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Differential gene expression and enrichment analysis in *Longissimus Dorsi* tissue of Nelore cattle with divergent water loss by cooking using RNA-Seq

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Perguntei ao céu tão lindo,
— Por que é todo cor de anil?
Ele me disse, sorrindo:
— Eu sou o céu do Brasil!

Renato Sêneca de Sá Fleury

Dedicated

To my sister Eva Serna García, for the support at all times, for teaching me that the fear of erring is natural and can be overcome by always make decisions with respect, for transmitting her passion, respect and values for the science, for her sensitivity, creativity and effort that teach me to be a better person and a better professional every day.

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ABSTRACT

Differential gene expression and enrichment analysis in *Longissimus Dorsi* tissue of Nelore cattle with divergent water loss by cooking using RNA-Seq

Meat cooking treatments are a major problem due to the shrinkage of the products resulting of the loss in water content and having a great impact on the quality attributes of the meat, such as juiciness and tenderness of the final product. Beef is characterized by a high and uncontrolled variability on its quality, which is one of the reasons for consumer's dissatisfaction.

Brazil is the second meat consumer country and the second world meat exporter, improving the quality of its meat could open new international markets to satisfy consumers and with the economic implications which this entails. One of the factors that most influences the loss of water is the genotype of the animal, which is the reason why studies have focused on improving the quality of Nelore races, because they are the most used breed in this country and also because they contain Zebu genes that reduce their meat quality with the Taurine breeds. The measurement of this characteristic can only be done after the sacrifice of the animal which hinders its improvement. Thanks to the development of genomics and new technological tools such as RNA -seq the beef industry is calling for muscle biomarkers to predict the ability of animals to produce high-quality beef.

The objective of this work was to identify differentially expressed genes related to the loss of water from meat, after cooking, in Nelore cattle and therefore to find possible biomarkers to improve meat quality through RNA-Seq.

In the UNESP the mRNA of 20 samples of *Longissimus dorsi* muscle of bovine Nelore was sequenced. After the cooking procedure of 132 samples of *Longissimus dorsi* muscle from Nelore cattle, 10 samples wich had little loss of water were selected, as well as other 10 samples with much loss of water after cooking. All the animals belonged to the same management group and were slaughtered the same day. 32 differentially expressed genes were found related to the loss of water by cooked meat, being 28 of these genes with described functions. From the differentially expressed genes, 25 were downregulated and 7 of them were upregulated.

Genes were found related to biological processes such as response to glucocorticoid, regulation of cell growth, JAK-STAT cascade involved in growth hormone signaling pathway, response to food, as well as master regulators such as cytokines and MAPK, and cell processes such as the increase of adipogenesis, water retention and immune system.

The results of this work will allow the generation of genetic biomarkers in Nelore cattle, for the selection of loss of water in the meat, to produce better quality meat for the consumption and will also bring economic benefits to the Nelore cattle farms.

Keywords: Production, Bovine, RNA-Seq, Transcriptome, Meat quality, *Logissimus Dorsi*, Nelore

RESUMO

Expressão gênica diferencial e análise de enriquecimento no tecido *Longissimus Dorsi* de bovinos Nelore divergentes para perda de água por cozimento usando RNA-Seq

O cozimento da carne é um problema importante, já que provoca o encolhimento da fibra muscular, devido à perda de conteúdo de água. O que causa grande impacto sobre caracteristicas de qualidade na carne, como marmóreo e maciez do produto final. A carne de bovino tem uma variabilidade elevada e descontrolada de sua qualidade, que é uma das razões da insatisfação do consumidor.

O Brasil é o segundo país consumidor de carne e o segundo exportador mundial, e melhorar a qualidade da carneproduzida, pode abrir novos mercados internacionais que satisfaçam os consumidores mais exigentes. Um dos fatores que mais influenciam à perda de água é o genótipo do animal, razão pela qual os estudos se concentraram na melhoria da qualidade da raça Nelore, já que essa raça é a mais utilizada neste país e, por ser de origemZebuína apresenta reduzida qualidade de carne quando comparadaàs raças Taurinas.

A avaliação das perdas de água por cozimento da carne só pode ser feita após o sacrifício do animal, que dificulta sua melhora. Graças ao desenvolvimento da genômica e de novas ferramentas tecnológicas, como o RNA-seq, tornou-se possível a busca por biomarcadores que estimem a capacidade dos animais de produzir carne de bovino de alta qualidade, sem a necessidade de abate.

O objetivo deste trabalho foi identificar genes diferencialmente expressos relacionados à perda de água da carne porcozinhimentoem gado Nelore e, por tanto, encontrar possíveis biomarcadores para melhorar a qualidade da carne através de RNA-Seq.

Na UNESP, o mRNA de 20 amostras do músculo *Longissimus Dorsi* do bovino Nelore foi sequenciado. Após o procedimento de cozimento de 132 amostras do músculo *Longissimus dorsi* do gado Nelore, foram selecionadas 10 amostras que tinham baixa perda de água e 10 amostras de alta perda de água após o cozimento. Todos os animais pertenciam ao mesmo grupo de contemporâneos e foram abatidos no mesmo dia. Foram encontrados 32 genes diferencialmente expressos relacionados à perda de água por cozimento, sendo 28 genes com funções descritas. Dos genes expressos diferencialmente, 25 foram induzidos e 7 reprimidos com relação ao grupo de altas perdas de água por cozimento.

Foram encontrandos genes relacionados com processos biológicos como: resposta ao glucocorticoide, regulação do crescimento celular, cascata JAK-STAT envolvido na via de sinalização do hormonio do crescimento, resposta a alimentos, assim como reguladores principais como as citoquinas e MAPK, e processos celulares como o aumento da adipogénesis, retenção de água e sistema imune.

Os resultados deste trabalho revelaram possíveis biomarcadores genéticospara a seleção para perda de água na carne em bovinos Nelore, com o intuito deproduzir carne de melhor qualidade para consumo e trazer benefícios econômicos para as fazendas brasileiras.

Palavras-chave: Produção, Bovino, RNA-Seq, Transcriptoma, Qualidade da carne, *Logissimus Dorsi*, Nelore.

RESUMEN

Estudio de expresión diferencial y análisis de enriquecimiento en tejido *Longissimus Dorsi* para evaluar la pérdida de agua por cocinado en la calidad de la carne de bovino Nelore divergentes mediante uso de RNA-Seq

Los tratamientos de cocinado de la carne es un problema mayor debido al encogimiento de los productos por la pérdida en el contenido de agua teniendo gran impacto sobre atributos de calidad en la carne como jugosidad y ternura del producto final. La carne de vacuno se caracteriza por una alta e incontrolada variabilidad de su calidad que es una de las razones de la insatisfacción de los consumidores.

Brasil es el segundo país consumidor de carne y el segundo exportador mundial de carne, la mejorar de la calidad de su carne podría abrir nuevos mercados internacionales que satisfaga a los consumidores con las implicaciones económicas que conlleva. Uno de los factores que más influye en la perdida de agua es el genotipo del animal por eso los estudios se han concentrado en mejorar la calidad de las canales en razas Nelore por ser raza más utilizada en este país y por contener genes de origen cebú que reducen su calidad de la carne en relación con las razas taurinas. Para la medida de esta característica solo se puede realizar después del sacrificio del animal lo que dificulta su mejoramiento. Gracias al desarrollo de la genómica y nuevas herramientas tecnológicas como RNA –seq la industria de la carne de vacuno está pidiendo biomarcadores musculares para predecir la capacidad de los animales para producir carne de vacuno de alta calidad.

El objetivo de este trabajo fue identificar genes diferencialmente expresados relacionados con la perdida de agua de la carne tras su cocinado en bovinos raza Nelore y por tanto encontrar posibles biomarcadores para mejorar calidad de carne mediante RNA-Seq.

En la UNESP fue secuenciado el mRNA de 20 muestras de musculo *Longissimus Dorsi* de bovino Nelore. Después del procedimiento de cocinado de 132 muestras de musculo *Longissimus Dorsi* de bovinos Nelore fueron elegidas 10 muestras que contuvieron poca perdida agua y 10 muestras de mucha perdida agua tras su cocinado. Todos los animales pertenecían al mismo grupo de manejo y fueron sacrificados el mismo día. Fueron encontrados 32 genes diferencialmente expresados relacionados con la perdida de agua por cocinado de la carne, siendo 28 genes con funciones descritas. De los genes diferencialmente expresados 25 fueron infraexpresados y 7 sobreexpresados.

Se encontraron genes relacionados con procesos biológicos como; respuesta al glucocorticoide, regulación del crecimiento celular, cascada JAK-STAT involucrada en la vía de señalización de la hormona del crecimiento, respuesta a los alimentos, así como reguladores principals como las citoquinas y MAPK, y procesos celulares como el aumento de la adipogénesis, retención de agua y sistema inmune.

Los resultados de este trabajo permitirán generar biomarcadores genéticos en bovino Nelore para la seleccion de perdida de agua en la carne para produccir carne de mejor calidad para el consumo y traer beneficios económicos a las explotaciones de bovino Nelore.

Palabras clave: Producción, Bovino, RNA-Seq, Transcriptoma, Calidad de la carne, *Logissimus Dorsi*, Nelore.

RESUM

Estudi d'expressió diferencial i anàlisi d'enriquiment en teixit *Longissimus Dorsi* per a avaluar la pèrdua d'aigua per cuinat en la qualitat de la carn de boví Nelore divergentes per mitjà d'ús de RNA-Seq

Els tractaments de cuinat de la carn és un problema major a causa de l'encolliment dels productes per la pèrdua en el contingut d'aigua tenint gran impacte sobre atributs de qualitat en la carn com a suculència i tendresa del producte final. La carn de vaquí es caracteritza per una alta i incontrolada variabilitat de la seua qualitat que és una de les raons de la insatisfacció dels consumidors.

Brasil és el segon país consumidor de carn i el segon exportador mundial de carn, la millorar de la qualitat de la seua carn podria obrir nous mercats internacionals que satisfaça els consumidors amb les implicacions econòmiques que comporta. Un dels factors que més influïx en la perduda d'aigua és el genotip de l'animal per això els estudis s'han concentrat a millorar la qualitat de les canals en races Nelore per ser raça més utilitzada en este país i per contindre gens d'origen zebú que reduïxen la seua qualitat de la carn en relació com les races taurines. Para la mesura d'esta característica només es pot realitzar després del sacrifici de l'animal el que dificulta el seu millorament. Gràcies al desenrotllament de la genòmica i noves ferramentes tecnològiques com RNA -seq la indústria de la carn de vaquí està demanant biomarcadores musculars per a predir la capacitat dels animals per a produir carn de vaquí d'alta qualitat.

L'objectiu d'este treball va ser identificar gens diferencialment expressats relacionats amb la perduda d'aigua de la carn després del seu cuinat en bovins raça Nelore i per tant de trobar possibles biomarcadores per a millorar qualitat de carn per mitjà de RNA-Seq.

En la UNESP va ser seqüenciat el mRNA de 20 mostres de múscul *Longissimus Dorsi* de boví Nelore. Després del procediment de cuinat de 132 mostres de múscul *Longissimus Dorsi* de bovins Nelore van ser triades 10 mostres que van contindre poca perduda aigua i 10 mostres de molta perduda aigua després del seu cuinat. Tots els animals pertanyien al mateix grup de maneig i van ser sacrificats el mateix dia. Van ser trobats 32 gens diferencialment expressats relacionats amb la perduda d'aigua per cuinat de la carn, sent 28 gens amb funcions descrites. Dels gens diferencialment expressats 25 fueron infraexpressats i 7 sobre expressats.

Es van trobar gens relacionats amb processos biològics como; resposta al glucocorticoide, regulació del creixement cel·lular, cascada JAK-STAT involucrada en la via de senyalització de l'hormona del creixement, resposta als aliments, així com reguladors principals com les citoquinas i MAPK, i processos cel·lulars com l'augment de l'adipogènesi, retenció d'aigua i sistema immune."

Els resultats d'este treball permetran generar biomarcadores genètics en boví Nelore per a la selecció de perduda d'aigua en la carn per a produccir carn de millor qualitat per al consum i portar beneficis econòmics a les explotacions de boví Nelore.

Paraules clau: Producció, Boví, RNA-Seq, Transcriptoma, Qualitat de la carn, *Logissimus Dorsi*, Nelore.

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Abbreviations

CL	Cooking Loss
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DE	Differentially Expressed
FPKM	Reads Per Kilobase of exon per million reads Mapped
GC	Guanine-Cytosine
LD	*Longissimus Dorsi*
NGS	Next-Generation Sequencing
PCA	Principal Component Analysis
RNA-Seq	RNA-Sequencing
SD	Standard Deviation
TEC	*Tonelada Equivalente Carcaça*
WHC	Water-Holding Capacity

I. Introduction

1. Beef cattle production in Brazil

The cattle population in Brazil consists of 218.22 million heads, being the second country with the largest amount of catlle heads (Figure 1) and the first for human consumption in the world (FAO, 2018).

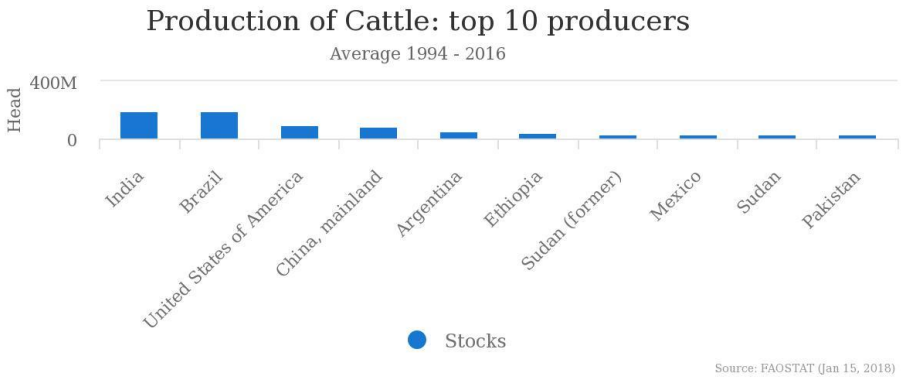


Figure 1: Production of Cattle: top 10 producers of the world.FAO 2018.

The growth trend experienced by Brazil in the number of cattle heads over the last few years has resulted in becoming the leader in word production (Figure 2) fighting with India for the first places (Figure 1). The 218.22 million heads are distributed in 196 million hectares, being 1.11 heads per hectare. (FAO, 2018).

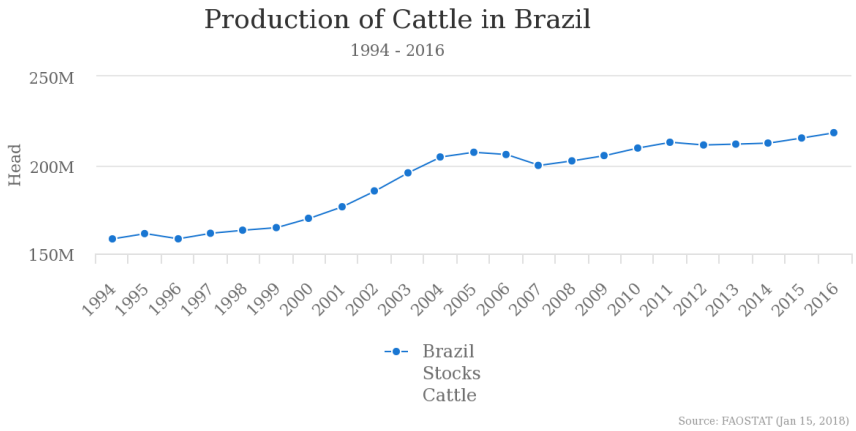


Figure 2: Production of Cattle in Brazil.FAO 2018.

According to the "Associação Brasileira das Indústrias Exportadoras de Carnes" (ABIEC), the Brazilian production of bovine meat (Equivalent Tonnes of Carcass (TEC)) was 9.56 million tons, of a total of 39.16 million head slaughtered beef cattle. Only 13% of the slaughtered animals were finished in confinement, this is due to the fact that given the extension of Brazil, it has a mostly extensive management of cattle (Figure 3).

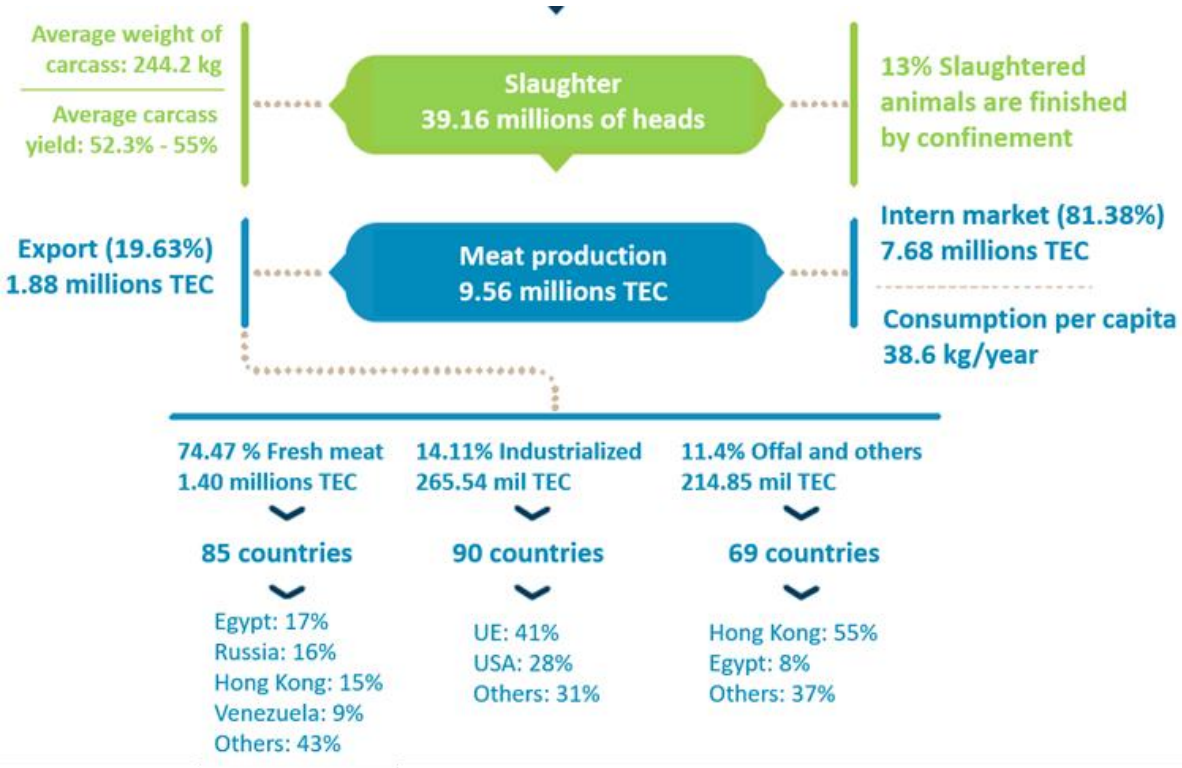


Figure 3: Cattle profile of Brazil, ABIEC 2016.

The export was of 1.88 million TEC representing the 19.63% of the total production of meat in Brazil, the internal market was responsible for the consumption of the 81% of the meat produced (Figure 3).

Eventhough Brazil only exports 19.63% of its meat production, Brazil is the second world exporter after India and followed by Australia and the USA (ABIEC, 2016). Brazil exports to the whole world, although the largest export is fresh meat, which is the 74% of its exports of beef (Figure 3). Hong Kong is the country that receives the most volume of beef, that is the 21% of the final volume of meat exported by Brazil (Figure 4).

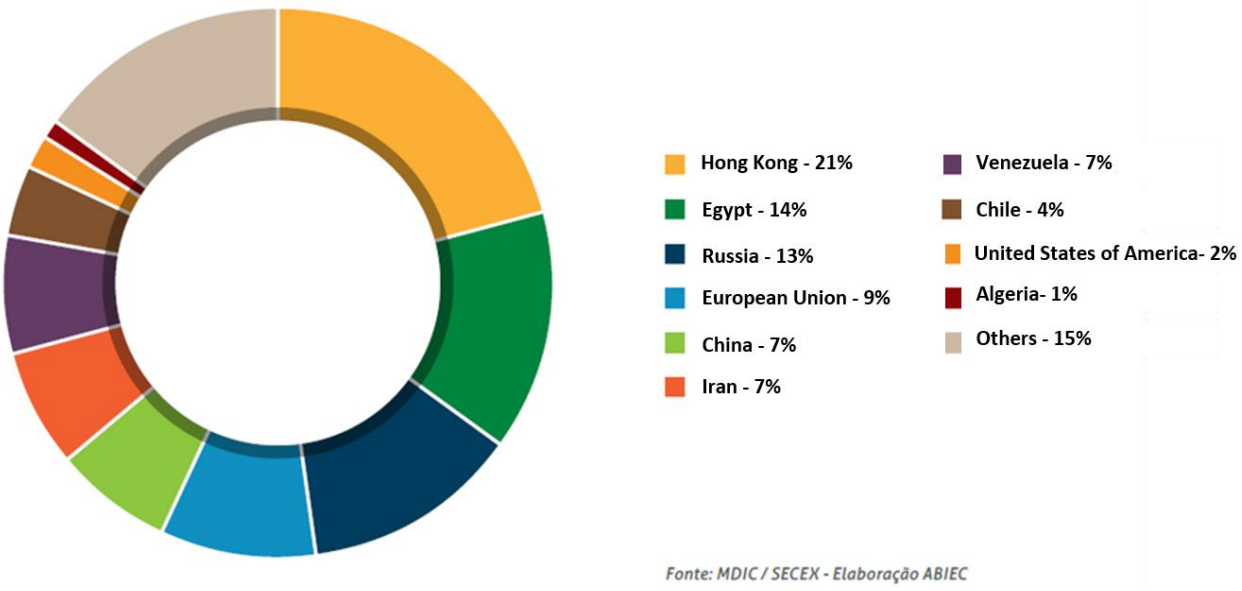


Figure 4: Main destinations of Brazilian beef exported in 2015 by volume, ABIEC 2016.

Brazil is the second largest world beef consumer, after the US and followed by China and Argentina. In addition, Brazil is the third largest beef world consumer per capita, with 38.6 kg / year after Australia and Argentina (88.3 kg / year and 64.6 kg / year respectively) (ABIEC, 2016).

2. Genetic improvement of meat quality

The need to produce meat to satisfy consumers, has made markets more and more demanding. This has generated a growing concern from the productive meat chain members, and it is also generating new researches dedicated to study factors related to the improvement of meat desirable characteristics. (Koohmaraie, M. et al., 2003). The studies have focused on identifying animals to improve the quality of specialized breeds meat with ideal standards for the consumer (Berg, R. and Walters, L. 1983; Kempster, A. 1989; Renand, G. et al., 2001; Vanek, J. et al., 2008) as well as in the improvement on the consumers´ health (Luciano, F. 2009).

In Brazil the Nelore breed is the most commonly used, about 72% of the total cattle and more than 150 million heads of this breed are destined to meat consumption (ABIEC,2016). Therefore many breeding studies are using Nelore.

2.1 Race Nelore (*Bos taurus indicus*)

The Nelore breed (Figure 5) arrived in Brazil in the middle of the 19th century brought from India. It is characterized by its excellent adaptation to tropical conditions and its rapid weight gain. Its resistance to heat makes it very suitable for handling in an extensive system, which is the predominating in Brazil. They are also resistant to skin parasites (Bianchini, E. et al., 2006, Canavez, F. et al., 2012) and have excellent maternal abilities (Cundiff, L. 2004, Bianchini, E. et al., 2006).

Figure 5: Images of the Nelore Breed. A) Profile image of a beef cattle of the Nelore breed. (Associação dos Criadores de Nelore do Brasil, 2018). B) Image of a group of beef cattle of the Nelore breed. (Apoio Genetica, 2018).

The genetic improvement for the consumption of meat is essential in this breed, since it frecuently shows genes of Zebu origin which reduce the tenderness of the meat when compared with others breeds. An example is the intramuscular fat, that is observed specifically in the Nelore breed, wich has a lower rate compared with animals of the *Bos taurus* breed (Crouse, J. et al., 1993; Leal, J. 1994; Shackelford, S. et al., 1994; Sherbeck, J. et al., 1995; Gesualdi, A. et al., 2000; Restle, J. et al., 2003; Cundiff, L. 2004). This intramuscular fat isn´t also heterogeneous, which is a problem for the meat quality of this breed, as well as for a greater hardness and little juiciness (Shackelford, S. et al., 1994; Restle, J. et al., 2003; Cundiff, L. 2004).

2.2 The texture of the meat

Texture is a sensorial property (Szczenesniak, A. 1963) and tenderness is the most valued and desired attribute by consumers. Therefore it is a factor that determines the meat quality, and there are countless factors that can influence it (Koohmaraie, M. et al., 2002).

Specifically, the tenderness lack in beef as the biggest problem encountered by the meat industry (Savell, J. and Shackelford, S., 1992). There is a positive relationship between the meat cuts and their tenderness with their respective prices in the market, and the inconsistency in its prediction is a relevant problem for the meat industry, and it is considered a highly variable and complex feature to take into account within the animal genetic improvement programs (Torres, J. and Botero, M., 2012).

In a study among cattle breeds, it was observed that approximately 46% of the tenderness variations of the beef are due to animal genetics. (Koohmaraie, M. et al., 2003). Genetic factors may explain the biological characteristics of skeletal muscle in meat, as well as its tenderness and general taste (Bernard, C. et al., 2007; Renand, G. et al., 2001). Beef is characterized by a high and uncontrolled variability on its quality, which is one of the reasons for customers´ dissatisfaction.

Therefore, the beef industry is asking for muscular markers to predict the ability of animals to produce high quality beef. Due to the development of genomics and new technological tools, some recent researches allowed the identification of a great number of these markers simultaneously, to evaluate the tenderness of the meat (Hocquette, J. et al., 2009). Brazil plays a very important role as it is the second world meat exporter, and improving the quality of its meat could open new markets, because at national level, producers are not remunerated for any meat improvements, but the international market considers it essential for the product determination. (Paz, P. and Lucchiari Filho, A., 2000).

2.3 Factors that influence the tenderness of the meat

The most determinating and important factors that affect the tenderness of the meat are the genotype, the age and the sex of the animal.

During the *post-mortem* meat there are two things taking place: softening and quality improves. That myofibrillar degradation is carried out due to the proteolytic enzymes such as calpains. It has been observed that the more calpastatin, which is a calpain inhibitor, the meat will be harder and the higher the cutting force (Geesink, G. et al., 2005). Some studies observed differences in the amount of calpastatins and calpains found *post-mortem*, among bull and cebuina breeds. Which there is greater expression of calpastatin than in European breeds, it results indicates less degradation of the myofibrillar proteins and therefore in a greater hardness of the meat (Shackelford, S. et al., 1994; Wulf, D. et al., 1996; Pringle, D. et al., 1997; Pringle, D. et al., 1999).

Other decisive factors the influence the tenderness of the meat are the length of the sarcomeres, the connective tissue, and others such as the number and diameter of the fibers, the intramuscular fat, the pH and water holding capacity and the cooking treatments.

The length of the sarcomeres influences the initial tenderness, but it is not so important during the *post-mortem* period. It has been observed that when the muscles go into rigor mortem in relaxation, the length of the sarcomeres is greater and the meat is more tender (Wheeler, T. and Koohmaraide, M., 1999).

The connective tissue is also an important factor, due the collagen amount and the cross-linking degree of the collagen molecules; the more cross-linking the harder the meat is. It is also determining metabolism muscular state in relation with the number and diameter of the muscular fibers.

The existence of intramuscular fat correlates directly with the tenderness of the meat, that is because, the more intramuscular fat, the more tender the meat. It has been observed that more juiciness the less hardness exists (Hernández, P. et al., 2000). It has also been observed that the values of shear strength and water retention capacity in the meat are important to determine the range between beef hardness and tenderness (Miller, M. et al., 2001). When treating zebu origin animals, the deposition of intramuscular fat needs to be improved since it has a low index in these breeds. (Fernandez, X. et al., 1999; Magolski, J. et al., 2013). A scale from 0 to 10 is used in order to measure the intramuscular fat. In this scale, 0 wil mean total absence of intramuscular fat and 10 will mean abundant existence of intramuscular fat. (USDA – Quality and Yield Grade, 2000) In Brazil, this scale only goes up to 6, due to the low rate of muscle fat that Brasilian cattle have. Silva-vignato, B. et al., 2017 and some other coworkers illustrate some of the involved molecular

processes in the fat and muscle deposition. In their transcriptomic study and functional enrichment analysis, it was observed how important was the MAPK (Mitogen-Activated Protein Kinase) pathway, responsible for processes such as growth, differentiation and hypertrophy in cattle Nelore.

The pH, is generally also considered, as a key factor to control the ability of the meat to contain water, which was revised by Bendall, J. and Swatland, H. (1988). When the pH is higher in *post-mortem*, the calpains activity is better and it favors the softening and therefore the ability to contain water making the meat more tender. The casing cooling and the speed of the pH decrease, will influence the quality of the product. (Felício, P. 1997; White, A. et al., 2006).

Finally, cooking treatments are very important mainly because of the reached temperatures that directly affect the proteins and connective tissue and therefore the capacity of water retention will be greater and the meat will also be more tender.

3. Role of water in the quality of meat

Meat cooking processing is a main problem since the water content of meat products is one of the quality parameters, and this is because the shrinkage of the products also has a great impact on the attributes of the texture, as well as the juiciness and tenderness (Bertram, H. et al., 2000; Lawrie, R. 1998; Oeckel, M. et al., 1999; Toscas, P. et al., 1999), and its relation with the final product yield (Bertram, H. et al., 2003; Rosenvold, K. and Andersen, H. 2003) and the involved economic implications. This is often expressed as loss of drip, loss during the cooking and loss during the cooling, depending on the stage of the process in which it was measured. The meat ability to retain inherent water is defined as water retention capacity (WHC) and the loss of water during different cooking processes (CL) (Grau, R. and Hamm, R., 1956).

Several methods have been developed for the determination of the WHC of meat, including the gravimetric bag method (Honikel, K. 1998), the NMR (nuclear magnetic resonance), the relaxation measurement (Bertram, H. et al., 2001), the filter paper press (Kauffman, R. et al., 1986), and other methods based on centrifugation (Honikel, K. 1998).

Lean meat may contain up to 75% water, with the remaining 20% of protein, and approximately 2% of fat and about 3% of secondary components (Pedersen, D. et al., 2003). The water is maintained in the spaces between the thick and thin filaments of the *post-mortem* muscle cell, while a small proportion of water in the muscle is also maintained because of the electrostatic attraction between proteins (Bond, J. et al., 2004).

This loss of drip arises predominantly from the longitudinal channels through the flesh between the fiber bundles, that is, through the capillaries of the largest pores. (Offer, G. et al., 1989). Consequently, severe water loss will reduce the product, the consumers´ acceptability and the sales value, consequently, the meat industry has a great interest in improving the WHC and the CL on the meat products (Maribo, H. et al. 1998).

Many factors affect the loss of meat water during the growth and development of animals. The genotype affects the losses of water in the meat (Uytterhaegen, L. et al., 1994), and together the animal diet, have a direct influence on the muscle characteristics. The same can be applied to the stress of the animal, the fasting, or the different methods of stunning.

On the other hand, the livestock feeding, is considered as one of the most important factors affecting the WHC and LC. Vitamin E supplementation improves the WHC and therefore reduces the drip loss in meat, other measures such as magnesium supplementation, vitamin D3, expanded sunflower seed, conjugated linoleic acid (CLA), or decreasing the amounts of digestible carbohydrates, can also reduce the WHC and LC. (Cheng, Q. and Sun, DW., 2008).

The meat is cooked before eating, during this process, some structural changes that affect the quality of the product take place. These aspects have been summarized mainly in the review of (Tornberg, E. 2005).

Davey, C. and Gilbert, K., 1974 defined cooking as heating the meat to a high temperature in order to denature or alter the proteins. The main part of cooking the meat is the water loss that is produced (Heymann, H. et al., 1990). Water is lost due to the denaturation of the protein induced by the heat during the cooking process, which causes that less water is trapped inside the proteins retained by the capillary forces.

It was seen that the losses due to cooking in bulls with the DM (double-muscled) gene, were superior to those bulls with normal conformation. In the work of (De Smet, S. et al., 2000), it is also reported higher drip losses in DM. To further investigate the meat with the effect of the presence of the homozygous (mh / mh) or heterozygous DM gene (mh / +) (Oliván, M. et al., 2004) it was also reported that the retention of raw meat was significantly lower in mh / mh (homozygous) animals, which showed higher losses. However, no differences were found in cooking losses between genotypes in these study. Several factors could explain the previous results. Since the losses in meat decrease with the amount of connective tissue (Gariepy, C. et al., 1999) a reduction in tissue content could partly explain the meat of mh / mh animals. The lower amount of intramuscular fat could be another factor that leads to higher losses from dripping of meat (Oliván, M. et al., 2004). In addition, metabolism of the glycolytic muscle more in mh / mh (homozygous) could also contribute to higher drip losses (Uytterhaegen, L. et al., 1994; Gagniere, H. et al., 1999). Another author says that DM animals are more susceptible to diseases, they are very thin and their intramuscular fat content is lower. The collagen content of the meat is lower, so the meat of animals with double musculature is mostly tender. However, the tenderness of the meat, the color and its juiciness may not always improve because of different glycolytic myofibers (Fiems, L. 2012).

According to a transcriptomic study (Hyun-Jung, K. et al., 2007) it was determined that protein metabolism, specifically mitochondrial energy, could be the key to the increase of the WHC and therefore to reduce of the CL in order to obtain more tender meat. It was identified that protein metabolism was the only dominant biological factor category among the WHC markers, detecting some ubiquitin-specific protease (USP) genes. And Eventhough a functional analysis of the genes correlated with CL was performed, no dominant functional category was detected between the gene markers.

So, the low cooking temperature, the rate of heating, and the final temperature point in the center of the beef, will result in less loss of food and its quality. However, we are eager to challenge the proper cooking methods, to mitigate the loss of food, have a good quality and safety, being efficient and economical (Qiaofen, C. and Da-Wen, S., 2008).

4. Importance of the loss of water by cooking for the quality of the meat

In the study we focused on water losses and more specifically on the loss of water by cooking, these procedures of cooking (Figure 6). The way final meat products are cooked can also affect the WHC and LC of the product, the rate of heating and cooling, cooking temperature and endpoint temperature (Qiaofen, C. and Da-Wen, S. 2008). The *Longissimus Dorsi* (LD) is generally the muscle region is generally chosen for the study method, this is because it has a high economic value in the market, and many efforts are therefore ongoing to improve their taste-related tenderness, juiciness and taste of this muscle (Moloney, A. et al., 2001).

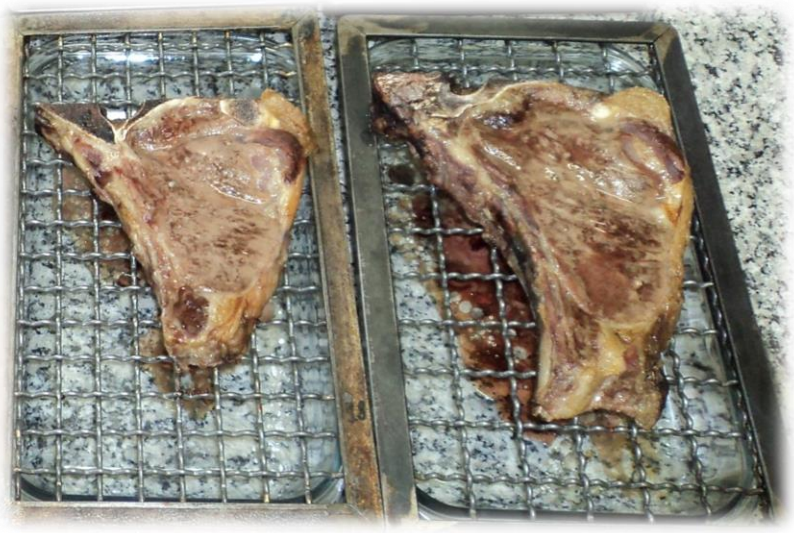


Figure 6: Samples of beef tissue *Longissimus Dorsi* after cooking in the oven at UNESP.

To measure the loss of water by cooking we have to take into account that the measurement is done post-mortem, as well as the meat tenderness measurement. This makes it difficult and expensive for the improvement programs, as well as an increase in the interval of generations.

The use of new technologies for the study of the gene expression at a global level, called high throughput, which promotes the mRNAs sequencing in platforms, and is capable of generating information about millions of base pairs in a single reading, such as RNA-Seq, bring us the possibility of understanding the molecular mechanisms involved in complex characteristics and the possible identification of potential markers (Tizioto, P. et al., 2015).

5. Transcriptomic analysis by the RNA-Seq

We currently distinguish the genetic studies in two eras (Figure 7): the pre-genomic era, in which the gene is studied individually, by in situ hybridization techniques, Northern blotting (gel-based methods) or RT-PCR (real-time polymerase chain reaction), used for the quantification of small-scale genes in meat studies of economic interest characteristics (Kelly, A. et al., 2011; Fonseca, L.F.S. et al., 2015) and / or for validation of the genes found by means of the global gene expression technique (Driver, A. et al., 2012; He, H. and Liu,X., 2013; Piorkowska, K. et al., 2015, Fonseca, L.F.S. et al., 2017).

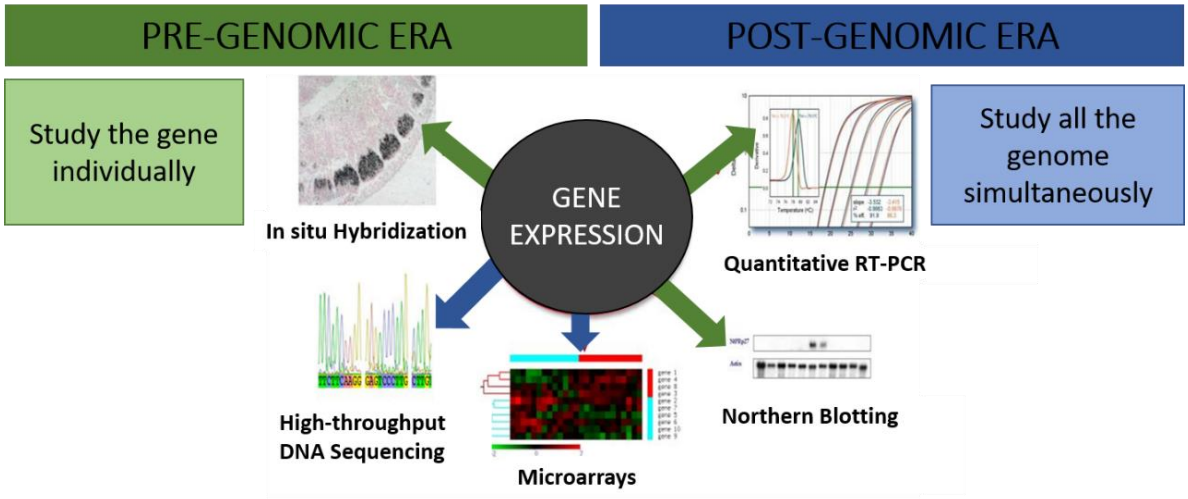


Figure 7: Tools that allow the analysis of gene expression.

The second era, the so-called post-genomic, allows to study simultaneous the whole genome. The technologies allow us to analyze expression global profiles in a single test to obtain information about all the genes from the organism, under specific conditions. We can distinguish two techniques that analyze the global gene expression (Figure 6): the massive sequencing and the microarrays. The latter are more widely used, but they have the disadvantage of having a limited identification of express transcripts in small quantities and can not detect splicing or other new genes that are not present in the microarray (Tang, F. et al., 2011). In addition, the comparison of expression levels between different experiments is very laborious and requires a complex standardization methodology. (Wang, Z. et al., 2008).

The introduction of high-performance last-generation sequencing (NGS) technologies revolutionized the transcriptomics. This technological development eliminated many challenges set out the microrrays based on hybridization and on the approaches on Sanger sequencing which had been previously used to measure gene expression. The increase of the knowledge about the action mechanisms of the genes linked to economic characteristics of interest, allow the complementation of the improvement quantitative methods (Ledur, M. 2001; Cassar-Malek, I. et al., 2008).

In this work we used the RNA-Seq technology, which consists of isolating RNA, to convert it into complementary DNA (cDNA), preparing the sequencing library and sequencing it on an NGS platform.

5.1 RNA-Seq tecnique

The RNA-seq allows to quantitatively measure the gene expression, with the identification of differentially express genes and isoforms. It is an efficient method for the identification of SNPs and, also to identify sites of splicing, which is the most important source of phenotypic diversity in eukaryotes (Malone, J. and Oliver, B., 2011), as well as being able to identify unknown genes. (Wang, Z. et al., 2008). The search for genes differentially expressed by means of this technique has been used to understand the biological mechanisms related to economic interest characteristics, in several species included in cattle. (Ramayo-Caldas, Y. et al., 2012; Jin, W. et al., 2012; De Jager, N. et al., 2011; Ramayo-Caldas, Y. et al., 2014; Tizioto, P. et al., 2015; Cesar, A. et al., 2015; Gonçalves, T. 2015; Piorkowska, K. et al., 2015; Fonseca, L.F.S. et al., 2017).

RNA sequencing (RNA-Seq) uses the capabilities of high-throughput sequencing methods to provide information about the genome transcriptome. In comparison with Sanger's previous methods of sequencing and microarrays, RNA-Seq offers a much greater coverage and a higher resolution of the dynamic nature of the transcriptome. Due the fact that it allows direct access to messenger RNA sequences, avoids variations due to hybridizations and allows the sequencing as well as the quantification of transcripts independently of their size, it also requires a smaller amount of RNA.

Recent advances in the RNA-Seq workflow, from sample preparation to library construction and data analysis, have allowed researchers to further elucidate the functional

complexity of transcription. In addition to the polyadenylated messenger RNA (mRNA) transcripts, RNA-Seq can also be applied to investigate different RNA populations, including total RNA, previous mRNA and non-coding RNA, such as mRNA and long ncRNA.

The general mechanism of RNA-Seq is summarized in Figure 8. First, the RNA is extracted from the biological material chosen (e.g., cells, tissues). Second, subsets of RNA molecules are isolated using a specific protocol, such as the poly-A selection protocol to enrich the polyadenylated transcripts or a ribo-deletion protocol to eliminate ribosomal RNA. The RNA is then converted into complementary DNA (cDNA) by reverse transcription and the sequencing adapters are ligated to the ends of the cDNA fragments. After PCR amplification, the RNA-Seq library is ready for sequencing.

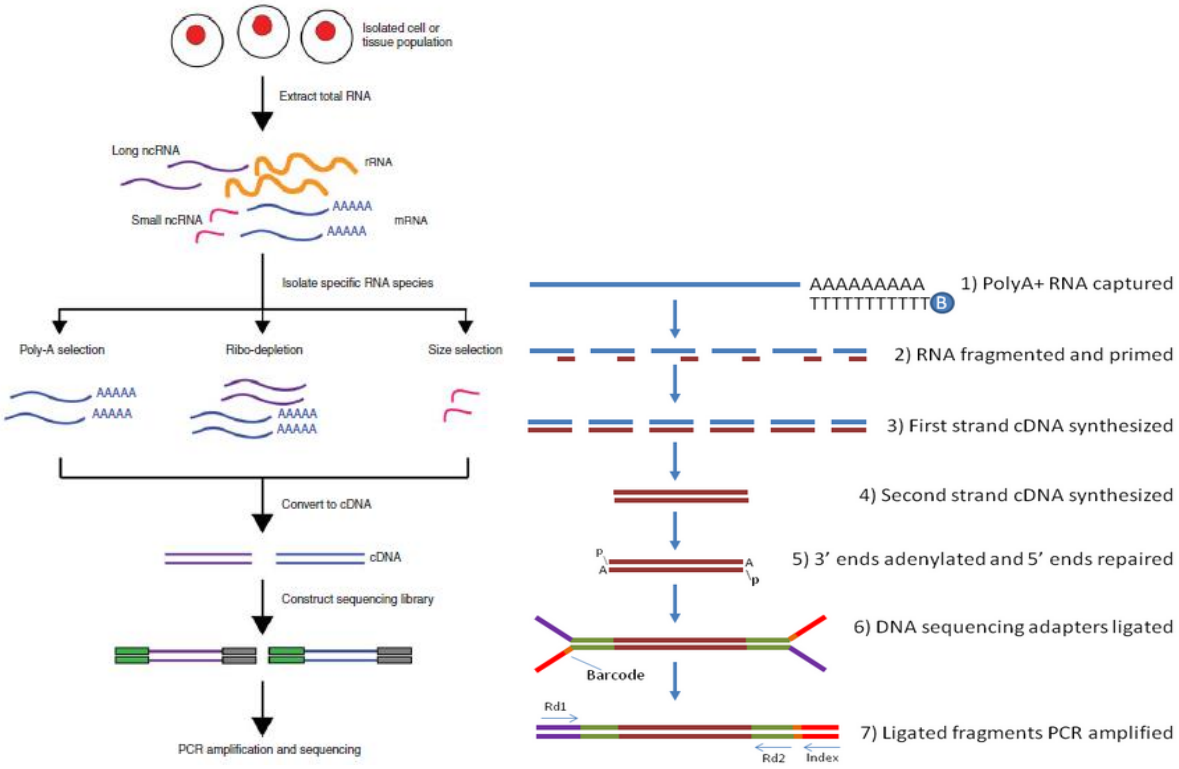


Figure 8: RNA Sequencing and Analysis. (Kukurba, KR. and Montgomery, SB., 2015).

The Solexa Illumina platform is a second generation sequencing platform in which sequencing is performed by synthesis, through the DNA polymerase enzyme and terminator nucleotides labeled with different fluorophores (Carvalho, M. and Silva, D., 2010). After the

Illumina libraries (Figure 9) are mounted with adapters on the extremities, the fragments are cloned in vitro on a solid platform (flow cell). Each flow cell has eight channels (lanes), in the first 7 lanes, samples are deposited and the eighth channel is used for the control that comes in the kit and it is essential for the calibration and normalization of the sequencing. Readings of up to 300 bp of bases can be obtained (Carvalho, M. and Silva, D., 2010; Illumina ,2017).

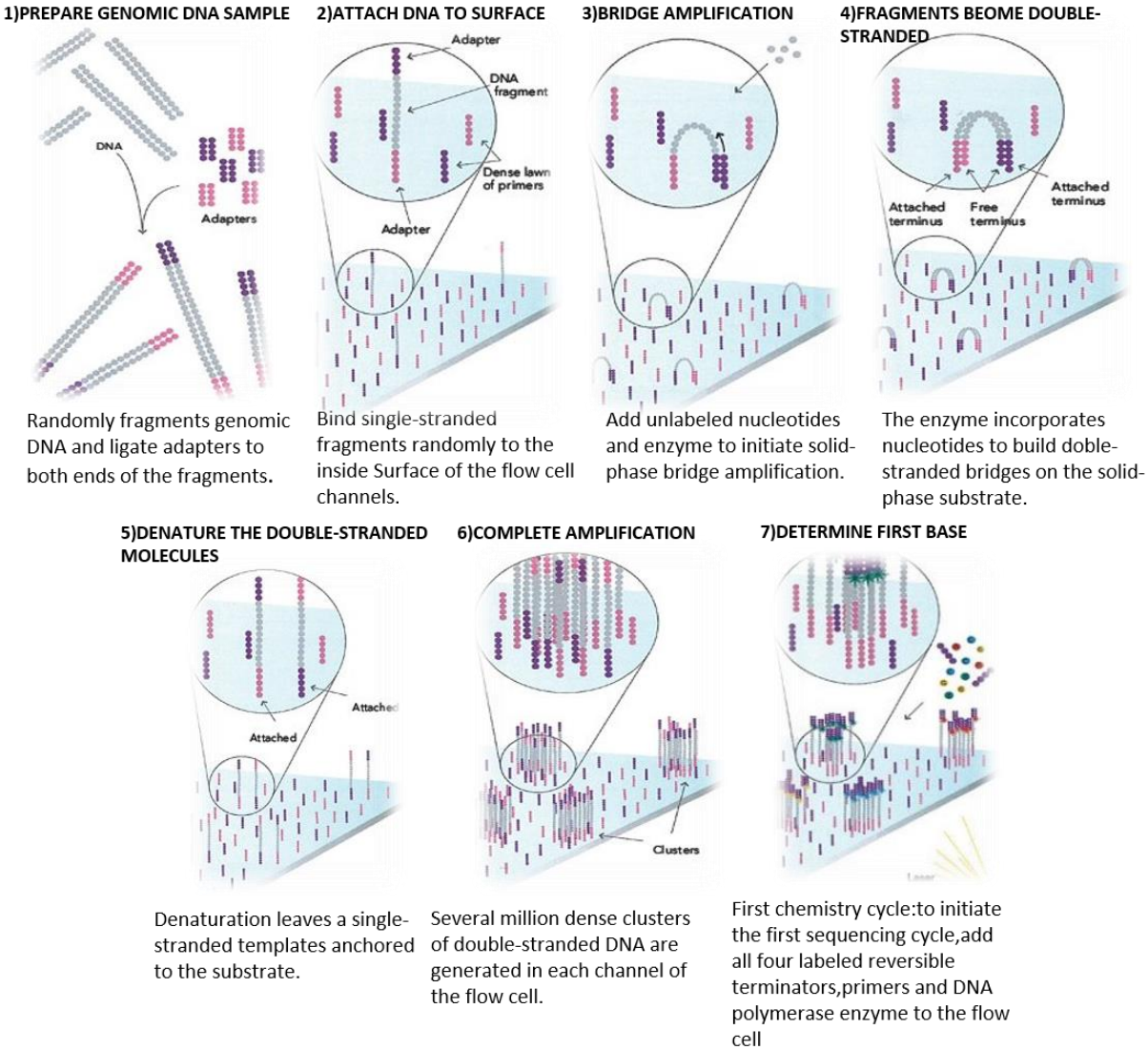


Figure 9: Construction of Illumina protocol libraries.

5.2 Analysis of RNA-Seq data

Using the Solexa Illumina Platform and after the sequencing, we obtain small nucleotide sequences, called reads, corresponding to each fragment belonging to the library of cDNA fragments obtained. The number of reads varies depending on the expression of a particular gene, that is, if a gene is more expressed, it will have more cDNA and, therefore, it is more likely to have a greater number of reads. These reads are collected in files fastq, which include, mainly an identifier, the size, the quality for each nucleotide and the sequence it self.

The first step in the RNA-Seq data analysis is the quality analysis of the reads through the fastq files. Once the corresponding quality study has been carried out, these sequences are aligned and mapped against the reference genome. If there are some samples with low quality, other programs to filter data, can also be used.

Then next step is the alignment of the transcripts. There are different tools for the alignment of the data: TopHat, TopHat2, HPG Aligner, HitSat2 ...and so on. These programs generate bam extension files. In each of these files, there is the position of each read in the reference genome, indicating the chromosome, the gene and the corresponding transcript, as well as the sequence and quality.

Once the alignment is done, we proceed to count the number of reads per gene or transcript for our sample. There are numerous tools to proceed with their respective analysis, within them, the methodology of the program Cuffdiff2 uses the T test to calculate the value of p for differentially express genes, between the two interest groups. The method assumes that the data present normal distribution (Rapaport, F. et al., 2013).

After that some files must haven been generated, from which we can extract the information of differentially expressed genes, isoforms, splicing etc. And if they exist in our study samples, we would proceed to analyze the results of the transcripts, perform analysis and general tables and figures of interest for the study as for example: vulcans, boxplot, PCA, ...and so on, for this, there are different programs available, one quite used is CummeRbund (Trapnell, C. et al., 2012), which is a package of the program R.

Finally, enrichment analyzes are carried out, for which we have at our disposal countless free and licensed programs. They are used to identify the molecular function and the biological significance of lists of genes and / or proteins from the gene expression study performed. These programs work with biological databases and analytical tools.

In Figure 10 we highlight the different free programs used today.

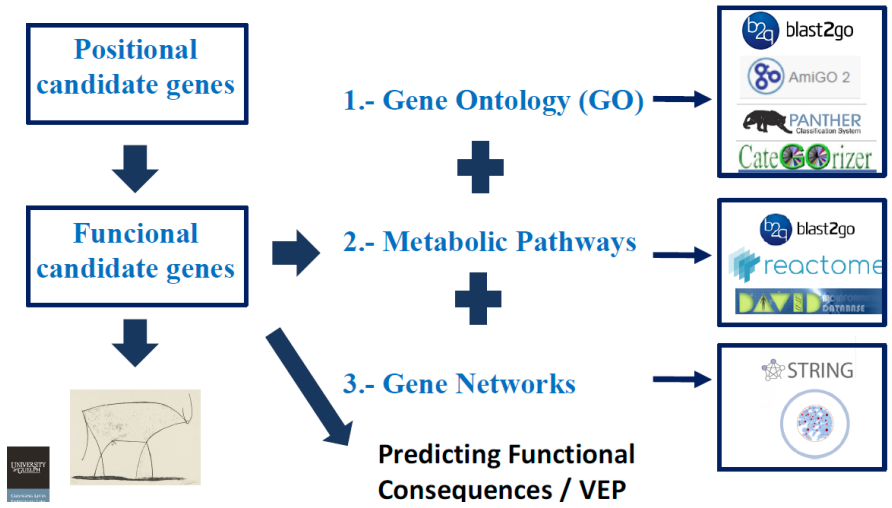


Figure 10: Fine mapping and identification of funcional candidates' genes (Prepared by the Dra Angela Canovas, University of Guelph, Canada.Course systems biology: how to integrate tructural and functional genomic data, UNESP,2018).

Out of the free programs, we can highlight Blast2go as the most robust in the analysis of the data (Figure 11)

Figure 11: Gene Ontology (GO) Analysis. (Prepared by the Dra Angela Canovas, University of Guelph, Canada.Course systems biology: how to integrate tructural and functional genomic data, UNESP,2018).

There are many types of licensed programs, each institution or platform can work with one or more. Among those I know I would recommend the Ingenuity Pathways Analysis mapping software (IPA, Ingenuity Systems, Redwood City, CA, www.ingenuity.com), and the Pathway Studio (Ariadne Genomics software, Elsevier Inc, Rockville, MD). (Figure 12)

Figure 12: Licensed programs. A) Ingenuity Pathways Analysis.B) Pathway Studio.

Objectives

II. Objectives

- **Transcriptomic analysis in muscle tissue (*Longissimus Dorsi*) of Nelore bovine to evaluate the water loss at cooking through the use of RNA-Seq (RNA sequencing).**

- **Analysis of the enrichment of the relevant genes and to obtain information on the biological mechanisms related to the loss of water at cooking in the Nelore beef meat.**

Material and methods

III. Material and methods

1. Collection of biological material

Meat samples were collected from 132 whole cattle of the Nelore breed, belonging to *Fazenda Capivara* (Piacatu, São Paulo) (Figure 13), participant of the genetic improvement program "Nelore Qualitas". We have been used 20 samples from Nelore bonives for this analysis.

Figure 13: Fazenda Capivara, ABS Pecplan,2009.

These animals belong to the same group of contemporaries, that is, the animals have experienced a similar environment regarding to the expression of a trait, from birth to death. Therefore, the results depend only on its functional genetic component.

All animals were finished in confinement for 90 days, slaughtered the same day, and in the same conditions, aged approximately 24 months and an average weight of 277 kg warm carcass.

Immediately after the sacrifice, samples were collected for the RNA extraction muscle tissue (*Longissimus Dorsi*) from the region 12th and 13th ribs of each half left carcass. The material was stored in a 15 mL falcon tube containing 5 mL of RNA holder (BioAgencia, São Paulo, SP, Brazil), conditioned in isopor with ice and transported to the Animal Science Department of the Faculty of Agricultural and Veterinary Sciences of the São Paulo State University (UNESP-FCVA) (Figure 2), Campus of Jaboticabal -SP, Brazil.

The samples were frozen at -80 ° C until the moment of total RNA extraction.

1.1 *Universidade Estadual Paulista* (UNESP-FCVA)

The UNESP-FCAV was created in 1966. It offers five undergraduate courses in Administration, Agronomy, Biological Sciences, Veterinary Medicine and Animal Science, 9 post-graduate programs, with master's and doctoral degrees, in 12 concentration areas (Figure 14).

Figure 14: Campus of UNESP-FCAV, Jaboticabal, Brazil 2018. A) panoramic view B) Main entrance.

The campus of Jaboticabal city is located in one of the richest regions of Brazil in the São Paulo State, mainly of agricultural production. The campus has an area of 828.9 hectares from which approximately 13 hectares are occupied by the physical structure, 680 hectares by agricultural activities (vegetable production 340 hectares, animal production 220 hectares, field research 120 hectares). It is surrounded by 34 hectares of parks and gardens (Figure 15).

Figure 15: The interior of the Campus of UNESP-FCAV, Jaboticabal, Brazil 2018. A) Road between buildings. B) Unesp Aquaculture Center.

Academically it is distributed in 13 departments, among which the Department of Animal Genetic Improvement stands out. The cooking processes of the samples were carried out in it and the results were subsequently analyzed (Figure 16).

Figure 16: Members of the Department of Animal Genetic Improvement, 2018.

2. Method of cooking samples

The protocol used for the cooking the samples was proposed by Wheeler, TL. et al.,1995.

The tissue samples of *Longissimus Dorsi* have 2.54 cm thickness, they were obtained between from the 12th and 13th ribs of the left half carcasses of the animals, which were first refrigerated for two days.

Before starting the cooking procedure, the room temperature was left at 16h and the samples were weighed (Figure 17).

Figure 17: Samples of LD of beef Nelore weighed before cooking.

The oven of the animal genetic improvement unit of the UNESP was preheated to a temperature of 250 ° C, and the samples were introduced with probes (Figure 18) until they reached an internal temperature of 71 ° C.

Figure 18: Samples of LD of beef Nelore with the probes. A) the oven of the UNESP with the samples of LD in its interior. B) Samples of LD of beef Nelore in individual trays with the probes to measure the temperature.

After cooking the samples, we proceeded to weigh the Longissimus Dorsi samples of beef Nelore cooked to 71 degrees (Figura 19). We proceeded to calculate the percentage of losses of water by cooking the meat (CL).

Figure 19: Samples of Nelore beef *Longissimus Dorsi* cooked at an internal temperature of 71ºC.

To obtain the Cooking Loss (CL) data as a percentage, the evaporation losses and the drip losses were added. For this, empty trays and trays with uncooked samples were weighed. After the cooking process the trays were weighed with the cooked samples and the trays with the water requests resulting from the cooking.

Therefore, to calculate the evaporation losses, the difference between the weight of the trays with the samples before and after the cooking was made. For the calculation of drip losses, the difference between the weight of the trays with the samples cooked and without samples was made.

From this analysis, 20 samples from extreme animals were selected for Cooking Loss (CL) (10 with Low CL and 10 with High CL).and used for the analysis of RNA-Seq. The Student's T test was applied using software R environment (Table 1) to verify the difference between groups.

Table 1: Loss of water in the two study groups. Number of animals (N), Mean, Standard Deviations, Minimums and Maximums of 20 samples selected for CL.

	N	Mean (%)	Standard Deviations	Minimum (%)	Maximum (%)	p-value
Low CL	10	23.07	1.39	20.16	24.46	9.3E-10
High CL	10	33.51	1.76	31.88	37.62	

3. Total RNA isolation

From the samples selected, total RNA was extracted at Laboratory of Biochemistry and Molecular Biology of the Department of Technology of the Faculty of Agrarian and Veterinary Sciences of UNESP, Jaboticabal-SP.

For this purpose, an average of 50 mg of muscle tissue previously stored in RNA holder (BioAgency, São Paulo, SP, Brazil) was used. The samples were removed from a -80 ° C freezer and transported on ice to perform the extraction.

The protocol used for total RNA extraction, is based on the method described by the QIAzol Lysis Reagent, which is based on the phenol–chloroform (Chomczynski and Sacchi ,1987). The kit used for the extraction was RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, EUA)

The tissue breakage and the homogenization were performed with 1 mL of the QIAzol Lysis Reagent solution, in the Turrax tissue homogenizer. 200 µl of chloroform were added to the samples and they were incubated for 15 minutes at room temperature. Soon after, the samples were centrifuged at 12,000 x g for 15 minutes at 4 ° C. The aqueous phase was withdrawn and transferred to another microtube, where 600 µl of 70% ethanol were added and vortex homogenized. This solution was transferred to a column and centrifuged at 8,000 x g for 15 seconds, for the elimination of the residues. In this step, the mix containing 10 µL of DNase I (1500 Kunitz units) and 70 µL of RDD buffer, components of the RNase-Free DNase Set Kit (Qiagen, Valencia, CA, USA) was added to the membrane

and the samples were incubated at room temperature for 15 minutes. To conclude, 350 µL of RW1 buffer were added and the samples were centrifuged again at 8000 x g for 15 seconds. The column was washed with 500 µl of the RPE buffer twice and the column-bound RNA was eluted in 30 µl of RNase-free water by centrifugation at 8000 x g for one minute. The total RNA samples were then stored in a freezer at -80 ° C.

4. Quality control of the total RNA obtained

4.1 Integrity of samples

The integrity of the total extracted RNA is determined with the Agilent 2100 Bioanalyzer (Agilent Technologies), which offers a wide range of analysis kits. Agilent RNA 6000 Nano Chips kit (Agilent, Santa Clara, CA, USA) was used appropriate for the analysis of RNA samples. Each chip contains interconnected microchannels that are used for the electrophoretic separation of the nucleic acids according to the size. Chip-based analyzes offer different advantages over the existing technologies, including the reduced amount of necessary sample and hazardous materials, as well as the increase in the speed of the analysis and the accuracy of the data. The preparation of this type of chips is done according to the manufacturer's instructions.

For the determination of the integrity of the total RNA, the bioanalyzer uses fluorescence between 670 nm and 700 nm. For each sample, the software calculates the integrity value RIN (RNA Integrity Number) by means of an algorithm that evaluates different integrity parameters in the electrophoretic measurements of RNA (Agilent Technologies, 2005). The integrity of the RNA is a parameter of great importance in the studies of gene expression. Traditionally it has been evaluated by calculating the 28S / 18S ratio of ribosomal RNA (rRNA), since the RNA degradation causes a decrease in the band ratio between 28S and 18S ribosomes. However, a large number of studies have shown the inconsistency of this method. The RIN algorithm takes values between 1 (highly degraded sample) and 10 (integrated full sample), and allows direct comparison of the RNA samples and guarantees the reproducibility of the experiments (Figure 20). Values of RIN, equal to or greater than 6, are considered optimal for an analysis of the whole transcriptome.

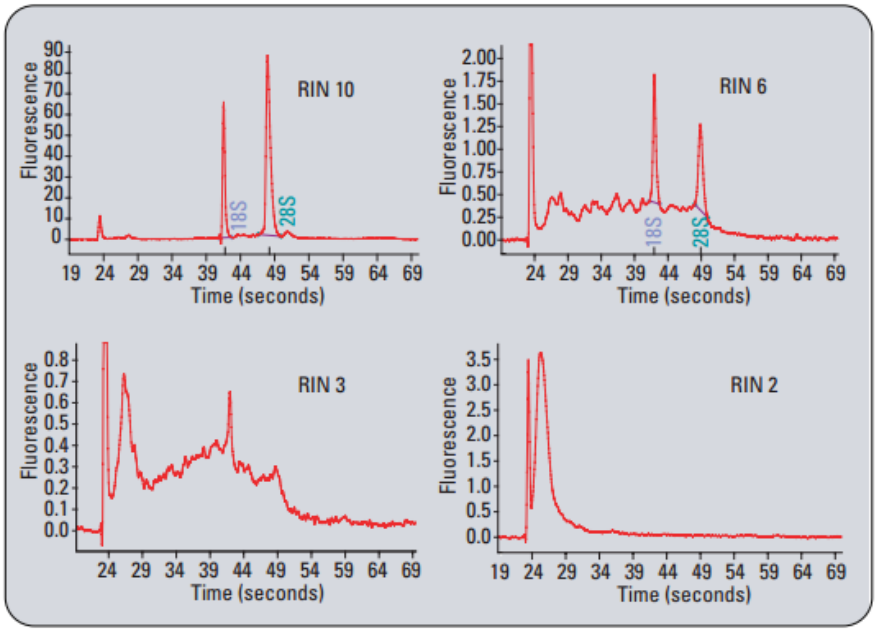


Figure 20: Example of electropherograms of RNA samples with different integrities and therefore with different RIN values. It can be observed from a sample with a total integrity (RIN = 10) to a sample with a great degradation (RIN = 2) (Mueller, Lightfoot, & Schroeder, 2004).

4.2 Concentration and purity of samples

The quantification of the concentration of RNA extracted and contamination by genomic DNA was measured by the Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA, 2010) using specific kits.

Nucleic acids efficiently absorb ultraviolet light due to the presence in their chains of nitrogenous aromatic bases. The absorption of ultraviolet radiation from DNA and RNA is a specific characteristic of these molecules, and is therefore used efficiently to determine their concentration.

The Qubit® 2.0 Fluorometer is a benchtop fluorometer for the quantification of DNA, RNA, and protein, using the highly sensitive and accurate fluorescence-based Qubit™ quantification assays. Use of the state-of-the-art dyes selective for dsDNA, RNA, and protein minimizes the effects of contaminants in your sample that affect the quantitation. Furthermore, the very latest illumination and detection technologies used in the Qubit® 2.0 Fluorometer for attaining the highest sensitivity, allow to use as little as 1 µL of sample and they still achieve high levels of accuracy, even with very dilute samples.

A determination of purity of RNA was carried out by spectrophotometer absorbance NanoDrop 1000 Spectrophotomether (Thermo Fisher Scientific, Santa Clara, CA, USA, 2007). The absorbance is measured spectrophotometrically at 260 nm (A260) and at 280 nm (A280), since while the nucleic acids have their maximum absorption at 260 nm, the proteins have it at 280 nm. In this way, observing the relationship between both absorbance values (A260 / A280) we can know if our total RNA extracted has a high purity or, if, on the contrary, it is contaminated with protein residues. An A260 / A280 ratio close to 2.0 indicates a high purity sample (Figure 21).

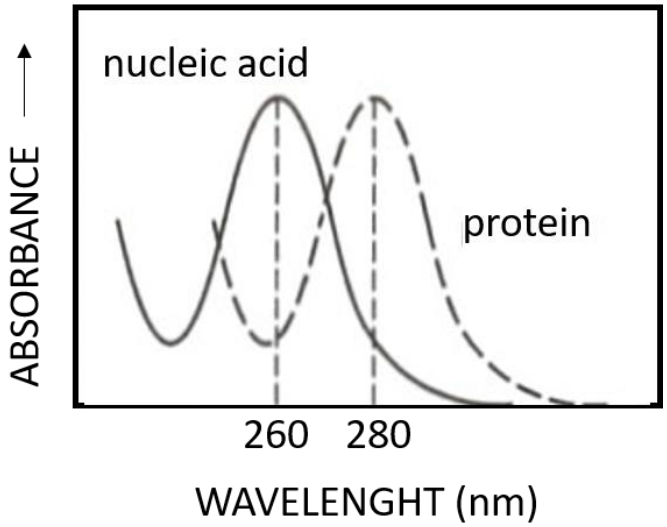


Figure 21: Graph of the absorbance in nm of nucleic acids and proteins using Spectrophotomether.

5. RNA Sequencing of new generation

The sequencing (RNA-Seq) was used to methodology "High output Run" of HiSeq 2500 System platform (Illumina) at the Biotechnology Laboratories of ESALQ / USP, Piracicaba, SP.

This methodology used flowcell wiht eight lanes. A single channel is used for the application of a check control (PHYX) which is a kit that is essential for calibrating and normalizing the run of the sequence.

From the total RNA of each sample obtained or RNA message, is used for the formation of libraries, enriched with oligo (dT) adapters linked to the extremities. Each molecule was sequenced in both extremities (paired end), with a 63X sequencing coverage. Obtain fragments of 100 base pairs (2x100).

6. Analysis of RNA-Seq data

6.1 Quality analysis and data filtering

The sequencing data generated by the HiSeq.System Illumina platform were concerned with the FastQ format and separated by libraries through the Casava software (www.support.illumina.com/sequencing/sequencing_sotfware/casava/documentation.ilmn) .

The computational analyzes were performed through the Cyverse platform (Goof, SA. et al., 2011).

The first step of our RNA-Seq data analysis is the quality analysis of our reads. For this, the FastQC program is used, a new generation of sequencing data quality control tool.

The main objective of FastQC is to provide a simple way to do some quality control checks of the reads obtained through the massive sequencing technologies (NGS). This tool provides a set of analyzes that allow us to obtain, mainly through graphics and tables, a first impression of possible problems in the reads, to be taken into account before continuing with our analysis, allowing their purification in case of being necessary.

Finally, the low quality sequenced fragments (reads) were filtered (trimmed) through the Sickle program (Figure 22) (github.com/najoshi/sickle).

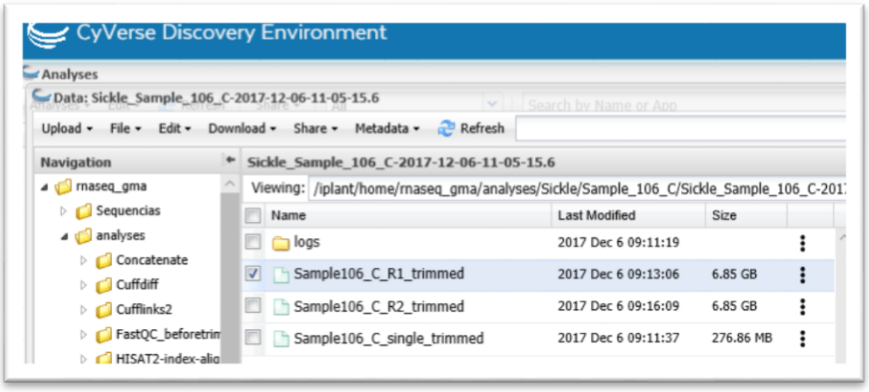


Figure 22. Example of a sample with extensión (“. trimmed").

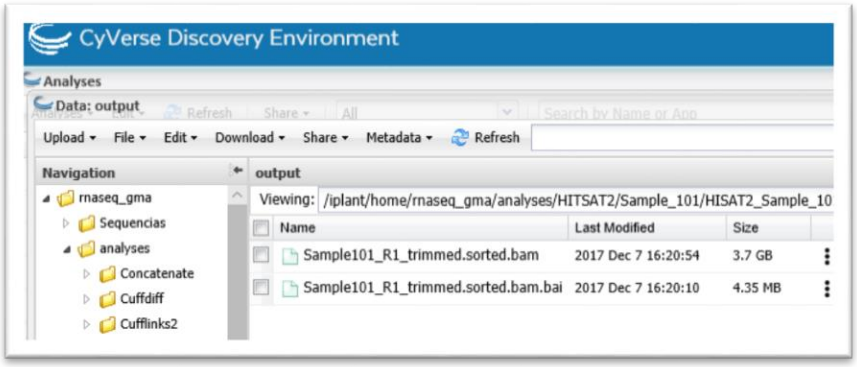
6.2 Alignment of the sequences (reads)

Later the HiSat program v2.0.9 (Trapnell, C. et al., 2009) was used to map the fragments, aligning with the reference genome (*Bos Taurus* UMD3.1), available in the NCBI data bank (http: // www. ncbi.nlm.nih.gov/genome/? term = bos + taurus). A file with extension ".bam" was generated for each library, containing the alignment of the fragments in relation to the reference genome.

Once the quality analysis of the reads was carried out, together with its respective depuration, comes the alignment and mapping of the reads against a reference genome. The fundamental objective of this step is to know the location of the corresponding reads regarding the all read mentioned reference.

The HiSat2 is a tool that allows to map reads by transcripts, among other functions, giving the possibility of discovering alternative splicing with this data. However, it may happen that in this alignment, some reads do not align / map to any sequence belonging to the reference genome, this can be either due to contamination problems of the reads or when we start from highly altered samples. TopHat tries to align these unaligned reads by means of a more precise algorithm that allows the existence of gaps in the alignment.

These tools generate extension files (". bam") (Figure 23). These are files that contain alignments data separated by tabulation. Each one of them presents the following structure by rows: name / identifier of the read, position of the mentioned read in the reference genome, indicating the chromosome in which it is located, the initial and final point of the place it occupies within the reference genome, allowing to identify the corresponding gene and transcripts; the sequence and the quality of the sequence.



. **Figure 23:** Example of a sample with extension (“. bam").

7. Transcriptional Analysis

The Cuffdiff2 v 2.1.1 program (Trapnell, C. et al., 2012; Trapnell, C. et al., 2013), was used to assemble the aligned fragments with extension (“. bam"), of each of the samples contained in a single file separated into two groups (high loss of water and low loss of water per cooking process) in beef Nelore.

The Cuffdiff2 program uses the statistical Student´s T-test to calculate the p-value. The corrections for false positive rates (FDR) were made by means of Benjamini-Hochberg methodology. A FDR less than 5% was considered.

7.1 Visualization of differentially express data

The CummeRbund package (Trapnell, C. et al., 2012), implemented in program R, was used for the exploration and visualization of the data obtained.

The flowchart of the data analysis performed for the samples can be visualized in figure 24.

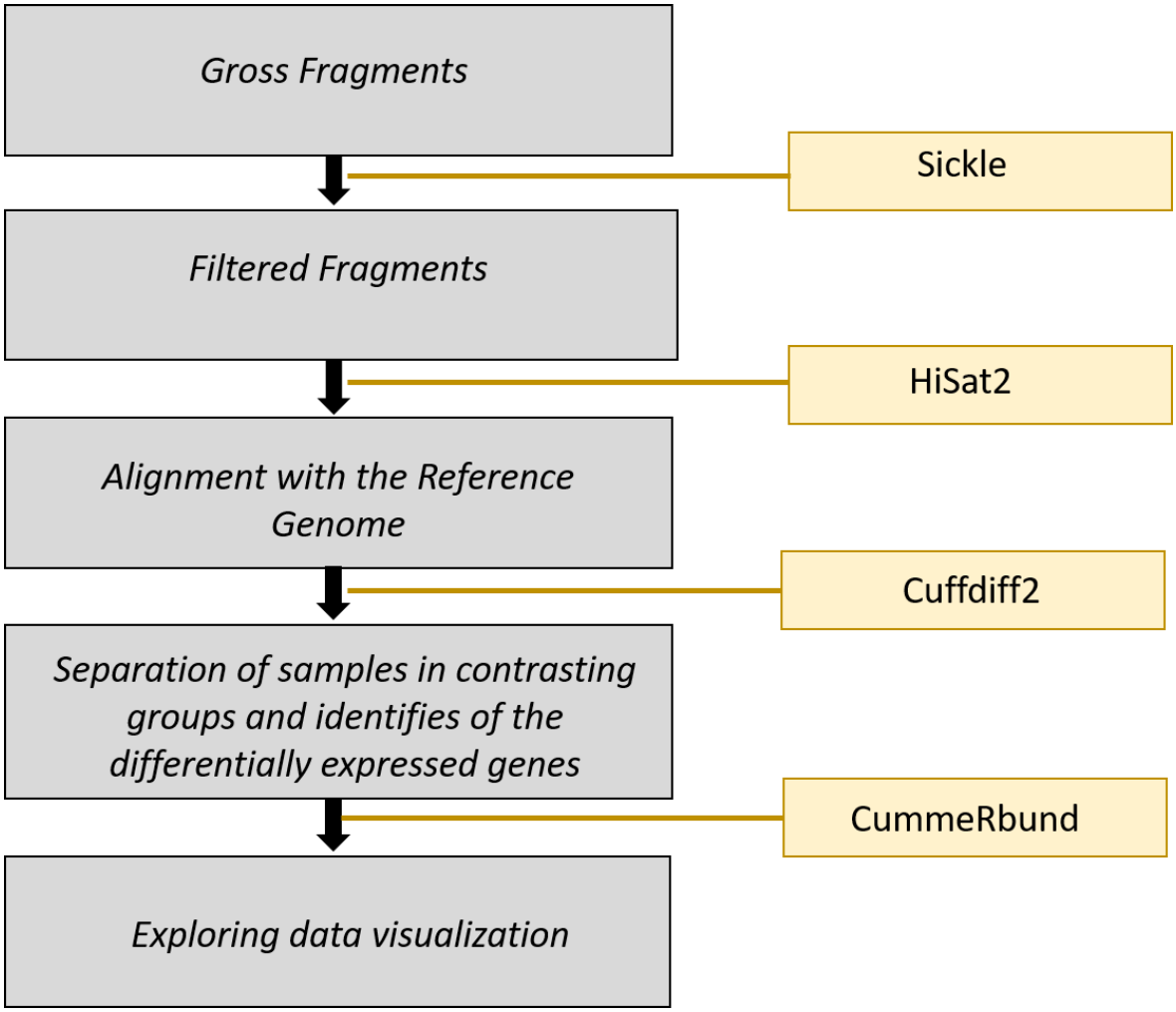


Figure 24: Flowchart used in the analysis of differential gene expression for the characteristic of cooking loss (CL).

8. Enrichment Analysis

Finally, the genes differentially expressed visualized with the Pathway Studio version 10, database resnet 11. (Ariadne Genomics software, Elsevier Inc, Rockville, MD) were used to identify the main biological processes between Low CL versus High LC and to determine main master regulators.

Some genes and biological pathways involved were checked using the DAVID v6.8 database (Database for Annotation, Visualization, and Integrated Discovery).

An enrichment p-value < 0.05 from biological analysis was used.

Results

IV. Results

1. Integrity of total RNA

Once the 20 samples of Nelore bovine LD tissue were collected, and the total RNA extraction of each of them was performed, the integrity of the samples was analysed by Agilent 2100 bioanalyzer (Agilent Technologies) to check their quality.

The electropherogram allows a visual inspection of the RNA integrity where the peaks observed corresponding to 5S, 18S and 28S. The RIN value is the parameter that integrates the area of the whole electropherogram in scales of 0-10, with values close to 10 those of greater integrity. The values obtained for the 28S / 18S ratio of each sample analyzed, confirmed that all the samples were integrated and free of contaminants, resulting in RIN values above 7.2 on average and 0.38 SD for the High CL group, for the Low CL group they were 7.5 on average and 0.40 SD. When purchasing the RIN values present in the two groups, similar and optimal patterns are observed for the study of the transcriptome (Table 3).

Table 2: RIN values of the samples. RIN (RNA Integrity Number) values obtained for the analyzed LD tissue samples belonging to Low CL and High CL.

N	Sample	RIN
1	High CL	7.3
2	High CL	6.3
3	High CL	7.5
4	High CL	7.0
5	High CL	7.1
6	High CL	7.4
7	High CL	7.5
8	High CL	7.5
9	High CL	7.1
10	High CL	7.5
	Mean High CL	7.2
	SD High CL	0.38
11	Low CL	7.9
12	Low CL	7.4

Continuation

13	Low CL	7.0
14	Low CL	7.8
15	Low CL	7.7
16	Low CL	7.8
17	Low CL	8.1
18	Low CL	7.3
19	Low CL	7.0
20	Low CL	8.1
	Mean Low CL	7.5
	SD Low CL	0.40

2. Purity and quantification of total RNA

The total RNA quantification of the 20 samples was performed using the Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA, 2010). The control of purity was performed by Nanodrop spectophotomer.

The values of the ratio A260 / A280 nm and 260/230 nm for all the samples are close to 2.00, indicating that the preparations have an optimum purity. The concentration of total RNA in the samples is similar, attributing it to a very homogeneous extraction process performance. When purchasing the values of purity and concentration of RNA present in two groups, similar and optimal patterns are observed for the whole study of the transcriptome (Table 3).

Tabla 3: Concentration and purity values of the samples. Concentration and purity values (A260 / A280 ratio) for the analyzed LD tissue samples belonging to Low CL and High CL.

N	Sample	Concentration (ng/ul)	Ratio A260/A280
1	High CL	178.6	2.08
2	High CL	158.0	2.07
3	High CL	550.0	2.12
4	High CL	93.0	2.03
5	High CL	56.0	2.09

Continuation

6	High CL	484.0	2.06
7	High CL	185.4	2.04
8	High CL	417.0	2.03
9	High CL	267.0	2.11
10	High CL	85.2	2.00
	Mean High CL	192.2	2.06
	SD High CL	176.4	0.04
11	Low CL	132.8	2.02
12	Low CL	360.0	2.05
13	Low CL	790.0	2.04
14	Low CL	620.0	2.06
15	Low CL	1000.0	2.04
16	Low CL	423.0	2.02
17	Low CL	652.0	2.02
18	Low CL	222.0	2.05
19	Low CL	110.0	2.11
20	Low CL	21.0	2,00
	Mean Low CL	356.5	2.04
	SD Low CL	302.3	0.03

3. Analysis of RNA-Seq data

3.1. Quality analysis and data filtering

We proceed to describe each of the quality checks of the study samples. As a global result, we obtained a good quality of the data, but we also decided to filter the data to ensure the quality and homogeneity of them. Ultimately, a procedure to follow is to cut the reads from those bases where we get very bad quality.

3.1.1. Basic Statistics

Figure 25 provides the name of the file. fastq, the number of reads processed, the length of those reads (they may have different size) and the percentage of GC (the content of the nucleotides GC from all the bases in all the sequences).

Measure	Value
Filename	106_C_R1
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	30560144
Filtered Sequences	0
Sequence length	101
%GC	52

Figure 25: Basic Statistics.Table of one of samples analyzed by the FastQC software.

This percentage of GC is considered acceptable when it exceeds 45%. In addition, it is advisable that the length of the reads is the same, to avoid difficulties in the rest of the quality checks. As we can see, our length is 101 nucleotides in all our samples, in addition the percentages of GC of the samples in our study are between 50 and 53%, with an average of 51.5%, therefore, they all exceed the acceptable percentages of 45%,

3.1.2. Per Base Sequence Quality

Figure 26 allows us to obtain an overview of the quality per base (nucleotide) on our reads. This is the most important quality check graph. This graphic presents a Box-Plot for each base. The red lines correspond to the median and the blue line to the average quality of the bases. The axis Y represents the quality of each base. The higher the value in axis Y, the better the quality of that base will be. The background of the graphic appears divided into three colors: in green, the zone of very good quality; in orange, the zone of reasonable quality; and in red, the area of poor quality.

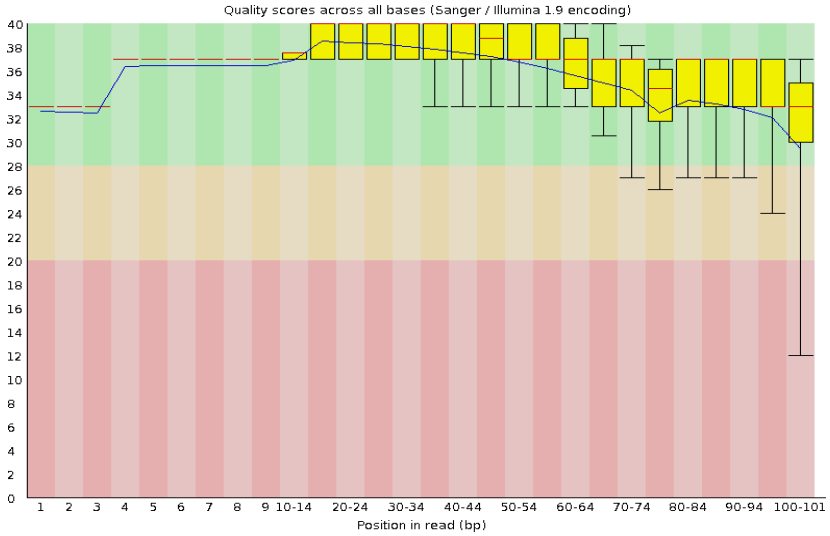


Figure 26: Per Base Sequence Quality. Vision of the quality by base (nucleotide) of a sample analyzed by the FastQC program.

We did not get a signal from the FastQC program for warning or failure for the samples.

The bases present high levels of quality, we can also observe that the smallest quartile of each level is in the green zone and they are never less than 10, and the medium ones do not take values lower than 20, therefore the program did not give a warning signal or failure.

It is common that as the sequencing progresses along the size of the read, it will make more mistakes. This is because the sequencing technology used by the Illumina platform, when incorporating nucleotides into the read, increases the possibility of error; As the process of sequencing goes on, it is more likely to fail.

3.1.3. Per Sequence Quality Scores

Figure 27 allows us to check the quality in subsets of reads. FastQC did not give a warning or failure signal.

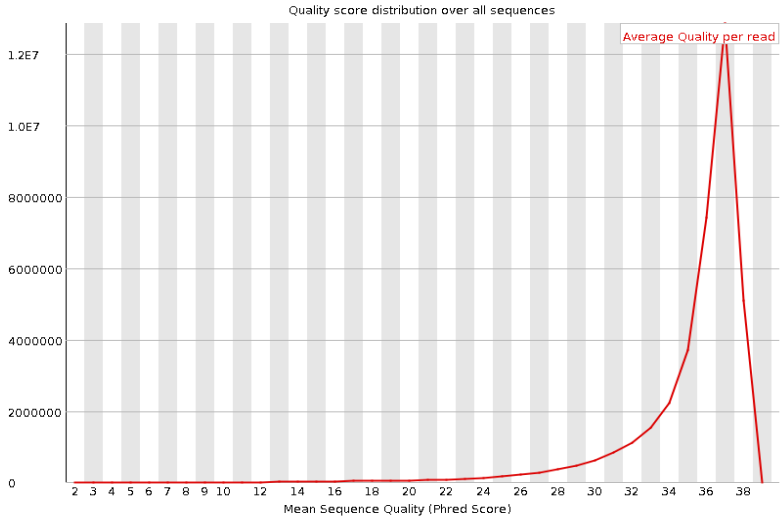


Figure 27: Per Sequence Quality Scores. Distribution of the average qualities of the set of reads.

The average quality observed more frequently was above 27, (the samples that were below this value would be equivalent to an error rate of 0.2%.) We also had no sign of failure taking into account that the given average quality most frequently observed was always above 20 (the samples that were below this value would be equivalent to an error rate of 1%).

3.1.4. Per Base Sequence Content

Figure 28 shows the proportion of DNA nucleotides (G, A, T, C) in each reads.

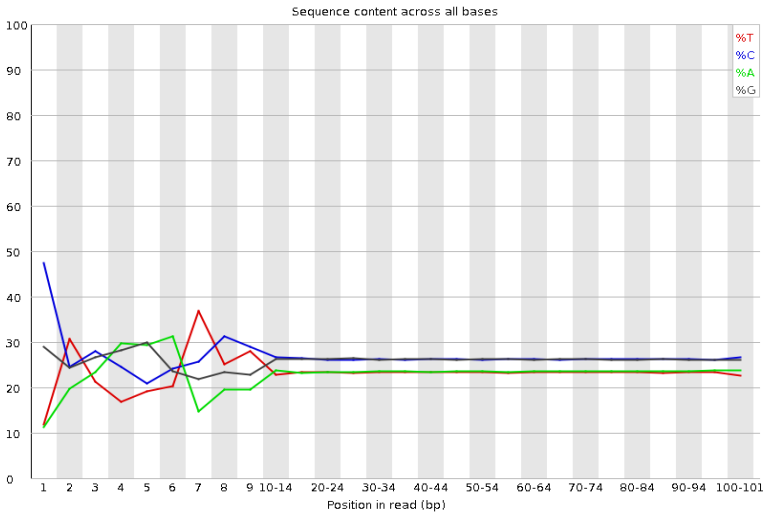


Figure 28: Per Base Sequence Content.Proportion of each base of the DNA nucleotides (G, A, T, C)

The recommendation is that the lines should be parallel. The amounts related to each base in each position of the reads should not be very unbalanced, since these quantities should reflect, in a certain way, the proportion of their bases in the genome.

FastQC gave a signal of failure since the difference exceeded 20% in every position. It is not very relevant because the filtering and correcting of the data is carried out right after. If the program had detected that it was greater than 10% in any position, it would give a warning signal.

3.1.5. Per Sequence GC Content

Figure 29 represents the average content of GC in the reads and compares that content with the normal distribution.

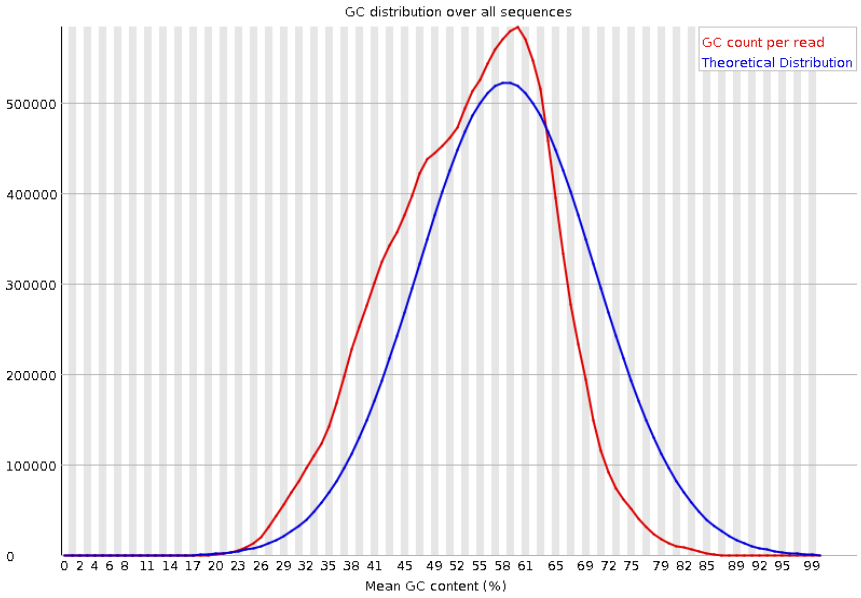


Figure 29: Per Sequence GC Content. Distribution of GC content in the reads.

We did not obtain a warning or failure signal from the FastQC program for the samples, since it is optimal to observe a practically normal distribution of GC content, with the central peak corresponding to the GC content of the genome.

The program will give a warning signal if the sum of the deviations from the normal distribution represents more than 15% of the reads, and will indicate a failure if the sum represents more than 30% of the reads.

3.1.6. Per base N Content

Figure 30 shows the proportion of N (unknown nucleotide) that is observed in each position of the reads.

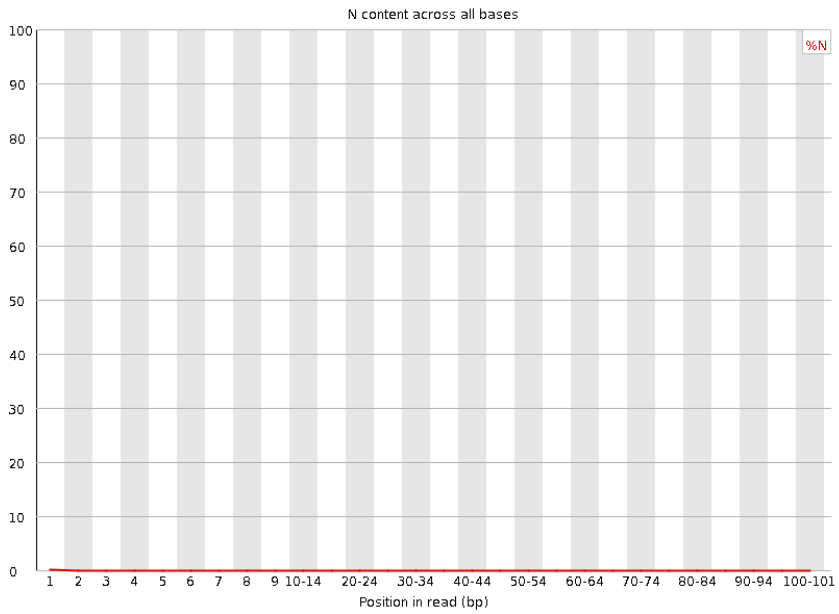


Figure 30: Per base N Content.Proportion of N in each reads.

When the sequencing tools fail to incorporate with certainty one of the bases of DNA nucleotides, they are forced to add an N in that position.

It is more likely that the appearance of Ns happens when we are advancing in the positions of the reads. This is because, as any sequencing tool advances in the procedure, there is a decrease in the quality of sequencing.

The program did not give a signal of failure or warning, as the percentage of N was less than 5% (the program warns when the percentage of N is superior to 20%).

3.1.7. Sequence Length Distribution

Figure 31 represents the size distribution of the reads.

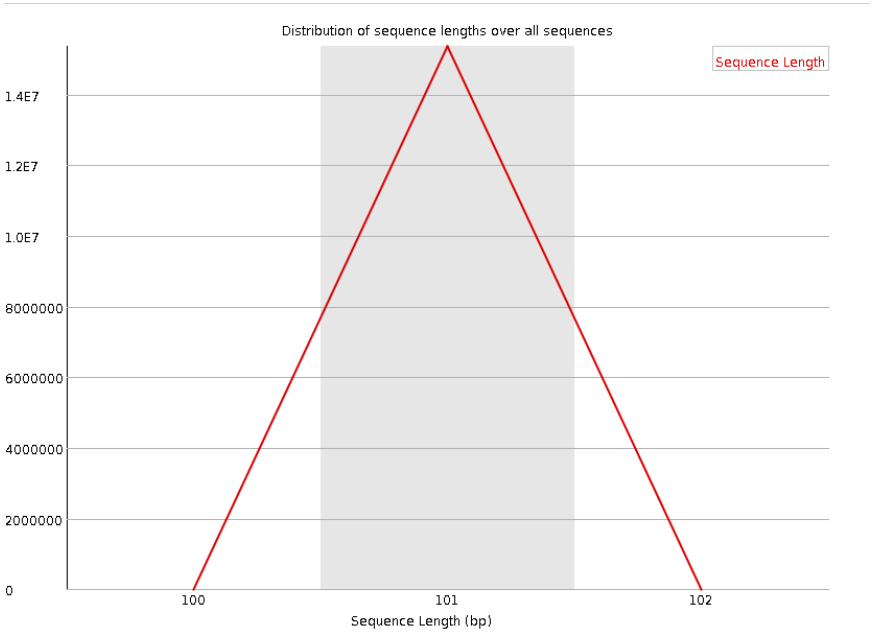


Figure 31: Sequence Length Distribution. Size distribution of the reads.

It is advisable to have reads of the same length. However, this graphic does not present special relevance because the fact of having reads of different sizes does not mean any problem for the later use of different tools.

The program did not give warning signal or failure, since all reads had the same length.

3.1.8. Kmer Content

The Figure 32 is composed of a set of sequences between 5 and 7 nucleotides that appear a greater number of times in the total of reads. In this analysis, once the mentioned sequences are determined, we can see the position that they occupy in relation to all the reads.

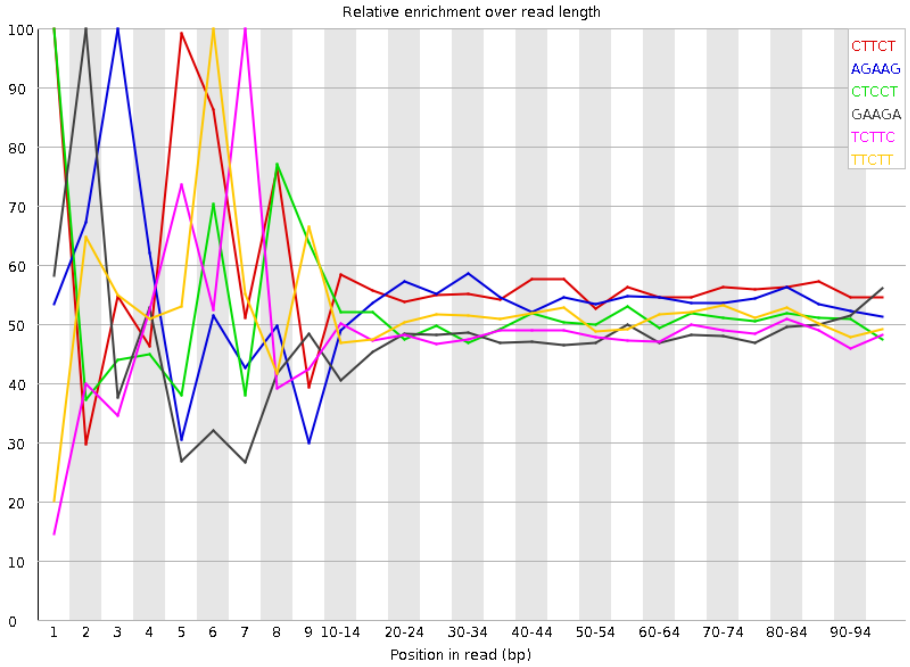


Figure 32: Kmer Content

The fact that a certain sequence appears repeated, for example, after an adapter in the majority of reads, can help us to detect possible problems in the sequencing, since the same nucleotides are being introduced in the reads after the adapter.

FastQC did not give a signal of failure, taking into account that any of the sequences that make up the graph does not appear repeated more than 5% over the length of the reads in a certain position. It didn´t give any warning signal because it also exceeded the 3%.

3.1.9. FastQC summary/ Report

We finally want to mention that in order to have a summary of the results of the quality of the samples, we must observe the summary and report (Figure 33) provided by the program to obtain an overview of the status of our data.

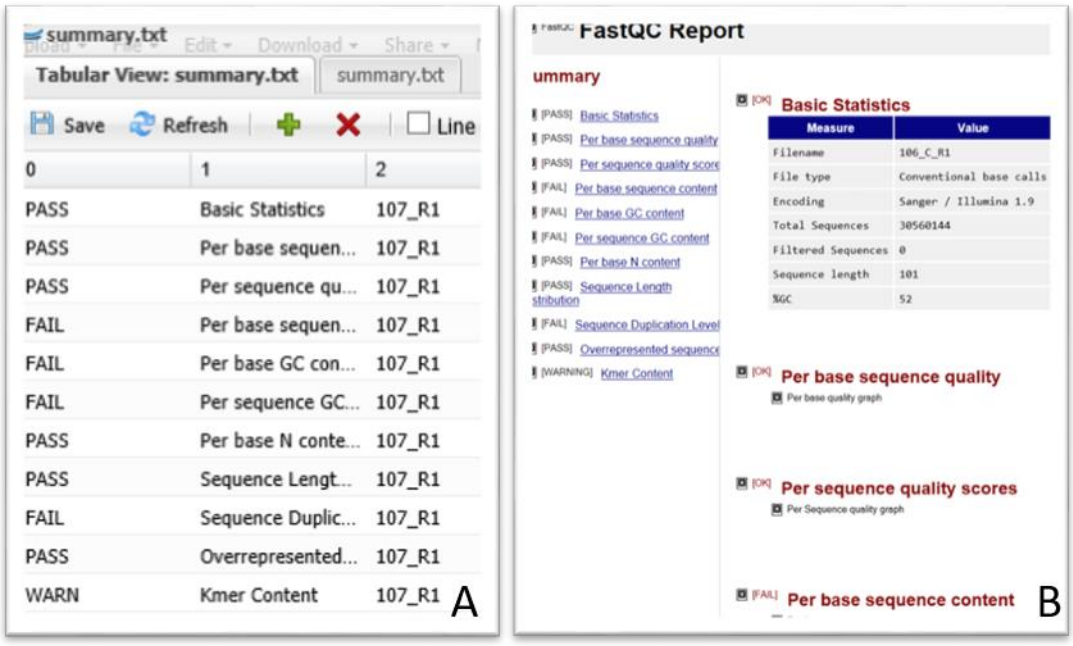


Figure 33: Summary of processes. A). Report of a sample. B) FastQC summary

3.2. Sequencing Alignment (reads).

Once the quality analysis of the reads was performed, along with the low quality sequenced fragments (reads), filtered with the Sickle program, the reads were aligned and mapped to a Bovine genome with the HitSat2 program.

In Table 4, we can see: the loss of water after the cooking of the samples, the number of aligned transcripts and the percentage of aliquoted transcripts. A total of 2.1 million reads (2x100 bp) were obtained, the coverage of the sequencing was 63X (coverage for all transcripts of all samples). The average was almost 1.6 million reads per sample, and 96.1% of the reads were mapped. A total of 24,616 genes were found. Therefore, we

obtained a good alignment of the transcripts of the study groups, giving robustness to the results.

Table 4: Alignment of the sequences. Number of sample (N), Sample classification, Cooking Loss (CL), Number of transcripts allotted in pairs (N Reads) and Percentage of transcripts allotted to pairs (% of reads).

N	Sample	Cooking Loss %	N Reads	% de reads
1	High CL	31.88	1815012	96.5
2	High CL	31.94	992569	95.4
3	High CL	32.13	4176228	95.6
4	High CL	32.30	1001703	95.7
5	High CL	33.13	755970	96.2
6	High CL	33.31	2023027	96.3
7	High CL	33.31	1640340	96.6
8	High CL	33.64	1571963	96.2
9	High CL	35.84	957113	96.2
10	High CL	37.62	1599460	97.0
	Mean High CL	33.47	1464867	96.2
11	Low CL	20.16	1833125	96.7
12	Low CL	21.63	2633029	95.4
13	Low CL	22.09	1831447	96.0
14	Low CL	22.17	805926	96.3
15	Low CL	23.66	1773799	96.4
16	Low CL	23.82	1948455	96.0
17	Low CL	23.97	1618227	96.7
18	Low CL	24.35	909486	96.1
19	Low CL	24.43	2026885	95.4
20	Low CL	24.46	1738743	96.4
	Mean Low CL	23.03	1624139	96.1
	Total Mean	27.76	1542449	96.1

3.3 Transcriptional Analysis

3.3.1 Analysis Boxplot

The Boxplot was generated by means of the cummerRbund package for the R program (Figure 34). It was observed that the distribution of the quartiles between the groups was consistent, which shows the high quality of the data, in addition, the medians between the groups were similar and close to -1 indicating that the level of sequentially coverage allowed the identification of genes with low expression (Chapple, RH. et al., 2013., Tizioto, P. et al., 2015). Therefore, the values for the two groups studied are comparable to each other and of optimum quality.

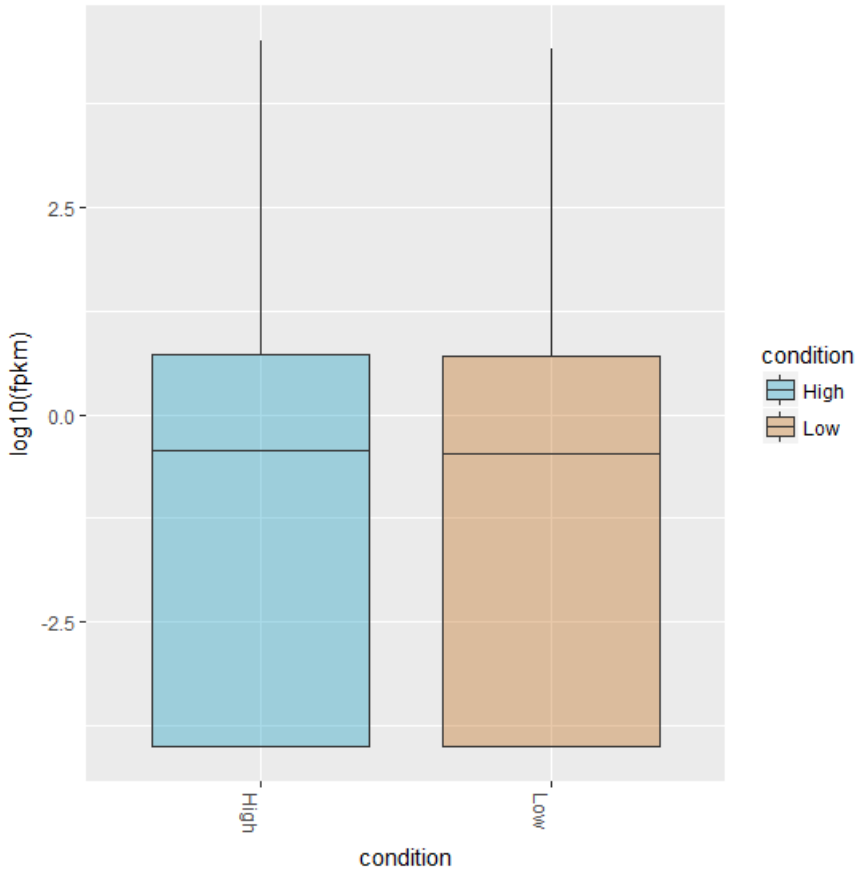


Figure 34: Boxplot of the log10 of FPKM (fragments per kilobase of exon model per million reads mapped) is a normalised estimation of the expression values for the groups studied groups, Low CL (red) and High CL (blue).

3.3.2 Principal Components Analysis (PCA)

Principal components analysis (PCA) graph was generated by means of the cummerRbund package for the program R (Figure 35).

The principal component analysis (PCA) was used to determine the significant sources of variability in the datasets. PCA reduces the complexity of high-dimensional data and simplifies the task of identifying expression patterns and sources of variability in a large dataset in a dimensional fashion.

The distance between any pair of points (represents independent variables in this case defined as groups of High CL and Low CL) is related to the similarity between the 2 samples in the high-dimensional space, that is, if the points are separated. They differ in a large number of variables and if they are close to each other, they are similar in a large number of variables. The analysis is orthogonal (perpendicular and not correlated) among themselves, avoiding redundant information (Ringnér, M, 2008).

In our analysis, we observe the space distribution from all the genes in the analyzed samples, indicating that there was a difference in the expression of some genes, between the low and high water loss groups, since the variables in both groups differed in the space.

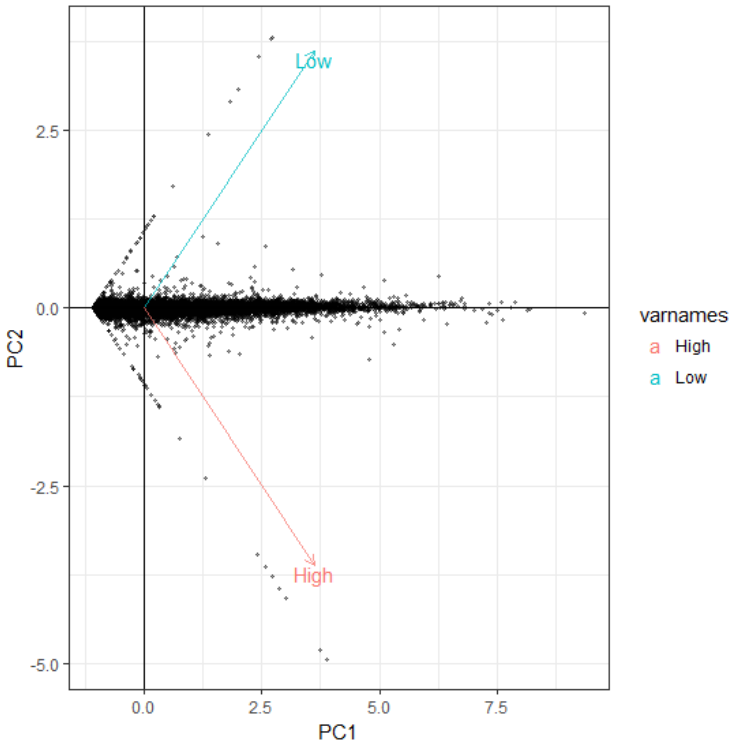


Figure 35: Principal component analysis (PCA) of the transcripts found in the Low CL (red) and High CL (blue) groups.

3.3.3 Differential Analysis

For the analysis of differentially expressed genes between Low Cooking Loss vs High Cooking Low, we used the Cuffdiff2 (Trapnell, C. et al., 2013) that robustly identified transcripts and differentially expressed genes and revealed differential changes between the Low CL and High CL population by means of the Student's t-test.

The number of differentially expressed genes obtained was 32 induced by CL beetwen them. The Table 5 represents the genes with different expression (p-value <0.05) for each of the samples studied.

Of these 32 differentially expressed genes 28 are genes well-annotated but 4 are potential new genes not annotated yet.

Table 5: Differentially expressed genes found in the samples for CL. Symbol of differentially expressed genes (Gene), location of genes in the *Bos taurus* genome, FPKM values obtained for High CL and Low CL, relative expression fold change, p-value and FDR.

Gene	Locus	CL High	CL low	log2 (fold_change)	p value	FDR<0.05
NF-M	8:73147852-73152844	0.137289	187.316	377	0,0001	0.0183769
POSTN	12:24241936-24276486	12.684	430.808	176	0,0001	0.0183769
OXT	13:52575277-52576188	37.364	107.027	152	0,0001	0.0183769
ALCAM	1:50332271-50558841	340.891	868.186	135	0,0001	0.0183769
CISH	22:50320204-50325617	111.005	226.453	103	0,0001	0.0183769
CCDC3	13:11543627-11636251	253.355	503.957	0,992141	0,0001	0.0183769
MLLT11	3:19762895-19767967	119.757	231.916	0,953491	0.0001	0.0434364
FGD5	22:58037232-58148743	366.205	220.131	-0,734289	0.0001	0.0434364
BOLA-DRB3	23:25458593-25476944	348.724	19.935	-0,806783	0,0001	0.0183769
-	23:28330538-28334072	298.626	168.895	-0,822214	0.0001	0.0434364
PPM1K	6:37876469-37898522	996.245	553.112	-0,848929	0,0001	0.0183769
TUBB6	24:43249499-43250564	235.426	12.917	-0,866001	0.0001	0.0329517
SGK1	9:73305312-73310869	152.168	834.765	-0,866219	0,0001	0.0183769
CILP	10:12099599-12114284	339.511	185.751	-0,87009	0,0001	0.0183769
MICAL2	15:41003242-41107850	132.243	692.906	-0,932464	0,0001	0.0183769
BEST3	5:43995904-44048333	81.338	416.534	-0,965497	0,0001	0.0183769

Continuation

SERPINE1	25:36198559-36206859	192.785	922.343	-106	0,0001	0.0183769
HCLS1	1:66720536-66754184	220.758	101.114	-113	0,0001	0.0183769
OTUD1	13:24655213-24656659	143.548	611.835	-123	0,0001	0.0183769
-	6:87555287-87556157	175.005	716.585	-129	0,0001	0.0183769
KIAA0226	1:70996326-71044754	368.289	134.262	-146	0,0001	0.0183769
SPP1	6:38120577-38127577	353.441	128.806	-146	0.0001	0.0329517
SOCS3	19:54458855-54459555	987.305	348.577	-150	0,0001	0.0183769
-	X:94701433-94701912	174.432	578.835	-159	0,0001	0.0183769
IFI27	21:59330562-59336752	178.284	546.978	-170	0.0001	0.0434364
GIPC2	3:66795566-66895377	46.086	134.079	-178	0,0001	0.0183769
IFI6	2:126246560-126250182	303.041	81.738	-189	0,0001	0.0183769
GEM	14:72386750-72399660	938.251	248.904	-191	0,0001	0.0183769
HBB	15:49022977-49024619	395.886	993.693	-199	0,0001	0.0183769
CCL1	19:16110979-16114069	859.592	210.378	-203	0.0001	0.0329517
FAIM2	5:30155762-30185025	232.214	0.376181	-263	0,0001	0.0183769
-	1:105171798-105172518	6.688	0.933634	-284	0,0001	0.0183769

The log2 (fold change), referring to the relative expression, was used to characterize the genes underexpressed and overexpressed, from the 32 genes, 7 were overexpressed genes 21.88%) and 25 genes (78.12%), were underexpressed. (Figure 36).

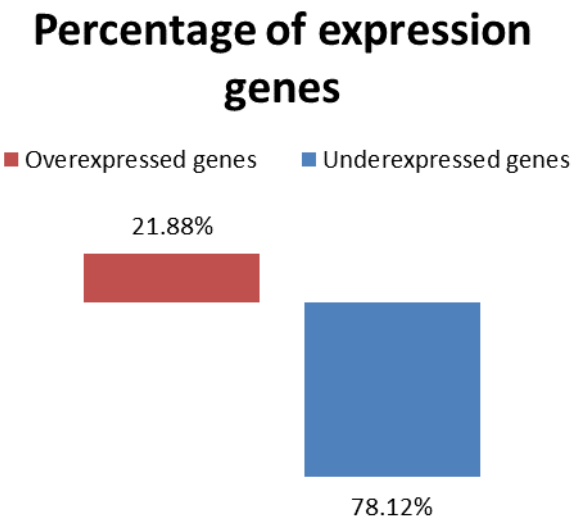


Figure 36: Differential expression for CL. Blue represents the underexpressed genes and red the overexpressed genes, obtained 25 of the 32 DE genes were underexpressed (78.12%) and 7 of the 32 DE genes were overexpressed (21.88%) for the CL.

4. Enrichment Analysis

To understand the biological meaning of the 32 significant genes among the Low CL group, compared with the High CL group, to identify the most relevant biological processes and the master regulators, we used Pathway Studio software version 10 (Elsevier Inc, Rockville, MD) with database resnet 11. The table 6 shows the 10 main biological processes and the 10 main regulators filtered in terms of p-value of enrichment.

Table 6: Top 10 biological process and master regulators in Terms of Enrichment p-value derived from the 32 significant genes using Pathway Studio v10. Genes represents the number of genes involved in each regulator or signaling pathway, belonging to the list of the 32 significant genes: NR4A3 : Nuclear Receptor Subfamily 4 Group A Member 3,IL3: Interleukin 3,LEF1 : Lymphoid Enhancer Binding Factor 1,PRKG1: Protein Kinase, CGMP-Dependent, Type I,Cytokine :small proteins important in cell signalling,GDF2 :Growth differentiation factor 2,SLC39A1: Solute Carrier Family 39 Member 1,EDN1 : Endothelin 1,MAPK3: mitogen-activated protein kinase 3,ROCK1: Rho Associated Coiled-Coil Containing Protein Kinase 1.

Low CL Versus High CL	No. Of Genes[a]	Genes	p-value
Biological Process			
Response to glucocorticoid	4	CCL1,OXT,SOCS3,SERPINE1	3.1E-06
Regulation of cell growth	3	SOCS3,CISH,SGK1	4.2E-05
JAK-STAT cascade involved in growth hormone signaling pathway	2	SOCS3,CISH	1.3E-04
Response to food	2	OXT,SOCS3	1.7E-04
Response to progesterone	2	OXT,SOCS3	6.6E-04
Positive regulation of leukotriene production involved in inflammatory response	1	SERPINE1	7.1E-04
Positive regulation of hindgut contraction	1	OXT	7.1E-04
Negative regulation of vascular wound healing	1	SERPINE1	7.1E-04
Negative regulation of collateral sprouting of intact axon in response to injury	1	SPP1	7,.E-04
Cell response to gravity	1	SERPINE1	7.1E-04
Master Regulators[b]			
NR4A3	4	CCL1,SPP1,OXT,SGK1	3.8E-06
IL3	6	POSTN,SPP1,SOCS3,CISH,SERPINE1,ALCAM	4.1E-06
LEF1	5	HCLS1,POSTN,SPP1,SGK1,FAIM2	4.3E-06
PRKG1	3	SPP1,SOCS3,SERPINE1	5.4E-06
Cytokine	1	HCLS1, POSTN, NEFM, CCL1, SPP1, OXT, SOCS3, CISH, SGK1,SERPINE1,ALCAM	1.3E-05
GDF2	4	POSTN,NEFM,SPP1,SERPINE1	2.0E-05
SLC39A1	2	POSTN,SPP1	2.4E-05
EDN1	6	SPP1,SOCS3,SGK1,PPM1K,SERPINE1,ALCAM	2,.E-05
MAPK3	7	POSTN,BEST3,SPP1,SOCS3,SGK1,SERPINE1,ALCAM	4.1E-05
ROCK1	4	POSTN,SPP1,SOCS3,SERPINE1	4.7E-05

a The number of genes that belong to each gene list that are involved in each signaling or network.
b Expression Targets of Master Regulators

In figure 37 we show the subnetworks of the differentially expressed genes filtered by the amount of genes involved. Therefore, it is observed, in the first and third place, that the majority of the differentially expressed genes are integrated in the Cytokine signaling pathways, and in the second and fifth place, differential genes are observed in the MAPK signaling pathways. Both the Cytokine signaling pathways and the MAPK signaling pathways emerge as relevant in the enrichment analysis filtered by the amount of differential genes involved, filtered by enrichment p-value (Table 6) and filtered by the quantity of differential genes (Figure 37).

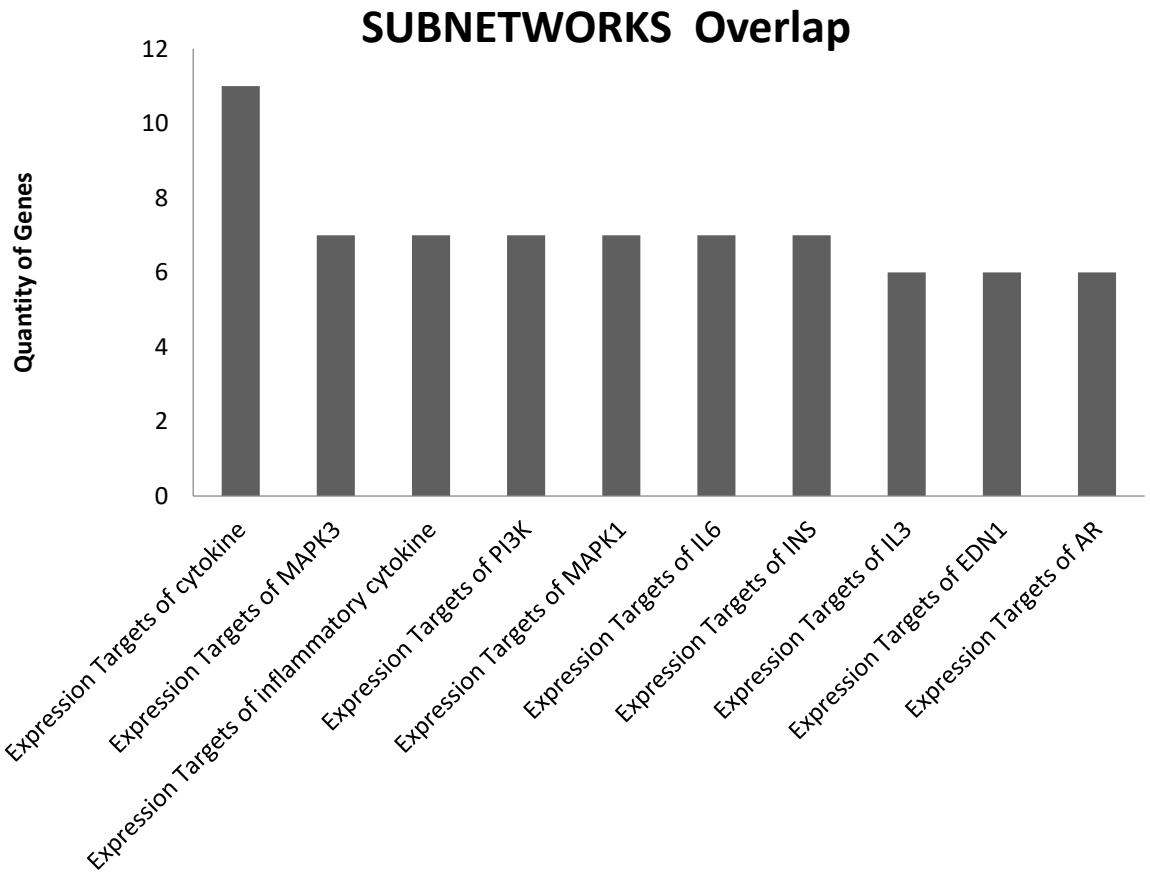


Figure 37: Graph subnetworks of our differential genes filtered by overlap.

We used Software David v6.8 (Database for Annotation, Visualization, and Integrated Discovery) using *Bos Taurus* as a reference, to compare different databases in the enrichment analysis, with the results obtained by the software Pathway Studio v.10.

Using the David v.6.8 software, a functional annotation analysis was performed, using all differentially expressed genes scored for the studied CL characteristic. We found 3 functional groups ("Annotation clusters" -Annex 1). These genes were classified according to their function. The differentially expressed genes involved in the biological pathways were also analyzed ("Functional annotation table" - Annex 2). The results of software David v6.8 were similar in term of biological processes as Pathway Studio v.10 were similar.

In Figure 38 we can observe the differentially expressed genes involved in the 10 main biological processes and in the 10 main master regulators filtered by the enriched p-value. Seven genes differentially expressed in common were found: CCL1 (Chemokine ligand 1), OXT (Oxytocin), SOCS3 (Suppressor of cytokine signaling 3), SERPINE1 (Plasminogen activator inhibitor-1), SGK1 (Serine/threonine-protein kinase), SPP1 (phosphoprotein 1), CISH (Cytokine-inducible SH2-containing protein) of them, three genes: OXT, SOCS3, SERPINE1 are directly involved in 9 of the 10 main biological processes and 8 of the 10 main regulatory masters. Therefore, we emphasize the importance of deepening in the study of these genes, as potential biomarkers candidates for the water loss in Nelore bovine LD as an improvement in the meat quality.

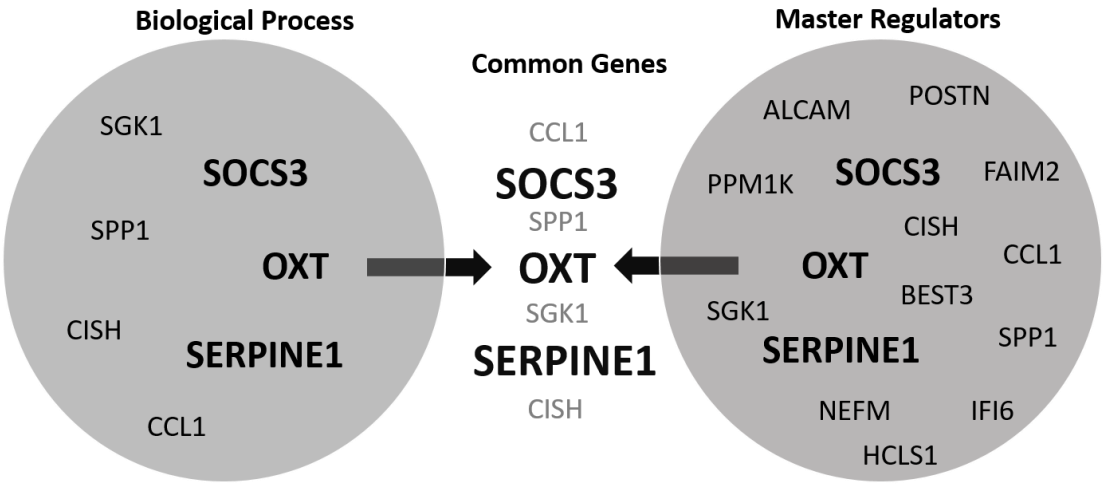


Figure 38: Genes overlapping. The sphere on the right, represents the genes differentially expressed in the 10 main master regularos and 10 main biological processes filtered by the enrichment p-value. The common genes are represented in the centre.

In Figure 39, we show the cell processes of the three differentially expressed genes for the CL study characteristic. We observed that the three genes (OXT, SOCS3 and SERPINE1) are related to the immune system and to the regeneration of cell damage. We highlight that the OXT is directly involved in the increase of water retention and development of muscle fibers, by stimulating the recruitment of glucose in the skeletal muscle cells.

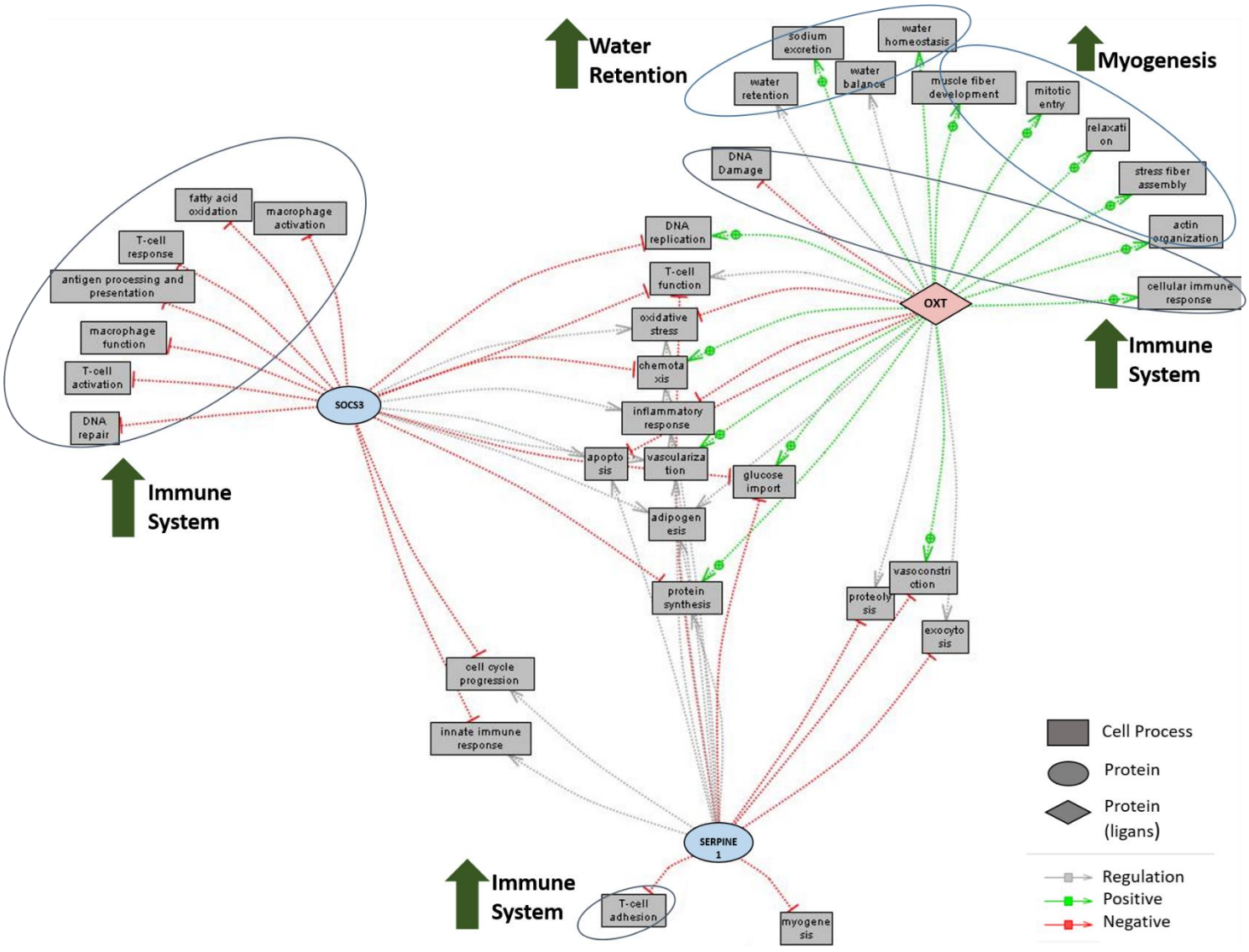


Figure 39: Cell processes of the three genes that are candidates for biomarkers. The red sphere represents overexpression and the blue sphere underexpression of the differentially expressed genes in the study of the Low CL vs High CL groups.

In Figure 40, we represente the cell processes in common to the three differential genes of interest. We observed that the three genes are involved in important cell processes in the deposition of fat in the muscle, such as the adipogenesis and protein synthesis, as well as in the amount of glucose, immune system and protein synthesis.

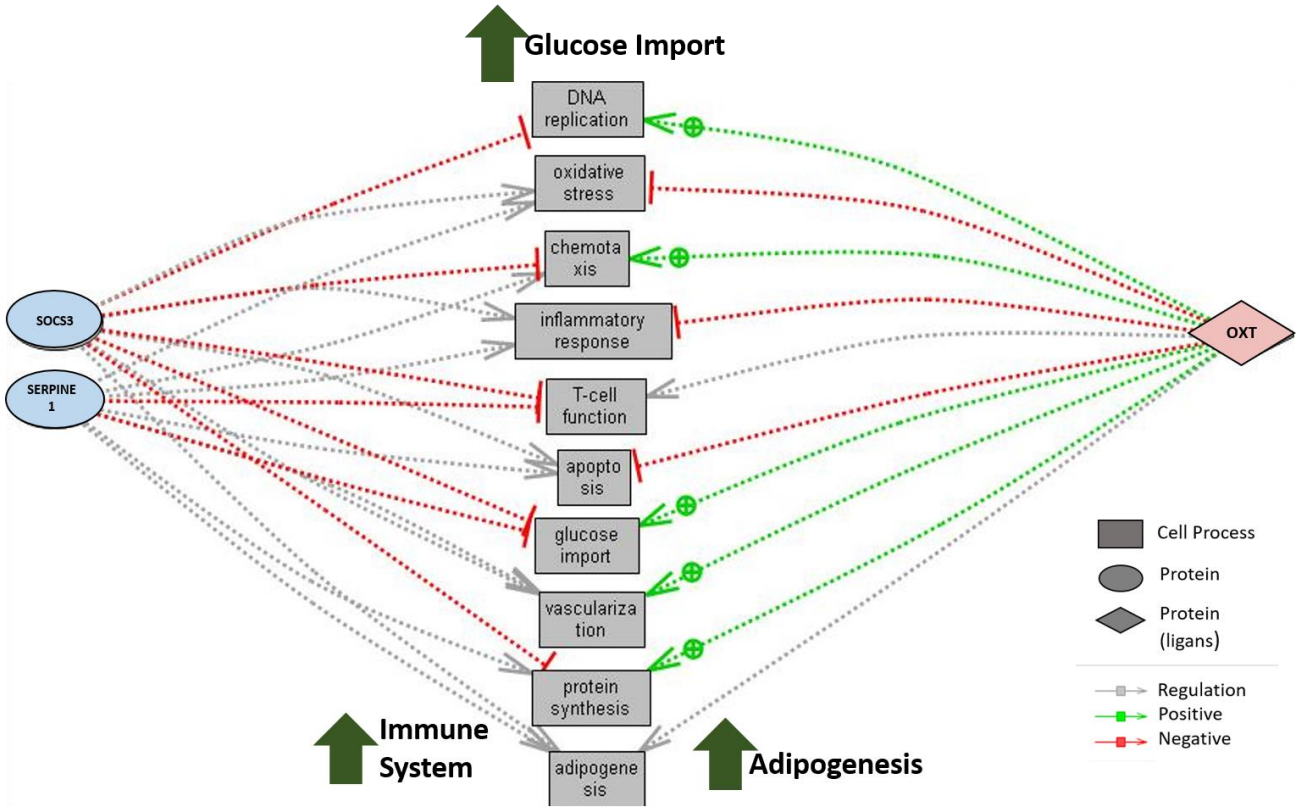


Figure 40: Common cell processes among the three genes which are candidates for biomarkers. The red sphere represents over expression and the blue colored sphere under expression of differentially expressed genes, in the study of the Low CL vs. High CL groups.

5. Discussion

There are numerous factors affecting the loss of water from the Nelore beef , during the growth and development of the animals, the one that has an importance of 46% its genetic value for meat quality .traitsThis fact makes the search for genetic improvement of beef in cattle very relevant , besides the Nelore breed, since is the most used in Brazil for the human consumption of meat and it presents a greater variability in its meat quality, being a problem for the consumer and the cattle production. All this makes the search of biomarkers a very important fact to characterize and improve the skeletal muscle obtaining a higher quality of the Nelore beef.

One of our hypotheses was to show that the group with the least water losses could be the interest group, since the greater retention of water after cooking, would give better quality to the meat, satisfying the consumer and increasing the economy of the bovine sector from the Nelore breed in Brazil.

According to the differential and enrichment study, we found 32 differentially expressed genes found, were involved in 10 relevant biological processes as:response to glucocorticoid,regulation of cell growth,JAK-STAT cascade involved in growth hormone signaling pathway,response to food,response to progesterone,positive regulation of leukotriene production involved in inflammatory response,positive regulation of hindgut contraction,negative regulation of vascular wound healing,negative regulation of collateral sprouting of intact axon in response to injury,cell response to gravity. And the 10 most important master regulators were: NR4A3, IL3, LEF1, PRKG1, cytokine, GDF2, SLC39A1, EDN1, MAPK3.

From the 32 differential genes, we found that 3 genes (OXT, SOCS3 and SERPINE1) involved in all biological processes and more relevant master regulators studied.

OXT

The gene OXT (Oxytocin-Neurophysin I, Preproprotein), is synthesized in the hypothalamus and stored in the back apophysis, it is water-soluble and that is why it is freely transported through the blood. This gene was the third one that most overexpressed in our differential study when in contrasted the group Low CL vs High CL.

It has been observed that the increase in water retention due to the OXT is much greater when other hormones are compared (Goldenberg, S. et al., 1983) as well as the

sodium secretion (Chae, HE. et al.,1998; Hatanaka, K. et al., 2006; Rinaman, L. et al., 1997; Stier, CT. et al., 1980). Therefore, it is a good regulator for the hydro-corporal balance (Chou, CL. et al., 1995; Hussy, N. et al., 2001; Murphy, D. et al., 1998) and it also participates in the osseous density and the appetite (Evans, SL. et al., 2014).

The oxytocine is also involved in some lipid response biological processes (Carbon, S. et al., 2009), taking part in the adipogenesis. It also participates in the storage system and in the lipids and water energy consumption in processes such as the lipolysis, excretory activity and plasticity (Fonseca – Alaniz, MH. et al., 2006).

The role of the oxytocine in the proteolysis is because of the calpain, which causes it to release oxytocine (Puliyanda, DP. et al., 2005). Orwig, KE., 1994 and his coworkers show that when the oxytocine increases, the calpains and the calpastatins action also increases. Nevertheless, it has been observed that the oxytocine mostly increases the proteins synthesis (Devost, D. et al., 2005; Devost, D. et al., 2008; Ma, D. et al., 2002; Petersson, M. et al., 2002) This has a direct implication in the actin organization (Wang, YF. And Hatton, GI., 2007) a protein which together with the myosin are determinants for the formation of the miofilaments which take part in the muscle contraction presenting between a 52% and a 56 % of the muscle proteins (Sgarbieri, VC. 1996).

The oxytocine influences the development of the muscle fibers, stimulating the glucose up-take in the skeletal muscle cells (Alizadeh, AM. And Mirzabeglo, P., 2012; Altszuler, N. et al., 1992; Noiseux, N. et al., 2012) and it stimulates the myoblasts blending and the myotubes formation (Gajdosechova, L. et al., 2014). De Jager, N., 2011 and his coworkers also relate the OXT with the formation of skeletal muscle and the muscle contraction.

The oxytocine participates helping the T cells, as well as, to the regeneration of the DNA damage from the gastric mucosa (Işeri, SO. et al., 2008) and the DNA replication (Gavrilenko, VG. et al., 2000).

It was also observed as the most overexpressed gene in the differential study related with the meat tendernes of the LD tissue in the Nelore beef cattle (Fonseca, L.F.S. et al.,2017 and it appears differentially expressed in another beef study (De Jager, N. et al., 2011).

SOCS3

The gene SOCS3 (Suppressor of cytokine signaling 3), also known as the suppressive of cytokines markers. This gene is underexpressed in the group low CL.

SOCS 3 plays an important role in the regulation of the fatty acids oxidation, (Luo, B. et al., 2011). The fall of the Socs3 can increase the fatty acids oxidation, and therefore the decrease of the lipogenesis (Gu, H. et al., 2009; Ye, J. et al., 2012). That means that the SOCS-3 causes adipogenesis and overweight (Ullah, M. et al., 2013; Zheng, RD. et al., 2013). It has been seen that besides the rise of the adipogenesis, the osteogenesis decreases (Li, J. et al., 2012). Ye, Y., 2014 and cooperators showed that if the gene SOCS3 was taken away, this influenced on the insulin resistance and on account of this, the adipogenesis was reduced. The overexpression of the SOCS3 on the fat cells (adipocytes) decreases the glucose absorption stimulated by the insulin in the adipocytes and damages the lipogenesis, which results in obesity resistance, as well as in resistance to the insulin adipocyte caused by a high fat diet (Singhal, NS. et al., 2007).

It was demonstrated that the expression of SOCS-3 affects the answer of the T cells (Kinjyo, I. et al., 2006; Knosp, CA. et al., 2011) prevents the arthritis development induced by the collagen in rats (Kuppan, G. et al., 2009). It has also been showed that the expression of SOCS-3 has a beneficial role in the reduction of the inflammatory answers in several illnesses (Hovsepian, E. et al., 2013; Nasreen, N. et al., 2013). as well as the decrease of the oxidative stress (Hilberath, JN. et al., 2011) and the promotion of the DNA repair (Sitko, JC. et al., 2008).

SERPINE1

The transcribed SERPINE1 (plasminogen-1 activator inhibitor), main inhibitor from the Plasmin (t-PA) tissue activator and the urokinase (uPA). This gene was infraexpressed in the group low CL.

In the study from Huang, W., 2017 and cooperators, it was observed that the SERPINE1 gene contributed directly in the adipogenesis and the lipometabolism. It was determined that a PAI-1 increased worsens the differentiation of the osteoblasts, the mineralization and the bone resorption, and it also promotes the adipogenesis in the bone tissues (Tamura, Y. et al., 2013).

The PAI-1 controls the physiological and pathological proteolysis (Jankun, J. et al., 2006). Therefore, the PAI-1 is a primary and negative regulator of the proteolysis powered by plasminogen (Kindzelskii, AL. et al., 2004; Małgorzewicz, S. et al., 2013) The PAI-1 expression as itself, is a direct influence on the proteolysis, the invasion and the accumulation of the extracellular matrix (Cho, HJ. et al., 2012) As there was no PAI-1, the angiogenesis was completely annulled, this demonstrates its importance on the proteolysis control mediated by plasmin (Devy, L. et al., 2002). The PAI-1 inhibits the tissue plasminogene activator as well as the uroquinase type plasminogen activator, which results in a reduced plasminogen activity and an attenuated fibrinolysis and proteolysis (Meyer, MW. et al., 2002).

The PAI-1 can alter the glucose uptake, especially in adipocytes (Liang, X. et al., 2006), its direct participation in the development of resistance to insulin has been questioned (Bernot, D. et al., 2004) The deficit in PAI-1 also improved the basal glucose uptake in the in vitro fat cells (Ma, LJ. et al., 2004).

The PAI-1 can regulate the natural or innate response, it enables the leucocytes adherence, it regulates the cell migration and the phagocytosis (Jeon, H. et al., 2012) it plays its role regulating the cell cycle and repairing injurie (Qi, L. et al., 2008) and it also regulates the inflammatory responses (Hua, F. et al., 2011). It could also be speculated that the lack of PAI-1 would avoid the collagen deposition, as it inhibits the leucocytes and the collagen productive cells migration to the respiratory tract, challenged with ovalbumin, which would result in a lower inflammatory response (Oh, CK. et al., 2002).

Therefore, in the differential expression of the Low CL vs High CL group, biological differences of interest between the two groups were observed, such as water retention, protein synthesis and the adipogenesis, which may directly influence on the quality of the meat after cooking, being of greater interest for the consumer.

The results of the cellular process were surprising: innate immunity and DNA damage repair, this could indicate that their immune system was different between the two study groups, and for that reason a biological process that has been seen as differential, is the answer to the cytokines, being the main messengers of the immune system to the brain and responsible for the organization of the cells responsible in the immune system response. Therefore, it might appear that meats with a low water loss could belong to animals with different immune systems with a possible tendency to a greater immune response than the group of higher losses of water, at the time of slaughter, what makes it

necessary to do more studies on this differential change in Nelore cattle immune system expression in Brazil.

According to our differential study, the differentially expressed genes between the two groups were involved in the MAPK pathways, according to Silva-vignato, B., 2017 and cooperators, it has been illustrated that some of the molecular processes involved in muscle and fat deposition are those marked by those pathways. Thus, our differential genes could be involved in muscle and fat depositions. In addition, Elabd, C., 2014 and his coworkers even speak about an improvement in the age of the cell activation/proliferation tissues through the activation of the MAPK pathways through oxytocin.

For the first time, it has been shown that, the differential genes of the group of less water loss after cooking could be directly involved in the tenderness of the meat and thus they could help to improve the quality of the meat. The differential genes are directly involved in cellular processes such as water retention and can demonstrate that meats with lower water losses after cooking treatments, can have a greater tenderness. Also, the differential genes obtained, can contribute in the fact that the skeletal muscle has intramuscular fat, due to its implication in the cell process of adipogenesis and thus being able to directly influence the quality of the meat by giving it more tenderness. Therefore, it is possible to conclude with the hypothesis that the group that had less water loss after cooking, could contain a possible fat disposal in the *Longissimus Dorsi* Nelore bovine tissue, being this a fact of interest for the quality of the meat and its production in Brazil. The same way that a difference in the immune system between the two study groups opens a possible door for future research.

Conclusions

V. Conclusions

- ***Longissimus Dorsi* tissue from Nelore bovine grouped into two groups; high and low water loss after cooking, show a different transcriptional profile through the analysis using RNA-Seq.**

- **We have detected 32 differentially expressed genes for water loss by cooking the *Longissimus Dorsi* tissue from Nelore bovine, from which 28 genes are well annotated and 4 are new sequences.**

- **The candidate genes for biomarkers for water loss by cooking are Oxytocin (OXT), Suppressor of cytokine signaling 3 (SOCS3) and plasminogen-1 activator inhibitor (SERPINE1) involved in the upregulation of cell processes such as adipogenesis, water retention and immune system.**

- **The group that have less water loss after cooking could have better quality of meat.**

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VII. ANNEXES

Annex 1: Functional Categories ("Annotation Clusters") of differentially expressed genes for CL by David Software.

Annotation Cluster 1	Enrichment Score: 0.9191887049001116											
Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
UP_KEYWORDS	Signal	9	31	0,042	CCL1,ALCAM,CCDC3,IFI6,BOLA-DRB3,OXT,POSTN,SPP1,SERPINE1	27	2817	17808	2,111	0,95751906	0,79389095	4
UP_SEQ_FEATURE	signal peptide	6	21	0,101	ALCAM,IFI6,BOLA-DRB3,OXT,SPP1,SERPINE1	17	1010	6267	2,190	0,99768774	0,99768774	7
GOTERM_CC_DIRECT	GO:0005615~extracellular space	4	14	0,135	CCL1,OXT,SPP1,SERPINE1	22	950	15461	2,959	0,99700478	0,94527144	7
UP_KEYWORDS	Secreted	3	10	0,364	OXT,SPP1,SERPINE1	27	868	17808	2,280	1	0,99974081	10

Annotation Cluster 2	Enrichment Score: 0.6229390886277377											
Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
UP_KEYWORDS	Glycoprotein	5	17	0,085	FAIM2,ALCAM,NEFM,SPP1,SERPINE1	27	1157	17808	2,850	0,99844726	0,88420241	6
GOTERM_CC_DIRECT	GO:0070062~extracellular exosome	5	17	0,337	GIPC2,ALCAM,SPP1,SERPINE1,TUBB6	22	2162	15461	1,625	0,99999993	0,99582771	10
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	4	14	0,473	FAIM2,ALCAM,SPP1,SERPINE1	17	988	6267	1,492	1	0,99999999	10

Continuation												
Annotation Cluster 3	Enrichment Score: 0.029381441944674 9											
Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
UP_SEQ_FEATURE	transmembrane region	3	10	0,865	FAIM2,ALCAM,IFI6	17	1269	6267	0,872	1	1	10
UP_KEYWORDS	Membrane	6	21	0,949	FAIM2,ALCAM,BEST3,IFI6,BOLA-DRB3,SGK1	27	5650	17808	0,700	1	0,999999999	10
GOTERM_CC_DIRECT	GO:0016021~integral component of membrane	4	14	0,950	FAIM2,ALCAM,IFI6,BOLA-DRB3	22	4185	15461	0,672	1	1	10
UP_KEYWORDS	Transmembrane helix	5	17	0,956	FAIM2,ALCAM,BEST3,IFI6,BOLA-DRB3	27	4935	17808	0,668	1	0,999999997	10
UP_KEYWORDS	Transmembrane	5	17	0,957	FAIM2,ALCAM,BEST3,IFI6,BOLA-DRB3	27	4953	17808	0,666	1	0,999999992	10

Annex 2: Biological Process ("Functional annotation table") of differentially expressed genes for CL by David Software.

Gene Name	GOTERM_BP_DIRECT
40S ribosomal protein S23(LOC787803)	
C-C motif chemokine ligand 1(CCL1)	monocyte chemotaxis,inflammatory response,protein coupled receptor signaling pathway,neutrophil chemotaxis,positive regulation of GTPase activity,lymphocyte chemotaxis,positive regulation of inflammatory response,chemokine-mediated signaling pathway,positive regulation of ERK1 and ERK2 cascade,cellular response to interferon-gamma,cellular response to interleukin-1,cellular response to tumor necrosis factor,

CONTINUATION	
FYVE, RhoGEF and PH domain containing 5(FGD5)	regulation of Rho protein signal transduction
Fas apoptotic inhibitory molecule 2(FAIM2)	response to ischemia,apoptotic process,cerebellum development,cerebellar Purkinje cell layer development,cerebellar granular layer development,cerebellar Purkinje cell differentiation,regulation of neuron apoptotic process,negative regulation of neuron apoptotic process,negative regulation of apoptotic signaling pathway,
GIPC PDZ domain containing family member 2(GIPC2)	
GTP binding protein overexpressed in skeletal muscle(GEM)	mitotic nuclear division,small GTPase mediated signal transduction,chromosome organization,metaphase plate congression,
OTU deubiquitinase 1(OTUD1)	protein K63-linked deubiquitination,
RUN and cysteine rich domain containing beclin 1 interacting protein(RUBCN)	negative regulation of phosphatidylinositol 3-kinase activity,negative regulation of endocytosis,negative regulation of autophagosome maturation,
activated leukocyte cell adhesion molecule(ALCAM)	adaptive immune response,cell adhesion,heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules,motor neuron axon guidance,retinal ganglion cell axon guidance,GO:0048846~axon extension involved in axon guidance,neuron projection extension,
bestrophin 3(BEST3)	negative regulation of ion transport,chloride transmembrane transport,
coiled-coil domain containing 3(CCDC3)	

CONTINUATION	
cytokine inducible SH2 containing protein(CISH)	negative regulation of protein kinase activity,protein kinase C-activating G-protein coupled receptor signaling pathway,protein ubiquitination,cytokine-mediated signaling pathway,intracellular signal transduction,regulation of growth,negative regulation of JAK-STAT cascade,negative regulation of insulin receptor signaling pathway,
family with sequence similarity 127, member A(FAM127C)	
hematopoietic cell-specific Lyn substrate 1(HCLS1)	negative regulation of transcription from RNA polymerase II promoter,positive regulation of cell proliferation,response to hormone,positive regulation of phosphatidylinositol 3-kinase signaling,actin filament polymerization,erythrocyte differentiation,regulation of actin filament polymerization,positive regulation of granulocyte differentiation,positive regulation of peptidyl-serine phosphorylation,positive regulation of tyrosine phosphorylation of STAT protein,positive regulation of transcription factor import into nucleus,positive regulation of macrophage differentiation,positive regulation of transcription from RNA polymerase II promoter,positive regulation of protein kinase B signaling,cellular response to cytokine stimulus,negative regulation of leukocyte apoptotic process,
hemoglobin, beta(HBB)	oxygen transport,
interferon alpha inducible protein 6(IFI6)	release of cytochrome c from mitochondria,negative regulation of cysteine-type endopeptidase activity involved in apoptotic process,~negative regulation of mitochondrial depolarization,negative regulation of extrinsic apoptotic signaling pathway in absence of ligand,
major histocompatibility	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II,immune response,

CONTINUATION	
complex, class II, DRB3(BOLA-DRB3)	
microtubule associated monooxygenase, calponin and LIM domain containing 2(MICAL2)	heart looping,cytoskeleton organization,heart development,positive regulation of transcription via serum response element binding,sulfur oxidation,actin filament depolymerization,oxidation-reduction process,
myeloid/lymphoid or mixed-lineage leukemia; translocated to, 11(MLLT11)	positive regulation of apoptotic process,positive regulation of transcription, DNA-templated,positive regulation of mitochondrial depolarization,positive regulation of release of cytochrome c from mitochondria,extrinsic apoptotic signaling pathway,~intrinsic apoptotic signaling pathway,
neurofilament, medium polypeptide(NEFM)	axon development,mitophagy in response to mitochondrial depolarization,
oxytocin/neurophysin I prepropeptide(OXT)	response to mechanical stimulus,response to food,response to external biotic stimulus,response to estrogen,
periostin(POSTN)	
protein phosphatase, Mg2+/Mn2+ dependent 1K(PPM1K)	protein dephosphorylation,
secreted phosphoprotein 1(SPP1)	ossification,osteoblast differentiation,cell adhesion,biomineral tissue development,response to vitamin D,positive regulation of bone resorption,
serpin family E member 1(SERPINE1)	chronological cell aging,angiogenesis,regulation of receptor activity,negative regulation of plasminogen activation,negative regulation of endopeptidase activity,negative regulation of smooth muscle cell migration,positive regulation of interleukin-8 production,negative regulation of cell adhesion mediated by integrin,positive regulation of leukotriene production involved in inflammatory response,positive regulation of angiogenesis,positive

	CONTINUATION regulation of receptor-mediated endocytosis,efense response to Gram-negative bacterium,negative regulation of fibrinolysis,negative regulation of vascular wound healing,cellular response to lipopolysaccharide,positive regulation of monocyte chemotaxis,negative regulation of extrinsic apoptotic signaling pathway via death domain receptorsnegative regulation of smooth muscle cell-matrix adhesion,negative regulation of endothelial cell apoptotic process,
serum/glucocorticoid regulated kinase 1(SGK1)	regulation of cell growth,cellular sodium ion homeostasis,apoptotic process,cellular response to DNA damage stimulus,positive regulation of sodium ion transport,peptidyl-serine phosphorylation,positive regulation of transporter activity,intracellular signal transduction,egulation of cell proliferation,regulation of apoptotic process,neuron projection morphogenesis,
suppressor of cytokine signaling 3(SOCS3)	negative regulation of protein kinase activity,JAK-STAT cascade,protein ubiquitination,cytokine-mediated signaling pathway,regulation of growth,negative regulation of tyrosine phosphorylation of Stat1 protein,negative regulation of tyrosine phosphorylation of Stat3 protein,negative regulation of apoptotic process,positive regulation of cell differentiation,negative regulation of JAK-STAT cascade,negative regulation of insulin receptor signaling pathway,negative regulation of inflammatory response,branching involved in labyrinthine layer morphogenesis,placenta blood vessel development,trophoblast giant cell differentiation,spongiotrophoblast differentiation,
tubulin beta 6 class V(TUBB6)	cytoskeleton organization,microtubule-based process,