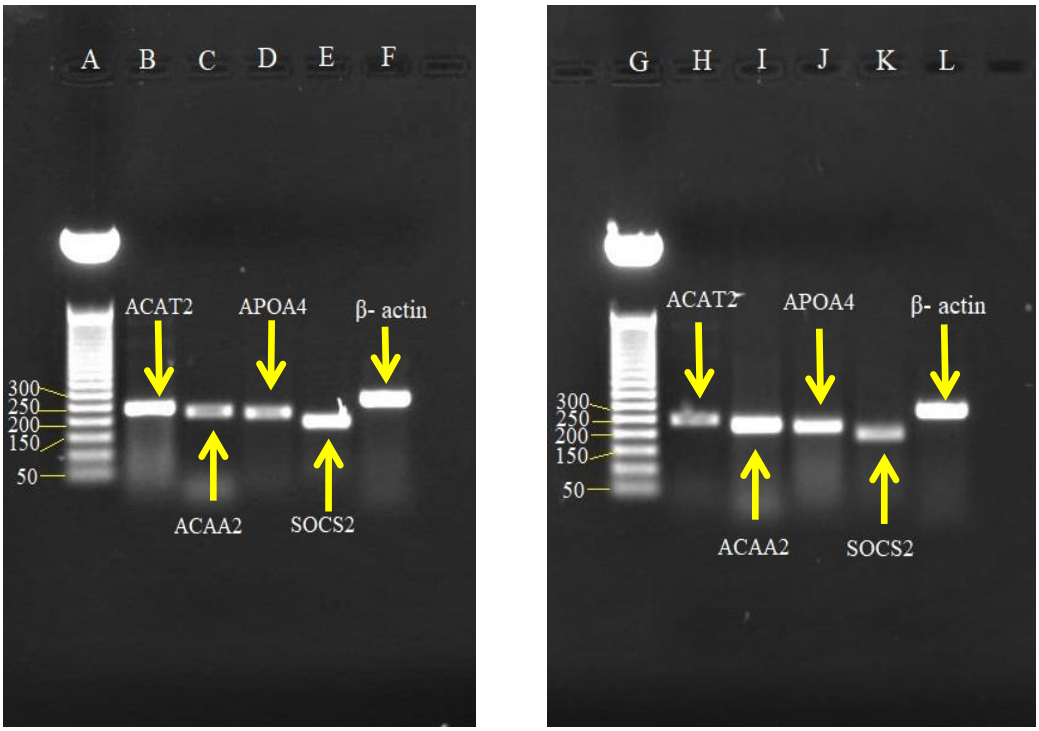


Figure 3. The bands in gel-doc image showing ligation of primers in PCR product. Wells "A" and "G" - 50 bp DNA ladder, wells "B" and "H"- *ACAT2* gene, wells "C" and "I"- *ACAA2* gene, wells "D" and "J"- *APOA4* gene and wells "E" and "L"- SOCS*2* gene and wells "F" and "L"- Beta Actin gene respectively from LWY and indigenous pigs.

4.2 mRNA expression of *ACAA2*

In the current study the mRNA expression of *ACAA2* was evaluated by using real-time PCR in liver tissue of LWY and indigenous pigs. The fold change expression of indigenous pigs is 0.83, which is lower than LWY (Table 11). It has been demonstrated that the gene is highly involved in beta-oxidation pathway and regulates the protein content in ovine milk (Miltiadou et al., 2017; Paten et al., 2015). *ACAA2* is also responsible for the high lean and low obesity in mice (Tomilov et al., 2016).

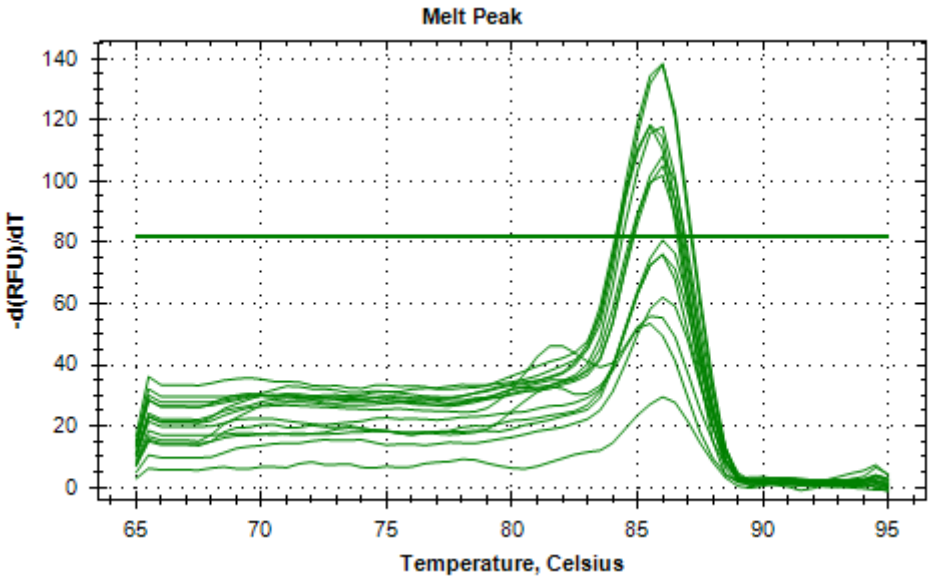
The overexpression of gene results in development of tumor as its expression inversely correlates with tumor suppressor microRNA (Yang et al., 2018). The other studies found that upregulation of gene leads to formation of brain tumor and energy metabolism disorders (Wu et al., 2020 a; Xia et al., 2016).

Sodhi et al., (2014 a) reported the lower expression of *ACAA2* in indigenous pig breed of Jeju island than Berkshire, which is support to the findings of current study. Bovo et al., 2018 done a comparative study between Italian Duroc pigs and Italian Large White pigs, found the higher expression of ACAA2 in Italian Duroc pigs. The other study conducted by Sodhi et al., (2014 b) retrieved data on *ACAA2* gene from Entrez gene of NCBI has lower expression in JNP than Bos taurus (Sus scrofa and bovine species; that are present on same clade). Although in the current study, no significant difference in the expression of *ACAA2* in both the breeds have been observed (Fig.2 and Fig.4) in present study. But yet, lower expression of *ACAA2* in indigenous pigs indicates that the lean meat is low and risk of tumor generation and metabolic disorders is less in indigenous pigs.

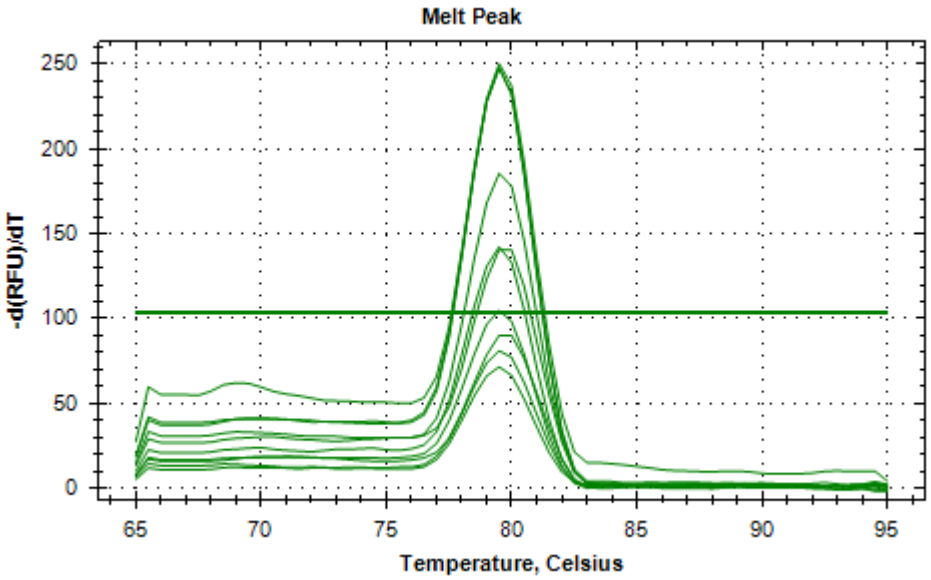
Table 11: Validation of expression of *ACAA2* mRNA using real time PCR (SYBR green)

Group	*ACAA2* (Average C_t)	β-actin (Average C_t)	ΔCT (C_t *ACAA2*- C_t β-actin)	ΔΔCT ΔCt of treated – ΔCt of untreated	Fold change
LWY	21.17 ± 0.19	19.43 ± 0.07	1.74 ± 0.17	0.00 ± 0.17	1.00
Indigenous	27.06 ± 0.16	25.05 ± 0.18	2.00 ± 0.22	0.26 ± 0.18	0.83

Values are Mean ± SE.



Beta-Actin



ACAA2

Fig. 4: Melt curve analysis of ACAA2

4.3 mRNA expression of *APOA4*

In the present study the mRNA expression of *APOA4* was evaluated by using real-time PCR in liver tissue of LWY and indigenous pigs. The fold change expression of indigenous breed is 0.01, which is 1% expression of gene in LWY (Table 12). A statistically significant difference (Fig.1 and Fig.4) in the transcript levels of *APOA4* between the two breeds has been supported by the findings of Sodhi et al., (2014 a).

In the earlier studies, it has been demonstrated that *APOA4* is responsible for lipid circulation and metabolism (Lu et al., 2006), carboxylic acid metabolic processes and immune response (Pashaj et al., 2013). Further, it also acts as an antioxidant, antiulcer and anti-inflammation factor that prevents the risk of various diseases (Okumura et al., 1995 and Natarajan et al., 2019). Also, *APOA4* regulates CD8+ T and CD4+ T lymphocytes (Wang et al., 2021) and recruit's macrophages and dendritic cells to prevent liver damage (Li et al., 2021 a).

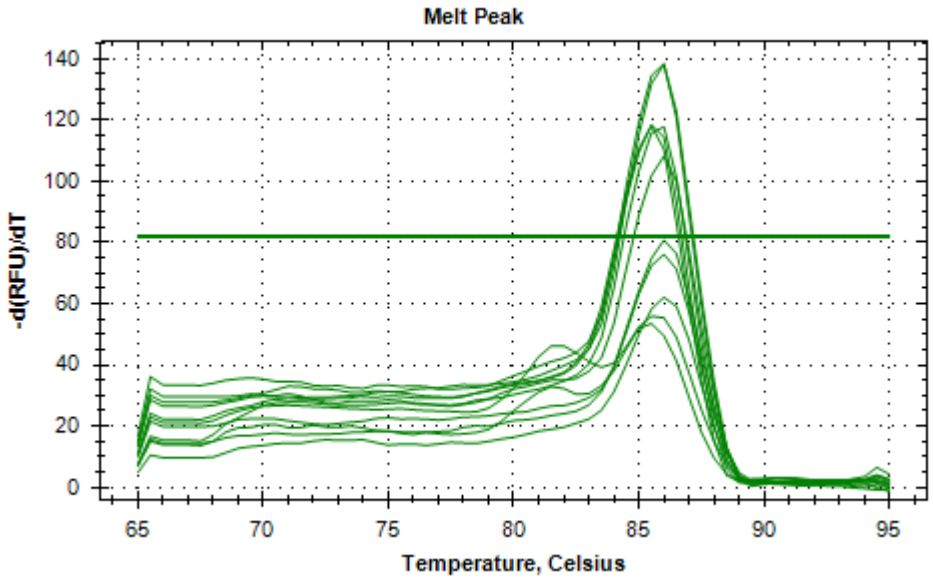
The growth performance is highly dependent on immune response (Adler et al., 2013), since good immune response leads to enhanced weight gain. Thus, the animal with good immune response should be selected for breed improvement purpose (Clapperton et al., 2006, Mallard et al., 1998).

Wu et al., 2018 conducted a study on rabbit and mouse models fed with high fat diet used for atherosclerosis research and reported the up-regulation of *APOA4*. Also, Wang et al., (2019) identified that in absence of *APOA4*, there is dysregulation of other hepatic genes result in increased *de novo* lipogenesis and enhancement in glycolysis and gluconeogenesis. Therefore, in accordance with present study it can be said that LWY pigs have good immune response which depicts chances of better weight gain than indigenous pigs.

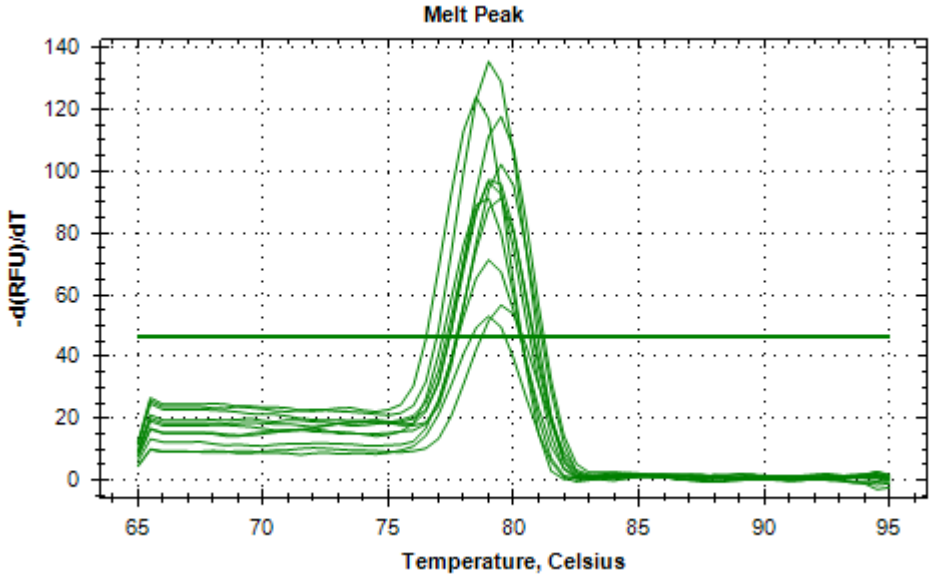
Table 12: Validation of expression of *APOA4* mRNA using real time PCR (SYBR green)

Group	*APOA4* (Average C_t)	β-actin (Average C_t)	ΔCT (C_t *APOA4*- C_t β-actin)	ΔΔCT (ΔCt of treated – ΔCt of untreated)	Fold change
LWY	22.62 ± 0.21	23.80 ± 0.26	-1.18 ± 0.24	0.00 ± 0.24	1.00
Indigenous	25.79 ± 0.20	19.91 ± 0.15	5.88 ± 0.29	7.05 ± 0.36	0.01*

Values are Mean ± SE. Mean's having superscript * shows significant difference at $p < 0.05$.



Beta-Actin



APOA4

Fig. 5: Melt curve analysis of APOA4

4.4 mRNA expression of *SOCS2*

In the present study the mRNA expression of *SOCS2* was evaluated by using real-time PCR in liver tissue of LWY and indigenous pigs. The fold change expression of *SOCS2* in indigenous breed is 0.70 (Table 13), which shows significantly lower expression of gene in indigenous pigs as compared to LWY (Fig.1 and Fig.5).

SOCS2 has vital role in differentiation of immune cells (Knosp et al., 2013; Esper et al., 2012; Brant et al., 2016). It also regulates lipid metabolism (Yang et al. 2013), CNS (Esper et al., 2012 and Brant et al., 2016) and prevents various diseases i.e. multiple sclerosis, systemic lupus erythematosus and rheumatoid arthritis (Cramer et al., 2019; Tsao et al., 2008). Therefore, the higher expression of *SOCS2* in LWY depicts more disease resistance than indigenous breed of pigs.

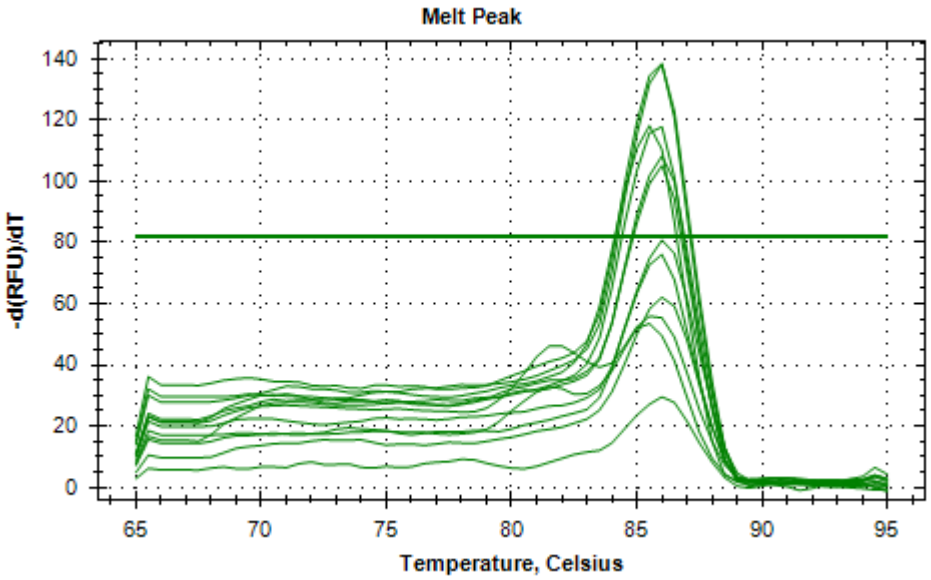
The findings of current study are supported by Sodhi et al., (2014 a) stating similar pattern of expression analysis in indigenous breed (JNP) with respect to Berkshire. Liu et al., (2006) conducted a study using three pig groups; Tongcheng pig at 90-day embryo stage, at adulthood and Landrace at adulthood. It has been found that expression of *SOCS2* in liver was higher at earlier stage than adult Toncheng pig whereas the gene showed upregulation in Toncheng adult pig in comparison to Landrace adult pig. Letellier et al., (2014) found that there is lower expression of *SOCS2* in human colorectal cancer cell lines in comparison to the healthy samples.

Therefore, on the basis of current findings it can be indicated that higher expression of *SOCS2* in LWY may be one of the reason for this breed to have improved status of resistance.

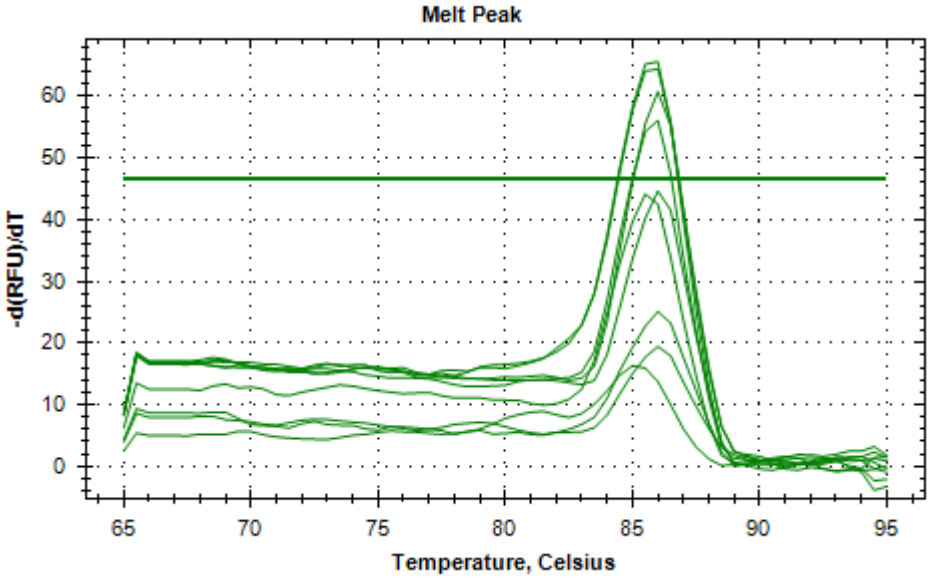
Table 13: Validation of expression of *SOCS2* mRNA using real time PCR (SYBR green)

Group	*SOCS2* (Average C_t)	β-actin (Average C_t)	ΔCT (C_t *SOCS2*- C_t β-actin)	ΔΔCT (ΔCt of treated – ΔCt of untreated)	Fold change
LWY	26.71 ± 0.21	24.68 ± 0.19	2.03 ± 0.29	0.00 ± 0.29	1.00
Indigenous	23.41 ± 0.23	20.87 ± 0.14	2.53 ± 0.22	0.51 ± 0.22	0.70^{*}

Values are Mean ± SE. Mean's having superscript * shows significant difference at $p < 0.05$.



Beta-Actin



SOCS2

Fig. 6: Melt curve analysis of SOCS2

CHAPTER V

SUMMARY AND CONCLUSION

Pigs are an important part of livestock of India. There are number of indigenous and non-descript pig breeds located in the different geographical areas of India. Pig rearing is one of the main occupation of lower classes of society which supports them economically and also provides good source of protein in the form of pork. The pork production is equal to consumption, therefore only 1% of trade is done in last two years. Mainly, indigenous breeds are used for pork production having good disease resistance, tenderer and juicy meat but with lower body growth rate. So, in last decade the trend of cross-breeding has been highly appreciated to improve the performance traits of indigenous pigs. The exotic breeds which are already adapted to the Indian environment are used for cross-breeding purpose. It has been observed that the consumption of pork is increased in the country. Liver is one of the important organ, in demand for the consumption as it has high nutritive value with good amount of proteins, vitamins and other nutrients. Liver is involved in metabolic processes which are regulated by number of genes including *ACAT2*, *ACAA2* and *APOA4*. Also, the immune factors are present in liver that eliminate the invading pathogens and reduce the risk of disease. *APOA4* and SOCS2 are key genes that regulate these immune factors.

The main objective of breeding methods is to increase growth rate with high disease resistance and less fat. Indigenous breeds have good disease resistance, juiciness and tenderness for pork but lacks in growth rate in comparison to exotic breeds.

Therefore, the current study is hypothesized to observe the expression of genes (*ACAT2*, *ACAA2*, *APOA4* and SOCS2) involved in metabolic process and immune response in indigenous pigs with respect to Large White Yorkshire breed.

In this study six liver samples of each breed were collected from slaughter house, following with RNA extraction and cDNA synthesis. Then quantitative Real-Time PCR was performed for observing differential expression of genes.

The comparative transcriptomic analysis showed that all genes have significant low expression in indigenous pigs except *ACAT2* with 3.78 fold change. Whereas, the

fold change values of *ACAA2*, *APOA4* and SOCS2 reported in indigenous pigs are 0.83, 0.01 and 0.70 significantly.

The present study presents the *ACAT2*, *ACAA2*, *APOA4* and SOCS2 as key genes play role in growth, disease resistance and quality of meat. Till now, this study is first of its kind on genes linked with metabolism and immunity in indigenous and Large White Yorkshire breeds of pigs. The good pork quality and quantity are the main concerns of industrial livestock market. Therefore, the specific markers must be identified and targeted to improve growth and immunity of pigs. The indigenous pig population of country have lower growth rate which enforces the breeders to target growth improvement by cross-breeding with economically viable exotic pig breeds with better growth rate.

Therefore, the following conclusions are made from current study:

1. The investigation of expression profiling of liver tissue can be one of the parameters to know the genetic differences of indigenous breeds with respect to commercially viable exotic breeds.
2. The current expression analysis will act as a platform in identification of regulatory genes involved in immunity and metabolic growth which may be used as selection markers in breed improvement programs.
3. The current comparative expression study can be further used in planning of *in-vivo* and *in-vitro* studies for the regulated expression of genes targeted in present study.

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