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# Effects of Extrinsic and Intrinsic Factors on Meat Quality and Muscle Biology of Livestock

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Effects of Extrinsic and Intrinsic Factors on Meat Quality and Muscle Biology of Livestock

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**Abstract**

In order to improve the meat quality of livestock, intrinsic and extrinsic factors that impact meat quality have been studied for a long time to optimize production quality. Nutrition, breed, and environment are the three most common factors to improve meat quality and muscle growth. The objective of this thesis is to perform a horizontal comparison of the effects of crude protein levels, breeds, and topsoil on the growth performance, muscle development, and meat quality of livestock, and also a longitudinal analysis using RNA-seq, RT-qPCR, and Western-blot to investigate the changes in genes and proteins related to muscle growth, and intramuscular fat deposition of livestock. Chapter II evaluated the effects of crude protein levels on the growth performance, meat quality, and genes expression of finishing lambs. For nine weeks, the lambs were assigned randomly to low-protein (LP; 8% crude protein) and high-protein (HP; 13% crude protein) diets. The results showed that a high-protein level diet promoted the growth performance of finishing lambs; however, the low-protein diet benefited the meat color of lambs. Chapter III evaluated the impact of breeds on the growth performance, meat quality, RNA-seq, and gene expression of pigs. Large Black pigs and Cross-bred commercial pigs were used to evaluate the differences, and they were fed with a common diet. The results showed that the Large Black pigs had more intramuscular fat content, and the Cross-bred commercial pigs had better growth performance. The genotype differences between these two groups of pigs may result in the meat quality and growth performance variance. Chapter IV evaluated the effects of topsoil on muscle growth, lipid droplet area, and gene and protein expression of piglets. A Total of 180 piglets were separately assigned to the No soil, Antibacterial soil, and Normal soil group (each group, n=60), and were fed ad libitum with typical antibiotic-free corn-soybean meal diets until day-31. These data suggest that early exposure to topsoil regulates muscle fiber growth,

modulates the expression pattern related to myogenesis, muscle fiber type, intramuscular fat metabolism, and increases the phosphorylation of mTOR and AMPK pathways.

**Keywords:** crude protein levels, breeds, topsoil, growth performance, meat quality, muscle development, gene expression, Western-blot

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**Published and Submitted Papers**

1. Chapter II has been published in the *Animal Science Journal*. Related publication (First author): Wang, Y., Shelby, S., Apple, J., Coffey, K., Pohlman, F., & Huang, Y. (2021). Effects of two dietary crude protein levels on finishing performance, meat quality, and gene expression of market lambs. *Animal Science Journal*, 92(1), e13641.

2. Chapter III has been published in the journal of *Animals*. Related publication (First author): Wang, Y., Thakali, K., Morse, P., Shelby, S., Chen, J., Apple, J., & Huang, Y. (2021). Comparison of Growth Performance and Meat Quality Traits of Commercial Cross-Bred Pigs versus the Large Black Pig Breed. *Animals*, *11*(1), 200

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# Chapter I. General Introduction

## Introduction

The meat industry has been trying to improve the meat quality of livestock for a long time (Andersen, Oksbjerg, Young, & Therkildsen, 2005). The characteristics of meat quality include meat color, tenderness, water holding capacity, pH, flavor, and nutrition, which determines the consumers' purchase willingness (K. G. Grunert, L. Bredahl, & K. J. M. s. Brunsø, 2004). The meat quality of animals is affected by several factors, such as intrinsic (breed, sex, age, etc.) and extrinsic (nutrition, environment, handling process, etc.) factors (Dashdorj, Amna, Hwang, & Technology, 2015).

Crude protein is an essential nutritional component of the animal diet. Decreasing crude protein levels in diets of small ruminants reduces excess nitrogen (N) excretion and feed cost for producers (Cole, Greene, McCollum, Montgomery, & McBride, 2003). It was reported that lambs need less crude protein content during the finishing period than immature stage (Council, 1985). The feasibility of providing the crude protein level that is lower than the requirement of finishing lambs without harming meat quality is still unknown.

Animal genotype effects on meat quality usually are higher than other factors (Trocino et al., 2015). In addition, comparing the transcriptome expression profile differences between different breeds could help us understand the principles of genes related to meat quality and biological muscle processes. In order to investigate the differences in gene expression between different breeds, the RNA-seq methodology is a valuable method (Simoneau, Dumontier, Gosselin, & Scott, 2021).

The feeding environment is another essential factor that can improve muscle growth and intramuscular fat deposition, resulting in different meat quality in the later growth stage of animals. Previous studies showed that feeding piglets outdoors during lactation improves growth

performance, carcass weight, and feed efficiency (Colson, Martin, Orgeur, Prunier, & behavior, 2012; L. Cox & Cooper, 2001). The result indicated that animals contacting with outdoor environment had a higher growth performance than the animals fed in the cage. The effects of the environment on the muscle fiber type and intramuscular fat deposition, which is related to meat quality, are rarely reported. Gene and protein expression are efficient ways to analyze the phenotype changes of animals caused by any treatment.

The objective of this thesis is to investigate the effects of intrinsic and extrinsic factors on the growth performance, muscle characteristics, and meat quality of animals using traditional and intensive technology.

## Literature review

### *Meat science*

#### *What is meat*

Meat is identified as a wide variety of animal tissues that can be used as food. Usually, most animals' tissues can be used as meat, especially skeleton muscles, and they come from domestic and aquatic animals, such as pigs, sheep, goats, cattle, rabbits, chickens, fish, oysters, lobsters, etc. Cattle, sheep, and pigs, the most popular livestock species of meat production, were domesticated by our ancestors thousands of years ago (Teletchea, 2019).

The current cattle breeds may come from these two major groups, *Bos taurus* from Europe and *B. indicus* from southwest Asia (MacHugh, Shriver, Loftus, Cunningham, & Bradley, 1997). The development of cattle throughout Asia, Africa, and Europe arose thousands of years, and the cattle breeding projects were popular in North American over hundreds of years ago (Ajmone-Marsan, Garcia, Lenstra, & Reviews, 2010). Nowadays, hundreds of cattle breeds exist across the world that yield good productivity, body composition, and meat quality. For example, Aberdeen Angus has been known as the premier breed for meat quality (Taylor, 1910), and it can provide a substantial amount of intramuscular fat and great tenderness. The two representative species of pigs were *S. vittatus*, which were domesticated in East Asia, and *Sus scrofa*, which was originated from Europe (Choi et al., 2014; Keuling et al., 2013). As livestock animals, pigs have good feed efficiency and short stage time compared to others. Pork production today uses several different breeds, such as Large White, Landrace, and Welsh, to produce meat or bacon for their proper purposes. Compared to pigs and cattle, sheep are the first and easiest animal that has been domesticated because of its small body shape and ease of handling. The original species of sheep is *Ovis aries* which is originated in Western Asia (J. Guo et al., 2005). There are about

200 breeds of sheep all over the world, and some of them, such as Suffold, have been improved to have more greater carcass yield and meat quality (Pavithra, Thangadurai, & Research, 2018).

***Meat production trends and development***

Usually, meat consumption increases with the increase of economic conditions and the income of families (Bereznicka & Pawlonka, 2018). As the consumption of meat increases, consumers tend to focus more on the meat quality (Font-i-Furnols & Guerrero, 2014). Meat is one of the most nourishing foods available for humans' consumption. It is especially rich in high-quality protein, iron, and vitamins, especially A and B (Mann, 2000). Above all, meat is one of the crucial nutritional sources for getting vitamin B12 (F. J. E. b. Watanabe & medicine, 2007). It is essential to get adequate protein nutrition for humans every day. As a good protein source, meat has all essential amino acids required by humans for physical and mental development (Elgasim & Alkanhal, 1992; Srinivasan, Moorjani, & Technology, 1974). Compared to animal protein's amino acids, animal protein tends to be more efficient and beneficial than plant protein, especially for young people during the growing period (Lim, Pan, Toh, Sutanto, & Kim, 2021; Shams-White et al., 2018). In addition, meat can also provide specific fatty acids, such as conjugated linoleic acid (CLA), which plays a vital role in human health (Dilzer, Park, & nutrition, 2012; Khanal & Olson, 2004; Koba, Yanagita, & practice, 2014).

Meat production and meat animal species vary all over the world in different countries. For beef and veal, the United States, Brazil, European Union, China, and India are the top five producers (Hocquette et al., 2018). For pork, China, the European Union, and the United States are the top three producers, and China produces nearly half of the pork globally (Chatellier,

2021; Oh & Whitley, 2011). Besides, the import and export of meat production are also diverse from regions and countries.

In developing countries, the meat industry focuses on improving the productivity of meat to meet the increasing demands of meat products. While, in the developed countries, there may be more challenges in improving the meat quality and the demands of consumers, such as organic meat, cage-free raised, sustainably raised products (Henchion, McCarthy, Resconi, & Troy, 2014; Strassburg et al., 2014). The popularity of meat as delicious and nutritious food will continue in the future, and sustainable agriculture may become the next topic for the meat industry.

### *Muscle biology*

#### *The structure, composition of muscle*

There is the carcass after removing blood, viscera, head, hair, skin, digestive tract, intestines, feet, hides, bladder, heart, trachea, lungs, kidney, spleen, liver, and adhering fatty tissue of livestock animals. Usually, the percentage of the carcass to live weight is about 50 for the sheep, 55 for the cattle, and 75 for the pigs (Dokmanovic et al., 2015; Pariacote, Van Vleck, & Hunsley, 1998; Sen, Santra, & Karim, 2004). The animal carcass consists of muscle, fatty tissues, bones, large blood vessels, and some connective tissues (McCormick, 1994). Among them, muscle tissues make up most of the carcass composition (Council, 1988).

There are three kinds of muscle tissues, skeletal muscle, smooth muscle, and cardiac muscle. Skeletal muscles are the most common tissues of the muscular system of the animal body, and they are attached to bones directly or indirectly (Cianferotti & Brandi, 2014). There are nearly six hundred skeleton muscles in the animal body, divided by shape, size, and action (Galasko & Bennett, 1976). Smooth muscle is another small portion of meat, and it is mainly

located in the surface of arteries, lymph vessels, and gastrointestinal tracts (Horowitz, Menice, Laporte, & Morgan, 1996).

The hierarchical fibrous structure is the general structure of skeletal muscle (Thorpe & Screen, 2016). For example, each level of the structure consists of several fibrous subunits. The whole muscle is surrounded by a connective tissue layer (epimysium), and the internal level -- fascicle was separated by another connective tissue (perimysium) (Purslow, Molecular, & Physiology, 2002). Fascicles are made up of tiny muscle fibers, which are individual cells. The muscle fiber diameter can be around 100 mm, and varies in different muscle fiber types and different ages (Frontera et al., 2008).

Red and white muscle fibers are categorized by the color of the muscle fiber (Beatty, Peterson, & Bocek, 1963). The muscle fibers can also be classified based on the ATPase activity as type I, IIa, IIb, and IIx (Ogilvie & Feeback, 1990). Type I and IIa fibers are red muscle fibers, and type IIb and IIx fibers are white fibers (Picard, Gagaoua, & chemistry, 2020). Type II fibers have higher fiber diameter, higher contraction speed, and shorter duration than type I fibers. Based on the functions, white fibers are mainly used for rapid actions, and red fibers are mainly used to maintain a posture. Besides, type I and type IIa are oxidative fibers, while type IIb and IIx are oxidative fibers (Ijkema-Paassen & Gramsbergen, 2005; Linssen et al., 1991; Van Wessel, De Haan, Van der Laarse, & Jaspers, 2010).

The skeletal muscle mainly consists of water, protein, fat, carbohydrate, and inorganic ingredients (Pearson, 2012). There is around 75% of water inside muscle tissues, and the water is the medium of chemical constituents between muscle fibers. Proteins make up about 20% of the muscle tissues, and they are mainly concentrated in sarcoplasmic, myofibrillar, and stromal. The intramuscular fat content is varied from 1.5 to 13 percent, and they primarily belong to

triglycerides and phospholipids. The muscle tissues are not abundant in carbohydrates, and the carbohydrate content is only about 1%. Besides, there is also some mineral substance inside muscle tissues, such as calcium, magnesium, potassium, sodium, phosphorus, sulfur, etc. (Gollnick & Matoba, 1984; Goodpaster, Thaete, & Kelley, 2000; Thorstensson & Karlsson, 1976).

### *Muscle growth*

There are three stages of muscle growth: 1) embryonic development, 2) fetal development, and 3) postnatal development (Allen, Merkel, & Young, 1979). The number of muscle fibers is determined during the embryonic and fetal stages, and it usually stays the same for the rest of the animal's life. During the postnatal period, there is only an increase of the muscle fiber volume (Millward, Garlick, Stewart, Nnanyelugo, & Waterlow, 1975; White, Biérinx, Gnocchi, & Zammit, 2010). Embryonic and fetal stage are essential for the growth of muscle fiber, and it requires a good maternal nutrition. In addition, the early nutrition status is highly related to the growth performance of the after-birth animals.

### *Muscle to meat*

### *Animal slaughter*

The transition from muscle to meat is a crucial procedure to determine the meat quality. After a long rearing period in the facility, animals are transported to the lairage or a place to rest until harvesting. Animals should have a rest time no less than two hours before the slaughter to reduce the stress because the stress of transportation negatively affects the meat quality (Owens & Sams, 2000). The first step of slaughter is stunning. There are usually three types of stunning, physical, electric, and carbon dioxide stunning (N. G. J. M. S. Gregory, 2005). The physical stunning, such as sled hammers and firearms, are typically used for cattle, sheep, and goats

(Nakyinsige et al., 2013). The electric stunning is becoming popular in swine and poultry processing, while the carbon dioxide stunning is usually used on pigs (N. Gregory, Moss, & Leeson, 1987; Lambooy, 1982; Marcon et al., 2019). After stunning, animals should be exsanguinated as soon as possible to remove blood from the body (Biswal & Kannurpatti, 2009). Scalding and evisceration are the next steps of slaughter. Finally, carcasses are washed using different methods, such as steam vacuum, organic acid rinses, hot water rinses, steam pasteurization, or antimicrobial chemicals (N. Cox et al., 2010; Lillard, 1988).

### *Aging*

After slaughter, another essential process, called aging, is used for the conversion of muscle to meat, especially for cattle and sheep. Aging is a simple process of holding a carcass at refrigerated temperatures (0-4°) in an enclosed and clean environment to improve the flavor and tenderness of meat (Hedrick, Stringer, & Clarke, 1993). Usually, the aging period is about 7 to 10 days, and 3 to 5 days for the meat with less or no fat (Campo, Sañudo, Panea, Alberti, & Santolaria, 1999). During aging, there are some biochemical changes inside the carcass. There is a rapid pH decline during the first 12 h after slaughter, and the pH level decreases from 7.4 to 5.4 ultimately (Geesink et al., 1992; McGeehin, Sheridan, & Butler, 2001). Rigor mortis is another dramatic physical phenomenon of the carcass. This phenomenon is caused by the lack of ATP that can break the permanent cross-bridges that form between actin and myosin filaments, and it is completed by the proteolytic degradation of specific myofibrillar proteins (Sandelowski, 1993). At the end of rigor mortis, the tenderness of the meat is at the lowest level, and it will increase gradually during aging (Devine, Payne, & Wells, 2002; Wheeler & Koohmaraie, 1994).

### *Meat quality and its impact factors*

Along with the economic and consumption growth, consumers are beginning to pay attention to the meat quality, especially the eating quality and nutrition quality of meat. There are some before and after cooking indexes of eating quality, such as meat color, water-holding capacity, tenderness, pH, and flavor. In addition, fatty acids composition, minerals, amino acids, and vitamins are attribute to the nutrition quality of meat.

### *Meat color*

The sensory quality of meat is related to consumer's willingness to purchase meat products, and the appearance of meat is the first perception that consumers can get (Resurreccion, 2004). The appearance of meat is influenced by physical and chemical factors (Qamar et al., 2019). The moisture on the meat surface is one of the physical factors that the light can be reflected by the water and then perceived by consumers (Mir et al., 2017). The myoglobin of meat, heme-containing protein, is the critical chemical factor of the meat color (Ranjith Ramanathan, Suman, Faustman, & chemistry, 2020). Besides, hemoglobin and cytochromes are also related to meat color changes (Ahn & Maurer, 1990). Concentration and stability of myoglobin are the two critical factors that impact meat color (Renerre, 2000).

Myoglobin is full of iron-containing protein, which provides oxygen for aerobic muscle metabolism (Braunlin, Wahler, Swayze, Lucas, & Fox, 1986). The muscle containing a high mount of myoglobin is slow-twitch muscle, also called red muscle or dark meat. In contrast, the muscle contains more fast-twitch fibers, less myoglobin, is regarded as white muscle or white meat (S. Baylor & Hollingworth, 2003; S. M. Baylor & Hollingworth, 2012). The concentration of myoglobin is affected by different muscle fiber types, species, age, diet, and living environment of animals. Compared to pigs, the concentration of cattle is more significant, so the

color of beef is red, and the pork is white (Joseph et al., 2010; Lindahl, Lundström, & Tornberg, 2001). Myoglobin concentration showed different levels in different breeds of animals, such as pigs, cattle, sheep, chickens (King et al., 2010; Newcom et al., 2004; Prasad et al., 2019; Suman, Joseph, Li, Steinke, & Fontaine, 2009). The content of myoglobin increases as the animal becomes older (Pethick, Hopkins, D'Souza, Thompson, & Walker, 2005). In addition, myoglobin concentration also showed different levels in different muscles of animals. Like chicken, there are more myoglobin in the leg and less myoglobin in the breast (Intarapichet & Maikhunthod, 2005). For external factors, animals feed with a concentration of more iron will accumulate more myoglobin in the muscles (Beard, 2001), and animals feed outside areas with more exercise have more dark meat (X. Guo et al., 2019).

There are four types of myoglobin with different colors, deoxymyoglobin, oxymyoglobin, carboxymyoglobin, and metmyoglobin. Usually, deoxymyoglobin is the normal status of myoglobin in fresh meat, and the color of deoxymyoglobin is purplish-red (Faustman, 1994). After cutting the meat, the meat's appearance contacts the oxygen of the air, and the deoxymyoglobin converts to oxymyoglobin showing red color (R Ramanathan, Mancini, Joseph, & Suman, 2013). Ultimately, the oxidation of heme iron in the myoglobin transfers the oxymyoglobin to the metmyoglobin, which is brown (Madhavi & CARPENTER, 1993). The carboxymyoglobin has a similar color to oxymyoglobin, and it forms by the combination of carbon monoxide and deoxymyoglobin when there is inefficient combustion of cooking (Alben et al., 1982).

There are some endogenous and exogenous factors that affect myoglobin redox stability, like mitochondria, lipid oxidation, pH value, temperature, diet, bacteria, light, etc. The oxygen consumption of mitochondria after slaughter decreases the relative oxygen partial pressure in

muscle, and it prevents or slows the rate of the oxidation of oxymyoglobin to metmyoglobin (Tang et al., 2005). The oxidation states of myoglobin indicate the lipid oxidation and leads to the flavor and color change of fat (Baron, Andersen, & chemistry, 2002). The pH of meat is usually around 5.6 after slaughter, and a lowered pH of meat leads to fast oxidation of ferrous (deoxymyoglobin and oxymyoglobin) to ferric (metmyoglobin) status (L. Zhu & Brewer, 2002). For exogenous factors, a higher temperature was reported to enhance myoglobin oxidation (Hoa et al., 2021). In contrast, the antioxidant chemicals in the feed, such as vitamin E, can reduce the oxidation rate of myoglobin in meat (FAUSTMAN & Wang, 2000). It is inevitable to deter the bacterial contamination of fresh meat during the slaughter, and the bacteria was reported to improve myoglobin oxidation (P. Li et al., 2016). The time of display under the light and the type of light in the retail shop also affect myoglobin stability. Cooper et al. (2016) reported that light improved beef myoglobin oxidation, and fluorescent had a more significant influence than LED (light-emitting diodes) light. Packaging is another important factor, and vacuum packaging without any gases can extend the stability of myoglobin and keep the fresh color (Q. Q. Fu, Liu, Zhou, Zhang, & Preservation, 2017).

***Tenderness***

The tenderness of the meat is defined as the force required to cut a standardized piece of meat in people's mouths (Tornberg, 1996). Consumers always desire a low shear force value of meat. Usually, a specific machine measures the shear force of meat after cooking, and the shear force value of the meat shows a negative correlation with meat tenderness (Caine, Aalhus, Best, Dugan, & Jeremiah, 2003). The tenderness of the meat is considered to be the most important character (Fletcher, 2002). The general impression of meat tenderness contains three characteristics: first, the ease of meat cut by the teeth; second, the ease that meat can be break

into pieces; third, the remaining content of meat after chewing (Bouton, Ford, Harris, & Ratcliff, 1975). The size of muscle fiber bundles, such as muscle fiber numbers and thickness, is one factor determining the tenderness (Albrecht, Teuscher, Ender, & Wegner, 2006).

The tenderness of meat negatively correlates with the size of muscle fiber bundles. For example, meat tenderness decreases with the increase of muscle fiber numbers or the muscle fiber diameter (Miller, Garwood, & Judge, 1975). In addition, there are three kinds of protein in muscle related to the tenderness of meat – connective tissue (collagen, reticulin, elastin, mucopolysaccharides of the matrix), sarcoplasm (sarcoplasmic proteins and reticulum), and myofibril (actin, myosin, and tropomyosin) (Küchenmeister, Kuhn, & Ender, 2005; D. Olson & Parrish Jr, 1977; Weston, Rogers, & Althen, 2002). Collagen is a kind of connective tissue protein of meat, and collagen molecules are bound together to form an intermolecular crosslink that provides structure and strength to muscle fibers (Mathews, 1965). The crosslink of collagen is becoming less soluble and tougher as the age of animals increases, finally increasing the toughness of meat (Weston et al., 2002). Besides age, the growth rate, nutrition, sex, and genetics can also affect the collagen content in animals' muscles (Henrickson, Ranganayaki, Asghar, Bailey, & Nutrition, 1984).

Some preslaughter factors can impact the tenderness of meat, such as genetics, age, gender, and the handling process of animals. For genotype effects, it has been reported by several previous studies. Hanzelková, Simeonovová, Hampel, Dufek, and Šubrt (2011) reported that breeds affect the tenderness of beef, and the Simmental had a significantly higher shear force than the other breeds. Jeleníková, Pipek, Miyahara, and Technology (2008) reported that Duroc had better tenderness than Large White and Landrace. Consistently, breeds affect the meat tenderness of sheep (Hopkins, Fogarty, & Mortimer, 2011). Among all the genes, *Bos indicus*

gene was determined to have the most significant impact on the meat tenderness in cattle, and this genotype increased the shear force of meat and decreased the meat tenderness of cattle (Carvalho et al., 2014; O'connor, Tatum, Wulf, Green, & Smith, 1997). Within a breed, age is another critical factor that impacts meat tenderness. Generally, the increase of age correlates with the decrease of meat tenderness. The tenderness tends to be significantly decreased after 18 months of cattle (Monsón, Sañudo, & Sierra, 2004). A previous study found that the mean tenderness of beef significantly decreased with age from 1 to 60 months old (Shorthose & Harris, 1990). Veiseth, Shackelford, Wheeler, and Koohmaraie (2004) reported that lamb longissimus dorsi muscle tenderness was affected by the age at slaughter, and the tenderness decreased with age. The percentage of acid- and salt- soluble collagens decrease in the muscle of cattle, but the content of inter- and intramolecular cross-linking structure, which attributes to the toughness of meat increases (Jonsson, Ranta, Strömberg, & surgery, 1985; Nian, Allen, Harrison, Kerry, & Agriculture, 2018). Within the same breed and age, gender shows a significant effect on the shear force of meat. Johnson, Purchas, McEwan, and Blair (2005) reported that the tenderness of meat of ram is significantly lower than the ewe of the same age. In cattle, it was found that the shear force value of bulls was higher than the steers (Rodriguez et al., 2014). The meat tenderness of boars was significantly lower than gilts after 2 days of aging; however, the tenderness became closer after 7 days of aging (Beattie, Weatherup, Moss, & Walker, 1999). Before the slaughter, livestock usually needs to be transported to the abattoir and rest for a while (Miranda-De La Lama, Villarroel, Liste, Escós, & María, 2010). Transportation and lairage stress impact the metabolism of the muscle of animals (Zhen et al., 2013). Muscle metabolism changes lead to a higher final pH value (6.0) of meat and increase the shear force (Poleti et al., 2018). Faucitano (2018) reported that transportation stress, stocking density, and the use of

electric prods had effects on meat quality, especially tenderness of the meat. A previous study also found that the lairage time had effects on the meat tenderness, and cattle slaughtered 3 hours after transportation had a better meat tenderness (Del Campo, Brito, De Lima, Hernández, & Montossi, 2010).

Post-slaughter factors also have significant impacts on meat tenderness. Postmortem glycolysis happens after slaughter, and it is related to tenderness. As mentioned above, rigor mortis appears after the death of animals because the insufficient of ATP leading to a contracted state of muscle filaments (Sandelowski, 1993). During the postmortem glycolysis, the pH value of meat decreases gradually to around 5.5 with the increase of hydrogen ions made by muscle glycolysis (Young, West, Hart, & Van Otterdijk, 2004). A remarkable decrease in pH value leads to a Pale Soft Exudative (PSE) meat in pigs which is not desirable for consumers (Barbut et al., 2008). If the pH value decreases slowly and simply to be higher than 6.0, there are DFD meat that is dark, firm, and dry, and the tenderness of meat decreases (Lesiów, Kijowski, & sciences, 2003). It was reported that the tenderness is slightest when the final pH value of meat is between 5.8 to 6.2 (Purchas & Aungsupakorn, 1993). The shear force of meat increases when the pH value increases from 5.5 to 6.0 (Purchas, Yan, & Hartley, 1999). The shortening of muscle sarcomere has a positive correlation with meat toughness, so increasing the length of the sarcomere is another way to increase the tenderness of meat after slaughter (Ertbjerg & Puolanne, 2017; Tornberg, 1996). An old method called tenderstretch is suspending carcass from the aitchbone of a split carcass or the pelvis of the whole carcass after slaughter (Ahnström, Hunt, & Lundström, 2012). This method increased the tension of most leg and loin muscles before rigor mortis and avoided sarcomeres' shortening (Sørheim, Hildrum, & Technology, 2002). Desmond and Kenny (2005) reported that pelvic suspension increased the meat

tenderness of the pork, particularly hams used for silverside. There is another method called "tendercut", which has been used for a few years in the United States, and this method is to cut the bones and connective tissue of carcass to stretch specific muscles before rigor mortis happens (Sørheim et al., 2002). It was found that tendercut improved the meat tenderness of the *longissimus dorsi* and *semimembranosus* muscle of goats, and it is easily applied to prerigor goat carcass (Basinger et al., 2019; Edwina S Toohey, Hopkins, & Bekhit, 2017). There are also some new methods to increase the meat tenderness, such as Pi-Vac Elasto Pack System, which is wrapping hot-boned muscle with elastic material, and SmartStretch, which using a flexible rubber sleeve with four inflatable bladders covered by an airtight chamber (M Taylor, L Hopkins, & agriculture, 2011; Edwina Skye Toohey, van de Ven, Thompson, Geesink, & Hopkins, 2012). Aging is another crucial process that improves meat tenderness after slaughter. Some previous studies showed that the shear force of meat from pigs, chicken, cattle, and sheep decreased with the aging (Hanzelková et al., 2011; Hopkins, Hegarty, Walker, & Pethick, 2006; H.-W. Kim, Kim, Seo, Setyabrata, & Kim, 2018; Thielke, Lhafi, & Kühne, 2005). Besides working on carcass, there are also some chemical enzymes that can be used for improving meat tenderness. Tantamacharik, Carne, Agyei, Birch, and Bekhit (2018) reviewed several plant proteolytic enzymes used for tenderization, such as papain, bromelain, ficin, and actinidin. In addition, the injection of organic acids such as acetic, citric, and lactic can also be used for meat tenderization. It was reported that lactic and citric penetration improved the tenderness value of middle-aged bovine muscle (Aktaş, Kaya, & Technology, 2001).

***Water-holding capacity***

Water-holding capacity (WHC) not only determines the loss of water during storage, processing, and cooking, but also determines the juiciness of meat, which is a part of meat

quality (R. J. M. o. W.-h. C. Warner, Objective, & Subjective, 2014). WHC also impacts the visual acceptability related to consumers' purchase willingness (Murphy, Gilroy, Kerry, Buckley, & Kerry, 2004). Water is blocked in cells by cellular membrane (sarcolemma), and there are several membrane pumps to control its movement (Houang, Sham, Bates, & Metzger, 2018). After slaughter, the water in the sarcolemma is moved to sarcoplasm from intramyofibrillar cells by the shrinkage of myofibrils, and keep stagnant until the decrease of pH (E. Huff-Lonergan & S. M. J. M. s. Lonergan, 2005). Usually, the loss of water during the processing of meat is defined as cook yield, such as drip loss, released water (Torres Filho, Cazedey, Fontes, Ramos, & Ramos, 2017). There are about 75% of water in the chemical composition of meat, which is significantly greater than protein ( around 20%), lipids (around 5%), carbohydrate (around 1%^), and vitamins and minerals (around 1%) (Cobos & Díaz, 2015). There is a negative correlation between the content of water and the lipids of meat, and a higher content of lipid of meat usually results in lower water content (Aaslyng et al., 2003). There are mainly three kinds of water inside the meat: (1) bond water, this kind of water is only about 1% of the total content of water which is tightly bound by proteins, and its low mobility makes it resistant from freezing and heating; (2) immobilized water, which is about 85% of total water, and this kind of water can be moved or lost by drying, cooking, and degradation of proteins; (3) free water, this water can be easily removed from or inside of the meat, and it is the rest of the total content of water (E. Huff-Lonergan & S. M. J. M. s. Lonergan, 2005; Puolanne & Halonen, 2010). The juiciness of meat is defined as the feeling of moisture and lubrication during chewing in the mouth (Guinard, Mazzucchelli, & Technology, 1996). A bad WHC of meat results in a higher loss of water during heating and cooking, reducing the juiciness of the meat, leading to negative reviews of consumers (Santos et al., 2021).

Several factors determine the WHC of muscle during pre- and post-slaughter. First, the decreased value and rate of pH caused by the postmortem anaerobic glycolysis of muscle is a significant determinant (M. Qiao, D. Fletcher, D. Smith, & J. J. P. s. Northcutt, 2001). Typically, muscle pH decreases from 7.0 to 5.5 after slaughter, and the water losses as the shrinkage of myofilaments (Guignot, Vignon, & Monin, 1993). A small postmortem drop of pH in muscle leads to DFD (dark, firm, and dry) meat formation, and the final pH of DFD meat is higher than 5.8 (Guàrdia et al., 2005). DFD meat is caused by a limitation of glycogen metabolism after slaughter, and the loss of water of DFD meat is less than usual meat (Adzitey & Nurul, 2011). In contrast, higher and more rapid glycolysis of muscle after slaughter causes the PSE (pale, soft, and exudative) meat, and the water loss of PSE meat is higher than standard meat because of the additional shrinkage of myofibrils (Barbut et al., 2008). Compared to beef and lamb, pork has a higher PSE or DFD meat rate due to the stress during transportation and handling (Andersen, Oksbjerg, & Therkildsen, 2005). R. D. Warner, Kerr, Kim, and Geesink (2014) reported that making the muscle stretched by preventing the shortening during rigor, cold, or heat can increase the WHC of meat. Electrical stimulation is a typical method to reduce the time of carcass rigor onset of cattle and sheep by accelerate the postmortem glycolysis; however, it was reported that this method can increase a little bit drip loss of semimembranosus muscle of ruminant animals, and a large drip loss of pork, so it is not recommended to use on pork industry (den Hertog-Meischke, Smulders, Van Logtestijn, & Van Knapen, 1997; C. Li, Li, Li, Hviid, & Lundström, 2011; Walukonis, Morgan, Gerrard, & Forrest, 2002). During the aging of carcass, the WHC increased with the increase of pH value (Boakye & Mittal, 1993). After processing, the packaging methods increased the water loss of meat. Compared to modified atmosphere packaging (MAP), vacuum packaging has a longer storage time for fresh meat. However, it

decreases the WHC of meat during storage by extracting liquid from the meat (Lagerstedt, Lundström, & Lindahl, 2011). In addition, the freezing and thaw during the pre-rigor state and the storage of meat decreased the WHC by increasing the intracellular spaces during freezing (Aroeira et al., 2016; Huff-Lonergan & Sosnicki, 2002).

***The flavor of meat***

The flavor of the meat is a complicated sensation that contains several different aspects, such as olfactory, gustatory, and trigeminal sensations (Delwiche & preference, 2004). It was defined that sour, sweet, bitter, salty, and umami are the five basic human tastes (Chandrashekar, Hoon, Ryba, & Zuker, 2006). The flavor of raw meat is only slightly sweet, sour, salt, or bitter caused by its original biochemical compositions (Dashdorj et al., 2015; Ramalingam, Song, & Hwang, 2019). However, the organoleptically desirable taste and odor of meat change during cooking, and the flavor molecules are released in the meantime (Liu et al., 2017). During cooking, these molecules attribute to the flavor changes include peptides, fatty acids, amino acids, nucleotides, thiamine, glycogen, sugars, and organic acids (Ramalingam et al., 2019). Amino acids, like Ser, Gln, Gly, Thr, Ala, Val, Met, Lys, Pro, Cys, contribute to sweet flavor; however, Thr, Asp, Glu, and Asn contribute to sour taste, and Val, Tyr, Met, Trp, Ile, Phe, His, Lue, Lys, Arg, Pro, and Cys contribute to bitterness (Kawai, Sekine-Hayakawa, Okiyama, & Ninomiya, 2012). Besides, the organic acids, like glutamate, aspartic acid, succinic acid, and tartaric acid, contribute to meat umami (Duan et al., 2020). The main chemical reactions during cooking are lipid degradation, Maillard reactions, Thiamine degradation, (Toldrá, Flores, & Hui, 2007), and Strecker degradation (Domínguez, Gómez, Fonseca, & Lorenzo, 2014; Resconi, Escudero, & Campo, 2013; Ruiz-Carrascal et al., 2019; A Watanabe et al., 2015).

With the development of identification methods of aroma compounds, gas chromatography (GC-O) takes the place of odor activity value (OAV), becoming the popular determination method due to its accuracy and feasibility (Baldovini & Chaintreau, 2020). During high-temperature cooking, like toast, the meaty flavor is released by incorporating sulfur into heterocyclic ring systems, and 2-methyl-3-furanthiol, 2-furfurylthiol, 2-mercapto-3-pentanone, and 3-mercapto-2-pentanone are the main odor compounds (Cerny, Davidek, & chemistry, 2003; Gibis & Safety, 2016). Furaneol is another vital flavor compound in beef generated from the Maillard reactions (Martins, Leussink, Rosing, Desclaux, & Boucon, 2010). Besides Maillard reactions, lipid oxidation is another crucial reaction that provides odor compounds of meat. The meat's PUFAs (polyunsaturated fatty acids) are oxidized to linear aldehydes, 2-alkenals, and 2,4-dienals during cooking due to their unstable inside double bounds (Frankel, 2014).

Some intrinsic and extrinsic factors impact meat flavor. It was reported that breeds affect the flavor of lambs, and the volatiles (pyrazines and sulfurs) generated from the Maillard reaction of *Soays* lambs were significantly higher than *Suffolks* lambs (Elmore, Mottram, Enser, & Wood, 2000). Warren, Scollan, Nute, et al. (2008) also found the effects of breeds on the flavor of beef, and the abnormal flavor of *Holstein-Friesian* was higher than *Aberdeen Angus*. Age is another crucial factor that impacts meat flavor. It was reported that the intensity of the flavor of meat increases with the rise of age in beef and lamb (Della Malva et al., 2016; Garza III et al., 2021; Stetzer, Cadwallader, Singh, Mckeith, & Brewer, 2008). The fat, which can be oxidized during cooking to produce meat aroma, especially intramuscular fat of meat, is higher in older animals than younger animals, and the percentage of PUFA and MUFA also changed with the increase of age (Pietro Lo Fiego, Macchioni, Minelli, & Santoro, 2010; Warren, Scollan, Enser, et al., 2008). During aging, the flavor contents change due to the variation of fatty acids

and amino acids (Virgili et al., 2007). Besides, the degradation of nucleotide, which generated ribose, hypoxanthine, phosphate, and ammonia with the split of adenosine diphosphate (ADP) and adenosine monophosphate (AMP), was reported to change the flavor of turkey meat during aging (Feng, Moon, Lee, & Ahn, 2016). The feeding method is a significant extrinsic factor that impacts meat flavor. Especially for ruminant animals, several previous studies found that the flavor of the meat is different between the grass-fed animals and the intensive-fed animals, and consumers are more likely to purchase meat of grass-fed animals (Daley, Abbott, Doyle, Nader, & Larson, 2010; Gwin, Durham, Miller, & Colonna, 2012; Xue, Mainville, You, Nayga Jr, & Preference, 2010). After the purchase of meat, the flavor is also affected by consumers' cooking methods. It was reported that the beef brothy flavor had the most intense flavor heated at 85℃, and there are significant differences in the amino acids compositions compared to the other groups in different temperature (Wood, Nute, Fursey, & Cuthbertson, 1995). D. Han et al. (2021) found that high-temperature stewed method improved the flavor of pork, especially high-temperature stewing with enzymatic degradation and Maillard reaction. It was also reported that different cooking methods produce different flavor compounds in cooked chicken (J. Bi et al., 2021).

***Nutrition quality of meat***

As mentioned above, meat is an excellent nutrition source for humans to get the nutrients required for body growth and daily activities. It can provide protein, vitamins, minerals, energy, and fatty acids critical to human health. Meat consumption has increased gradually year by year globally, and the nutrition quality of meat is becoming more and more noticeable (Henchion et al., 2014).

The proximate composition of meat contains water, protein, fat, and ash. About 70-75% of water is inside the meat (Wierbicki, Deatherage, & Chemistry, 1958). As a high protein food, protein is another main meat content, and it contains about 20-22% of protein (Kwasiborski et al., 2008). The fat content of meat varies widely, and it negatively correlates with water content (Chambaz, Scheeder, Kreuzer, & Dufey, 2003). Compared to beef, the fat content of pork is only about 2-4%, significantly low than beef (15-30%) (Prevolnik et al., 2005). It was reported that the fat content of Japanese Black steers (*Wagyu*) could reach 39% in *longissimus dorsi* muscle (Ueda et al., 2007). Ash is an inorganic meat substance, only about 2%, and it mainly contains several mineral elements (Perez & Andujar, 1981).

Amino acids are the constituents of meat protein, and they are essential for human muscles. The protein of meat is ideal amino acids sources because the amino acids composition of meat is similar to the human body (Rowan, Moughan, Wilson, Maher, & Tasman-Jones, 1994). Meat contains all of the nine essential amino acids (Iso, Leu, Lys, Met, His, Phe, Thr, Try, Val), which cannot be synthesized by human bodies and need to be gained from food (Ahmad, Imran, & Hussain, 2018). The content and composition of amino acids can be affected by feed, animal breed, age, different parts of the carcass, and even cooking methods. Koutsidis et al. (2008) found that diet and breed had significant effects on amino acid concentration and composition, and the animals fed on grass silage had a higher level of amino acids. Akira Watanabe, Ueda, and Higuchi (2004) pointed out that beef's free amino acids composition was significant between the age of 15 and 25 months. It was also reported that, compared to boiling, grilling increased most of the amino acids contents of veal, especially leucine (Lopes, Alfaia, Partidário, Lemos, & Prates, 2015).

Meat contains most of the required minerals for humans, and some of them (iron and zinc) are exceptionally high in meat products (Pereira & Vicente, 2013). Iron is required to synthesize myoglobin and some enzymes, and about 15% of daily iron intake comes from meat (Buratti, Gammella, Rybinska, Cairo, & Recalcati, 2015). It was also reported that the iron of meat, which is heme iron, are more absorbable than the non-heme iron of plants (Pizarro, Olivares, Hertrampf, Mazariegos, & Arredondo, 2003). Zinc is another essential mineral needed by the human body, and the amount of zinc in meat is higher than other food sources (Krebs et al., 2006). Besides, meat also contains plenty of sodium, potassium, and magnesium, which are required a higher amounts in diet (Reykdal, Rabieh, Steingrimsdottir, Gunnlaugsdottir, & Analysis, 2011).

Vitamins are highly recommended for daily intakes by health institutions worldwide to keep general body health and well-being (Geissler & Powers, 2017). Meat contains abundant vitamins, especially vitamin B series, required by the human body's metabolism system (Kennedy, 2016). For example, pork is rich in vitamin B1; vitamin B3 are abundant in all kinds of meat; lamb has a high amount of vitamin B5; beef, pork, and turkey have a lot of vitamin B6; vitamin B12 is numerous in beef, lamb, pork, and turkey (Czerwonka, Szterk, & Waszkiewicz-Robak, 2014; Koc et al., 2006; Mun et al., 2017; Van Heerden, Schönfeldt, Kruger, Smit, & Analysis, 2007). Besides, vitamins A, C, D, and E are also abundant in meat products, and the concentration of vitamin A is specifically high in the liver (Morrissey, Buckley, Sheehy, & Monahan, 1994; J. A. J. H. o. v. Olson, 1990).

The research of meat fatty acid composition was in great demand recently because the intake of respective fatty acid correlated with some diseases and human health. There are three types of fatty acids in the meat products, saturated fatty acids (SFA) with no double bound,

monounsaturated fatty acids (MUFA) with one double , and polyunsaturated fatty acids (PUFA) with more than one double bound (EFSA Panel on Dietetic Products & Journal, 2010). People are recommended to reduce the daily intake of SFA because it has been considered to increase the risk of heart disease (DiNicolantonio, Lucan, & O'Keefe, 2016). In contrast, PUFA is recommended in the human diet because of its benefits in lowering blood cholesterol (P. M. Kris-Etherton, Hecker, & Binkoski, 2004). It was recommended that humans take more n-3 PUFA, and the n-6:n-3 should be less than 4 for human health (Simopoulos, 2000). Besides the main *cis* configuration of double bonds, the trans configuration of fatty acids can be generated in the rumen by bacterial metabolism, and the conjugated fatty acids, a combination of *cis*- and *trans*-fatty acids, are formed in rumen (Mulvihill, 2001). In ruminant animals, meat products, such as beef and lamb, the primary conjugated linoleic acid (CLA) is 18:2 *cis*-9, *trans*-11 and 18:2 *trans*-10, *cis*-12 (Bauman, Corl, & Peterson, 2020). The studies focused on CLA are becoming popular recently due to the evidence that it benefits human health (Mcguire & Mcguire, 2000).

The fatty acid composition differs between species and breed, and is even affected by animal feed. Usually, ruminant meat products, such as beef and lamb, have the highest SFA level, but they also contain the highest level of CLA (Scollan et al., 2006). Compared to beef, pork has less SFA, but there is no CLA in the pork (Ripoche & Guillard, 2001). Like chicken and fish, the white meat was reported to contain the lowest level of total fatty acids and SFA, and the P:S (polyunsaturated fatty acids: saturated fatty acids) is higher than 1.2 (Milićević et al., 2014). Previous studies from our lab found that breeds had significant effects on the fatty acid composition. Compared to Simmental crossbreed cattle, Xuanhan Yellow cattle had a higher proportion of SFA, which is not recommended for human health (Yongjie Wang, Wang, et al.,

2021). It was also found that Large Black pigs had a higher MUFA content than commercial crossbred pigs, but less PUFA content (Yongjie Wang, Thakali, et al., 2021).

Dietary nutrition is another essential factor that has effects on the fatty acid composition of meat. It was reported that the dietary fat sources and a crude fat level of feed impacted the fatty acids composition of finishing pigs. The pigs fed with animal fat had great SFA and less PUFA than pigs fed with linseed oil (J. Kim et al., 2014).

**Summary**

Meat quality has become more and more important in the meat industry and significantly determines the consumer's purchase willingness. Thus, understanding the factors that impact meat quality is beneficial for improving meat quality in the future. Here we performed a comprehensive introduction of the meat quality and its impact factors, allowing us to understand better the intrinsic and extrinsic factors that can improve the meat quality.

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# Chapter II. Effects of Two Dietary Crude Protein Levels on Finishing Performance, Meat Quality, and Gene Expression of Market Lambs

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**Abstract**

Seventeen crossbred lambs were assigned randomly to low-protein (LP; 8% crude protein (CP); n=9) and high-protein (HP; 13% CP; n=8) diets for nine weeks. The final body weight, average daily feed intake (ADFI), and average daily gain (ADG) of the HP lambs were significantly higher ($P < 0.05$) than the LP lambs; however, gain to feed ratio (G:F) for the LP lambs was significantly higher ($P < 0.05$) than the HP lambs. Hot carcass weight (HCW), adjusted fat thickness, and drip loss of *longissimus dorsi* (LD) muscle were significantly higher ($P < 0.05$) for the HP than LP lambs. In contrast, instrumental color values L*, a*, b*, C*, and hue angle (H) of meat from the LP lambs scored significantly higher ($P< 0.05$) than the HP lambs. The LD muscle from HP lambs had significantly greater CLA of cis-9 trans-11 isomer ($P< 0.05$) than the LP lambs. The gene expression of metabolism and meat quality-related genes of LP was significantly higher than HP ($P< 0.05$). These results suggest that a higher dietary CP level promotes growth performance for finishing lambs, whereas lower dietary CP level is beneficial for meat quality, especially when evaluating color characteristics in the final product.

**Key words:** finishing lambs, crude protein level, growth performance, meat quality, gene expression

## Introduction

Recently, studying the resilience of animals facing environmental challenging is not only becoming critical in animal industry but also in ensuring sustainable agriculture (Wilkes, Hynd, & Pitchford, 2012). Decreasing crude protein levels in diets of small ruminants reduces excess N excretion and feed cost for producers (Cole et al., 2003). It was reported that lambs need less crude protein content during finishing period compared with immature stage (Council, 1985). The feasibility of providing the crude protein level which is lower than the requirement of finishing lambs is still unknown.

Animal performance and meat quality characteristics are two of the most important measures of production value because of the strong relationship between industry income and consumer preferences (K. G. Grunert, L. Bredahl, & K. Brunsø, 2004). Nutritional deficiencies in young, growing animals are reflected more quickly than in mature animals due to their higher basal metabolic rate (Nutrition, 1958; USDA, 1997). Energy and protein are two important nutrients related to growth, protein accretion, and meat quality traits in food animals. The amount of tissue deposited as carcass components is primarily determined by the nutrition available for growth and development, especially protein and energy nutrients (Burrin & Mersmann, 2005). In the final product, consumers judge and value the quality expectations of meat largely based on appearance when determining their intent to purchase. Meat color is a major criterion in this assessment, with strong consumer preference for cherry red (Grebitus, Jensen, & Roosen, 2013). Overall body composition of lambs can be affected by dietary protein level. The CP requirement for growing lambs varies from 10 to 18%, depending on life stage (NRC, 2007). According to N. Atti, H. Rouissi, and M. Mahouachi (2004), diets with a CP level of 130 g/kg increased muscle mass and decreased fat deposition of lambs as compared to a

greater CP level of 160 g/kg. Different levels of dietary CP may also impact the intramuscular fatty acid composition of lambs (Scerra et al., 2011). Dietary protein level is also one of the factors affect intramuscular fat (IMF) content in skeletal muscle, which is called marbling fat in meat products (S. J. Park et al., 2018). Studies in ruminant animals showed controversial effects of high and low CP levels on IMF deposition, but a consistent conclusion is that high CP level diet is not a key factor to meat quality (Bindon, 2004; Hwangbo et al., 2009; L. Li, Zhu, Wang, He, & Cao, 2014; Marino et al., 2009).

The aim of this experiment is to investigate the effects of low CP diet on growth performance, carcass characteristics and meat color of finishing lambs, and also the effects on the gene expression related with lipid metabolism, fatty acid composition, IMF content, and carcass quality traits.

## Materials and Methods

All procedures involving animals during the study were approved by the University of Arkansas's Institutional Animal Care and Use Committee before the initiation of research (AUP#:17085).

### *Animal management and diets*

Seventeen (17) cross-bred one-year-old male lambs were housed in individual, indoor digestibility pens with *ad libitum* access to water and diet. An adjustment period of 3 weeks was observed by offering a diet consistent with the finishing diet plus chopped hay, with gradual reduction in the proportion of chopped hay until only the finishing diet was offered. During the treatment period, *ad libitum* access to diet was determined at approximately 10% refusal, adjusted at each feeding. Rations were provided equally at 07:00 and 18:00. Before each morning feeding, the amount of both refused and offered feed was recorded. Lambs were

randomly assigned to low-protein (LP, 8% CP, n=9) and high-protein (HP, 13% CP, n=8) diets, with initial mean body weights tabulated (37.5±1.97 for LP group and 39.5±2.17 kg for HP group; *P*=0.25). Low and high protein values were formulated to provide 85% and 135% CP, respectively, for 30 kg lambs gaining 300 g/day using the following equation for CP:

$$CP = \text{metabolizable protein}/((64 + (0.16 \times \% \text{ UIP}))/100.$$

Undegradable intake protein (UIP) was assumed at 30% (NRC, 2007). Initial body weight (BW) was determined by averaging individual BW obtained before feeding on d 0. Diets (Table 1)-were formulated to meet or exceed the requirements of a finishing lamb (NRC, 2007). Samples of diet were analyzed for crude protein (CP), dry matter (DM), ether extract (EE), calcium and phosphorus following the procedures of Helrick (1990). The amount of neutral detergent fiber (NDF) and acid detergent fiber (ADF) was measured according with Van Soest, Robertson, and Lewis (1991). At the end of the experiment (63 days), the lambs were weighed individually with total gain, average daily gain (ADG), feed consumption, and gain to feed ratio (G:F) was calculated.

***Animal Slaughter and Sample Collection***

At the end of the experiment, lambs were harvested by nonpenetration stunning method with a captive bolt gun followed by exsanguination. Immediately after exsanguination (with 3 min of stunning), a 30g sample of the *Longissimus dorsi* (LD) muscle of each lamb was removed from the left side between the $12^{th}$ and $13^{th}$ rib before pelt removal. The samples were placed in a 2 mL cryopreservation vial and immediately frozen in liquid nitrogen. Then, the samples were stored at -80°C for RNA extraction and genes expression. Another two pieces of LD muscles (100g) were sampled, placed individually in small plastic Ziploc storage bags, 24 h after slaughter and were stored at -20°C, for meat quality determination and IMF/fatty acid content

evaluation respectively. The IMF content of LD muscle were evaluated according to Association of Official Analytical Chemists (AOAC) method (Horwitz & International, 2000), expressed as weight percentage of dry muscle tissue.

***Carcass Characteristics***

The non-carcass parts of the body were removed and hot carcass (with kidneys and internal fat included) weight (HCW) was calculated by the difference between slaughter weight and non-carcass components weights. After that, carcasses were chilled in a cooler at -2 ℃ to 1 ℃ for 48h. The fat thickness perpendicular of LD muscle was measured over the center of the ribeye between the $12^{th}$ and $13^{th}$ rib. The carcass quality grade data were collected according to (Burson & Doane, 1983).

***Meat Quality***

The drip loss was calculated as the quantity LD muscle stored at 4 ℃ for 48h divided by the initial sample weight, expressed as percentage (Honikel, 1998). A Minolta CR-400 Chroma meter (Minolta, Tokyo, Japan) was used to measure the meat color of LD muscle after aging carcasses for 48h at 4 ℃. The redness (a*), yellowness (b*), and lightness (L*) parameters were measured (Hunt et al., 1991). The chroma (C*) of LD muscle was calculated as (a*2+b*2)1/2 and hue angle (H) was calculated as tan-1(b*/a*) (Hunter, Harold, & Harold, 1987).

***Fatty Acid Composition***

The LD samples were pulverized to homogeneity in liquid nitrogen using a Waring blender (Waring Product Div., Conair Corp., Stamford, CT). Fatty acid methyl esters (FAME) were formed by base-catalyzed transesterification (Murrieta, Hess, & Rule, 2003). The lipid extract was dried under nitrogen. FAME derivatives were prepared by methylation of the fatty acid (Metcalfe & Schmitz, 1961). FAME separation was completed by gas-chromatograph (model

Shimadzu GC-14A) equipped with flame ionization detector and a 100 m fused silica capillary column (0.25 mm inner diameter) with a 0.2μm film thickness (RT-2560, Restek) and nitrogen as the carrier gas. Initial oven temperature was set at 150°C for 5 min, increased at a rate of 4°C/min to 194°C for 15 min, and then ramped at 2.5°C/min to 235°C for 16.25 min. Injector and detector temperatures were maintained at 250°C. Tridecanoic acid (C13:0) was used as internal standard. Peak areas were used to calculate fatty acid compositions. Fatty acids were expressed as weight percentage.

***RNA Isolation and cDNA Synthesis***

The total RNA of LD muscle was extracted using Direct-Zol$^{TM}$ RNA Miniprep Kit (Zymo Research, Cat. No. R2072). In order to eliminate genomic DNA contamination, the extracted RNA was treated with DNase I, RNase-free (Promega, Madison, WI, USA). RNA Nano 6000 Assay kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA) was used to measure the integrity of RNA. Finally, RNA of LD was subjected to reverse-transcription using Takara PrimeScript$^{TM}$ RT reagent Kit and processed for cDNA synthesis according to the instructions.

***Real-time PCR***

Real-time PCR was performed using cDNA as a template to measure the expression levels of *GRAMD2, CCDC158, CHAC1, PIK3R3, HSPA12A, ADRA2A, ATXN7L1, ELOVL6, GPR1* with GAPDH as a housekeeping gene. Primers were designed with Oligo 6.0 Software according to coded sequences in GenBank® (Table 2). Real time-PCR was performed in 25 μL reaction system (2 × Real Master Mix SYBR Green I): 7.5 μL; upstream and downstream primer (10 pmol/L): 0.3 μL respectively; cDNA: 1.2 μL, and then add water to 15 μL on iCycler IQ$^5$ (Bio-

Rad, USA). After amplification, melting curves were analyzed by a melt-curve program. Gene expression per sample was analyzed in triplicate by qRT-PCR.

***Statistical analysis***

Data was analyzed using t-test, ANOVA and Scheffe's test with SPSS 19.0 (Chicago, USA). The data were expressed as means ± SEM (Standard error of the mean). Statistical significance was determined at $P < 0.05$; tendencies were identified at $0.05 < P \leq 0.10$.

**Results and Discussion**

***Growth performance***

Some previous studies showed that crude protein levels had effects on the growth performance of lamb kids (N. Atti, H. Rouissi, & M. J. S. R. R. Mahouachi, 2004; Negesse, Rodehutscord, & Pfeffer, 2001). In our study, there was no significant difference of initial BW (d0) between LP lambs and HP lambs ($P > 0.05$; Table 3). However, final BW (d63) of the HP lambs was higher than that of LP lambs ($P$=0.031; Table 3). For the HP lambs, the ADFI and ADG were both greater than the LP lambs ($P < 0.05$ and $P < 0.01$, respectively; Table 3). These results indicated that dietary CP levels impacted growth performance of lambs during finishing stage, such as final body weight, ADG and ADFI. Titi, Tabbaa, Amasheh, Barakeh, and Daqamseh (2000) reported lambs with 16 and 18% CP ration had higher body weight and gain than lambs in 14 and 12% groups. Besides, a study indicated that there was no difference observed in F:G ratio within crude protein levels from 10 to 18%, but the 10% CP group had the lowest ratio (Haddad, Nasr, & Muwalla, 2001). In the present study, however, the G:F of the LP lambs was tended to be lower than that of HP lambs ($P < 0.1$; Table 3).

***Carcass characteristics***

The HCW and adjusted fat thickness of HP lambs tended to be greater than the LP lambs ($P < 0.1$; Table 4). The hot carcass weight is one of the most important factors that has a strong relationship with the income of meat industry, and it has a positive relation with final body weight (Mourad, Gbanamou, & Balde, 2001). Fat thickness is a useful method to evaluate the composition of carcass of lambs. There was an experiment reported that low crude protein level of diet caused the reduction in backfat depths (Kerr, McKeith, & Easter, 1995), and our results has the same tendency. However, there was no significant difference between LP and HP lambs in yield grade ($P > 0.05$; Table 4). The result indicated that different crude protein levels didn't result in significant changes in carcass yield grade.

***Meat quality***

Water holding capacity (WHC) is the ability of meat to retain its inherent moisture under the external conditions such as gravity, heating, centrifugation and pressing (E. Huff-Lonergan & S. M. Lonergan, 2005). It is also an important property of fresh meat as it affects both the yield and the quality of the meat product (M. Qiao, D. Fletcher, D. Smith, & J. Northcutt, 2001). It was observed that higher drip loss is associated with protein oxidation (Traore et al., 2012). The drip loss of the LD was tended to be greater for the HP lambs when compared to the LP lambs ($P < 0.1$; Table 4). It was also reported that high protein levels increased the drip loss of pork (Teye et al., 2006). The results indicated that lower protein level has positive effect on the water holding capacity of meat. However, Lebret (2008) concluded that drip loss is generally not affected by the crude protein level of diet.

Meat color is an important parameter which affect consumers buying desire of meat products, as it is associated with freshness (Velasco & Williams, 2011). Most of the research

articles reported using L*, a*, and b* values to measure lightness, redness, and yellowness (Tapp Iii, Yancey, & Apple, 2011). In addition, the meat color can also be evaluated by calculations using a* and b* value in the form of chroma ($C^* = (a^{*2}+b^{*2})^{1/2}$) and hue ($H^* = \tan^{-1}(b^*/a^*)$). There are numerous articles that have been published reporting the effects of protein levels on meat color, and the low crude protein level increased the meat color value especially L* compared to high protein levels (Cortese et al., 2019; Prado et al., 2015). In this experiment, the L*, a*, b*, C* and hue angle of LD from the LP lambs scored higher than the HP lambs ($P < 0.05$; Table 5). It was reported that the L* values of pork decreased with raising the protein content, because it made meat have more lean meat and less added water (Youssef & Barbut, 2009). Goerl, Eilert, Mandigo, Chen, and Miller (1995) observed that low protein diets increased the a* and b* values of muscle, and it is similar with our results. However, there were also studies showing that low protein level diets had no effect on meat color (Cisneros, Ellis, Baker, Easter, & McKeith, 1996; Essen-Gustavsson, Karlsson, Lundström, & Enfält, 1994; Witte, Ellis, McKeith, & Wilson, 2000).

***Fat content***

According to the result, the fat content of LP lambs was not significantly higher than HP lambs ($P>0.05$; Figure 1). However, it was reported by other experiments that feeding a lower level of protein in the diet resulted in increased fat content in the muscle compared with feeding diet with higher protein level (Bidner, Ellis, Witte, Carr, & McKeith, 2004; Cisneros et al., 1996; Witte et al., 2000; Wood, Nute, et al., 2004). Usually, inadequate dietary protein has a limitation on protein synthesis and increases energy available for fat deposition resulting in more fat contents in meat, especially during the finishing time. It was observed that animals produced more marbling fat but grew slowly when amino acid intake is not enough to meet protein

accretion rate (Pettigrew & Esnaola, 2001). There were no significant differences in intramuscular fat content between LP and HP, and it indicated that crude protein levels do not affect the intramuscular fat content during the finishing stage.

***Fatty acid composition***

The total fatty acid compositions of LD muscles of LP and HP expressed as percent of total fatty acid and total fatty acid content are shown in Table 6. The percentages of C18:3n3 and C18:2c9t11 of HP were greater than LP ($P < 0.05$; Table 6), and the percentages of C20:0 and C15:0 of HP lambs tended to be greater than LP lambs ($P < 0.1$; Table 6). However, the percentage of C18:1n9t and the ratio of n6:n3 of LP tended to be higher than HP ($P < 0.1$; Table 6). The results indicated that crude protein levels have no effects on the total SFA, MUFA and PUFA percentage of fatty aicds in lambs, however, the fatty acids composition of non-ruminant animals such as pigs were reported to be changed by different crude protein levels (Wood, Richardson, et al., 2004). The ratio of n6 to n3 is a very important evaluation index of the food health, as a lower ratio is considered to have human health benefits (Wood, Nute, et al., 2004), and the LP and HP did not show significant differences in the ratio of n6 to n3.

***Gene Expression***

For the Real-time PCR, the genes expression of *CHAC1, GRAMD, ELOVL6, PIK3R3, GPR1, HSPA12A, ADRA2A* and *ATXN7L1* in LP group were up-regulated in comparison with HP group (80.23±15.10%, $P < 0.05$; 67.15±23.13%, $P < 0.05$; 69.25±21.56%, $P < 0.05$; 60.96±13.38%, $P < 0.05$; 77.83±14.15%, $P < 0.05$; 55.18±20.50%, $P < 0.05$; 92.73±19.82%, $P < 0.05$; 88.17±18.41%, $P < 0.05$, respectively; Figure 2). However, CCDC158 was down-regulated in LP compared with HP (83.84±22.85%, $P < 0.05$; Figure 2). *GRAMD2A* exhibits robust positive correlations with genes involved in lipid metabolism (Besprozvannaya et al., 2018),

*PIK3R3* has a central role in regulation of metabolism (Engelman, Luo, & Cantley, 2006), *ADRA2A* is associated with metabolic alterations (Lima et al., 2007), and *ELOVL6* is related to fatty acid metabolism (Shimano, 2012). The significant differences of these genes indicated that different crude protein levels have effects on the muscle metabolism. *CCDC158* is associated with carcass quality traits including marbling, LD muscle traits, and cold carcass weight (J.-Y. Lee, Lee, Yeo, & Kim, 2013; Y. Lee, Oh, Lee, Yeo, & Lee, 2011), however, the carcass characteristics of HP was only tended to be higher than LP, but no significant differences. *ATXN7L1* is up-regulated in muscle with high IMF content and can be associated with marbling (Komolka et al., 2016). *CHAC1* is associated with fatty acid composition (Puig-Oliveras et al., 2014) and may be the reason for the significant difference seen between LP and HP. Both of *HSPA12A* and *GPR1* are related to lipid measurements. *HSPA12A* is connected with adipocyte differentiation (Zhang et al., 2019) and *GPR1* is related to lipogenesis (J. Huang et al., 2010). Upregulation of traits associated with metabolism, fat deposition, marbling, and carcass quality based on dietary provisions is beneficial when designed a finishing regimen for lambs. Further research is warranted to develop more precise feeding stages for finishing lambs to utilize the advantages that lower dietary CP levels provide in an early finishing stage with the benefits of higher dietary CP levels a later finishing stage. The increased genetic expression of traits of interest during a low CP level stage may potentially improve growth performance in a final higher CP level finishing stage.

**Conclusion**

In this study, the higher dietary CP level promoted growth performance for finishing lambs while the lower CP level was beneficial for meat quality, especially when evaluating coloration characteristics in the final product. Gene expression related to adipose metabolism of the LP

growth was higher than the HP group. This suggests muscle utilizes dietary crude protein as an energy resource for growth, but higher levels of dietary crude protein did not promote marbling or fat cover.

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**Table and Figures**

Table 1. Ingredients and chemical composition of diets fed to finishing lambs

| **Item** | Low Protein[1] | High Protein |
|---|---|---|
| Ingredients (% of DM) | | |
| Ground corn | 0.818225 | 0.820702 |
| Cracked corn | 83.75811 | 71.5971 |
| Cotton seed hulls | 11.88809 | 11.92409 |
| Soybean oil | 1.773227 | 1.778597 |
| CoCO3 | 4.07E-05 | 4.08E-05 |
| Soybean meal-49[2] | 0 | 11.97708 |
| Salt | 0.466639 | 0.468052 |
| limestone | 0.940348 | 1.077937 |
| Sel-Plex[3] | 0.004885 | 0.0049 |
| CuSO4 | 0.000247 | 0.000248 |
| Ca Iodate | 5.66E-05 | 5.67E-05 |
| ZnSO4 | 0.001303 | 0.001307 |
| Vit ADE premix[4] | 0.050315 | 0.050467 |
| Vit E premix[5] | 0.298523 | 0.299427 |
| Chemical composition | | |
| $NE_m$, Mcal/kg | 3.01 | 3.00 |
| $NE_g$ Mcal/kg | 1.28 | 1.29 |
| CP, % | 8.1 | 13.0 |
| NDF, % | 18.3 | 18.1 |
| Ca, % | 0.40 | 0.48 |
| P, % | 0.26 | 0.31 |

[1] Two treatments consisted of total mixed ration containing 8.1% and 13% CP, respectively.
[2] Soybean meal-49: 54% CP, 33% UIP (undegradable intake protein)
[3] Sel-Plex: Se 0.24 ppm
[4] Vit ADE premix: Vit A 8820 IU/g, Vit D 1764 IU/g, Vit E 1.1 IU/g.
[5] Vit E premix: Vit E 44.1 IU/g

Table 2. The primer sequences of house-keeping gene and target genes

| Gene[1] | Direction | Primer sequence | GenBank Accession No. |
|---|---|---|---|
| GAPDH | Forward | 5'- CCAACGTGTCTGTTGTGGAT -3' | HM043737 |
| | Reverse | 5'- CTGCTTCACCACCTTCTTGA -3' | |
| GRAMD2 | Forward | 5'- AAGAGGAATGCTCTGGGTGC -3' | XM_012129072 |
| | Reverse | 5'- GACTCGGTCTCAACACAGCA -3' | |
| CCDC158 | Forward | 5'-GGGAGGATATTTCCAGTAAGTGGT -3' | XM_012122875 |
| | Reverse | 5'- AGCACTGCTAGCTTTCTCGG -3' | |
| CHAC1 | Forward | 5'- CTCCTGCTTGACACCGACTT -3' | XM_012131990 |
| | Reverse | 5'- TGGGTAGCATGGGGACAGTA -3' | |
| PIK3R3 | Forward | 5'- ACTCAAAGCAGCAGGAAAGGT -3' | XM_012131714 |
| | Reverse | 5'- TGTCACAAACTCACCTTAGGCT -3' | |
| HSPA12A | Forward | 5'- CACACTCCTGATCCGCACC -3' | XM_012108783 |
| | Reverse | 5'- ACTCCCCATCACAGGGCTAT -3' | |
| ADRA2A | Forward | 5'- TGCAGATAACACAAGCCGGT -3' | XM_012128412 |
| | Reverse | 5'- TGCTGCCCGAGAGAAAAGAG -3' | |
| ATXN7L1 | Forward | 5'- GCCACTCCAAGGAGAACAGA -3' | XM_012137858 |
| | Reverse | 5'- GCAGGCCACAGTCTTCTACC -3' | |
| ELOVL6 | Forward | 5'- CGGAAACGCCTTTTGGACAC -3' | XM_012171533 |
| | Reverse | 5'- GACTGGCGGAGGGAATACAG -3' | |
| GPR1 | Forward | 5'- CAAGACTCTGGGTAGCTGCG -3' | XM_012157615 |
| | Reverse | 5'- CTTTTCCCCCAGGACTCCAC -3' | |

[1] GAPDH = Glyceraldehyde 3-phosphate dehydrogenase; GRAMD2 = GRAM domain containing 2; CCDC158 = coiled-coil domain containing 158; CHAC1 = ChaC glutathione specific gamma-glutamylcyclotransferase 1; PIK3R3 = phosphoinositide-3-kinase regulatory subunit 3; HSPA12A = heat shock protein family A (Hsp70) member 12A; ADRA2A = adrenoceptor alpha 2A; ATXN7L1 = ataxin 7 like 1; ELOVL6 = ELOVL fatty acid elongase 6; GPR1 = G protein-coupled receptor 1

Table 3. Effects of different levels of CP on growth performance of finishing lambs

| Item | Treatment [1] | | P-value |
|---|---|---|---|
| | LP | HP | |
| BW D0 [2] (kg) | 37.5±1.97 | 39.5±2.17 | 0.24 |
| BW D63 [3] (kg) | 50.5±1.98 | 56.2±2.00 | 0.03 |
| Total gain (kg) | 13.0±0.86 | 16.7±0.60 | < 0.01 |
| ADG [4] (kg/D) | 0.204±0.0136 | 0.264±0.0094 | < 0.01 |
| Feed consumption (kg) | 92.9±5.09 | 104.9±4.05 | 0.05 |
| ADFI [5] (kg/D) | 1.47±0.080 | 1.66±0.063 | 0.04 |
| G/F [6] | 0.141±0.0088 | 0.160±0.0082 | 0.06 |

[1] Treatments containing: LP (low protein level, 8.1%) and HP (high protein level, 13.0%).
[2] BW D0: bodyweight of day 0.
[3] BW D63: bodyweight of day 63.
[4] ADG: average daily gain.
[5] ADFI: average daily feed intake.
[6] G/F: average daily gain/average daily feed intake.
Data are presented as the differences between two treatments (means ± SEM), significant differences ($P < 0.05$).

Table 4. Effects of different levels of CP on carcass characteristics of finishing lambs

| Item | Treatment[1] | | P-value |
|---|---|---|---|
| | LP | HP | |
| HCW[2], kg | 23.89±1.03 | 26.22±0.97 | 0.06 |
| Fat thickness[3], cm | 8.89±0.77 | 10.45±0.76 | 0.08 |
| Yield grade | 4.3±0.35 | 3.9±0.25 | 0.18 |
| Drip loss, % | 1.52±0.19 | 2.09±0.42 | 0.09 |

[1] Treatments containing: LP (low protein level, 8.1%) and HP (high protein level, 13.0%).
[2] HCW: hot carcass weight;
[3] Fat thickness: perpendicular of *LD* over the center of the ribeye between the $12^{th}$ and $13^{th}$ rib.
Data are presented as the differences between two treatments (means ± SEM), significant differences ($P < 0.05$).

Table 5. Effects of different levels of CP on meat color of LD muscle of finishing lambs

| Item[2] | Treatment[1] | | P-value |
|---|---|---|---|
| | LP | HP | |
| L* | 43.23±0.63 | 40.71±0.54 | < 0.01 |
| a* | 24.61±0.23 | 23.83±0.21 | 0.01 |
| b* | 17.10±0.30 | 15.86±0.20 | < 0.01 |
| C | 29.97±0.35 | 28.63±0.27 | < 0.01 |
| H | 34.77±0.26 | 33.64±0.20 | < 0.01 |

[1] Treatments containing: LP (low protein level, 8.1%) and HP (high protein level, 13.0%).
[2] The L* values measure darkness to lightness, with a higher value indicating a lighter color; a* values are a measure of redness, with a high value indicating a more red color; b* values measure yellowness, with a higher value indicating a more yellow color; C values measure chroma, the degree of color saturation or intensity compared to a neutral gray, calculated as C= (a*2+b*2)1/2 chroma, with larger values indicating more saturation; H values describe the hue angle and color as it is perceived, calculated as H= tan-1(b*/a*).
Data are presented as the differences between two treatments (means ± SEM), significant differences ($P < 0.05$).

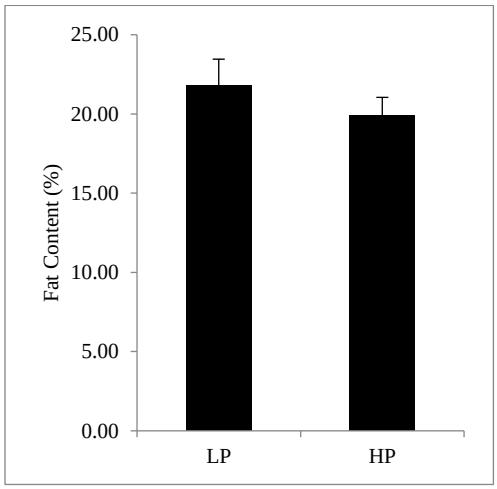


Fig 1. Effects of different levels of CP of finishing lambs fed low CP (LP, 8%) and high CP (HP, 13.0%) on fat content of LD muscle as a percentage of dry tissue weight. Data presented as the differences between two treatments (means ± SEM). Statistical significance* was determined at $P < 0.05$.

Table 6. Effects of different levels of dietary CP[1] on fatty acid composition of LD muscle of finishing lambs[2]

| Fatty acids | LP | HP | P-value |
|---|---|---|---|
| C10:0 | 0.14±0.01 | 0.14±0.01 | 0.53 |
| C12:0 | 0.07±0.01 | 0.06±0.01 | 0.24 |
| C14:0 | 2.10±0.11 | 1.89±0.13 | 0.35 |
| C14:l | 0.07±0.01 | 0.06±0.01 | 0.11 |
| C15:0 | 0.25±0.01 | 0.29±0.02 | 0.28 |
| C16:0 | 23.6±0.39 | 23.4±0.55 | 0.79 |
| C16:1t | 0.32±0.02 | 0.34±0.02 | 0.31 |
| C16:1c | 1.94±0.14 | 1.78±0.15 | 0.42 |
| C17:0 | 1.94±0.14 | 1.78±0.15 | 0.23 |
| C17:1t | 0.1±0.01 | 0.1±0.01 | 0.38 |
| C18:0 | 13.4±0.47 | 13.5±0.54 | 0.67 |
| C18:1t9 | 5.09±0.34 | 4.24±0.39 | 0.13 |
| C18:1c9 | 39.34±0.96 | 40.09±0.61 | 0.41 |
| C18:1c11 | 1.55±0.04 | 1.62±0.07 | 0.27 |
| C18:2c6 | 5.80±0.41 | 5.81±0.28 | 0.65 |
| C20:0 | 0.05±0.01 | 0.06±0.01 | 0.47 |
| C20:1 | 0.10±0.01 | 0.10±0.01 | 0.83 |
| C18:3n3 | 0.20±0.01[b] | 0.26±0.02[a] | 0.04 |
| C18:2c9t11 | 0.37±0.02[b] | 0.43±0.03[a] | 0.03 |
| C20:2 | 0.05±0.01 | 0.05±0.01 | 0.82 |
| C20:3n6 | 0.10±0.01 | 0.11±0.01 | 0.75 |
| C20:4n6 | 1.19±0.14 | 1.11±0.07 | 0.24 |
| C20:5 | 0.05±0.01 | 0.05±0.01 | 0.41 |
| C22:5 | 0.13±0.01 | 0.14±0.01 | 0.43 |
| Total SFA[3] (mg/g) | 41.55±0.92 | 41.12±0.87 | 0.55 |
| Total MUFA[4] (mg/g) | 48.51±0.79 | 48.33±0.95 | 0.49 |
| Total PUFA[5] (mg/g) | 7.89±0.42 | 7.96±0.35 | 0.21 |
| P/S[6] | 0.19±0.01 | 0.20±0.01 | 0.23 |
| N6/N3[7] | 18.46±0.52 | 15.71±0.55 | 0.06 |

[1] LP = low crude protein, 8%; HP = high crude protein 13%

[2] Data presented as percentage of dry tissue weight.

[3]SFA = saturated fatty acid

[4]MUFA = monounsaturated fatty acid

[5]PUFA = polyunsaturated fatty acid

[6]P/S = polyunsaturated fatty acid / saturated fatty acid

[7]n6/n3= n6-fatty acids / n3-fatty acids.

[a,b]Means±SE within the same row differ. Statistical significance was determined at $*P < 0.05$.

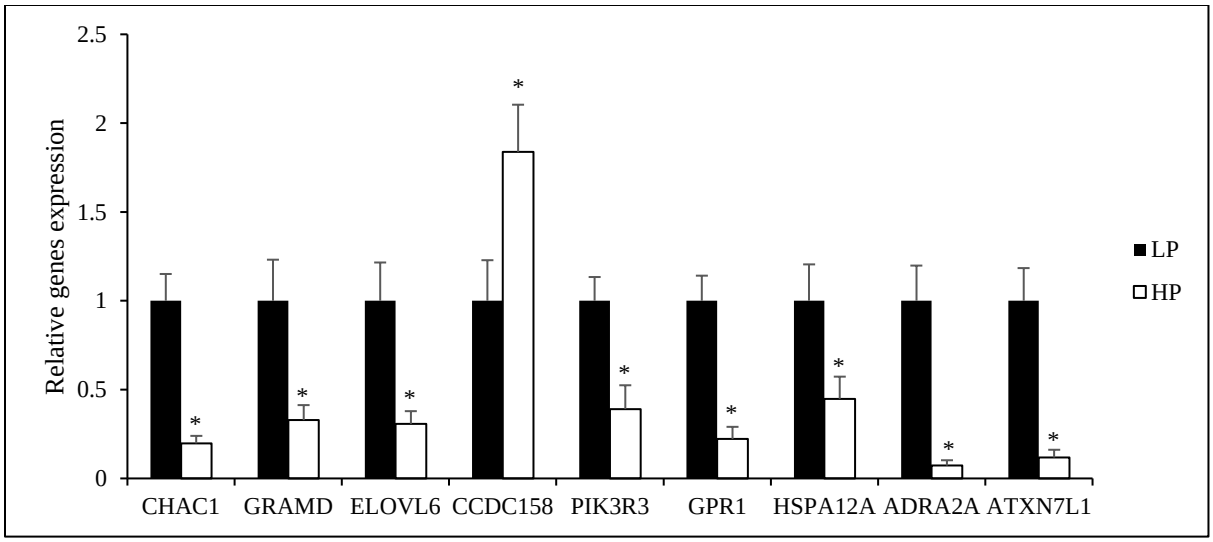


Fig 2. Gene expression of LD muscle of lambs fed low CP (LP, 8%) and high CP (HP, 13.0%). Data presented as the differences between two treatments (means ± SEM). Statistical significance* was determined at $P < 0.05$.

# Chapter III. Comparison of Growth Performance and Meat Quality Traits of Commercial Cross-bred Pig versus Large Black Pig Breeds

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**Abstract**

The meat quality of different pig breeds is associated with their different muscle tissue physiological processes, which involves a large variety of genes related with muscle fat and energy metabolism. Understanding the differences of biological processes of muscle after slaughter is helpful to reveal the meat quality development of different breeds. Therefore, 8 native Large Black pigs (BP), with high fat contents in meat, and 7 Cross-bred commercial pig (CP), which has a high feed efficiency with high lean meat, were used to investigate the differences of their meat quality and genotype. The average daily gain (ADG) and hot carcass weight (HCW) of CP were higher than BP, but the back-fat thickness of BP was higher than CP ($P < 0.05$). The CP had higher a* (redness) but lower h (hue angle) than BP ($P < 0.05$). The metmyoglobin (MMb) percentage of CP was higher ($P < 0.05$) than BP. The fat content and oxygen consumption of *longissimus dorsi* (LD) muscle in BP were higher ($P < 0.05$) than CP. BP had higher monounsaturated fatty acids (MUFA) content, but CP had higher polyunsaturated fatty acids (PUFA) content ($P < 0.05$). The RNA-seq data highlighted 201 genes differentially expressed between two groups ($P < 0.05$), with 75 up-regulated and 126 down-regulated genes in BP compared with CP using the FC and FDR thresholds. The real-time PCR was used to validate the results of RNA-seq for 8 genes, and the genes related with lipid and energy metabolism were highly expressed in BP ($P < 0.05$). Based on the results, BP had superior intramuscular fat content to CP, while the growth performance of CP was better, and the genotype differences between these two groups of pigs may cause the meat quality and growth performance variance.

**Keywords:** Commercial cross-bred; Purebred; Meat quality; Intramuscular fat; RNA-seq

## Introduction

Increasing carcass weight and leanness of pigs have been a strong emphasis on production efficiency in swine breeding programs for many years. The researchers concentrated on improving pork production such as growth rate and feed conversion ratio, and they also aimed at decreasing carcass fat content and backfat thickness. However, pork quality has received a prime focus on pig production as consumers demand better meat quality, for example, tenderness, marbling, color, and water holding capacity (WHC) (Dransfield et al., 2005). The interactive effects of pig breeds, environmental situations, pre-harvest management and post-harvest process result in different meat quality (Rosenvold & Andersen, 2003). However, the effects of genotype on meat quality are generally higher than external feed conditions (Bonneau & Lebret, 2010). It was reported that meat quality was also related to postmortem meat metabolism, and oxygen consumption of muscle tissue is one of its metabolic phenotypes (Herrera-Mendez, Becila, Boudjellal, Ouali, & Technology, 2006). In addition, a large variety of genes related to both muscle structures and metabolic substances could affect the postmortem physiological developments inside the muscle cells. Comparing the transcriptome expression profile differences between different breeds could help us to understand the principles of genes related with meat quality and muscle biological processes.

Commercial cross-bred pigs (CP) are widely used in pig industry for its great feed efficiency and higher average daily gain (ADG), with high productivity of lean meat and less fat content. In contrast, the Large Black pigs (BP) are British native pigs with long and deep-bodied shape. They are well known for its high-fat content and high meat quality characteristics (Dohner, 2008; Ekarius, 2008).

The aim of this experiment was to investigate the carcass and meat quality traits of Large Black Pigs (BP) and Commercial Cross-bred pigs (CP) in relation to their transcriptome profiles, and consequently clarify the phenotypic and genotypic differences between these two groups of pigs. The outcome of the study provided new sights into the adoption of Large Black breed in pig industry. In order to investigate the differences of gene expression between BP and CP, the RNA-seq methodology, which is widely used to provide a comprehension transcriptomic profile of analyzed tissue has been applied (Bonnet et al., 2011; Freeman et al., 2012). On the base of RNA-seq results, functional analysis of Gene Ontology (GO) and biological process were used to highlight enriched relevant biological pathways (Martin & Wang, 2011; Ozsolak & Milos, 2011). In addition, the real-time PCR was used to support the RNA-seq data.

## Material and Methods

### *Animals*

The University of Arkansas's Institutional Animal Care and Use Committee approved all procedures of the experiment involving animals during the study (ethical approval code: 18000). Eight Large Black pigs and seven commercial Cross-bred pigs (PIC 29 dam × PIC 380 boar) were allocated to BP group and CP group, and their initial mean body weights were tabulated (23.31±1.93 kg for BP group and 18.82±1.41 kg for CP group). They were fed *ad libitum* and kept individually in digestibility pens for 101 days. The final body weight of CP groups was determined at the end of 101-day experiment. However, due to low growth performance of the BP, the average body weight of BP group was 115.4 kg, lower than the average market live weight which was 127.9 kg (https://www.pork.org/facts/stats/consumption-and-expenditures/typical-market-pig-today). The BP group was kept for 108 days. The difference value between initial and final body weight was used to calculate ADG. Animals were fasted 12

h prior to slaughter, with access to water. Then, they were rendered unconscious by a nonpenetration stunning method.

***Carcass characteristics and sampling***

Immediately after stunning and completion of exsanguination, hot carcass weight (HCW) and backfat thickness (midline, between the $4^{th}$ and $5^{th}$ lumbar vertebra level) were determined. Then, muscle samples of *longissimus dorsi* (LD) of left carcass was removed, and the samples were snap frozen at -80°Cin liquid nitrogen for RNA isolation process. The carcasses were chilled at -9°C for two hours and then kept at 4°C for 24h. Another piece from LD muscle was obtained (between the *12*$^{th}$ and $13^{th}$ *rib*) and transported under refrigeration until being processed for meat quality. The muscles were cut into small pieces, vacuum-packed and stored at -20°C before analyzing the fatty acid profile.

***Meat quality***

The intramuscular fat (IMF) content of *longissimus dorsi* muscle was determined by using Soxhlet apparatus to extract ether without previous acid hydrolysis (Sukhija, Palmquist, & chemistry, 1988). A 100g thick slice cut from LD muscle was placed into a polypropylene bag and then stored in a vacuum package for 24 h at 4°C, and the weight difference of samples were regarded as drip loss showed as percentage (Christensen, 2003). In order to ensure stable data of color measurements, samples were bloomed for 20 min before further analysis. The Lightness (L), red to green (a*), and yellow to blue (b*) color values were determined by a CR-400 Chroma Meter. Then, the hue angle ($h=\arctan(b^*/ a^*)$) and chroma ($C^*=(( a^*)^2+( b^*)^2)^{0.5}$) were calculated using the L, a*, and b* values.

***Fatty acid composition***

The fatty acid composition of LD muscle IMF was determined by fat extraction (O'Fallon, Busboom, Nelson, & Gaskins, 2007). Ten grams of minced meat were homogenized at 3000 rpm for 1.5 min by UltraTurrax using 0.003% butylhydroxytoluene (BHT) with 200 ml Folch solution (chloroform-methanol mix 2:1). After paper-filtering (Whatman No. 1) the homogenized liquid, Folch solution (50ml) was added. After filtering, the solution was poured out into a decantation infundibulum mixed with 8% sodium chloride (80ml) for 24 h. The solvent, collected from lipidic phase, was evaporated. After evaporation, the fatty acids composition was analysed using a gas chromatography (Agilent 6890 N Network GC System). As a carrier gas, helium was used at a division ratio of 1:50 with a 3.2 ml per minute flow rate. The undecanoic acid methyl ester was used as an internal standard to quantify the methyl esters of fatty acids.

***Oxygen consumption and myoglobin calculation***

Approximately 20 mg of tissue from *longissimus dorsi* (LD) muscle from each sample was put into a respiration buffer (1.1 mM sodium pyruvate, 25 mM glucose in PBS and 2% BSA). The Oxygen consumption rate (OCR) of every sample was measured by an Orion Dissolved Oxygen platform (Scientific) about 25 minutes, repeat 3 times. A full reflectance spectral analysis was also taken on each muscle and wavelength ratios were used to calculate relative deoxymyoglobin (474 nm:525 nm), oxymyoglobin (610 nm : 525 nm), and metmyoglobin (572nm : 525nm) concentrations (Zou et al., 2018).

***RNA extraction and cDNA synthesis***

The total RNA of *longissimus dorsi* muscle was extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) with mechanic homogenization by using the Precellys Evolution

homogenizer (Bertin Technologies, Rockville, MD, USA). The total RNA, extracted from the muscle, was then treated with DNase I (Promega, Madison, WI, USA) to eliminate the contamination of genomic DNA, following the directions of manufacturer. The concentration of total RNA was assessed by NanoDrop (Agilent Technologies, CA, USA). The same RNA extracts were used both for real-time qPCR and RNA sequencing later. The decontaminated RNA samples were reverse-transcripted using Takara PrimeScript$^{TM}$ RT reagent Kit, and then prepared for cDNA synthesis according to the instructions of Takara PrimeScript$^{TM}$ RT.

***Real-time qPCR***

According to the recorded sequences showed in GenBank, the primers of *SLC26A7, TKTL2, ACBD7, THRSP, SLPI, FADS1, ACSL6, FOS* and *GAPDH* were designed using Oligo 6.0 Software (Table 1). GAPDH gene has been widely used as housekeeping gene normalizer (Q. Li et al., 2011), and was thus selected as reference in the present study. The cDNA was used to perform Real-time PCR in order to obtain the expression level of *SLC26A7, TKTL2, ACBD7, THRSP, SLPI, FADS1, ACSL6, FOS.* Real time-qPCR was performed using 15 μL reaction system: 7.5 μL 2 × Real Master Mix; 0.75 μL upstream and 0.75 downstream primer (10 pmol/L); 3 μL cDNA; and 3 μL water. The reaction liquid was added on iCycler IQ$^{5}$ (Bio-Rad, USA). Technical duplicates were applied for the qPCR analysis. The PCR procedure is denaturing the DNA at 95℃ for 3 mins, followed by 40 cycles of denaturation at 95℃ for 15s, annealing/extension at 55℃ for 30s.Relative expression levels were normalized to GAPDH gene and expressed as fold change (Y. Huang et al., 2010). The software CFX manager (Bio-Rad, USA) was used to process Ct values, and delta-delta Ct method was used to calculate the fold change.

***RNA sequencing and functional analysis***

The RNA was isolated from *longissimus dorsi* muscle using Trizol reagent as described above. Three micrograms of total RNA was used to isolate poly-A RNA using the Dynabeads mRNA Direct Kit (Thermo Fisher Scientific, Waltham, MA) (Saben et al., 2014). Three biological pools were made for each breed of pig. In the commercial cross-bred pigs, the number 1, 2, and 3 pigs were pooled, the number 4 and 5 were pooled, and the number 6 and 7 were pooled. In the Large Black pigs, the number 1, 2 and 3 pigs were pooled, the number 4, 5, and 6 were pooled, and the number 7 and 8 were pooled. The RNA fragmentation, library prep and adapter ligation were performed using the NEBNext Ultra Directional Library Prep kit for for Illumina (New England Biolabs, Ipswich, MA). Library size and quality were assessed using the High Sensitivity DNA Analysis Kit for the Agilent Bioanalyzer (Agilent, Santa Clara, CA) and library concentration was determined using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). Single-read 75-bp sequencing of libraries were performed using a NextSeq500 (Illumina, San Diego, CA). The FastQC app in BaseSpace was used to assess read quality and the FASTQ Toolkit was used for adapter trimming, base trimming low quality reads at the 3' end, and filtering using a minimum mean quality score of 30 (Illumina) (http://basespace.illumina.com/apps/). Alignment was performed using the Star Alignment app in BaseSpace and aligned to the pig genome (sus scrofa 11.1_v91) (Dobin et al., 2013). The resulting Bam files were uploaded into SeqMonk (v.1.37.1, http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/.) for differential expression analysis using the EdgeR package in SeqMonk ($p<0.05$ after FD correction) (Robinson, McCarthy, & Smyth, 2010). The lists of differentially expressed genes were further analyzed for

GO of biological function enrichment using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool (Dennis et al., 2003; Huang, Sherman, & Lempicki, 2009).

### *Statistical analysis*

All the data analysis was performed by SPSS version 19.0 software (SPSS Inc., Chicago, IL). The differences of mean values of these two groups were carried out using *t*-test of independent samples. The results were given as means and SD in the text, and the differences was considered significant when $P < 0.05$.

## Results and Discussion

### *Growth performance and carcass measurements*

The growth performances in terms of initial and final body weight and the quality of carcass are presented in Table 2. The initial body weights of CP and BP groups showed no significant differences ($P > 0.05$). However, CP group had higher final body weight ($P < 0.05$), ADG ($P <0.05$) and hot carcass weight ($P < 0.01$) compared with BP group. According to Bessa et al. (2013), the fat deposition of the Large Black pig is higher than the crossbreed pig. Correspondingly, the BP group showed a strong fat deposition ability with a significantly higher ($P < 0.01$) back fat thickness values compared to CP.

### *Meat quality*

Meat quality traits of CP and BP groups are summarized in Table 3. Drip loss is one of the important characteristics of water holding capacity (WHC). There is no significant difference showed in drip loss between CP and BP ($P > 0.05$). For the meat color, L* indicates lightness, a* is about red/green coordinate, b* shows the yellow/blue coordinate, C* means chroma, and h is the hue angle (Eagerman, Clydesdale, & Francis, 1977). There was no significant difference of

L*, b* and C* between these two groups ($P > 0.05$). In contrast, the a* value of CP is higher ($P < 0.01$) than BP, but the h value of BP is higher ($P < 0.05$) than CP.

The IMF content of these two breeds was significantly different ($P < 0.05$), meaning that IMF of BP is higher than that of CP pigs. However, the different fat content did not affect the drip loss. G. Watanabe, Motoyama, Nakajima, and Sasaki (2018) pointed out that pork IMF content was not correlated with drip loss, and our result had the same trend. The fat content of CP is similar with other reports about white pig breeds (De Vries, Van der Wal, Long, Eikelenboom, & Merks, 1994; Lo, McLaren, McKeith, Fernando, & Novakofski, 1992), and the fat deposition of BP is much higher than these commercial pigs. The research compared Iberian pigs and commercial cross-bred pigs showed a strong correlation between IMF and backfat thickness (Bressan et al., 2016). Our data showed higher IMF and backfat deposition in the Large Black pigs, which demonstrate the similarity fat deposition pattern in local native pigs that is different from commercial cross-bred pigs.

***Oxygen consumption***

The OCR is positively correlated with mitochondrial concentration (Tang et al., 2005). In addition, mitochondria present in the postmortem muscle can remain active and impact on the meat color through oxygen consumption (Ranjith Ramanathan, Mancini, & Biology, 2018). The OCR of BP was higher than that in CP ($P < 0.01$; Fig 1). It was reported that muscle with more β-red fibers tended to have higher OCR than muscle with more α-white fibers (Hunt & Hedrick, 1977). However, the relationship between oxygen consumption and meat quality needs to be further evaluated to understand color variations between the two groups of pigs. A study of the muscle fiber type of Korean native pig reported higher portion of type I muscle fibers than that of the commercial cross-bred pigs (Cho et al., 2019). The type I slow twitch fiber has more

content of mitochondria for oxidative phosphorylation. This report was consistent with what we found that the local native pigs have higher mitochondrial metabolism correlated with the higher content of type I muscle fiber.

***Myoglobin calculation***

The color of meat after slaughter is primarily affected by myoglobin which is a sarcoplasmic heme protein (Livingston & Brown, 1981). This protein can exist as deoxymyoglobin (DMb), oxymyoglobin (OMb) and metmyoglobin (MMb) in fresh meat (Ranjith Ramanathan et al., 2018). Our results showed differences in the percentage of myoglobin between CP and BP breeds (Figure 2). The MMb (metmyoglobin) percentage of CP was higher ($P < 0.05$) than BP, but there were no significant differences between CP and BP in DMb (deoxymyoblobin) and OMb (oxymyoglobin). The chemistry and functions of myoglobin in live muscles and meat may be different, but the functions of myoglobin as the oxygen binder and oxygen deliverer to keep the physiological functions of mitochondria continues normally (Wittenberg & Wittenberg, 2003). In addition, myoglobin is related to red meat color, which is a main meat purchasing factor for consumers (G.-D. Kim et al., 2010).

***Fatty acid composition***

The fatty acid composition of LD muscles from CP and BP breeds are listed in Table 4. There was a significant fatty acid composition difference between CP and BP in most comparisons ($P < 0.05$). The total polyunsaturated fatty acids content of CP was higher ($P < 0.05$) than BP, such as C18:2n-6, C18:3n-3, C20:2, C20:3n-6, C20:4n-6, and C22:5. However, BP had higher total monounsaturated fatty acid content along with higher C:10, C:20, C18:1, and C20:0 contents compared to CP. The overall saturated and unsaturated fatty acids contents of CP and BP had no significant differences ($P > 0.05$).

The fatty acid composition has a strong relationship with the IMF content and backfat thickness (Bosch, Tor, Reixach, & Estany, 2012). It was reported that intramuscular and backfat would increase the percentage of saturated, especially monounsaturated fatty acids, and the data of our experiment is in agreement with their findings (Olivares, Daza, Rey, & Lopez-Bote, 2009). The fatty acid composition variations in CP and BP are probably attributed to the difference in fat deposition between these two groups of pigs. The SFA level of intramuscular fat in loin has negative correlation with meat sensory quality, such as acid flavor (Straadt, Aaslyng, & Bertram, 2013). However, there was no significant differences of SFA between BP and CP. It was reported that monounsaturated fatty acids are the major fatty acids component in the Mediterranean diet which benefits lowering the risk of cardiovascular disease (P. M. J. C. Kris-Etherton, 1999), considering the IMF content in BP LD muscle is about 1.6 times higher than CP (10.02 ± 1.20:6.24 ± 1.53, $P < 0.05$. Table 2), which might indicate that BP pork products have higher healthy value with more content of monounsaturated fatty acids.

***Differentially expressed gene analysis***

High-throughput sequencing is a powerful way to identify the differences in gene expression, which is recently used in the study of different breeds to compared the difference of genes expression related with meat quality (Zhao et al., 2011). By comparing LD muscle transcriptome differences between BP and CP, we found that there was a total of 363 differentially expressed genes found between these two breeds, in which 201 are highly differentially expressed (log2 Fold change ≥1 or ≤ -1; *P*-value <0.05). Compared with CP, BP had 75 up-regulated and 126 down-regulated genes (Fig.3.). The functional category of these 201 differentially expressed genes, of which 75 are highly expressed in BP and 126 are highly expressed in CP, were determined by querying associated gene ontologies, and they were

classified into biological process, cellular component, and molecular function (Fig.4-6), by using DAVID bioinformatic resources. GO analysis showed the functional enrichment of these differentially expressed genes, and the different genes expression may cause the diversities of carcass characteristics and meat quality between these two groups of pigs. For biological process, 5 highly expressed genes in BP were related with fat cell differentiation (Fig.4). This might have led to a higher IMF content of LD muscle in BP, compared with CP. In molecular function, we found that 6 genes, which were highly expressed in CP, were responsible for oxidoreductase activity, and these genes' higher expression may cause the different OCR of the LD muscle between BP and CP.

***Gene expression***

In order to verify our RNA-seq expression profile data, we utilized real-time quantitative RT-PCR to determine eight genes related to lipid deposition or metabolism, and the results (shown in Figure 7.) partly validated the transcriptome profiles of RNA-seq. Results are presented as numerical relative gene expression values (using *GAPDH* as a housekeeping calibrator). The expression of lipid and energy metabolic related genes (*TKTL2*, *ACBD7*, *ACSL6*, and *FOS*) were higher in CP than in BP ($P < 0.05$). In addition, CP exhibited higher ($P < 0.05$) mRNA abundance of *THRSP* gene which is related to medium-length fatty acid chains (YH Wang et al., 2005). However, the gene expression of SLPI which involved in epithelial immunity was higher in BP ($P < 0.05$) is consistent with the characteristic of stronger environmental tolerance in BP as a native pig breed compared to CP (Si-Tahar, Merlin, Sitaraman, & Madara, 2000). The gene related to energy metabolism and unsaturated fatty acids composition (*SLC26A7* and *FADS1*) were higher expressed in CP. Pigs of different breeds always have the different condition of metabolism and lipid deposition. *SLC26A7* is strongly related to energy metabolism

(Irvin et al., 2011). *TKTL2* is related with lipid transport and metabolism, and the genetic distance increased from local pig breeds after selective seep caused by the selection of energy-rich pork production (Y. Fu et al., 2016). *ACBD7* is one of the gene family related with intracellular lipid-binding proteins (Neess, Bek, Engelsby, Gallego, & Faergeman, 2015). *FADS1* gene is associated with the unsaturated fatty acid composition (Pena et al., 2013). It was reported that *ACSL6* contributes to lipid synthesis (L. O. Li, Klett, Coleman, & Lipids, 2010). Reiner, Heinricy, Müller, Geldermann, and Dzapo (2002) reported that FOS is an important gene correlated with skeletal muscle fiber and metabolism. Above all, the differential of gene expression related to lipid and energy metabolism might cause the different fat deposition ability of CP and BP.

**Conclusion**

In conclusion, the growth performance of CP was higher than BP, but the intramuscular fat content of BP was higher than CP, which indicates higher meat quality in the BP group. The meat oxygen consumption rate of LD muscle in the BP group was also high, therefore, the Large Black pigs have higher metabolic rate and possibly more type I muscle fiber The RNA-seq and gene expression data proved an efficient sight about the differences of transcriptome profiles and genotype of these two groups of pigs. Comparing BP with CP, 201 significantly differentially expressed genes in LD muscle were identified. The analysis of the genes between the two groups of pigs may provide a novel strategy in swine genetic selection for both high growth performance and high meat quality specially marbling and flavor.

**List of Abbreviation**

| | |
|---|---|
| BP | Black pigs |
| CP | Cross-bred commercial pig |
| ADG | Average daily gain |
| HCW | Hot carcass weight |
| MMb | Metmyoglobin |
| DMb | Deoxymyoglobin |
| OMb | Oxymyoglobin |
| LD | Longissimus dorsi |
| SFA | Saturated fatty acid |
| UFA | Unsaturated fatty acid |
| MUFA | Monounsaturated fatty acid |
| PUFA | Polyunsaturated fatty acid |
| WHC | Water holding capacity |
| GO | Gene Ontology |
| PCR | Polymerase chain reaction |
| IMF | Intramuscular fat |
| BHT | Butylhydroxytoluene |
| RNA | Ribose nucleic acid |
| cDNA | Complementary deoxyribonucleic acid |
| SLC26A7 | Solute carrier family 26 member 7 |
| TKTL2 | Transketolase like 2 |
| ACBD7 | Acyl-CoA binding domain containing 7 |

| | |
|---|---|
| THRSP | Thyroid hormone responsive |
| SLPI | Secretory leukocyte peptidase inhibitor |
| FADS1 | Fatty acid desaturase 1 |
| ACSL6 | Acyl-CoA synthetase long chain family member 6 |
| FOS | Fos proto-oncogene, ap-1 transcription factor subunit |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| DAVID | Database for annotation, visualization and integrated discovery |
| OCR | Oxygen consumption rate |

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## Table and Figures

Table 1**.** Primer information for gene chosen for confirmation of expression using quantitative real-time PCR.

| Gene | Direction | Primer sequence | GenBank Accession No. | Product length (bp) |
|---|---|---|---|---|
| GAPDH | Forward<br>Reverse | 5'- TCGGAGTGAACGGATTTGGC -3'<br>5'- TGACAAGCTTCCCGTTCTCC -3' | NM_00120659.1 | 183 |
| SLC26A7 | Forward<br>Reverse | 5'- GAAAATGCCAGCAATCAGCCA -3'<br>5'- AGGGCCACAGTTCCCATTG -3' | XM_021089130.1 | 155 |
| TKTL2 | Forward<br>Reverse | 5'- CTGGCCTTTGCATCCCACTA -3'<br>5'- GTATCCATGCAGTGCGCAAG -3' | XM_013978706.2 | 197 |
| ACBD7 | Forward<br>Reverse | 5'- GGAAGATGCCATGAGTGCCT -3'<br>5'- CTGAGGGCTTCAAAAGGCAAA -3' | XM_003357745.4 | 130 |
| THRSP | Forward<br>Reverse | 5'- GTAGCCTCGGACTCTAGGCA -3'<br>5'- CTGCAGGTCCAGGTCTTTCT -3' | NM_001244376.1 | 178 |
| SLPI | Forward<br>Reverse | 5'- CAAGTGCACAAGTGACTGGC -3'<br>5'- GGCCATAGACCACTGGACAC -3' | NM_213870.1 | 149 |
| FADS1 | Forward<br>Reverse | 5'- GTCACTGCCTGGCTCATTCT -3'<br>5'- AGGTGGTTCCACGTAGAGGT -3' | NM_001113041.1 | 122 |
| ACSL6 | Forward<br>Reverse | 5'- GAATACGGGCACTCTCTGGC -3'<br>5'- CCTAGGACCCCAGTTTGCAG -3' | XM_021084743.1 | 187 |
| FOS | Forward<br>Reverse | 5'- GACTGCTATCTCGACCAGCC -3'<br>5'- CTGGCATGGTCTTCACGACT -3' | NM_001123113.1 | 164 |

Table 2. Effect of breeds on the carcass characteristics (CP: Cross-bred commercial pigs, BP: Large Black pigs).

| **Parameters** | **CP** | | **BP** | | ***P*-Value** |
|---|---|---|---|---|---|
| | Mean | sd | Mean | sd | |
| Initial body weight (kg) | 18.82 | 1.41 | 23.31 | 1.93 | 0.1097 |
| Final body weight (kg) | 130.04 | 8.16 | 121.17 | 2.80 | 0.0341 |
| ADG[a] (kg) | 1.10 | 0.05 | 0.91 | 0.01 | 0.0452 |
| Hot carcass weight (kg) | 77.95 | 9.81 | 63.63 | 0.95 | 0.0060 |
| Back fat thickness (cm) | 0.72 | 0.07 | 1.42 | 0.22 | 0.0004 |

[a] ADG: Average daily gain

Table 3. Effect of breeds on the meat quality of pork (*longissimus dorsi*) (CP: Cross-bred commercial pigs, BP: Large Black pigs).

| **Parameters** | **CP** | | **BP** | | ***P*-Value** |
|---|---|---|---|---|---|
| | Mean | sd | Mean | sd | |
| Drip loss (%) | 3.38 | 0.11 | 3.41 | 0.17 | 0.9571 |
| L*[a] | 57.78 | 4.52 | 60.24 | 2.43 | 0.3072 |
| a*[a] | 16.72 | 1.73 | 14.58 | 1.09 | 0.0063 |
| b*[a] | 14.31 | 1.68 | 13.73 | 0.67 | 0.4192 |
| C*[a] | 22.02 | 2.28 | 19.99 | 1.09 | 0.0951 |
| h[a] | 40.49 | 2.13 | 43.47 | 1.64 | 0.0176 |
| *Proximal composition*: | | | | | |
| Total fat (%) | 6.24 | 1.53 | 10.02 | 1.20 | 0.0431 |

[a] L*: Lightness; a*: red-green; b*: yellow-blue; C*: bright-dull; h: hue.

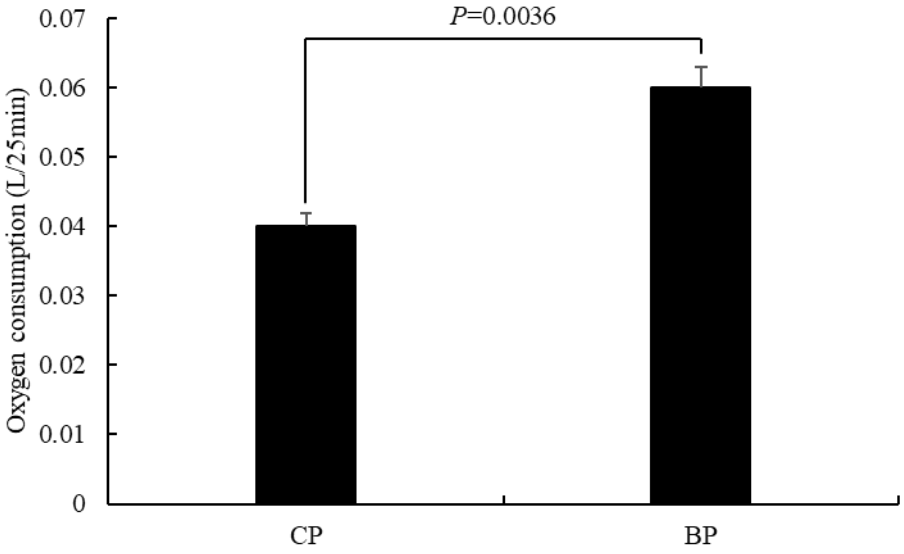


Figure 1. Effect of breeds on the oxygen consumption (*longissimus dorsi*) (CP: Cross-bred commercial pigs; BP: Large Black pigs).

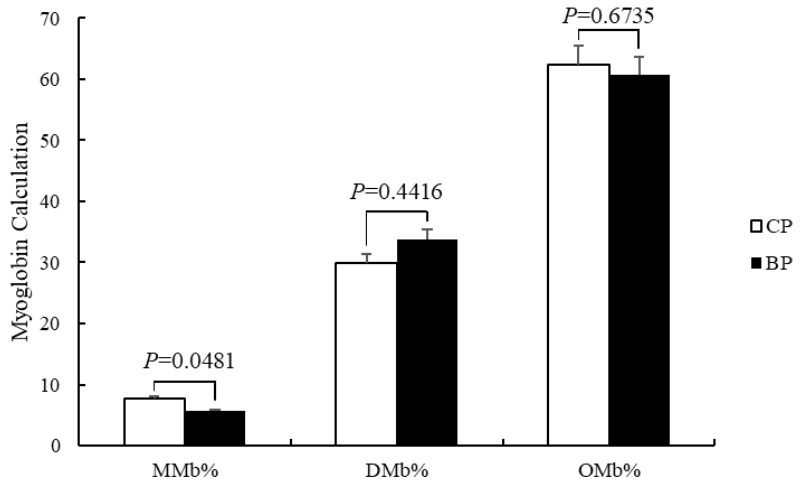


Figure 2. Effect of breeds on the myoglobin calculation (*longissimus dorsi*) (CP: Cross-bred commercial pigs, BP: Large Black pigs; MMb: Metmyoglobin, DMb: Deoxymyoblobin, OMb: Oxymyoglobin).

Table 4. Effect of breeds on the fatty acid profile of intramuscular fat (*Longissimus dorsi*) (CP: Cross-bred commercial pigs, BP: Large Black pigs).

| | CP | | BP | | *P*-Value |
|---|---|---|---|---|---|
| | Mean | sd | Mean | sd | |
| C10:0 | 0.08 | 0.01 | 0.10 | 0.01 | 0.0074 |
| C12:0 | 0.06 | 0.01 | 0.07 | 0.01 | 0.2895 |
| C14:0 | 1.14 | 0.17 | 1.20 | 0.08 | 0.5063 |
| C16:0 | 23.76 | 0.66 | 24.21 | 0.73 | 0.3495 |
| C16:1 | 3.06 | 0.46 | 3.32 | 2.41 | 0.2189 |
| C17:0 | 0.23 | 0.03 | 0.24 | 0.02 | 0.5706 |
| C18:0 | 11.74 | 0.79 | 11.95 | 0.73 | 0.4128 |
| C18:1 | 41.81 | 3.20 | 46.21 | 1.05 | 0.0153 |
| C18:2n-6 | 12.67 | 2.74 | 8.74 | 1.40 | 0.0352 |
| C18:3n-3 | 0.27 | 0.02 | 0.23 | 0.04 | 0.0496 |
| C20:0 | 0.13 | 0.01 | 0.19 | 0.02 | 0.0013 |
| C20:1 | 0.51 | 0.10 | 0.70 | 0.07 | 0.0007 |
| C20:2 | 0.29 | 0.04 | 0.23 | 0.02 | 0.0061 |
| C20:3n-6 | 0.35 | 0.11 | 0.22 | 0.05 | 0.0085 |
| C20:4n-6 | 2.82 | 0.87 | 1.53 | 0.48 | 0.0032 |
| C22:5 | 0.28 | 0.09 | 0.14 | 0.04 | 0.0162 |
| SFA[a] | 37.07 | 1.09 | 37.96 | 1.40 | 0.7946 |
| UFA | 62.04 | 1.05 | 61.25 | 1.34 | 0.5138 |
| MUFA | 45.32 | 3.61 | 50.18 | 1.21 | 0.0006 |
| PUFA | 3.75 | 1.06 | 2.11 | 0.55 | 0.0004 |
| n6:n3 | 27.59 | 3.63 | 28.35 | 3.08 | 0.0901 |

[a] SFA: saturated fatty acid; UFA: unsaturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; n6/n3: the ratio of n-6 fatty acids to n-3 fatty acids.

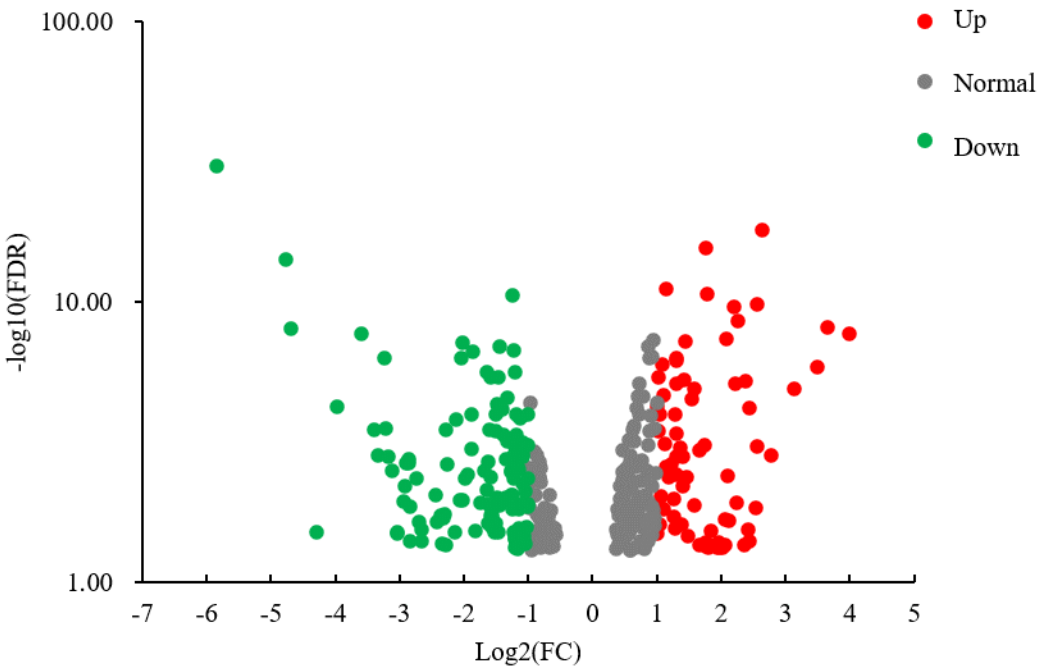


Figure 3. Volcano figure of RNA-seq (BP vs. CP; CP: Cross-bred commercial pigs, BP: Large Black pigs; Up: up regulated in BP compared with CP; Normal: No significant differences; Down: down regulated in BP compared with CP).

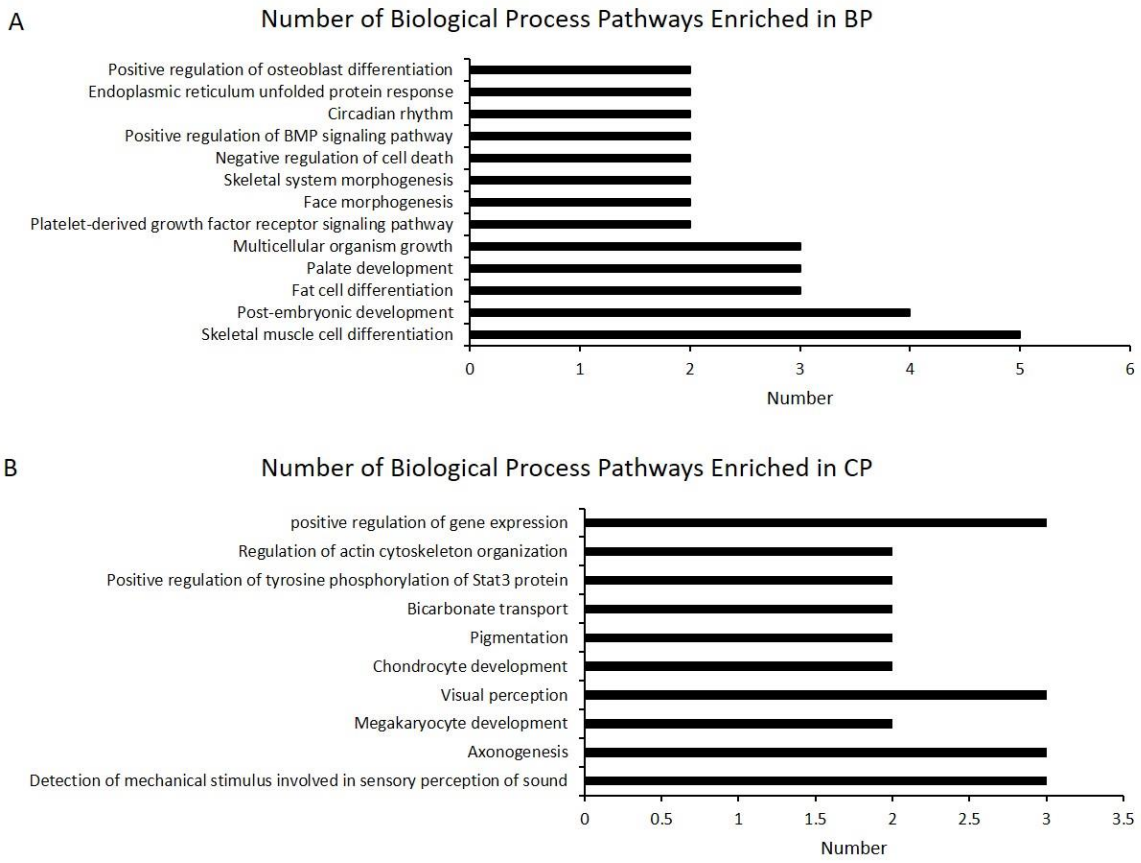


Figure 4. Gene ontology (GO) analysis of differentially expressed genes highly express in BP (A) and CP (B). The identified differentially expressed genes were classified in to Biological Process. The numbers of genes the GO term is shown above (BP vs. CP; CP: Cross-bred commercial pigs, BP: Large Black pigs).

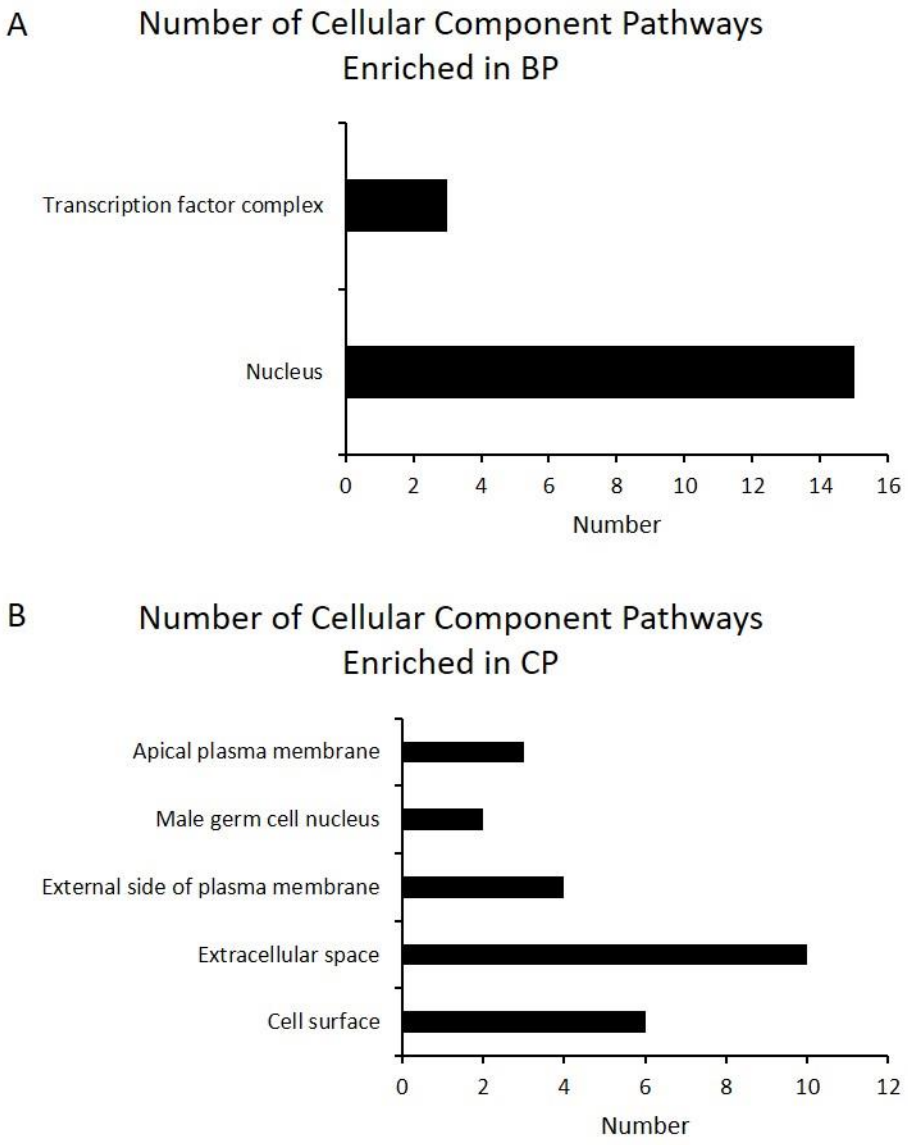


Figure 5. Gene ontology (GO) analysis of differentially expressed genes highly express in BP (A) and CP (B). The identified differentially expressed genes were classified in to Cellular Component. The numbers of genes the GO term is shown above (BP vs. CP; CP: Cross-bred commercial pigs, BP: Large Black pigs).

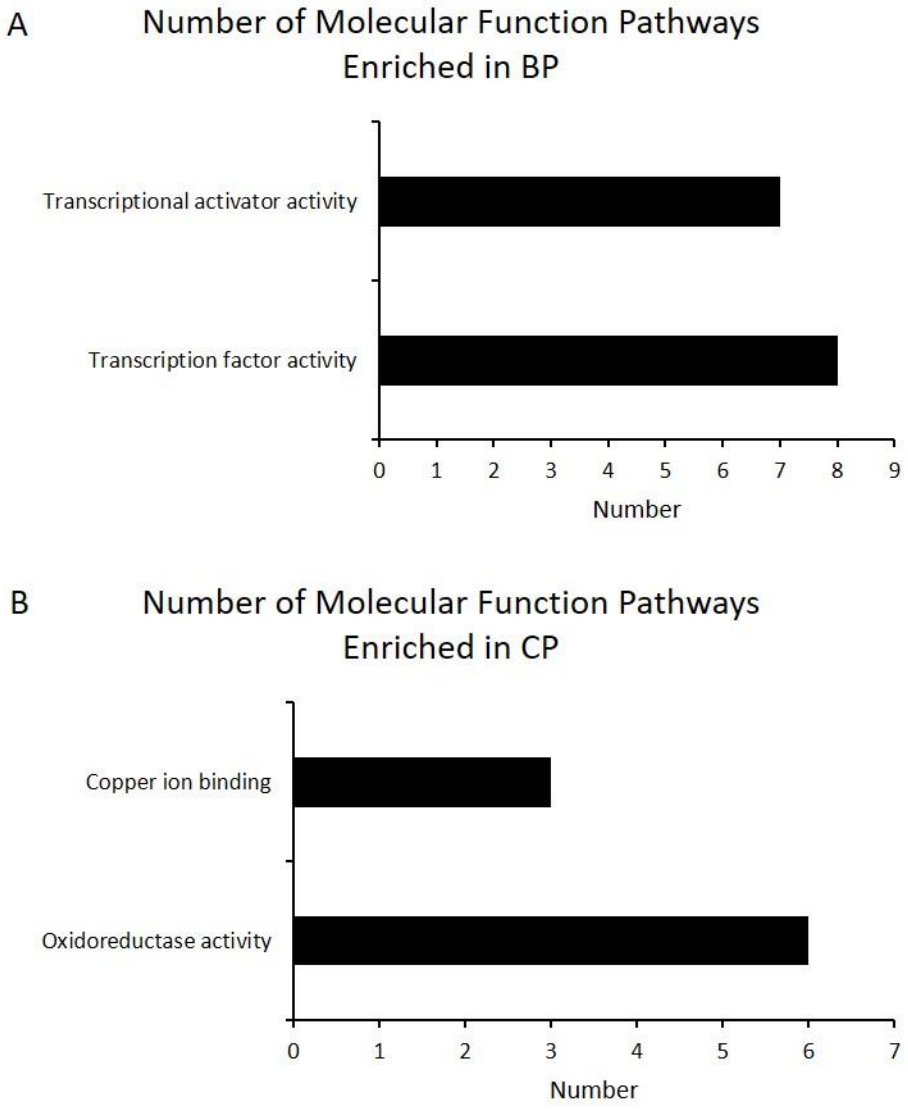


Figure 6. Gene ontology (GO) analysis of differentially expressed genes highly express in BP (A) and CP (B). The identified differentially expressed genes were classified in to Molecular Function. The numbers of genes the GO term is shown above (BP vs. CP; CP: Cross-bred commercial pigs, BP: Large Black pigs).

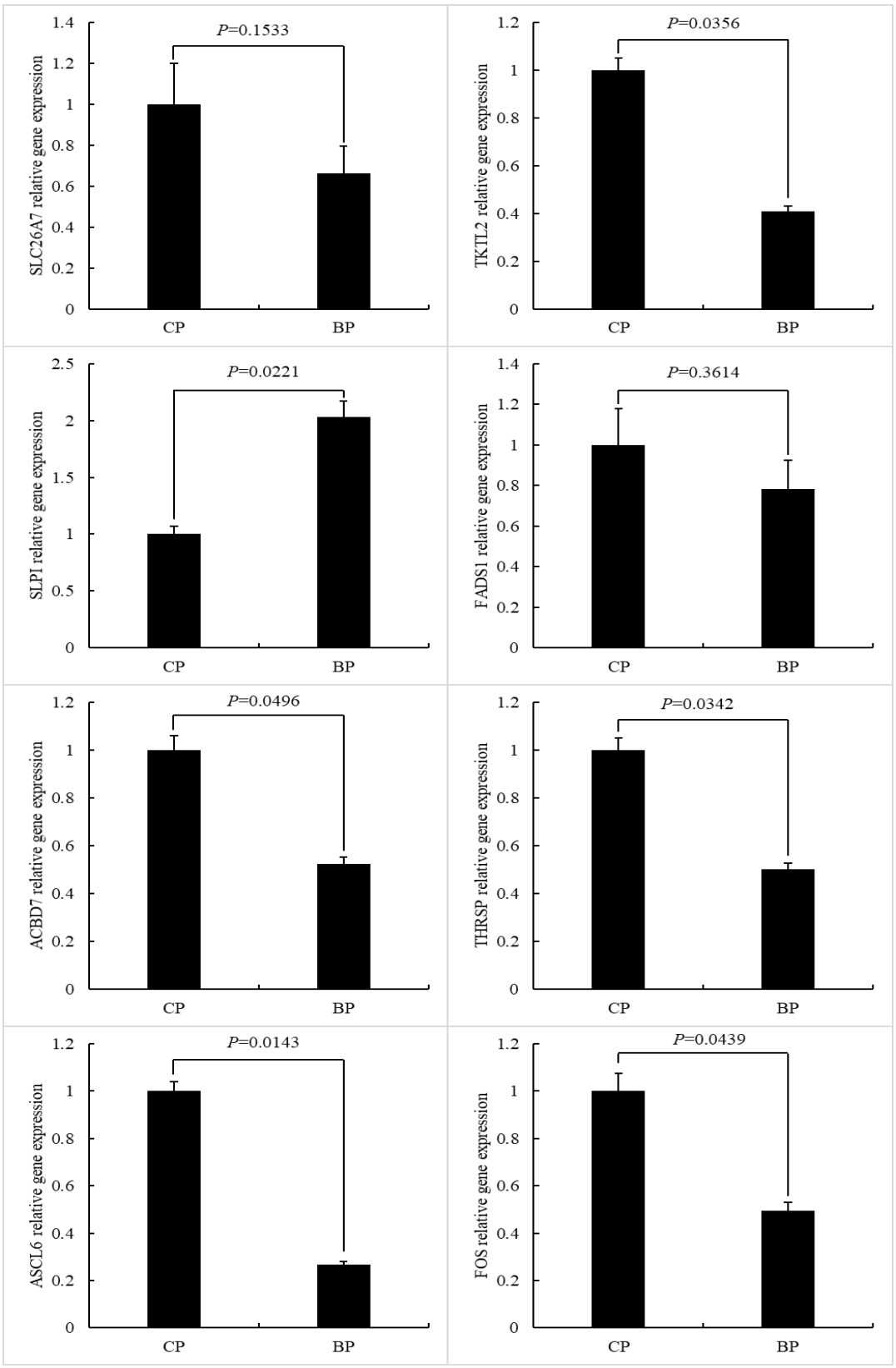


Figure 7. Effect of breeds on the relative gene expression (BP vs. CP; CP: Cross-bred commercial pigs, BP: Large Black pigs).

# Chapter IV. Effects of early-life exposure to topsoil on the muscle fiber characteristics and gene expression of weaned piglets

**Abstract**

The objective of this experiment was to investigate the influence of early exposure to topsoil on muscle fiber characteristics and transcription related myogenesis, intramuscular fat metabolism, muscle fiber types, and mTOR signaling pathway of weaned pigs. A Total of 180 piglets were separately assigned to No soil, Antibacterial soil, and Normal soil groups (each group, n=60), and were fed *ad libitum* with common antibiotic-free corn-soybean meal diets until day-31 after weaning. Ten pigs from each group with similar body weight were selected to be slaughtered, and the longissimus dorsi (LD) muscle samples were collected for histological analysis and measurements of gene and protein expression levels. The diameter and the area of muscle fiber in the Normal soil was significantly higher than No soil group ($P < 0.05$), but the lipid area of the Normal soil group was lower than the No soil group ($P < 0.05$). The Normal soil significantly upregulated the gene expression of *MyoG* compared to No soil and Antibacterial soil groups ($P < 0.05$). The gene expression of *CD36* and *CPT-1* of Normal soil group was significantly lower than No soil group ($P < 0.05$), while *HSL* expression of Normal soil group was significantly higher than Antibacterial and No soil groups ($P < 0.05$). The *MyHC I* of Normal soil group was significantly higher than No soil group ($P < 0.05$), but the expression *MyHC IIa* was lower than No soil group ($P < 0.05$). The protein expression expressed the similar result with gene expression. In addition, the Normal soil significantly increased the AMPK and mTOR phosphorylation compared to No soil and Antibacterial soil groups ($P < 0.05$). These data suggest that early exposure to topsoil regulates the muscle fiber growth, modulates the expression pattern related to myogenesis, muscle fiber type, intramuscular fat metabolism, and increases the phosphorylation of mTOR and AMPK pathways.

**Keywords:** top soil; muscle fiber characteristics; muscle growth; gene expression; Western blot

## Introduction

In the modern pig industry, fewer than 6% of pigs raised in the United States are housed in pasture of dirt pens, and most of them have been moved from outdoors into slatted-floor indoor systems (Bush, 2002). This phenomenon was caused by the increased space requirements as well as a need for more complex management practices for outdoor systems compared to indoor systems (Dourmad et al., 2014). Pigs fed indoors rarely have the opportunity to come in contact with soil, which is a complex system consists of air, water, minerals, organic matter, and biota (Sing, Sing, & Fromes, 2008). The effects of this environmental change are still understudied for the fact that the outdoor system is not a feasible means of swine production for the majority pig industry. However, previous studies show that feeding piglets outdoors during lactation improves growth performance, carcass weight, and feed efficiency (Colson, Martin, Orgeur, Prunier, & behavior, 2012; Cox & Cooper, 2001). In addition, it was reported that early exposure to agricultural soil positively modulates the gut microbiota maturation of pigs during their early life (Vo, Tsai, Maxwell, & Carbonero, 2017). Despite these discoveries, many questions remain in regards to the role of exposure to outdoor soil in regulating myogenesis, muscle fiber type, and intramuscular fat metabolism.

A previous investigation confirmed that muscle fiber characteristics could be affected by the animal rearing environment (Gentry, McGlone, Miller, & Blanton Jr, 2004). The muscle fiber characteristics (diameter, number, area) and fiber type composition are closely related to each other (Ryu, Rhee, & Kim, 2004), and are a reflection of animal growth rate (Larzul et al., 1997). Usually, muscle fiber types are defined by the isoforms of myosin heavy chain (MyHC) as MyHC I, IIa, IIb, and IIx, based on their different ATPase types (Pette, Staron, & technique, 2000). The gene expression related to intramuscular fat metabolism, such as *PPARγ*, *CPT1*,

*SREBP1*, etc., was affected by rearing systems (Guo et al., 2020). Different rearing environments may also impact lipid, protein, and energy metabolism in skeletal muscle by modulating the mechanistic target of the rapamycin (mTOR) signaling pathway (Lallès & Guillou, 2015).

The objective of this experiment was to explore the effects of exposure to topsoil during pre-weaning on the muscle fiber characteristics and expression patterns of genes and proteins related to myogenesis, muscle fiber type, intramuscular fat metabolism, and mTOR pathway.

## Material and Methods

### *Animals and experimental design*

The University of Arkansas's Institutional Animal Care and Use Committee approved all experimental procedures involving animals during the study (ethical approval code: 18059). All 30 sows used in the study were blocked by parity and farrowing body weight. Piglets from each litter were cross-fostered within 24 hours across three sows (within similar parity and body weight). Six piglets with similar body weight from each litter (n=180) were individually transferred to the same pen in the nursery facility and kept with their littermates for the entire trial. Piglets were equally allocated to the No soil group, Antibacterial soil group, and Normal soil group (60 piglets in each group). During the pre-weaning stage, the No soil group was exposed to an empty pan, Antibacterial soil group was exposed to a pan with 1 kg of irradiated topsoil (Sterigenics, Fort Worth, TX) to kill bacteria in the soil, and Normal soil group was exposed to a pan with 1 kg of topsoil (Sod Store, Inc., Tontitown, AR). After weaning (day-21), all the piglets were fed *ad libitum* with common antibiotic-free corn-soybean meal diets in littermates for another 31 days.

***Animal slaughter and sampling***

The day prior to harvest, all pigs were weighed, and the pigs of median weight form each pen (10 piglets per group) were selected for sampling on day 31. Following a 12 h period of fasting prior to slaughter with the access of water, the pigs were transported to the University of Arkansas red meat abattoir. Piglets were euthanized by a captive bolt and immediately followed by exsanguination. Then, the muscle samples from *longissimus dorsi* (LD) of left carcass were removed and subsequently snap frozen at -80℃ in liquid nitrogen for RNA isolation and protein extraction process. Another piece of LD muscle from each pig was cut into 0.5 × 0.5 × 1.0 cm cube, and immediately fixed in 10% buffered neutral formalin solution for the histological experiment.

***Histological analysis***

The LD muscle samples, fixed in 10% buffered neutral formalin solution, were dehydrated in alcohol, cleared in xylene, infiltrated, embedded in paraffin (Meng et al., 2014), and then were cut in to 3 μm thickness. H&E (hematoxylin and eosin) staining was used to treat the thickness for histological study (Fischer, Jacobson, Rose, & Zeller, 2008), and Oil red O staining was used to identify the lipid area (Lillie, 1944). Stained cross-sections were viewed and photographed at 175 × by ZOE$^{TM}$ Fluorescent Cell Imager (Bio-rad, Hercules, CA, USA). Five photographs of each cross-section of LD samples were taken, and then they were analyzed using Image-J software (National Institutes of Health, Bethesda, MD, USA). The average muscle fiber numbers per area were obtained by counting the total number of fibers in five areas (700,000 μm$^2$ for each area). The muscle fiber diameter (μm) and area (μm$^2$) of each LD muscle was measured by using 300 fibers in five cross-sections of each sample. The total lipid area (μm$^2$) of each LD muscle was measured by using 5 photographs of each sample.

***RNA isolation and cDNA synthesis***

The samples, stored in liquid nitrogen, were homogenized using the Precellys Evolution homogenizer (Bertin Technologies, Rockville, MD, USA) with TRIzol™ Reagent (Thermo Fisher Scientific, Cat. No. 15596026). After homogenization, Direct-Zol™ RNA Miniprep Kit (Zymo Research, Cat. No. R2072) was used to extract the total RNA from each sample of LD muscle. To eliminate genomic DNA contamination, resultant total RNA was then treated with DNase I, RNase-free (Promega, Madison, WI, USA), in accordance with manufacturer's instructions. The RNA concentration was assessed by NanoDrop (Agilent Technologies, Santa Clara, CA, USA). Finally, the purified RNA samples were subjected to reverse-transcription using iScriptTM Cdna Synthesis Kit (Bio-rad, Cat. No. 1708890) following the instructions given.

***Real-time qPCR***

According to the recorded sequences showed in GenBank, the primers of *β-actin, MyoG, Myf5, MSTN, PPAR-γ, FAS, SREBP-1, CD36, CPT-1, ATGL, LPL, HSL, MyHC I, MyHC IIa, MyHC IIb, MyHC IIx* were designed using GenBank for pig genes (Table 1). *β-actin* was chosen as the house-keeping gene to normalize target gene levels. Real-time qPCR was performed by using iQ™ SYBR® Green Supermix (Bio-rad, Cat. No. 1708890) along with the manufacturer's instructions. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression, and the relative expression was normalized and expressed as a ratio to the expression in the No soil group.

***Western Blot***

Total proteins of LD muscle samples were extracted using T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Cat. No. 78510) according to the guidelines. The concentration of protein samples was measured by Micro BCA™ Protein Assay Kit (Thermo

Fisher Scientific, Cat. No. 23235). Protein samples were separated on 7.5% Mini-PROTEAN® TGX™ Precast Protein Gels, 15-well, 15 µl (Bio-rad, Cat. No. 4561026), and then transferred onto Trans-Blot Turbo Midi Nitrocellulose Transfer Packs (Bio-rad, Cat. No. 1704159). Non-specific antibodies were excluded by blocking the membrane with 5% BSA (Bovine Serum Albumin) as blocking buffer for 120 min at 25℃. The membranes were incubated with primary antibodies over night at 4℃ after blocking, and then were rinsed and incubated with secondary antibodies for 60 min at 25℃. The antibodies, including *PPARγ, HSL, Myf5, MSTN, MyHC I, MyHC IIa, MyHC IIb, MyHC IIx, p-AMPK, AMPK, p-mTOR, mTOR, GAPDH* and HRP-conjugated anti-rabbit Ab were purchased from ABclonal Technology Inc. (Woburn, MA, USA). The Clarity™ Western ECL Substrate (Bio-rad, Cat. No. 1705060) was used to visualize bands, and then the bands were detected by ChemiDoc™ Touch Gel Imaging System (Bio-rad, Hercules, CA, USA). The density of bands was normalized according to *GAPDH* content. The expression levels of different proteins were quantified using Image Lab Software (Bio-rad, Hercules, CA, USA) (Herrero et al., 2005).

***Statistical analysis***

All data were analyzed by one-way ANOVA with SPSS 22.0 statistical software (SPSS Inc., Chicago, IL, USA). The results were expressed as the Mean ± SEM, and a $P < 0.05$ was used to determine statistical significance.

**Results**

***Muscle fiber characteristics***

The histochemical section of LD muscle is shown in Figure 1. The muscle fiber characteristics consisted of muscle fiber diameter, fiber area, and the number of fibers, and are shown in Table 2. The muscle fiber diameter of Antibacterial soil group was significantly higher

than Normal and No soil groups ($P < 0.05$), and the Normal soil group had a significantly higher diameter than the No soil group ($P < 0.05$). The muscle fiber area of Antibacterial soil and Normal soil groups was significantly greater than No soil group ($P < 0.05$). However, there were no significant differences in muscle fiber number among each group.

***Intramyocellular lipid area***

The lipid area of each LD muscle is shown in Figure 2. The intramyocellular lipid area of No soil group was significantly higher than Antibacterial soil group ($P < 0.05$), and the Antibacterial soil group had a significantly higher lipid area than the Normal soil group ($P < 0.05$).

***Myogenesis relative gene expression***

The gene expression related to myogenesis (*MyoG, Myf5*, and *MSTN*) in the LD muscle was measured by RT-PCR is presented in Figure 3. The Normal soil significantly upregulated the gene expression of *MyoG* of LD muscle compared to the No soil and Antibacterial soil groups ($P < 0.05$). However, there was no significant gene expression difference in *Myf5* and *MSTN* between each group ($P > 0.05$).

***Intramuscular fat relative gene expression***

The gene expression related with intramuscular fat deposition and lipolysis are shown in Figure 4 and Figure 5. For fat deposition, the gene expression of *CD36* in No soil group was significantly higher than Normal soil group ($P < 0.05$), but there was no significant difference between No soil group and Antibacterial soil group ($P > 0.05$). There were no significant differences of gene expression of *PPARγ, FAS,* and *SREBP-1* between each group ($P > 0.05$). The gene expression related with lipolysis such as *HSL* was significantly upregulated in the Normal soil group compared to Antibacterial soil and No soil group ($P < 0.05$). The *LPL*

expression of Normal soil group was significantly higher than No soil group ($P < 0.05$), yet no significant difference compared with Antibacterial soil group ($P > 0.05$) was noticed. However, the *CPT1* was down regulated in Normal soil group compared with No soil group ($P < 0.05$). Additionally, there was no significant difference of *ATGL* expression between each group ($P > 0.05$).

***Muscle fiber type relative gene expression***

The expression levels of *MyHC I, MyHC IIa, MyHC IIb* and *MyHC IIx* are llustrated in Figure 6. The *MyHC I* of Normal soil group was significantly higher than No soil group ($P < 0.05$), but no significant difference compared to Antibacterial soil group ($P > 0.05$). However, the *MyHC IIa* of Normal soil group was significantly down regulated compared with No soil group ($P < 0.05$). There were no significant differences of *MyHC IIb* and *IIx* between each group ($P > 0.05$).

***Protein expression related with intramuscular fat and myogenesis***

*HSL, PPARγ, Myf5*, and *MSTN* expression were further measured using Western blot, and the results are displayed in Figure 7. The protein abundance of *HSL* in Normal soil group was significantly higher than Antibacterial soil and No soil group ($P < 0.05$). In addition, *PPARγ* and *MSTN* protein abundances were significantly decreased in Normal soil group compared with the other two groups ($P < 0.05$). These protein expressions were consistent with the PCR results. However, there was no significant difference of *Mfy5* abundance between each group ($P > 0.05$).

***Muscle fiber type protein expression***

The protein abundances of *MyHC I, MyHC IIa, MyHC IIb* and *MyHC IIx* were determined via Western blot (Figure 8). The protein expression of *MyHC I* and *MyHC IIa* was comparable with the PCR results. The *MyHC I* of Normal soil group was significantly greater than No soil

and Antibacterial soil group ($P < 0.05$), and the *MyHC IIa* of Normal soil group was significantly lessened ($P < 0.05$). The *MyHC IIx* protein abundance in Normal soil group was also significantly decreased compared with other two groups ($P < 0.05$). Consistently, the *MyHC IIb* protein abundance was not significantly different among all groups ($P > 0.05$).

***Protein expression related with mTOR pathway***

AMPK and mTOR signaling pathways were determined using Western blotting analysis, and the outcomes were demonstrated in Figure 9. The results showed that the Normal soil significantly increased the AMPK and mTOR phosphorylation compared to No soil and Antibacterial soil groups ($P < 0.05$), and no significant differences were obvious between No soil and Antibacterial soil groups ($P > 0.05$).

**Discussion**

In relation to previous studies, muscle fiber characteristics (muscle fiber diameter, number, cross section area, and muscle fiber type) were affected by intrinsic factors (breed, sex, and age) and extrinsic factors such as nutrition value (C. Dwyer, Fletcher, & Stickland, 1993; C. M. Dwyer, Stickland, & Fletcher, 1994; Miller, Garwood, & Judge, 1975). Based on the results, our study indicated that different topsoil treatments also had effects on the muscle fiber characteristics and the transcription related with myogenesis and muscle fiber type. Larzul et al. (1997) pointed out that the muscle fiber cross section area had a positive relationship with the pig growth rate. Relevantly, the muscle fiber area of Normal soil group and Antibacterial soil group was significantly higher than No soil group and indicated a better muscle growth rate. In addition, the muscle fiber diameter is positively related to the cross-section area (Miller et al., 1975), and our results were consistent with this theory.

The adipose cells are reported to deposit within or between muscle fibers (Malenfant et al., 2001). The fat deposition can also be affected by the breed, sex, age, nutrition, and even environment (Park et al., 2018). According to our results, the topsoil treatment decreased the intramuscular fat deposition. During the early stage of animals, the energy intake is supposed to be used for muscle and bone growth, and the fat deposition usually happens at the final or later stage of animal growth (Van Es & Metabolism, 1977). The higher lipid deposition of No soil group may have a correlation with its small muscle fiber area. Besides, there is rare report about the effects of topsoil on the intramuscular fat deposition.

Hyperplasia (increasing muscle fiber number) and hypertrophy (increased fiber size) are two main processes that regulate muscle growth (Zhu et al., 2014). MRFs are a family of helix-loop-helix transcription aspects, including *MyoG, Myf5*, etc., that modify muscle hyperplasia and hypertrophy (Zammit, 2017). *Myf5* is the primary MRFs which directly proliferates myogenic progenitor cells towards a myogenic lineage, and *MyoG* is the secondary MRFs which regulates the differentiation and fusion of myoblasts to form myofibers (Conboy & Rando, 2002; Dhawan & Rando, 2005). The expression of *MSTN* is mainly in skeletal muscle and its expression has a negative relationship with muscle growth (McPherron, Lawler, & Lee, 1997). It was also reported that the mutation of *MSTN* increased the muscle growth and muscle mass in many animals including pigs, sheep, rabbits, and cattle (Bi et al., 2016; Grobet et al., 1997; Lv et al., 2016; X. Wang et al., 2016). This experiment confirmed that exposure to topsoil during the pre-weaning stage changed the transcription related with myogenesis. Exposure to Normal soil upregulated the gene expression of *Myf5*, while downregulated the *MSTN* transporter. The results indicated that exposure to topsoil had a positive impact on the myogenesis of piglets.

According to our results, the transcription of different muscle fiber types of weaned pigs was also affected by the topsoil. In general, there are four different muscle fiber types (type I, type IIa, type IIb, and type IIx) which can be detected in pig skeletal muscle, and they are distinguished by different types of myosin heavy chain (MyHC I, IIa, IIb, and IIx) (Pette et al., 2000). Type I is slow-oxidative fiber, type IIa is fast oxide-glycolytic fiber, and both type IIb and IIx are fast glycolytic fibers (Zierath & Hawley, 2004). Different muscle fiber types represent different ATPase characteristics of fibers (Kushmerick, Moerland, & Wiseman, 1992), for example, type I fiber is rich in mitochondria which provides ATP by its fatty acid oxidation, and type II fibers, which are classified as IIa, IIb, or IIx by its myosin heavy chain (MyHC) isoforms expression, utilize glucose to supply energy (Pette et al., 2000; Schiaffino & Reggiani, 2011). The muscle fiber types are impacted by complicated intrinsic and extrinsic factors such as breed, gender, age, nutrient level, and physical activity (Lee, Joo, & Ryu, 2010). Based on this theory and our transcription results, exposure to the topsoil, as an extrinsic factor, also altered the muscle fiber type composition and the energy utilization forms of weaned pigs, and contacting with Normal soil increased the proportion of type I fiber which relies on the oxidative activity while decreased the type II fibers which have more glycolytic metabolism. The changes of muscle fiber characteristics of weaned pigs may also affect the postmortem metabolism or even the consequential meat quality.

In addition, the gene and protein expression related with intramuscular fat deposition and fat removal of piglets were influenced by the exposure to topsoil. For the intramuscular fat deposition, exposure to Normal soil downregulated the *CD36* gene and *PPARγ* protein expression. *CD36* is a primary fatty acid transporter expressed in animal skeletal muscle (Bonen, Luiken, Arumugam, Glatz, & Tandon, 2000; Ibrahimi, Abumrad, & Care, 2002), and its

abundance on the plasma membrane of obesity animals has a positive correlation with the rate of fatty acid uptake (Aguer et al., 2010). *PPARγ* is a ligand-activated transcription factor, expressed in many tissues (skeletal muscle and adipocytes) which accelerates adipogenesis and insulin sensitivity (Hevener et al., 2007; Matsusue, Peters, & Gonzalez, 2004), and its expression regulates the stimulation of adipocyte differentiation and fat deposition (Janani, Kumari, Research, & Reviews, 2015). In terms of lipolysis, Normal soil unregulated the *HSL*, *LPL* expression, while downregulated the *CPT1* gene expression. *HSL* detached fatty acids from intracellular triacylglycerol for oxidation and exportation (Dolinsky et al., 2004). *LPL* is a rate-limiting enzyme for the hydrolysis of triacylglycerol, and its catalyzed reaction products, fatty and monoacylglycerol, are used by adipose tissue and skeletal muscle as element of neutral lipids (H. Wang, Eckel, & Metabolism, 2009). *CPT1* was reported to be correlated with mitochondrial fatty acid oxidation (Sebastián et al., 2009).

The mTOR pathway plays an important role in modulating amino acid metabolism and reflecting the availability of amino acid (Han et al., 2016; Jewell, Russell, & Guan, 2013). In addition, AMPK is a key energy sensor which regulates the cellular energy (Hardie, Ross, & Hawley, 2012), and it can also modulates oxidative stress and mitochondrial function (Wu et al., 2018). In this investigation, we found that exposure to topsoil increased the mTOR and AMPK signaling pathway in weaned pigs, and it activated their phosphorylation. The results indicated that the amino acid and cellular energy metabolism of piglets were modulated by the exposure to topsoil during weaning period.

## Conclusion

The current experiment proved that exposure to topsoil promotes the myogenesis, decreases the intramuscular adipogenesis, modulates the transcription related with muscle fiber types, intramuscular fat deposition and lipolysis, and regulates the AMPK and mTOR signaling pathway of weaned piglets.

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**List of Abbreviations**

AMPK: AMP-activated protein kinase

ATGL: adipose triglyceride lipase

CD36: cluster of differentiation molecule

CPT-1: carnitine palmitoyltransferase 1

FAS: Fas cell surface death receptor

H&E: hematoxylin and eosin

HSL: hormone-sensitive lipase

LD: longissimus dorsi

LPL: lipoprotein lipase

mTOR: mammalian target of rapamycin

MSTN: myostatin

MyHC-I: myosin heavy chain I

MyHC-IIa: myosin heavy chain IIa

MyHC-IIb: myosin heavy chain IIb

MyHC-IIx: myosin heavy chain IIx

Myf5: myogenic factor 5

MyoG: myogenin

PPAR-γ: peroxisome proliferator activated receptor gamma

SREBP-1: sterol regulatory element binding transcription factor 1

**Table and Figures**

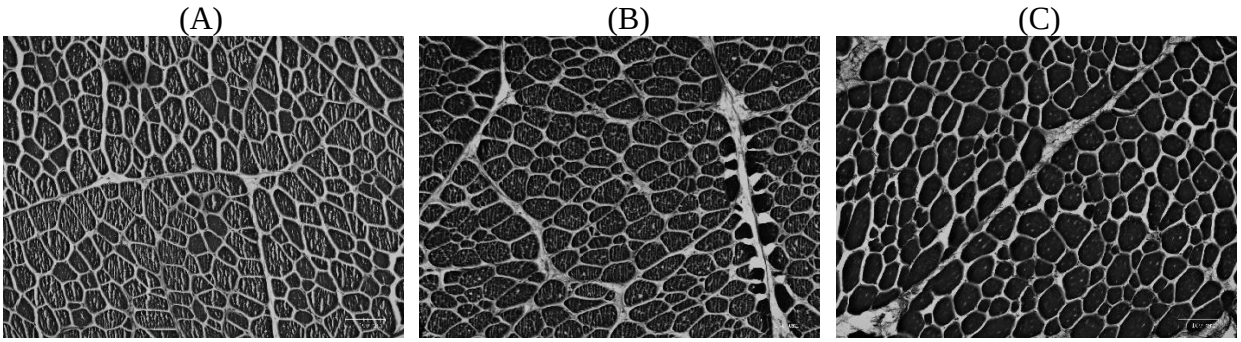
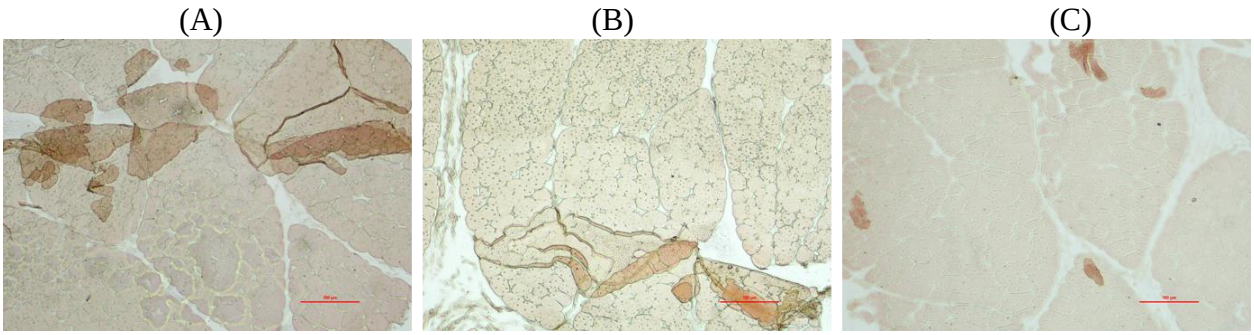



Figure 1. Histological cross-section of *Longissimus dorsi* muscles in piglets. (A) No soil; (B) Antibictorial soil; (C) Normal soil. Bar on lower right corner of each panel = 100 µm.



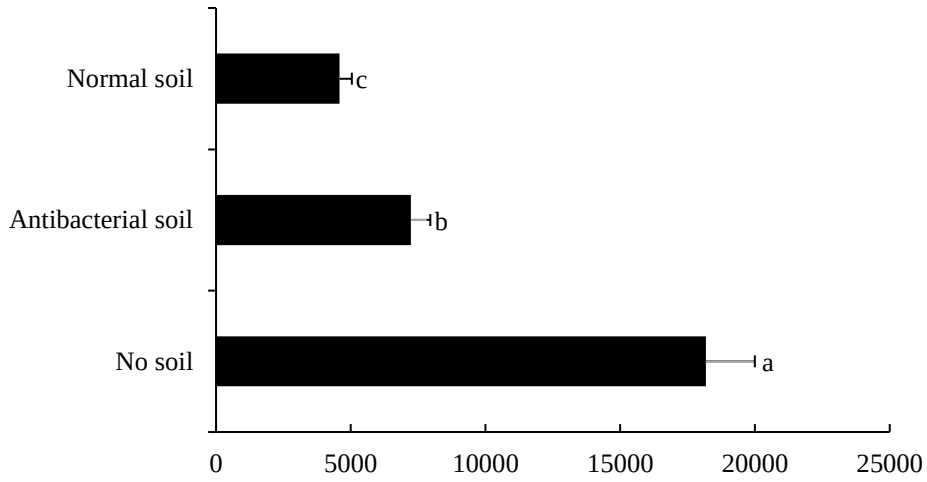




Figure 2. Red oil staining cross-section area of *Longissimus dorsi* muscles in piglets. (A) No soil; (B) Antibictorial soil; (C) Normal soil. Bar on lower right corner of each panel = 100 µm. Values are the least square mean ± standard error of the mean. Within a row, means with no superscripts or with a common superscript letter are not significantly different ($P < 0.05$).

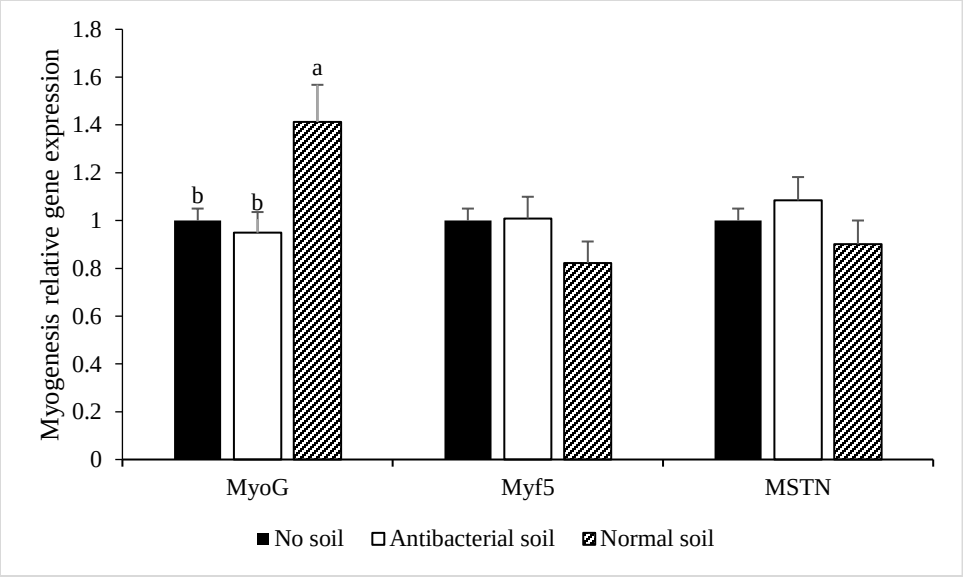



Figure 3. Effect of topsoil on gene expression related with myogenesis of weaned piglets. Values are the least square mean ± standard error of the mean. Within a row, means with no superscripts or with a common superscript letter are not significantly different ($P < 0.05$).

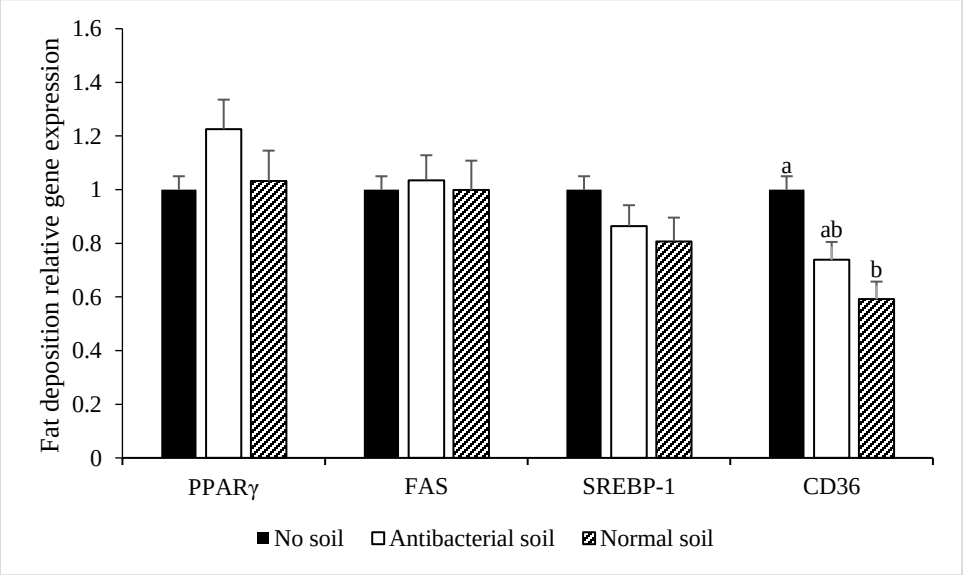



Figure 4. Effect of topsoil on gene expression related with intramuscular fat deposition in weaned piglets. Values are the least square mean ± standard error of the mean. Within a row, means with no superscripts or with a common superscript letter are not significantly different ($P < 0.05$).

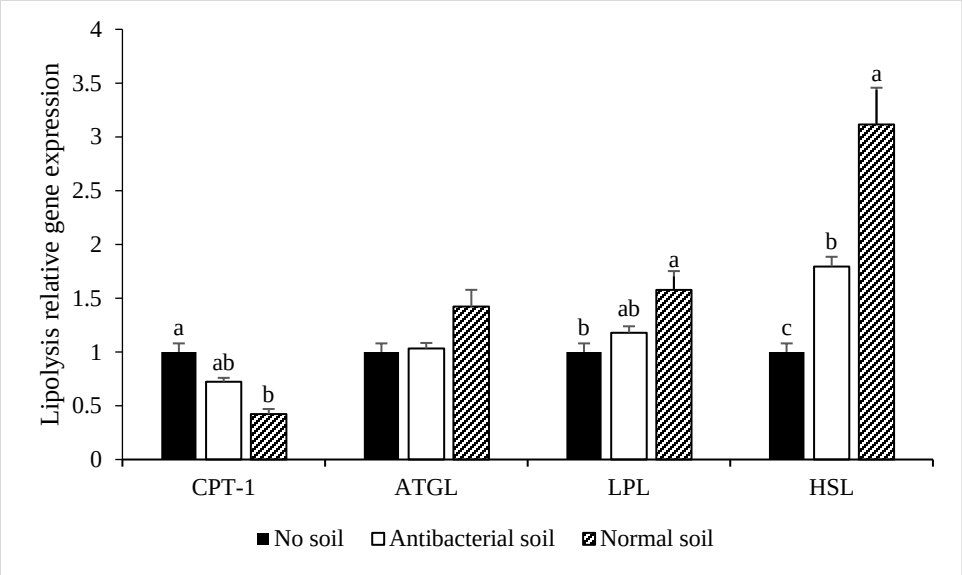



Figure 5. Effect of topsoil on gene expression related with intramuscular fat lipolysis in weaned piglets. Values are the least square mean ± standard error of the mean. Within a row, means with no superscripts or with a common superscript letter are not significantly different ($P < 0.05$).

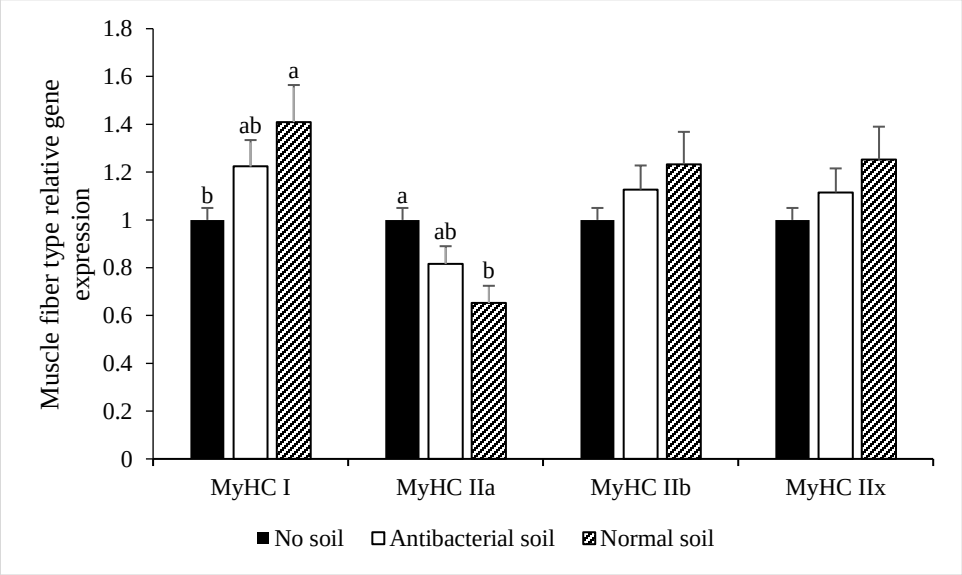



Figure 6. Effect of topsoil on gene expression related with muscle fiber type of weaned piglets. Values are the least square mean ± standard error of the mean. Within a row, means with no superscripts or with a common superscript letter are not significantly different ($P < 0.05$).

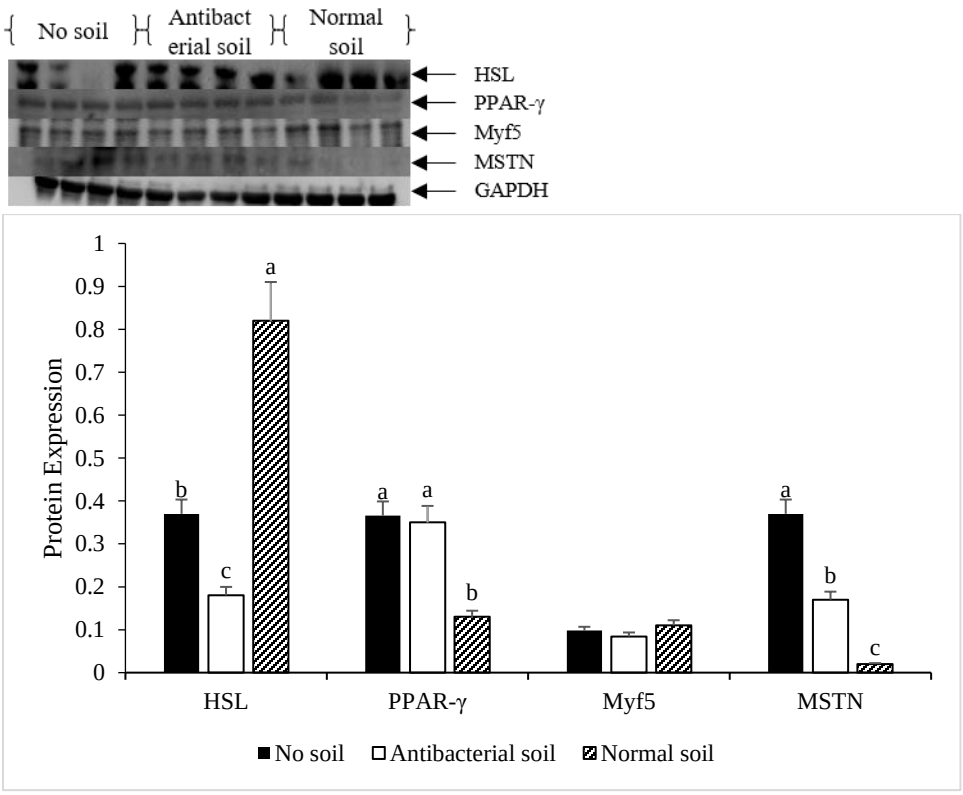



Figure 7. Effect of topsoil on protein expression related with intramuscular fat and myogenesis of weaned piglets. Values are the least square mean ± standard error of the mean. Within a row, means with no superscripts or with a common superscript letter are not significantly different ($P < 0.05$).

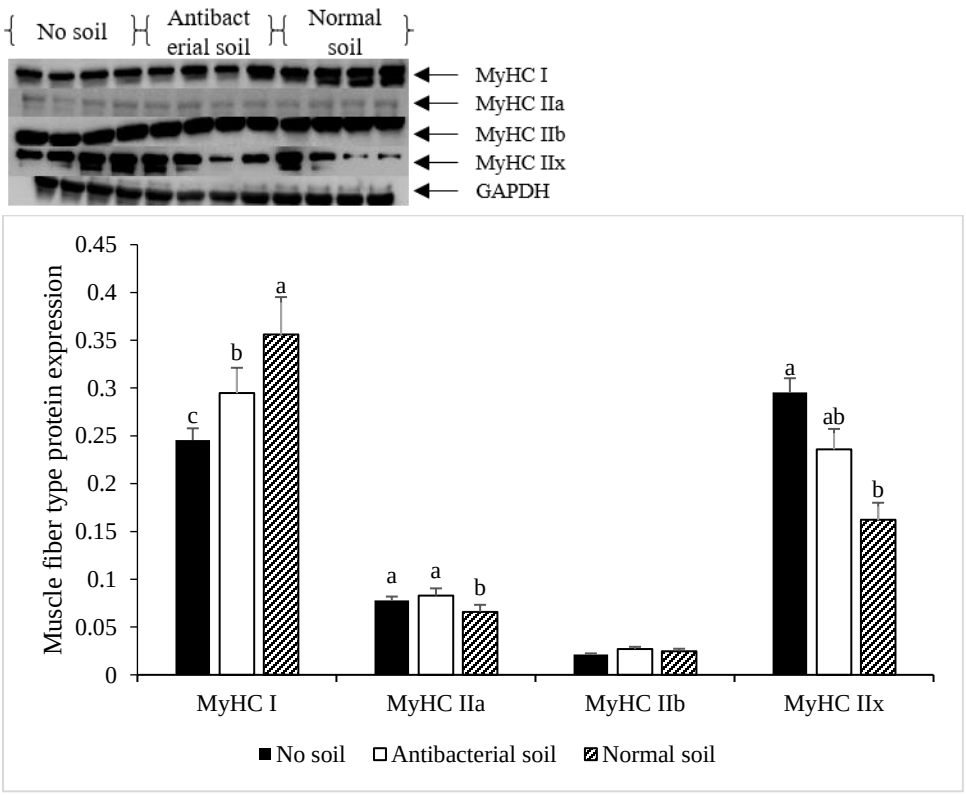



Figure 8. Effect of topsoil on muscle fiber type protein expression of weaned piglets. Values are the least square mean ± standard error of the mean. Within a row, means with no superscripts or with a common superscript letter are not significantly different ($P < 0.05$).

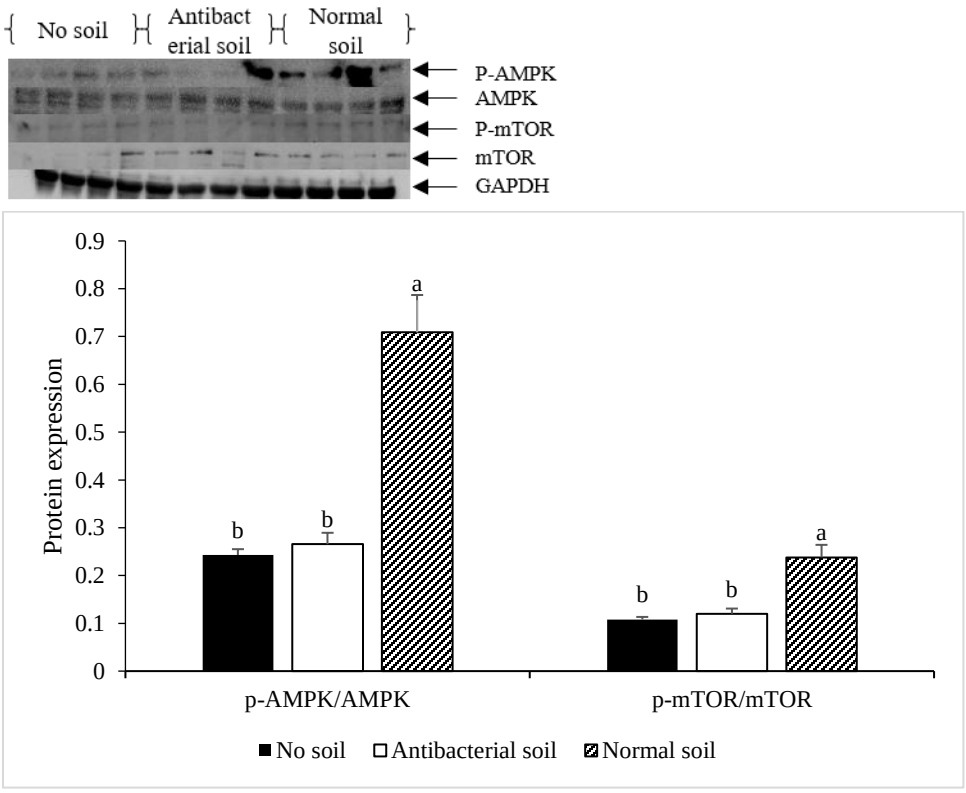



Figure 9. Effect of topsoil on AMPK and mTOR signaling pathways in the muscle of weaned piglets. Values are the least square mean ± standard error of the mean. AMPK: AMP-activated protein kinase; mTOR: mammalian target of rapamycin. Within a row, means with no superscripts or with a common superscript letter are not significantly different ($P < 0.05$).

Table 1. Primers used in this study

| Gene[1] | Direction | Primer sequence | GenBank Accession No. |
|---|---|---|---|
| β-actin | Forward | 5’- GGATGCAGAAGGAGATCACG -3’ | DQ845171 |
| | Reverse | 5’- ATCTGCTGGAAGGTGGACAG -3’ | |
| MyoG | Forward | 5’- TCT ATG ACG GGG AAA ACT AC -3’ | NM_001012406 |
| | Reverse | 5’- TGG AGC CAG AGT GGT GTA TC -3’ | |
| Myf5 | Forward | 5’-GAGGATATTTCCAGTAAGTGGT -3’ | NM_001278775 |
| | Reverse | 5’- AGCACTGCTAGCTTTCTCGG -3’ | |
| MSTN | Forward | 5’- CTCCTGCTTGACACCGACTT -3’ | NM_214435 |
| | Reverse | 5’- TGGGTAGCATGGGGACAGTA -3’ | |
| PPAR-γ | Forward | 5’- ACTCAAAGCAGCAGGAAAGGT -3’ | NM_214379 |
| | Reverse | 5’- TGTCACAAACTCACCTTAGGCT -3’ | |
| FAS | Forward | 5’- CACACTCCTGATCCGCACC -3’ | NM_213839 |
| | Reverse | 5’- ACTCCCCATCACAGGGCTAT -3’ | |
| SREBP-1 | Forward | 5’- TGCAGATAACACAAGCCGGT -3’ | AY338729 |
| | Reverse | 5’- TGCTGCCCGAGAGAAAAGAG -3’ | |
| CD36 | Forward | 5’- GCCACTCCAAGGAGAACAGA -3’ | NM_001044622 |
| | Reverse | 5’- GCAGGCCACAGTCTTCTACC -3’ | |
| CPT-1 | Forward | 5’- CGGAAACGCCTTTTGGACAC -3’ | AF284832 |
| | Reverse | 5’- GACTGGCGGAGGGAATACAG -3’ | |
| ATGL | Forward | 5’- CAAGACTCTGGGTAGCTGCG -3’ | EU373817 |
| | Reverse | 5’- CTTTTCCCCCAGGACTCCAC -3’ | |
| LPL | Forward | 5’- CCAACGTGTCTGTTGTGGAT -3’ | NM_214286 |
| | Reverse | 5’- CTGCTTCACCACCTTCTTGA -3’ | |
| HSL | Forward | 5’- CCAACGTGTCTGTTGTGGAT -3’ | AY559451 |
| | Reverse | 5’- CTGCTTCACCACCTTCTTGA -3’ | |
| MyHC-I | Forward | 5’- CCAACGTGTCTGTTGTGGAT -3’ | NM_213855 |
| | Reverse | 5’- CTGCTTCACCACCTTCTTGA -3’ | |
| MyHC-IIa | Forward | 5’- CCAACGTGTCTGTTGTGGAT -3’ | NM_214136 |
| | Reverse | 5’- CTGCTTCACCACCTTCTTGA -3’ | |
| MyHC-IIb | Forward | 5’- CCAACGTGTCTGTTGTGGAT -3’ | NM_001123141 |
| | Reverse | 5’- CTGCTTCACCACCTTCTTGA -3’ | |
| MyHC-IIx | Forward | 5’- CCAACGTGTCTGTTGTGGAT -3’ | NM_001104951 |
| | Reverse | 5’- CTGCTTCACCACCTTCTTGA -3’ | |

MyoG: myogenin; Myf5: myogenic factor 5; MSTN: myostatin; PPAR-γ: peroxisome proliferator activated receptor gamma; FAS: Fas cell surface death receptor; SREBP-1: sterol regulatory element binding transcription factor 1; CD36: cluster of differentiation molecule; CPT-1: carnitine palmitoyltransferase 1; ATGL: adipose triglyceride lipase; LPL: lipoprotein lipase; HSL: hormone-sensitive lipase; MyHC-I: myosin heavy chain I; MyHC-IIa: myosin heavy chain IIa; MyHC-IIb: myosin heavy chain IIb; MyHC-IIx: myosin heavy chain IIx

Table 2. Effect of topsoil on muscle fiber characteristics of weaned piglets

| Item | No soil | Antibacterial soil | Normal soil | *P*-value |
|---|---|---|---|---|
| Muscle fiber diameter / μm | $45.02 \pm 0.53^{c}$ | $50.53 \pm 0.69^{a}$ | $48.75 \pm 0.66^{b}$ | 0.047 |
| Muscle fiber area / $\mu m^2$ | $1703.24 \pm 41.68^{b}$ | $2188.73 \pm 61.82^{a}$ | $2037.1 \pm 58.42^{a}$ | 0.043 |
| Muscle fiber number | $229.91 \pm 7.69$ | $210.02 \pm 8.04$ | $216.75 \pm 12.62$ | 0.197 |

Values are the least square mean ± standard error of the mean. Within a row, means with no superscripts or with a common superscript letter are not significantly different ($P < 0.05$).

# Chapter V. Conclusions

This dissertation answered several important questions regarding meat products such as, 1) can a low-protein diet impact the growth performance and meat quality of finishing lambs compared to a high-protein diet; 2) what is the growth performance and meat quality differences between Large Black pigs and Commercial Cross-bred pigs, and what is the genotype differences caused this phenotype variance; 3) and whether topsoil can improve the muscle fiber development and intramuscular fat deposition, and what is the changes of the gene and protein expression caused by the treatment?

Our results indicated that 1) low-protein level diet can be used in the finishing stage of lambs to reduce the feed cost and improve the meat color characteristics; 2) compared to Commercial Crossbred pigs, Lager Black pigs have a higher meat quality and intramuscular fat, and their phenotype differences may explain the variance; 3) early exposure to topsoil improved the muscle fiber development, but decrease the lipid droplet deposition in cells.

This investigation has laid a foundation for a general strategy to improve growth performance, muscle development, and meat quality by modulating diet protein levels, animal breeds, and contacting environments.