Abstract

Nowadays consumers demand for meat includes traits like healthiness, palatability, and tenderness; for this reason those traits are becoming important for industries and breeders.

Limousine is an highly specialized beef cattle breed producing animals with a well muscled carcasses without excessive fat cover, features that are in addition to the great calving ease and maternal distinct attitudes for which the breed is universally known. In Italy this breed is widespread, in particular in the north-east and centre of the country.

Genome research in farm animals progressed rapidly in recent years. The goal of genomic technologies is the characterization and mapping of the loci that affected quality trait that is an important economic parameter in farms. The recent development of high-density single nucleotide polymorphism (SNP) genotyping microarrays has opened new selection perspectives for the possibility of estimating the breeding value.

This research is focused on factors involved in tenderness, that is an important feature of meat quality traits. Three different factors influencing the tenderness have been selected: muscular hypertrophy, *post-mortem* proteolysis, and fat deposition. Phenotypic data used were physical, chemical and fatty acid analyses of meat samples collected from 97 Limousine steers. We also selected genes involved in the above mentioned processes selecting 10 different SNPs on the whole.

Seven genes have been investigated: myostatin gene (with F94L and Q204X SNPs), calpain and calpastatin genes with respectively CAPN316, CAPN530, CAPN4751, and CAST82 SNPs, Diacylglycerol-O-acyltransferase gene (DGAT1), thyroglobulin gene (TG), fatty acid binding protein gene or FABP4 and the leptin gene (LEP). The total SNPs analyzed are 10.

The results obtained confirm that CAPN316 and CAPN530 are functional SNP for meat tenderness, while the CAPN4751 shows a contrasting results for this trait. For CAST282 the C allele is associated with low shear force for meat, a reduction of *Longissimus dorsi* area, lean yield and free water content, but it increases lightness and fatty acid deposition. DGAT1 allele association was detected only for few fatty acids. FABP4 SNP confirms its important role in lipid

metabolism of intramuscular fat. Mutation in the bovine LEP gene has not always shown associations with physical and fatty acids traits in beef cattle. F94L, Q204X, and TG were fixed in our population so it was impossible to investigate their effects on the traits analyzed.

Riassunto

I consumatori di oggi richiedono carni con caratteristiche di alta qualità quali: carni fresche, appetibili e tenere. Per questo motivo tali tratti stanno diventando importanti caratteristiche economiche per il mondo delle industrie e allevatoriale.

La Limousine è una razza bovina altamente specializzata, destinata alla produzione di carne, che presenta le seguenti caratteristiche specifiche: carcasse con un buon sviluppo della massa muscolare e senza copertura eccessiva di grasso, grande facilità di parto e buona attitudine materna. In Italia questa razza è molto diffusa, in particolare nel nord-est e nel centro del paese. Per tali ragioni si è scelto di studiare la razza Limousine.

La ricerca sul genoma per gli animali di interesse zootecnico è progredita rapidamente negli ultimi anni, ponendosi l'obiettivo di caratterizzare e mappare i loci che determinano la qualità della carne, data la sua elevata importanza a livello economico da parte del mondo allevatoriale. Il recente sviluppo dell'impiego degli SNP -polimorfismo a singolo nucleotide- ha aperto nuove prospettive di selezione, data la possibilità di stimare il valore genetico in tempi minori rispetto a quelli impiegati oggi dalla maggior parte delle associazioni allevatoriali.

Questa ricerca è focalizzata sull'analisi dei fattori coinvolti nella produzione di carne tenera, importante caratteristica della qualità della carne.

Sono stati individuati tre fattori che influenzano tale processo: l'ipertrofia muscolare, la proteolisi *post-mortem* e la deposizione di grasso. Sono stati analizzati 97 vitelloni provenienti dalla stessa azienda e sul campione di bistecca prelevato sono state eseguite analisi fisiche, chimiche e riguardanti la composizione degli acidi grassi. Per ognuno dei processi prima ricordati sono stati selezionati alcuni dei più importanti geni coinvolti e per ciascuno di essi sono stati individuati alcuni polimorfismi di interesse.

Nello specifico sono stati studiati 7 geni: il gene della miostatina (F94L e Q204X SNPs), i geni della calpaina e calpastatina (rispettivamente gli SNPs CAPN316, CAPN530, CAPN4751 e CAST82), il gene Diacylglycerol-O-aciltransferasi (DGAT1), il gene tireoglobulina (TG), il gene acido grasso della proteina o FABP4 e il gene della leptina (LEP). Gli SNPs totali analizzati sono stati 10.

I risultati ottenuti confermano che CAPN316 e CAPN530 sono SNPs funzionali per la tenerezza della carne, mentre il CAPN4751 mostra risultati contrastanti per questa caratteristica. Per CAST282 l'allele C è associato ad un basso sforzo al taglio, ad una riduzione dell'area del muscolo *Longissimus dorsi*, ad una maggior resa in carne magra e ad una minore capacità di ritenzione dell'acqua libera nella carne, inoltre influenza la deposizione degli acidi grassi. Il DGAT1 è risultato associato solo a poche variabili. Lo SNP FABP4 conferma il suo ruolo importante nel metabolismo inerente la produzione di lipidi nel grasso intramuscolare. Il LEP non ha mostrato effetti sui caratteri analizzati. Infine gli SNPs F94L, Q204X e TG risultano essere fissati all'interno della popolazione studiate e per cui non è stato possibile analizzarne gli effetti.

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General introduction

Meat quality and tenderness

Nowadays consumers demand meat with high quality characteristics like healthiness, palatability, and tenderness; for this reason those traits are becoming important for industries and breeders.

Because of that the major challenge facing the beef industry and cattle breeders is to improve the eating quality of beef. Eating quality includes various taste related traits such as tenderness, flavour and juiciness, and also appearance (including meat colour, fat deposition and distribution) and nutrient content.

Beef tenderness has significant impact on consumer satisfaction, in particular in Tuscany where steak is one of the most important dishes and the consumer wants and searches for tender meat, because tenderness could be considered the most important component of meat quality and consumer is willing to pay a premium for this characteristic.

It is important to know that the tenderness is determined by different factors like fat deposition, muscle structure (myofibril and connective tissue), breed, slaughter age, duration of post-mortem period before the consumption.

The medium heritability of tenderness suggests that this trait could be successfully improved by selection in beef cattle. However the difficulty and costs associated with collecting direct measurement remains a constraint to the genetic improvement of this trait. In addition, only phenotypes of relatives can be used to estimate breeding values, which limits their accuracy. These limitations make tenderness trait and also other meat quality traits ideal candidates for the use of molecular tools, such as DNA markers, through marker-assisted selection.

Meat quality in Limousine breed

The history of Limousine cattle may very well be as old as the European continent itself. Cattle were found in cave drawings estimated to be 20000 years old in the Lascaux Caves near Montignac.

These golden-red cattle originated in the West of the Massif Central between Central and South West France, a rather rainy region with harsh climatic conditions and poor granite soil. It was in these unfavourable conditions that the breed developed. As a result of their environment Limousine cattle evolved into a breed of unusual sturdiness, health and adaptability.

Since those early days the breed has developed from a working meat animal into a highly specialised beef producing animals with a well muscled carcasses without excessive fat cover, features that are in addition to the great calving ease and maternal distinct attitudes for which it is universally known. Nowadays, Limousine cattle are still referred as the “butcher’s animal” in France. Increasing demand for quality beef production has witnessed the breed becoming established all around the world. In fact, today the Limousine breed can be found in seventy countries from the northern-most herds of Finland and the Commonwealth of Soviet States to countries such as Cuba, South Africa and China.

Moreover Limousine breed is:

- the largest breed in the UK
- the third largest breed in the USA
- the fifth largest breed in Canada
- the seventh largest breed in Australia

In Italy this breed is widespread, in particular in the north-east and centre as we can see from the figure 1.1.

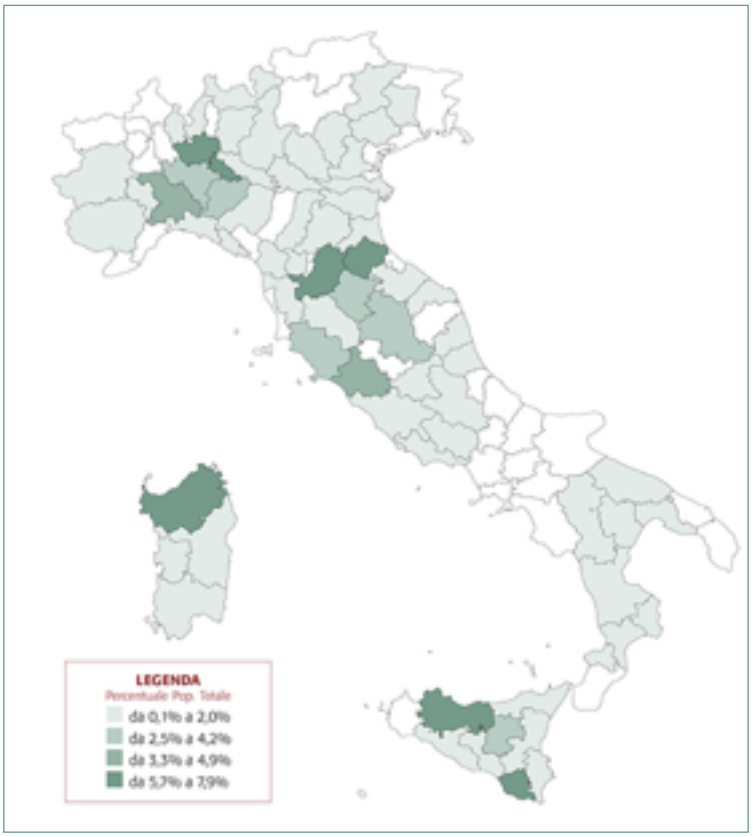

Figure 1.1 – Diffusion of Limousine breed in Italy.

This breed is characterized by the versatility in farming systems, the yield at the slaughterhouse and the earliness in terms of commercial maturity.

With its good rustic character, the Limousine has an extraordinary flexibility and a capacity to turn effectively unrefined feeds into beef. Therefore, this breed is an ideal breed for open air breeding.

Limousine calves grow very quickly and so they can reach the commercial maturity very early, in fact for this breed the French Limousine Breed Association has recorded the following data :

- birth weight of female calves: 39 kg,
- 120 day weight of females calves: 162 kg,
- birth weight of male calves: 42 kg,
- 120 days weight of male calves: 173 kg.

The Limousine breed also offers the best weaning weight of calves. The 210 days weight of male calves is 286 kg and 258 kg for female calves.

Furthermore the longevity of Limousine cows is considerable. The morphology of female cows reaches its full development between 6 and 8 years of age, and remains relatively constant until 10-12.

Limousine cattle are described as having a moderate increase in muscling compared with *B. taurus* breed, such as Hereford, Angus or Piedmontese, but it is not considered a double-muscled breed and does not suffer from increase in dystocia compared with Hereford cattle (*Short et al., 2002*).

However another important Limousine's feature is the fineness of the meat texture. In fact, in the Limousine breed the meat is always very tender, because the collagen content is not so high although the breed is known for this moderate double-muscling phenotype.

For all of these reasons Limousine is an important breed in Italy and its success is due to its meat's qualities and aptitude on breeding.

Molecular genetics and selection schemes

Meat quality is one of the most important economic traits in farm animals. Meat quality trait has a multi-factorial background and is controlled by an unknown number of quantitative trait loci. Genome research in farm animals progressed rapidly in recent years, moving from linkage maps to genome sequence. The goal of genomic technologies is the characterization and mapping of the loci that affected quality trait. The main outcome of genomics technologies is the determination of physical effect of the genes on phenotype (*Koopaei et al., 2011*).

Improvement of meat quality traits (such as colour, water-holding capacity, tenderness that are influenced by several factors) with traditional breeding programs are very difficult, because of low heritability of the desired traits (*Koopaei et al., 2011).*

For meat production the interest has been focused on genes involved in growth and meat quality, but only for a limited number of genes the effects of their polymorphism have been investigated, often in a single breed.

On the other hand, the recent development of high-density single nucleotide polymorphism (SNP) genotyping microarrays has opened new selection perspectives for the possibility of estimating the breeding value of animals with no phenotypic records (*Meuwissen et al., 2001*).

One justification for molecular genetics research on livestock and crop species is the expectation that the information at the DNA level will lead to faster genetic gain than the achieved based on phenotypic data (*Meuwissen et al., 2001*). Therefore incorporating information of markers SNP in the breeding programme of cattle might increase the rate of genetic gain for some of the traits in the breeding goal of population (*Lisa et al., 2013*).

Genetic effects on tenderness

A considerable number of mutations with phenotypic effects where investigated and are suitable for a marker assisted selection. Animal breeding selection is focused on economically important traits like meat quality, so these mutations are mainly located in genes that control different aspect of it.

With our research we are focused on factors involved in tenderness, that is an important part of meat quality.

We selected three different factors influencing the tenderness which are: muscular hypertrophy, post-mortem process, and fat deposition. We selected genes involved in those different processes and for every one of them we found SNPs.

Muscular Hypertrophy

Doublemuscling or muscular hypertrophy is an inherited condition in cattle. The condition is a syndrome, implying that it is associated with many physical, physiological and histological characteristics other than muscular hypertrophy (*Arthur, 1995*). Cattle showing the double-muscled syndrome are characterised by hypertrophy of muscles, especially in the regions of the proximal fore and hind quarter (*Ménissier, 1982*).

Compared with normal cattle, double-muscled cattle have less bone, less fat, more muscle, improved feed conversion, reduced feed intake and a higher production of “expensive” cuts meat *(Ménissier, 1982; Shahin and Beg 1985; Arthur, 1995).* Unfortunately the syndrome is associated with production problems such as reduced fertility, dystocia, low calf viability and increased stress susceptibility (*Ménissier, 1982).*

Muscular hypertrophy, that occurs in many European cattle breeds, shows an high frequency in some breeds, while in others the frequency is low, and double-muscled in individuals are rare. The double muscling is caused by an allelic series of mutations that cause a loss of function of

the myostatin gene – GDF8 – (*Grobet et al, 1998; Marchitelli et al, 2003; Dunner at al. 2003*).

The GDF8 has been mapped to the centromeric end of chromosome 2 (*Charlier et al., 1995; Dunner et al., 1997; Smith et al., 2000*). The expression of the gene results in the production of myostatin, a protein that suppresses both the proliferation and differentiation of myogenic cells (*Grobet et al., 1998*). Therefore mutations that make the gene inactive or decrease the activity of the protein cause a marked increase in muscle mass.

In cattle, 20 different mutations have been found in the myostatin gene (GDF8 is commonly known by the alias *myostatin* MSTN, and we will use MSTN here) of so-called double muscled cattle, 14 of them located in the coding sequence and 6 located in the intronic sequences of gene (*Stinckens et al, 2010*).

In Limousine there was no clear evidence for the role of MSTN in double muscling. *Sellick et al.* (2007) confirmed the absence of any loss-of-function or novel variants in the MSTN, whereas analysing additive and dominance effects of a non-disruptive phenylalanine (F) to leucine (L) missense mutation (F94L g.433 C>A - AF320998, *MCPherron et al, 1997*) found a strong evidence for the association of this mutation with the increased carcass weight and muscling, reduced fat content and altered fatty acid profiles. This suggested that either the F to L mutation or a DNA variant in close linkage disequilibrium with this SNP is responsible for a partial loss-of-function of MSTN gene in the Limousine breed (*Sellick et al. , 2007; Esmailizadeh et al, 2008; Alexander et al, 2009; Lines et al, 2009; Stinckens et al, 2010*).

The mutation Q204X (AB076403), is a C->T substitution at nucleotide position 610 in the second exon, generating a premature stop codon in the N-terminal latency-associated peptide at AA position 204. The presence of this mutation involves negative effects on fitness and maternal traits. The French Limousine breed association has decided to

eradicate this mutation and choose only animals without this mutation (*Allais et al, 2009*).

Post-mortem processes

The post-mortem meat tenderization process is the calpain proteolytic system, this process produces a tender meat that it is what a consumer looks for.

The calpain (CAPN) is responsible for the breakdown of myofibrillar proteins, which are closely related to meat tenderness (*Wheeler and Koohmaraie, 1994*). Calpastatin (CAST) inhibits the μ-calpain activity, therefore also it has important function in the tenderization process. Increased post mortem CAST activity is correlated with reduced meat tenderness (*Page et al., 2002; Casas at al., 2006; Schenkel et al., 2006; Pinto et al 2010*).

For these reasons these genes are considered functional candidates for meat quality trait related to tenderness.

The CAPN gene is located on chromosome 29, and three SNPs were used. The CAPN 316 is located in exon 9 and causes the substitution of cytosine with guanine (g.5709 C>G, AF252504), that produce an amino acid substitution of alanine (C allele) with glycine (G allele) according to *Page et al, 2002.* The CAPN 530 (g.4558 A>G, AF248054) is an adenine/ guanine polymorphism in exon 14 of the gene that produces an amino acid substitution of isoleucine (A allele) with valine (G allele) according to *Page et al, 2002.* The CAPN 4751 (AF248054) is a mutation located on intron 17 and on the base g.6545 C>T (*White et al., 2005*).

The CAST (AY008267) gene is located on chromosome 7, the mutation investigated is between the intron 5 and 6 where C mutated in G at base 282 (*Schenkel et al., 2006).* Studies showed that the C allele is favourable and the G allele is dominant, therefore, animals with CC genotype have more tender meat than CG or GG animals (*Schenkel et al., 2006; Lisa et al., 2009; Pinto et al., 2010*).

Fat deposition

The level of intramuscular fat content and fatty acid composition is among the main factors determining meat palatability and consumer satisfaction. For that intramuscular fat content, also assessed as marbling meat, represents an important beef quality trait.

Diacylglycerol-O-acyltransferase (DGAT1), thyroglobulin (TG) and fatty acid binding protein (FABP4) genes were previously hypothesised to be genetic factors influencing IMF deposition in muscle (*Barendse, 1999; Michal et al., 2006; Thaller et al., 2003; Pannier et al., 2010*), all of these genes are mapped to the centromeric region of bovine chromosome (BTA) 14.

The gene encoding TG has been proposed as a positional and functional candidate gene for QTL, because its product is the precursor of hormones that affect lipid metabolism (*Barendse, 2002*). The C422T SNP of the TG gene (accession number X05380) has been associated with an improvement in overall fattening and could be used as a gene marker for marbling deposition in beef cattle. Animals with the TT genotype had significantly higher marbling scores than those expressing the CC and CT genotypes (*Barendse, 2002; Thaller et al., 2003).*

Diacylglycerolacyltransferase (DGAT) catalyzes the terminal step in triacylglycerol (TAG) synthesis via the acyl-CoA- dependent acylation of sn-1,2-diacylglycerol and the level of DGAT activity may have a substitution effect on the quantity of triacylglycerol deposition in fat-faming tissue (*Mayorek et al., 1998*). This gene has been associated with increased milk yield and milk fat content (*Grisart et al., 2001, 2004*) and also it has been reported results on its effect on fat deposition in beef cattle (*Moore et al., 2003; Thaller et al., 2003; Gill et al., 2009*). The SNP studied here is an A in G nucleotide substitution causing a K (lysine) to A (alanine) amino acid substitution (named K232A) in the protein (accession number AY065621). This polymorphism was shown to be significantly associated with milk fat yield and fat percentage where AA animals, with the lysine amino acid, had increase levels for both traits

(*Grisart et al., 2001*). *Gill et al.* (2009) found that the polymorphism was also associated with sirloin weight and fat depth in commercial cross breed cattle.

Fatty acid binding proteins are a family of small highly conserved cytoplasmic proteins that bind long chain fatty acid and other hydrophobic ligands (*Kaikaus et al*., 1990). *Fatty acid binding protein 4* (FABP4, g. 7516 G>C – number X89244) is a gene that has not been confirmed for marbling or intramuscular fat (IMF) in all studies in cattle so far. Initially identified as a gene affecting IMF in mice and soon thereafter shown to be a gene affecting IMF in pigs. It was not until quite recently that the first report of an effect of variation at FABP4 on IMF cattle was published (*Michael et al., 2006; Jurie et al., 2007; Barendse et al., 2009*).

Leptin (LEP), the hormone product of the obese (leptin) gene located on BTA 4 (*Stone et al., 1996; Pomp et al., 1997)*, is considered to play a role in the regulation of appetite, energy partition, and body composition (*Houseknecht et al., 1998*). Leptin is synthesized and expressed predominantly by adipocytes (*Houseknecht et al., 1998*) and relates to the feedback system that regulates long-term body fat weight and composition (*Hossner, 1998*). Studies have found associations of serum leptin concentration with carcass adipose depots and carcass characteristics of beef cattle (*Geary et al., 2003*). A cytosine to thymine transition that encodes an AA change of an arginine to a cysteine (Arg25Cys) was identified in exon 2 of the leptin gene and this mutation is causative for a major body fat deposition (*Buchanan et al., 2002; Kononoff et al., 2004; Schenkel et al., 2005, Woronouk et al, 2012*). The Leptin accession number is AF120500.

Aim of the thesis

We focused on 7 genes that were selected for the effects that they have on meat quality traits and in particular on tenderness: myostatin gene (with F94L and Q204X SNPs), calpain and calpastatin genes with respectively CAPN316, CAPN530, CAPN4751, and CAST82, Diacylglycerol-O-acyltransferase gene , thyroglobulin gene, fatty acid binding protein gene or FABP4 and the leptin gene. The total SNPs analyzed were 10.

We performed the following analysis:

- allelic and genotypic frequencies and Hardy–Weinberg equilibrium for the SNPs analyzed,

- General Linear Models (GLM) Analysis using SNPs as fixed effect and sire as random effect,

- analysis of SNPs effect including the pedigree information.

The aim of this study is to estimate firstly the frequencies of the SNPs in the Limousine population, secondly their association with traits recorded at the slaughter and on meat samples in order to evaluate the effect of the genotypes and haplotypes on meat quality traits.

It is also studied how the effect of a pedigree could contribute to give a new information about the SNPs association with the studied traits.

The data produced from this thesis could be used to assess the inclusion of these SNPs in future genetic selection schemes. In fact the possibility to use these genes for estimating the breeding value of an animal and to increase genetic selection of a specific breed at low cost are an important goal for the breeder associations.

In fact the use of marker-assisted selection could contribute for breed selection reducing the time of evaluation of a future sire or dam to be employed in farms.

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Materials and Methods

Animals and samples

Data used in this study were from 97 Limousine steers, which are the progeny of 9 sires belonging to the same farm “Lippi Alessandro e Nocentini Roberto S.S. Società Agricola”. These bulls were submitted to the same environmental conditions (they were fattened in the same farm). Animals were slaughtered at the same slaughterhouse from September 2010 to March 2013. The animals were slaughtered at an average live-weight of 481 kg and at an average age of 596 days.

Table 2.1 shows the numbers of offspring for each sire.

Sire Name	Offspring numbers
Sire 1 - ASTERIX	13
Sire 2- ARAMIS	15
Sire 3 - APOLINAIRE	21
Sire 4 - APACHE	10
Sire 5 - BAMBOU	14
Sire 6 - ALEJANDRO	7
Sire 7 - VAURIEN	8
Sire 8 - CHARMEIRE	5
Sire 9 - DIOCLEZIANO	4

Table 2.1 – Offspring numbers for each sires.

Steak samples taken from sixth thoracic vertebra of each steer were collected. The samples were collected 8 days after slaughtering and immediately transferred to the laboratory of Animal Science Section of Department of Agrifood Production and Environmental Sciences - Università degli Studi di Firenze. The range of weight of samples was from 1.5 to 2.8 kg.

Blood or hair samples for each sire were collected and submitted to the DNA analysis with the exception of one that is dead at the beginning of the project .

Physical analysis

Once arrived at the laboratory steaks were dissected into the main tissues: *Longissimus dorsi*, other lean cuts, backfat, intra-muscular fat, bones and the following analysis were performed:

ALD - Longissimus dorsi area, the area of *Longissimus dorsi* muscle was measured transferring the area on a transparent paper and analyzing it with a graphic software (Photoshop 6.0 - Adobe).

Dissection of sample cut, we recorded the weight of *Longissimus dorsi*, other lean cuts, backfat, intra-muscular fat and bones.

Cooking loss percentage, a sample of *Longissimus dorsi* with an average weight of 127 g, was sealed off in a polyethylene bag and put in a water bath to an internal temperature of 88°C until the sample reached the 75°C (*Boccard et al., 1981*). The cooking loss percentage was calculated as the ratio "(steak weight before cooking – steak weight after cooking)/ steak weight after cooking *100".

Shear force, Shear force was measured by "Zwick/Roell Z2.5" with a Warner Bratzler and Cut shear attachments, 200-kg load cell. Two cylindrical cores of 1 inch of diameter taken parallel to muscle fibres from the steak sample were obtained and data of fresh and cooked samples were recorded.

Colour was measured on four different positions on a beef slice obtained after removing a 1 to 2 cm slice from the surface. The slice was wrapped with a breathable polyethylene film and, after 45 minutes of exposing to air at 4°C, colour was measured with a Minolta Chromameter CR-200 that gives the three colour parameters L*, a*, b* (*Boccard et al., 1981*). L* (lightness) is measured as the amount of incident light reflected by a surface, ranged between 0 (black) and 100 (white). Redness parameter (a*) is between a range to -60 (green) from +60 (red). Yellowness parameter (b*) where negative values represent blue (-60) and positive values represent yellow colour (+60). The parameters a* and b* gives the Hue (arctan (a*/b*)) and Chroma or saturation index $(a^2+b^2)^{½}$).

Free water, was used the method of Grau & Hamm (1952) modified: a 0.3 g of *Longissimus dorsi* was put in a filter paper and submitted to a pressure of 20 atm for 5 minutes between two Plexiglas plates. The free water content is given by the surface of area (cm^2) coming out by the pressure that is circumscribed by the external and internal perimeter.

pH, was measured with pH meter Delta Ohm-Do 9505- on a fresh side of sample at two random positions, using a penetrating electrode and a temperature sensor. The pH-meter was calibrated before using.

Chemical analysis

A part of *Longissimus dorsi* muscle was stored and dehydrated in order to perform the chemical analysis.

Moisture. About 60g of meat has been freeze-dried until constant weight.

Ether extract, 2 g of sample were treated in a "Soxhlet" apparatus. The ether extract was extracted by the petroleum ether. The residual has been dried at 105°C until a stable weight.

Crude protein, the samples were analyzed with the Kjeldahl method. The nitrogen mineralization was made with hot-treatment using concentrate sulphuric acid. Afterwards ammonia nitrogen distillation was carried out, and boric acid were used for titration.

Ash, was determined by incinerating the samples at 550°C .

Dry matter, is the difference of "1- moisture".

Fatty acid analysis

The total lipids have been determined by the Folch method (*Folch et al., 1957*), using the extraction of 0.5 g of sample of dehydrated *Longissimus dorsi* and hydrated with 1.5 ml of water, with a chloroform methanol mixture (2:1 v/v).

The fatty acid determination of total lipids have been made in the following way: the methyl esters of fatty acids have been obtained by esterification of *Morrison and Smith* (1964) method. Later the samples have been analyzed using the gas-chromatograph (GC) "Varian 430-GC". It was used the C19:0 as internal standard and the FAME MIX C8-C22 was used in order to create the calibration curve and to identify the output peaks thanks to their times of retention. The fatty acids have been separated by capillary column "Varian Select FAME", the column is long 100 meters, 0.25 mm of diameter, and film thickness optimized. The program temperature is: initial temperature is 40°C (isotherm 4 minutes), increase of 10°C/min until second temperature of 120°C (isotherm 1 minute), second increase of 5°C/min, the third temperature is 180°C (isotherm 18 minutes) with the third increase of 2°C/min. The fourth temperature is 200°C (isotherm 1 minute) with an increase of 2°C/min. The last temperature is of 230°C (isotherm 19 minutes), the total duration of running is 88 minutes. The Detector FID temperature is 300°C. The injection split/splitess is of 1 µl. The He carrier is a stable flux of 36.2 psi, aux N_2 5ml/min, air 300ml/min, H_2 30ml/min.

Molecular analysis

DNA from meat, blood and hair samples was extracted using the Promega Kit for a total of 110 samples at the laboratory of Animal Science Section of Department of Agrifood Production and Environmental Sciences - Università degli Studi di Firenze.

The 96-well plates were made with a minimum of 1.5 µL of DNA at 3.3ng /µL (quantified by Picogreen analysis) per SNP assayed, there was for each sample an extra amount for pipetting with robotics (as the industry required), a total volume was of 5 µL for sample. A total of 10 SNPs were investigated involving 7 different genes (table 2.2). The sample plates were made at the Department for Innovation in Biological, Agro-food and Forest systems (DIBAF) – Università degli Studi della Tuscia.

Genotyping was performed by LGC Genomics Kbioscience (London -UK-). The SNP sequence is created by 50 bases long either side of the mutation point as requested by the company.

Gene	Bovine Chromosome	SNP name	SNP location	SNP description
MSTN	Chromosome 2	F94L	Exon 1	AF320998 - C>A
		Q204X	Exon 2	AB076403 - C->T
CAPN	Chromosome 29	CAPN316	Exon 1	AF252504 - C>G
		CAPN530	Exon 14	AF248054 - A>G
		CAPN4751	Intron 18	AF245054 - C>T
CAST	Chromosome 7	CAST282	Intron 5	AY008267 - C>G
FABP	Chromosome 14	FABP4	Exon 1	X89244 - G>C
DGAT	Chromosome 14	DGAT1	Exon 8	AY065621 - A>C
LEPT	Chromosome 47	LEP	Exon 2	AF120500 - C>T
TG	Chromosome 14	TG	Exon 1	X05380 - C>T

Table 2.2 – SNPs studied.

Statistical analysis

All analyses were performed within the R open-source environment for statistical programming.

Allelic and genotypic frequencies for each polymorphism were tested using the Package “Hardy Weinberg” –Version 1.4– (*Graffelman, 2009*). It is a package for exploring bi-allelic marker data.

The general model used was:

x <- c(28, 44, 24)

names(x)<- c("AA", "Aa", "aa")

x.test <- HWChisq(x, verbose=TRUE)

The results were the p-value of the chi-square test for Hardy-Weinberg equilibrium and the allelic frequency of A. Genotypic frequencies were calculated from the allelic frequencies.

Where possible (SNPs on same chromosome), haplotypes were constructed using the PHASE software – Version 2.1– (*Stephens et al., 2001 and 2003*). The program PHASE implements a Bayesian statistical method for reconstructing haplotypes from population genotype data. The file output gives as result the haplotype frequencies estimates and the most likely haplotypes pairs for each individual.

Two haplotypes were constructed, one for chromosome 14 where the TG, DGAT1 and FABP4 gene are linked. The gene positions are the following:

- DGAT1 = 1.801.129 cM
- TG = 7.901.412 cM
- FABP4 = 41.879.116 cM

The second one on chromosome 29 where calpain gene is located. For the CAPN gene the SNPs positions are shown below:

- CAPN316 = 44,068,109 cM
- CAPN530 = 44,078,958 cM
- CAPN4751 = 44,080,945 cM

The relationship between the different genotypes of each SNP and the various traits recorded was evaluated using a linear mixed model for single-marker. Data were analyzed using the R package "lme4" (*Bates et al., 2013*).

They were used two statistical models:

1. linear mixed model using the sire as random effect,

2.linear mixed model using the additive relationship matrix.

The first model included fixed effects of SNP, year, season, slaughter age, carcass weight and sire as random effect.

The general model used for the traits analyzed was as follows:

$Y= \mu + A_j + B_k + C_l + D_m + F_n + S_p + e_{jklmnp}$

where:

Y= is the trait measured on the individual *i*

μ= is the overall mean for the trait

A_j = is the fixed effect of *SNP* genotype j (3 levels),

B_k = is the fixed effect of *year* of birth k (4 levels),

C_l = is the fixed effect of *season* of birth l (4 levels),

D_m = is the covariate of *slaughter age* m,

F_n = is the covariate of *carcass weight,* it is the animal weight at the slaughter n*,*

S_p = is the random effect of sire p (9 levels),

e_{jklmnp} = is the residual term associated with the observation.

The script was as follows:

modx <-lmer(ald ~ SNP + year + season + slaughter age+ carcass weight + (1|sire), data=fenotipi)

P-values were obtained by likelihood ratio test of the single fixed effects comparing a *null model* (without the factor that we were interested in) with a *full model* (with the factor that we were interested in), the likelihood of these two models was analyzed using the *ANOVA()* function:

modx <-lmer(ald ~ year + season + slaughter age+ carcass weight + (1|sire), data=fenotipi)

mody <-lmer(ald ~ SNP + year + season + slaughter age+ carcass weight + (1|sire), data=fenotipi)

anova(modx, mody)

The estimates of each SNPs from the model were obtained using the *fixef* function, it is important to note that *lmer()* function takes the factor comes first in the alphabet to be the reference level. In our study the homozygote genotypes were named “1” and “-1” and the heterozygote 0, so the genotype named “-1” is taken by the function as reference level and its value is zero in the output data.

fix<- fixef(lmer(ald ~ year + season + slaughter age + carcass weight + (1|sire), data=fenotipi))

The allele substitution effect was estimated as the difference between the mean of the two homozygotes divided by two.

Forty-three traits obtained through the analyses described above, have been processed by the models just described (see appendix 1). In the following chapters only variables where polymorphisms or haplotypes were significant will be discussed.

The linear mixed models used was also tested incorporating the effects of the pedigree (instead of the sire effect) using the *pedigreem* function of the *pedigreemm* package (*Bates and Vazquez, 2013*).

The full archive of the Limousine breed consisted of 156,659 records from which the genealogies of the 97 animals included in the model has been recovered using a pruning algorithm (Pieramati, personal communication). Following the pruning the dataset of the animals and their direct ancestors consisted of 645 records.

The model used was:

pedmody <-pedigreemm2(ald ~ factor(SNP) +factor(year) + factor (season) + age + carcass weight + (1|anim), data=lim2, pedigree=list (anim=pedok))

The object pedigree represents a set of individuals that can have two parents including the parent-child relations. This function was used only where the SNPs have shown to be significant.

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Webgraphy

- http://www.anacli.it/WEBSITE/

The allelic and genotypic frequencies and Hardy–Weinberg equilibrium for the SNPs analyzed

Introduction

The aim of this chapter was to investigate the allelic and genotypic frequencies in the studied Limousine population and to verify the Hardy – Weinberg equilibrium .

Materials and Methods

Animals and tissue samples

Data used in this study were from 97 Limousine steers, which are the progeny of 9 sires bred from the same farm “Lippi Alessandro e Nocentini Roberto S.S. Società Agricola”.

Meat samples were collected during the three year of trial**.**

Molecular analysis

DNA was extracted from meat, blood and hair samples using the Promega Kit. Then the 96-well plates were made with a minimum of 1.5 µL of DNA at 3.3ng/µL (quantified by Picogreen analysis) per SNP assayed. A total of 10 SNPs were investigated in 7 genes (chapter 2).

Results and Discussion

The allelic and genotypic frequencies of all polymorphisms are summarised in tables 3.1 and 3.2. No significant departures from Hardy –Weinberg equilibrium were identified for the genotypes, except for the CAST282 (P=0.012). The data is in agreement with those reported by *Schenkel et al.* (2006) that was P=0.001.

Polymorphism	Allele	Frequencies
CAPN316	G	0.84
	C	0.16
CAPN530	T	0.61
	C	0.39
CAPN4751	G	0.48
	A	0.52
CAST282	C	0.74
	G	0.26
DGAT1	G	0.93
	A	0.07
FABP4	G	0.64
	C	0.36
LEP	C	0.7
	T	0.3
TG	C	1
	T	0
F94L	A	1
	C	0
Q204X	C	1
	T	0

Table 3.1 - Allele frequencies of the analyzed SNPs.

The F94L and Q204X are fixed in our sample of Limousine breed. The fixation for the Q204X SNP it is probably due to the selection programme that the French Limousine Breed Association have made during these years on their animals. In fact, parents of sire analyzed in this study have French origin as it was possible to see from the ANACLI genealogy, and they were influenced by the French selection scheme where the causative mutations of muscular hypertrophy are not desired for the future breeding animals.

Indeed the Q204X is a disruptive mutation and causes fitness problems like dystocia, low fertility, stress susceptibility. For this adverse effects on fitness and maternal traits, the French Limousine breed association decided to eradicate the Q204X mutation together with other mutations like nt821 (*Allais et al., 2010*).

The F94L mutation is fixed in our population and this situation was also observed by *Allais et al.* (2010) where they did not investigate this mutation because it was almost fixed in the French Limousine breed even if the results of other studies like *Esmalizadeh et al.* (2008), *Lines et al*. (2009), *Alexander et al.* (2009), and *Stinckens et al.* (2010) showed a significant effect of the F94L mutation in this breed, where the allele C presents low frequencies. The A allele is considered a favourable allele because it was found a strong evidence for the association of this allele with the increased carcass weight and muscling, reduced fat content and altered fatty acid profiles. This mutation causes an intermediate phenotype because Limousine cattle are described as having a moderate increase in muscling compared with other *B. taurus* breeds., but are not considered a double-muscled breed and do not suffer from an increase in dystocia compared with hypertrophic breeds.

Also TG SNP is fixed for the C allele in contrast to other works of *Barendse et al*. (2002), *Thaller et al.* (2003), *Casas et al.* (2005), and *Panier et al.* (2010) where the TG SNP had low frequencies of T allele and TT genotype, allowing thus to study effects of this SNP on meat quality.

Polymorphism	Genotype	Frequencies
CAPN316	GG GC CC	0.69 0.30 0.01
CAPN530	AA AG GG	0.38 0.47 0.15
CAPN4751	TT TC CC	0.25 0.46 0.29
CAST282	CC CG GG	0.49 0.49 0.02
DGAT1	GG GA AA	0.87 0.13 0
FABP4	GG GC CC	0.38 0.51 0.10
LEP	CC CT TT	0.48 0.45 0.07
TG	CC CT TT	1 0 0

F94L	AA AC CC	1 0 0
Q204X	CC CT TT	1 0 0

Table 3.2 - Genotype frequencies of analyzed SNPs in Limousine.

For DGAT1 no AA animals were observed, in fact, the frequency of allele A is below 1% this data is in agreement with *Li et al* (2013).

The TT genotype frequency of LEP is low as the T allele frequency that is 3%.

Moreover the CC genotype of CAPN316 and GG of CAST282 have a low frequencies in the sampled animals instead the frequencies of the alleles C and G that are not below 15%.

Various studies (*Casas et al., 2006; White et al., 2005; Smith et al., 2009; Lisa et al., 2009, Pinto et al., 2009; Cafe et al.; 2010*) have not found the CC genotype for the CAPN316 (in Nellore, Brahman and Piedmontese breeds) whereas this favourable genotype for meat tenderness is present in our Limousine population. CAPN316 was associated with beef tenderness by *Page et al.* (2004), *White et al.* (2005), *Casas et al.*(2006), and *Cafe et al.* (2010) both on *Bos taurus* and *indicus*. In the above mentioned studies the C allele was favourable for more meat tender steaks.

Various researches (*Page et al., 2004*; *White et al., 2005*; *Corva et al.*, *2007*; *Pinto et al., 2009*) showed that the GG genotype of CAPN530 resulted fixed or with a very high frequency. Animals with G allele showed a lower shear force than animals with A allele (*Page et al., 2004*; and *White et al., 2005*); whereas *Corva et al.*(2007) found that the A

allele was favourable for the shear-force, but few animals showed the AA genotype. In the present work the AA genotype presents medium frequency (38%) whereas the GG a frequency of 15%.

It is well known that the allele C of CAPN4751 SNP is favourable for meat tenderness in *Bos taurus* cattle (*Page et al., 2004; White et al., 2005*) and in *Bos indicus* (*Van Eenennaam et al., 2006; Pinto et al., 2009*). Nevertheless the genotype CC of CAPN4751 SNP resulted extremely rare or absent (*White et al., 2005; Casas et al., 2006; Smith et al., 2009*) whereas in the present research it showed medium frequency (29%).

Considering the CAST282 it can be observed that the C allele showed high frequency and this result is in agreement with those obtained from other Authors (*Schenkel et al. 2006, Van Eenennaam et al., 2006; Pinto et al., 2009*) which demonstrated that C allele is favourable for meat tenderness in *Bos indicus* and *Bos taurus*. Moreover *Schenkel et al.* (2006) demonstrated that the C allele was associated with meat tenderness (measured as shear force) but it tended to reduce the LM area and lean yield and increase fat yield.

The frequencies of GG genotype and the G allele for the DGAT1 are in agreement with the results obtained by *Casas et al.* (2005) and *Gill et al.* (2009). These last Authors found that the A allele was associated with an increase in sirloin weight after maturation and with an increase in sirloin fat depth when compared the G allele. Animals with heterozygous genotype had values close to the homozygous GG animals, indicating a strong degree of dominance of the G allele over the A allele (*Gill et al.* , *2009*).

In the current analysis the G allele is the most common allele for the FABP4, this data is in agreement with *Avilés et al.* (2013) that shows a G allele frequencies equal to 0.64, but it is in contrast with the results coming from *Michal at al.* (2006), *Pannier at al.* (2010), and *Curi et al.* (2011) that working on *Bos taurus* and *indicus* breeds where they found frequencies of both G allele and GG genotype very low.

Our Limousine animals showed low frequencies of TT genotype and T allele for LEP. This result is in agreement with those found by *Lusk* (2007), *Fortes et al.* (2009), *Pannier et al.* (2008) but in contrast with *Buchanan et al.* (2002) and *Schenkel et al.* (2005). It has been suggested that the cysteine amino acid variant (T allele) of the leptin proteins interferes with the binding of the leptin molecule to its receptor (*Buchanan et al., 2002*; *Kononoff et al., 2004; Schenkel et al., 2005, Woronouk et al, 2012*) which could account for less circulating functional leptin, consequently resulting in fatter carcasses in some breed. This thesis is refuted by *Pannier et al.* (2009), *Fortes et al.* (2009), and *Avilés et al.* (2013). It is clear that the results for this SNP are conflicting. Environmental factors such as feeding regimes are likely to play a large role in intramuscular fat level regulation, and failing to control this factors could confound results leading to non-significant findings (*Pannier et al., 2009*).

The SNPs investigated in this work have been studied by other authors in crossbreed and in small Limousine populations. These works usually studied SNPs involved in only one process like muscular hypertrophy, post-mortem process, or fat deposition. This is the first study where a purebred Limousine population with a relevant number of animals has been investigated for all these SNPs involved in different biological processes. However it is important to notice that our steers are offspring of sires that have French originating. Therefore, it might be advisable to implement the number of animals analysed to have a greater number of data to assess more reliable allele frequencies.

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General Linear Models (GLM) Analysis using SNPs as fixed effect

Introduction

The aim of this study was to test previously identified associations between SNPs from five genes and economically important meat quality traits. The SNP tested are located in CAPN, CAST, LEP, FABP4, DGAT1 genes.

Materials and Methods

Animals

Phenotypic data used were physical, chemical and fatty acid analyses of meat samples collected from 97 Limousine steers. The animals come from the same farm "Lippi Alessandro e Nocentini Roberto S.S. Società Agricola". Animals were slaughtered at the same slaughterhouse from September 2010 to March 2013.

Tissue samples

Individual samples of *Longissimus dorsi* muscle were collected 8 days after slaughtering between the 5^{th} and 6^{th} thoracic vertebra and were immediately analyzed in the laboratory of Animal Science Section of the Department of Agrifood Production and Environmental Sciences.

Meat samples for each steer and sire's blood were collected and submitted to the DNA analysis.

Physical, chemical and fatty acid analysis

Among the forty-three variables analyzed, already reported in Materials and Methods, hereafter only the variables with significant SNPs effect will be discussed.

Steaks were dissected into the main tissues and the following information were determined: *Longissimus dorsi* area (**ALD)**, *Shear force* of raw meat (**wbSF)**, *Longissimus dorsi* weight (**Ld**), other lean cuts (**LCS**), cooking loss percentage (**CLP**), free water (**FW**), colour average for parameters L*, a*, b* (**LC, AC**, **BC)** (Chapter 2).

A part of *Longissimus dorsi* muscle was stored and dehydrated in order to perform the chemical analyses (Chapter 2).

The fatty acid composition of meat has been detected by the method explained elsewhere (chapter n.2), analyzed data were: total saturated fatty acid percentage (**SFAP**), monounsaturated fatty acid percentage (**MUFAP**), polyunsaturated fatty acid percentage for n6 and n3 series (**PUFAP**), n6-n3 ratio (**n6/n3**), sum of C18:1 n9 plus C18:1 n7 (**C18:1**), palmitic acid C16:0 (**C16-0**), stearic acid c18:0 (**C18-0**), linoleic acid (**C18-2 n6**), oleic acid (**C18-1 n9**).

The following traits have been expressed as %: **Ld, LCS, CLP** and all fatty acids.

Molecular analysis

DNA from meat, blood and hair samples was extracted using the Promega Kit for a total of 110 samples at the laboratory of Animal Science Section of Department of Agrifood Production and Environmental Sciences - Università degli Studi di Firenze.

Then the 96-well plates were made with a minimum of 1.5 µL of DNA at 3.3ng /µL (quantified by Picogreen analysis) per SNP assayed. A total of 10 SNPs were investigated in 7 genes (chapter 2).

Statistical analysis

The statistical model included fixed effects of single SNP, year, season, slaughter age, carcass weight and sire as random effect. P-values were obtained by likelihood ratio test of the single fixed effects comparing a null model with a full model. The estimates of each SNPs from the model were made using the 'f*ixef*' function. The allele substitution effect was estimated as the difference between the mean of the two homozygous divided by two.

Results and Discussion

Single genotype – trait association

Year effect was significant for most of the traits analyzed. Environmental factors are likely to play a large role for all the traits analyzed, and for that reason the *Year* effect gives this result.

Fixed effects and covariate of *season, slaughter age,* and *carcass weight* are not significant for most of the traits analyzed.

None SNPs shows significant association with the chemical parameters.

Before commenting the results it has to be pointed out that, even if not reported in the tables, the standard error of the estimates was of the same order of the estimates. This is probably due to two different reasons, namely the reduced number of phenotypes recorded.

CAPN316 does not segregate at appreciable frequencies in Limousine for the C allele. Only one animal shows the CC genotype, this situation is probably due to the sires' genotypes. Offspring's sires have been genotyped and five sires show the GG homozygous genotype and only three sires the GC genotype. For the ninth sire could not be possible to genotype the SNP analyzed.

Table 4.1 shows that the heterozygote GC has a higher value for different traits (**ALD, LCS, AC, BC, CLP**) instead the homozygote form. This situation is possibly due to a dominance effect, but it is also important to notice that the data are unbalanced and GG genotype is present in 65 animals out of 97. In agreement with several studies (*Page et al., 2004; White et al., 2005; Casas et al., 2006; Corva et al., 2007; Gill et al, 2009 and Cafe et al., 2010*) CC genotype shown significantly lower **wbSF** values, and therefore more tender meat, than TT animals (the additive effect for C>G substitution is 4.635 N). The present knowledge suggests that the C allele has a strongest linkage disequilibrium with a putative tenderness allele (*Corva et al., 2007*). For that reason it is possible to suppose that the C allele has been submitted to a natural selection.

FW shows a positive value for the GG genotype, so it's possible that the C allele is a favourable allele for meat quality because it is involved in a major capacity to retain water in the tissues.

There is a significant effect for the fatty acid composition, the CG genotype shows a decrease for **SFAP** and **C16-0.** This effect is important for human health in particular for palmitic acid with its known negative effects. The heterozygote form influences for a deposition of **MUFAP, C18-1 n9,** and **C18:1**. As reported in literature the C18:1 fatty acid is involved in vascular diseases.

Animals with homozygous G genotype at marker 530 (also known as CAPN530) had lower shear force (P= 0.003) than animals with AG and AA genotypes and this is in agreement with *Page et al.* (2004) and *White et al.* (2005). The value of the substitution effect for the A allele is +1.7265 N Force. The A allele is associated with increase of **ALD** (table 4.1). There was a putative association of CAPN530 with **BC** trait.

The AG genotype (table 4.2) shows for the **SFAP** and **C16-0** the same effect of heterozygote CAPN316 genotype. While for the **C18-1 n9** and **C18:1** the AG affects for a minor fatty deposition of these fatty acids.

CAPN4751 showed significant association with shear force (P=0.001). The TT animals had a lower value for the **wbSF**, and therefore more tender meat then CC or CT animals. The additive effect of C>T substitution is -2.578 N. This data is in contrast with the results related by *Page et al.* (2004), *White et al.* (2005), *Smith et al.* (2009), *Pinto et al.* (2010), and *Cafe et al.* (2010). Moreover the TT genotype shows lower values for the **ALD** and **Ld** traits, it seems that this genotype has a negative effect for these traits because it decreases the area and the weight of *Longissums dors*i.

Whereas the C allele is a favourable allele for the **FW** trait as shown in table 4.1.

C18-2 n6 presents a higher value for the TT and TC genotypes for marker 4751, that is important because the fatty acid C18:2 is known for its anticancer action.

The TC forms presents a effect of decrease of these traits: **C18-1 n9** and **C18:1.**

Traits	Gene											
	CAPN316				CAPN530				CAPN4751			
	Genotype			p-value	Genotype			p-value	Genotype			p-value
	GG (65)	GC (29)	CC (1)		AA (15)	AG (45)	GG (37)		TT (24)	TC (44)	CC (28)	
ALD	9.270	9.986	0	<0.001 ***	0.548	0.098	0	0.003 **	-1.596	-1.2	0	0.009 **
Ld	0.412	-0.389	0	0.001 **	-0.498	-0.496	0	0.019 *	-0.371	-0.366	0	0.022 *
LCS	3.715	4.252	0	<0.001 ***	1.449	1.452	0	0.011 *	-0.823	0.522	0	0.021 *
LC	-5.198	-4.509	0	0.0019 **	0.808	0.788	0	0.037 *	-0.581	0.511	0	0.037 *
AC	-1.848	-2.482	0	0.005 **	-0.751	-0.589	0	0.036 *	1.048	0.420	0	0.016 *
BC	-1.636	-1.784	0	0.022 *	-0.077	0.378	0	0.078 .	-0.375	0.484	0	0.077 .
FW	4.286	3.945	0	<0.001 ***	-0.673	1.655	0	<0.001***	1.091	1.049	0	0.011 *
CLP	16.409	22.894	0	<0.001 ***	-2.476	-4.125	0	0.009 **	-0.281	-1.511	0	0.010 *
wbSF	10.492	9.752	0	<0.001 ***	3.453	0.147	0	0.003 **	-5.156	-0.681	0	0.001 **

Table 4.1- Genotype at the calpain gene for physical analysis

Traits	Gene											
	CAPN316				CAPN530				CAPN4751			
	Genotype			p-value	Genotype			p-value	Genotype			p-value
	GG (65)	GC (29)	CC (1)		AA (15)	AG (45)	GG (37)		TT (24)	TC (44)	CC (28)	
SFAP	-1.844	-2.531	0	0.003 **	0.744	0.863	0	0.036 *	-0.217	-0.107	0	0.044 *
MUFAP	0.951	1.921	0	0.001 **	0.285	-0.144	0	0.031 *	-1.153	1.363	0	0.008 **
C16-0	-1.019	-1.161	0	0.006 **	0.385	0.493	0	0.048 *	0.042	0.031	0	0.035 *
C18-0	-0.048	0.403	0	0.005 **	0.109	0.195	0	0.047 *	-0.302	-0.041	0	0.050 .
C18-2 N6	0.496	0.276	0	0.003 **	0.846	0.547	0	0.026 *	1.203	1.225	0	0.004 **
C18-1 N9	1.395	2.323	0	0.001 **	-0.134	-0.280	0	0.041 *	-0.830	-1.064	0	0.009 **
n6/n3	0.232	-0.103	0	0.002 **	-0.327	-0.486	0	0.028 *	0.195	-0.08	0	0.049 *
PUFAP	0.943	0.727	0	0.002 **	-1.147	-0.766	0	0.020 *	1.559	1.620	0	0.002 **
C18:1	1.552	2.501	0	0.001 **	-0.149	-0.293	0	0.038 *	-0.818	-1.06	0	0.011 *

Table 4.2- Genotype at the calpain gene for chemical analysis.

Furthermore, the **CLP** shows higher values of heterozygous genotypes (GC, AG, CT) for all the SNPs, but only the CAPN316 has a higher value instead the other two show a lower values.

Calpain markers are significantly associated with fatty acids traits but the additive effect is of relative magnitude.

In conclusion the calpain system gene markers for tenderness presents conflicting results. The CAPN316 and CAPN530 are in agreement with the literature for this topic while the CAPN4751 shows a result that is in contrast with other scientific works. It has to be pointed out that none of the studies have made an investigation only on Limousine purebred for CAPN4751.

Hence it is confirmed that CAPN316 and CAPN530 are functional SNPs for meat tenderness, in particular CAPN530 that is more reliable than CAPN316, where the low number of animals (one) with the CC genotype in this study made it difficult to get an accurate estimate of its effect.

Markers 316, 530 and 4751 have few other effects in quality meat parameters as fatty acids and the physical traits.

A significant effect (P=0.001) was found for the CAST282; CC genotype is associated with more tender meat than CG and GG genotypes. The shear force value for the C allele is -1.9635 N. This data confirmed the results of *Schenkel et al.* (2006), *Van Eenennaam et al.* (2006), *Quaas et al.* (2006) and *Pinto et al.* (2010). Moreover the C allele tends to reduce the area of *Longissimus dorsi*, in fact the additive effect is -5.6115 cm^2 and the significance for this traits is P=<0.001; this data is in agreement with *Schenkel et al.* (2006). For that animals with CC genotype show a minor percentage of *Longissimus dorsi* muscle than animals with heterozygous and homozygous genotypes.

The estimated substitution allele effect for CAST282 indicated that the C allele was associated with a +0.67% increase in **LC** trait.

The CAST282 shows a significant effect for free water (**FW**) parameter (P=0.003), where the CC genotype shows a lower value.

The CC genotype of CAST282 shows a lower value for the **SFAP, C16-0** and **n6/n3** traits. The first two traits have a significance of P=0.008 and P=0.006 respectively, furthermore they present high negative substitution allele effect value for each of them (table 4.7).

However the C allele is involved in the fatty acid deposition. In particular for the **C18-2 n6** that has known for a positive influence in the human health, where the C allele is involved in this biological process.

The alleles of CAST single nucleotide polymorphism identified in this study were segregating in the Limousine population with an overall greater frequency for the C than G allele. Allele C is associated with low shear force for meat, a reduction of *Longissimus dorsi* area, lean yield and free water content, but it increases lightness and fatty acid deposition traits.

The combined effect of favourable alleles for CAPN and CAST markers resulted in reduction of shear force, so this study provides further evidence that selection based on the CAST and CAPN gene markers improves meat tenderness in Limousine cattle. It also appears that selection for the favourable alleles at these SNPs would have also a favourable effect for other important economically traits like free water content and fatty acid deposition. It is important to notice that this selection would have a negative effect on **ALD** and **Ld** traits.The LEP SNP shows significant association only with the **ALD** and **Ld** traits. Therefore C allele is involved in lean yield and confirmed the results found by *Buchanan et al.* (2002) and *Schenkel et al.* (2005), but it is to notice that this is a putative effect.

No significant association between LEP polymorphism and fatty acid composition has been found. This data is in contrast with the results of *Buchanan et al.* (2002), *Kononoff et al.* (2005) and *Schenkel et al.* (2005).

Mutation in the bovine LEP gene has not always shown associations with physical and fatty acids traits in beef cattle (*Pannier et al., 2008; Fortes et al., 2008; Curi et al., 2010; Avilés et al., 2013*), suggesting both that SNP effect is small and environmental factors play a large role. Thus failing to

Traits	Gene							
	CAST282				LEP			
	Genotype			p-value	Genotype			p-value
	CC (44)	CG (44)	GG (1)		CC (47)	CT (43)	TT (7)	
ALD	-11.223	-7.400	0	<0.001 ***	5.825	0.473	0	0.026 *
Ld	-2.263	-1.538	0	0.005 **	1.398	0.339	0	0.084 .
LCS	0.608	-0.724	0	0.013 *				
LC	1.340	1.264	0	0.026 *				
AC	-0.528	-0.629	0	0.059 .				
BC	0.124	0.396	0	0.031 *				
FW	-0.855	0.223	0	0.003 **				
CLP	0.173	0.356	0	0.008 **				
wbSF	-3.927	2.469	0	0.001 **				

Table 4.3- Genotypes at the calpastatin and leptin genes for physical analysis.

Traits	Gene							
	CAST282				LEP			
	Genotype			p-value	Genotype			p-value
	CC (44)	CG (44)	GG (1)		CC (47)	CT (43)	TT (7)	
SFAP	-2.012	-1.253	0	0.008 **				
MUFAP	0.845	0.858	0	0.037 *				
C16-0	-2.213	-1.822	0	0.006 **				
C18-0	0.704	0.887	0	0.053 .				
C18-2 N6	0.737	0.223	0	0.029 *				
C18-1 N9	0.891	0.841	0	0.035 *				
n6/n3	-0.813	-0.037	0	0.013 *				
PUFAP	1.347	0.558	0	0.014 *				
C18:1	0.988	0.944	0	0.036 *				

Table 4.4- Genotypes at the calpastatin and leptin genes for chemical.analysis.

control these factors could confound the results leading to non-significant findings.

G is the most common allele for the DGAT1 SNP, this is true also in the genotyped sires where only one sire shows a heterozygous GA genotype, all the others are GG genotype.

DGAT1 SNP shows to be significant only for the **LCS** physical trait with P=0.036 (table 4.5).

The DGAT1 effect has a higher values for the **SFAP** and **C16-0** traits. In other hand the GG genotype presents a lower values for the rest of the variables **C18-2 n6**, **n6/n3**, and **PUFAP.** The significance of this SNP is P= < 0.001 for these variables. This result is in agreement with *Gill et al.* (2009) where the A allele is considered favourable for fat deposition.

The mutation at the DGAT1 gene has been confirmed as being responsible for increased milk yield, fat yield, fat content, protein yield and content in dairy cattle (*Grisart et al., 2001; Winter et al., 2002; Grisart et al., 2004*). In contrast to the consistent results regarding the effect of DGAT1 polymorphism on dairy cattle traits, discrepant results regarding the association between DNA mutations and carcass fat deposition (both intramuscular and subcutaneous) have been described in the literature (*Thaller et al., 2003, Casas et al., 2005, Renand et al., 2007; Gill et al., 2009; Curi et al, 2010*). In the present study, association of DGAT1 alleles was detected only for few fatty acids variables. None of animals showed the homozygous genotype for the A allele; this is probably due to the fact that the investigated population is of limited size. For that the power to detect association is low.

At the FABP4 locus, a significant association was observed with almost all the physical variables. Animals with GG genotype have a lower values for the **ALD, Ld** traits (the additive effect are -3.8555 cm^2 and -0.835 % respectively). The G allele tends to increase the lean cuts with a substitution effect of the G allele equal to 1.0765 % point.

The heterozygous genotype of FABP4 presents an heterotic effect for the **LC, FW**, and **CLP.**

The GG genotype of FABP4 presents discordant results for fatty acid composition. In fact, it shows high values for the **SFAP** and **MUFAP** (also for palmitic and oleic acid) at the same time; whereas for **C18-2 n6** and **PUFAP** it presents low values.

This data confirms that FABP4 SNP plays an important role in lipid metabolism of intramuscular fat and it is in agreement with other association studies (*Michal et al., 2006; Avilès et al., 2013*).

In conclusion, fat content is a complex trait that has an important influence on meat quality. It has been demonstrated that the effect of markers FABP4, DGAT1, and CAST are involved on fat deposition. But it will be good to investigate for other SNPs with a reduced effect in the expression of the traits that have not been monitored in this study.

Trait	Gene							
	DGAT1				FABP4			
	Genotype			p-value	Genotype			p-value
	GG (84)	GA (13)	AA (0)		GG (36)	GC (49)	CC (10)	
ALD					-7.711	-3,818	0	<0.001 ***
Ld					-1.67	-0.805	0	<0.001 ***
LCS	-2.801	0	-	0.036 *	2.153	1.689	0	<0.001 ***
LC					-1.313	- 1.566	0	0.0011 **
AC					0.426	-0.089	0	0.003 **
BC					-0.996	-0.842	0	0.005 **
FW					0.579	-1.165	0	<0.001 ***
CLP					0.994	-2.360	0	<0.001 ***
wbSF					-0.896	0.806	0	<0.001 ***

Table 4.5- Genotypes at the DGAT and FABP4 genes for physical analysis.

Trait	Gene							
	DGAT1				FABP4			
	Genotype			p-value	Genotype			p-value
	GG (84)	GA (13)	AA (0)		GG (36)	GC (49)	CC (10)	
SFAP	2.482	0	-	0.004 **	0.554	0.384	0	0.001 **
MUFAP					1.757	0.853	0	<0.001 ***
C16-0	1.676	0	-	0.018 *	0.931	0.289	0	0.001 **
C18-0					-0.479	0.028	0	0.005 **
C18-2 N6	-2.979	0	-	<0.001 ***	-1.480	-0.634	0	<0.001 ***
C18-1 N9					1.154	0.492	0	<0.001 ***
n6/n3	-2.483	0	-	<0.001 ***	0.246	0.041	0	0.001 **
PUFAP	-3.667	0	-	<0.001 ***	-1.997	-0.912	0	<0.001 ***
C18:1					0.960	0.302	0	<0.001 ***

Table 4.6- Genotypes at the DGAT and FABP4 genes for chemical analysis.

Allele substitution effect							
Traits	**a**						
	CAPN316	**CAPN530**	**CAPN4751**	**CAST282**	**DGAT1**	**FABP4**	**LEP**
ALD	4,635	0,274	-0,798	-5,6115		-3,8555	2,9125
Ld	0,206	-0,249	-0,1855	-1,6315		-0,835	0,699
LCS	1,8575	0,7245	-0,4115	0,304	-	1,0765	
LC	-2,599	0,404	-0,2905	0,67		-0,6565	
AC	-0,924	-0,3755	0,524	-0,264		0,213	
BC	-0,818	-0,0385	-0,1875	0,062		-0,498	
FW	2,143	-0,3365	0,5455	-0,4275		0,2895	
CLP	8,2045	-1,238	-0,1405	0,0865		0,497	
wbSF	5,246	1,7265	-2,578	-1,9635		-0,448	
SFAP	-0,922	0,372	-0,1085	-1,006	-	0,277	
MUFAP	0,4755	0,1425	-0,5765	0,4225	-	0,8785	
C16-0	-0,5095	0,1925	0,021	-1,1065	-	0,4655	
C18-0	-0,024	0,0545	-0,151	0,352		-0,2395	
C18-2 N6	0,248	0,423	0,6015	0,3685		-0,74	
C18-1 N9	0,6975	-0,067	-0,415	0,4455		0,577	
n6/n3	0,116	-0,1635	0,0975	-0,4065	-	0,123	
PUFAP	0,4715	-0,5735	0,7795	0,6735	-	-0,9985	
C18:1	0,776	-0,0745	-0,409	0,494		0,48	

Table 4.7- Allele substitution effect.

Haplotype – trait association

The best haplotype reconstruction for each individual in the Chromosome 14 are shown in table 4.8. Chromosome 14 presents the DGAT1, TG and FABP4 genes. Table 4.8 shows also the number of haplotypes estimated that the Phase v2.1.1 software had reconstructed.

Reference	Haplotype	Frequencies
A	ACC	119
B	ACG	62
C	CCC	4
D	CCG	9

Table 4.8- Haplotype reconstruction for chromosome 14.

The most likely haplotype pairs after the reconstruction are shown in table 4.9 (letters refer to the list of haplotypes given above).

Reference	Combination	Number of animal with this combination in our population
1	AA	32
2	AB	44
3	AC	4
4	AD	7
5	BB	8
6	BD	2

Table 4.9- Haplotype combinations for chromosome 14.

Studying the haplotype effect the animals with BD combination were not considered since only two animals present this combination.

Trait	Haplotype chromosome 14					
	Combination					p-value
	1 (acc, acc)	2 (acc, acg)	3 (acc, ccc)	4 (acc, ccg)	5 (acg, acg)	
Ld	0	0.7499	-1.539	1.141	3.037	0.077 .
BC	0	0.126	0.619	0175	1.058	0.088 .
Tinta	0	0.012	0.014	0.022	0.049	0.011 *
FW	0	-1.749	-0.532	-2.674	-1.634	0.045 *

Table 4.10 - Haplotype effect on chromosome 14 for physical traits.

Trait	Haplotype chromosome 14					
	Combination					p-value
	1 (acc, acc)	2 (acc, acg)	3 (acc, ccc)	4 (acc, ccg)	5 (acg, acg)	
SFAP	0	-0.201	-3.353	-2.900	-0.800	0.055 .
C16-0	0	-0.691	-2.407	-2.260	-0.953	0.071 .
C 18-2 N6	0	0.677	2.862	3.322	0.990	0.007 **
C 20-3 N6	0	0.012	0.091	0.102	0.030	0.042 *
C 20-4 N6	0	0.101	0.429	0.498	0.213	0.016 *
C22-4 N6	0	0.015	0.022	0.045	0.057	0.001 **
C18-1 N7	0	0.031	0.277	0.234	0.145	0.030 *
n6/n3	0	0.451	3.559	2.635	0.542	0.019 *
PUFAP	0	0.383	3.459	4.088	1.328	0.009 **
sum C-14	0	-0.105	-0.499	-0.511	-0.248	0.099 .

Table 4.11- Haplotype effect on chromosome 14 for chemical traits.

Table 4.10 shows that the **FW** presents for each haplotype lower values. In particular haplotypes “ACC, ACG” and “ACC, CCG” (combination 2 and 4 respectively) produce a less loss of free water content than the other haplotype combinations from raw meat. These values are lower when the FABP4 is present with the heterozygote form, in fact also the heterozygous genotype shows a negative value for this trait. It could be supposed thus a strong FABP4 influence on this trait.

The “ACG, ACG” haplotype (5) presents the highest value for the *Longissimus dorsi* weight equal to 3.037% increase. To notice that it is only a putative effect.

Haplotype combinations resulted also significant for some fatty acids (**C 18:1 n7**, **C20:3 n6**, **C20:4 n6**, and **C22:4 n6)** where the single SNPs did not show any significance. These fatty acids are present in low concentration in the Limousine population and the haplotype effects is limited.

Haplotypes influence the fat deposition, in particular the combinations “ACC, CCC” (haplotype 3) and “ACC, CCG” (haplotype 4) give an important contribution for the **C18:2 n6**, **n6/n3** and **PUFAP** synthesis equal to 2.862, 3.559, and 3.459 and 3.322, 2.635 and 4.088 respectively for the former and the latter haplotype. The linoleic acid is on average 7% of the total fatty acids in the studied population, and it is an important fatty acid for its positive effects on human health.

An high n6/n3 ratio is associated to a risk factor for cancer and coronary heart diseases, therefore it is an important factor for the human diet. The best haplotype for n6/n3 ratio seems the “ACC, ACC” combination followed by the haplotypes number 2 and 5, whereas animals with combination “ACC, CCC” (haplotype 3) and “ACC, CCG” (haplotype 4) present the highest and the worst values.

The **SFA**, **C-16** and **C14:0** present lower values, except for the combination “ACC, ACC”. The combination “ACC, CCC” (haplotype 3) and “ACC, CCG” (haplotype 4) show the most lower values for the traits than the other combinations. On the other hand the FABP4 and DGAT1 SNPs show a positive contribution for this traits.

The “ACC, ACC” and “ACC, ACG” haplotypes (combinations 1 and 2 respectively) are the most represented within the population, but their contribution for the physical and fatty acid traits are not so high except for the FW trait at the combination “ACC, ACG”.

There is no significant variation for haplotypes data compared to single SNP analysis, this may be explained by the fact that only FABP4 SNP had a significant effect on almost all the traits analyzed while DGAT1 just for few and the TG SNP is fixed in this population.

The best haplotypes reconstruction for each individual in the Chromosome 29 are shown in table 4.12. CAPN316, CAPN530 and CAPN4751 genes are located on chromosome 29. Table 4.12 presents also the number of haplotypes estimated that the Phase v2.1.1 software had reconstructed.

Reference	Haplotypes	Haplotypes estimated
A	GAT	72
B	GGT	15
C	GGC	76
D	CAT	4
E	CGT	2
F	CGC	25

Table 4.12- Haplotype reconstruction for chromosome 29.

The most likely haplotypes pairs after the reconstruction are shown in the second table (letters refers to the list of haplotypes given above).

Reference	Combination	Number of animal with this combination in our population
1	AA	11
2	AB	6
3	AC	30
4	AD	4
5	AE	1
6	AF	9
7	BB	1
8	BC	3
9	BF	3
10	CC	16
11	CF	11
12	FF	1
13	BE	1

Table 4.13- Haplotype combinations for chromosome 29.

Studying the haplotype effect the animals with AE, BB, BE and FF haplotypes combinations were not considered because they are present in just one animal.

Traits	Haplotype chromosome 29									
	Combination									p-value
	1 gat gat	2 gat ggt	3 gat, ggc	4 gat cat	6 gat cgc	8 ggt ggc	9 ggt cgc	10 ggc ggc	11 ggc cgc	
AC	0	-1.349	-1.374	-2.229	-2.191	-0.782	-0.759	-2.205	-2.606	0.035 *
Chroma	0	-1.744	-1.123	-2.337	-1.974	-1.069	-2.276	-2.129	-2.655	0.055 .
FW	0	2.560	0.869	-0.012	2.085	-3.571	-3.803	-0.012	0.524	0.074.
CLP	0	-6.173	-4.917	3.950	2.968	-5.341	1.321	-1.633	4.252	0.086.

Table 4.13- Haplotype effect onchromosome 29 for physical and chemical traits.

AC and **Chroma** traits have lower values for all haplotype combinations. The “GAT, CAT” and “GGC, CGC” (combination 4 and 11) show lower value for these traits. It is possible that the low values for a* parameter influence the Chroma trait. To note that **AC** trait present a lower value also for the CAPN316 and CAPN530.

Free water presents a discordant data. “GAT, GGT” and “GAT, CGC” produce an increase of free water loss that leads to a worsening of meat quality. On the other hand haplotypes “GGT, GGC” and “GGT, CGC” (number 8 and 9) show a low values which in turn means better meat quality.

The same situation is for the cooking loss percentage. Also for this trait the results are conflicting. Combination “GAT, GGT” and “GGT, GGC” (haplotype 2 and 8) have a positive effect on the parameter in fact they present low values. While “GAT, CAT” and “GGC, CGC” (combination 4 and 11) have a high value that are not good for meat quality. These haplotype combinations do not show a specific effect for the parameter analyzed.

The haplotype combinations for BTA 29 have shown any significance effect associated with *Shear force* of raw meat and fatty acid profiles instead the CAPN SNPs.

Using the haplotypes did not give any extra information for the SNP-traits combinations. This may be explained by the fact that the Limousine population was not wide-ranging. In conclusion, incorporating haplotype information would not improve the performance of marker-assisted selection.

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Pedigree Analysis

Introduction

The aim of this study was to test the effect of the SNPs on the traits analysed incorporating the pedigree information within a standard BLUP analysis.

Materials and Methods

Animals

Phenotypic data used were physical, chemical and fatty acid analyses of meat samples collected from 97 Limousine steers. The steers were raised in the same farm "Lippi Alessandro e Nocentini Roberto S.S. Società Agricola". Animals were slaughtered at the same slaughterhouse from September 2010 to March 2013.

Tissue samples

Individual samples of *Longissimus dorsi* muscle were collected 8 days after slaughtering between the 5^{th} and 6^{th} thoracic vertebra and were immediately analyzed in the laboratory of Animal Science Section of the Department of Agrifood Production and Environmental Sciences.

Meat samples for each steer and sire's blood were collected and submitted to DNA analysis.

Physical, chemical and fatty acid analyses

The same variables analyzed in chapter 4 have been considered in this study.

Statistical analysis

Data were analyzed using a linear mixed model which incorporated the effect of the pedigree through the 'pedigreem2' function of R package. From the data output it has been possible to estimate the heritability, the trend of the SNPs effect on the traits analyzed and the SNP effects,

The h^2 was calculated following the formula:

$$\frac{animal\ variance}{(animal\ variance + residual\ variance)}$$

The formula to estimate SNP effect (*Falconer et al. 1996*) is:

$$V=2pqa^2$$

where:

- p and q are the frequencies of the minor and major alleles,

- a is the allelic substitution effect.

This formula is used in absence of dominance effect of hetorozygote. The data output from this study presents in some cases a dominance effect, for this reason the results are to be kept with caution.

Results and Discussion

Table 5.1 shows the additive variance and heritability for the parameters analyzed. Estimates reported in *italic* are to be kept with caution because of the values are out of the normal range found in literature for these parameters. In fact **ALD** shows too much high values and the same happens with **CLP.**

LCS, FW, **wbSF, C18-0,** and **n6/n3** parameters present a reduced genetic component.

Ld, **C18-2 n6**, and **PUFAP** showed consistent heritability in agreement with the literature and within the normal range. In fact the heritability for these traits is from low to medium which in turn means values ranging from 0.1 to 0.4.

Traits	Additive variance	h^2	Traits	Additive variance	h^2
ALD	120.007	*0.84*	SFAP	2.0826	0.23
Ld	3.8852	0.40	MUFAP	1.1485	0.10
LCS	3.3084e-08	0	C16-0	1.0550	0.18
LC	1.1698	0.18	C18-0	0.00	0
AC	0.7252	0.14	C18-2 N6	3.0348	0.44
BC	0.1971	O.11	C18-1 N9	2.3127	0.24
FW	0.00	0	n6/n3	0.00	0
CLP	118.324	*0.89*	PUFAP	4.7940	0.41
wbSF	0.00	0	C18:1	2.5498	0.27

Table 5.1 - Additive variance and heritability for the parameters.

Before commenting the results it has to be pointed out that, even if not reported in the tables, the standard error of the estimates was of the same order of the estimates. This is probably due to two different reasons, namely the reduced number of phenotypes recorded and the small pedigree depth.

DGAT1 and LEP show the same trend shown in “General Linear Models (GLM) Analysis using SNPs as fixed effect” Chapter 4. This data confirm the relationship between such SNPs and their effects on the traits analyzed.

FABP4 SNPs presents some contradictory effects on **CLP** and **n6/n3** parameters (see table 5.2). The other traits have the same trend effect.

The **CLP** and **n6/n3** showed different trends for the two models employed (GLM or pedigree); GG genotype seems to decrease the level of the traits in the pedigree model whereas on GLM model the genotype acts on opposite way. Moreover the heterozygote genotype has higher value in the pedigree model than in the other one.

The FABP4 SNP shows the same trend for “other lean cuts” trait but the GC genotype has a values more higher than the homozygous genotype as it is possible to see in table 5.2, this is in contrast with data shown in the previous chapter. In fact in table 4.5 the heterozygous genotype has lower values than the homozygous form.

This conflicting data in FABP4 SNP could be explained, as stated before, by the small depth of the pedigree.

Trait	Gene					
	DGAT1 Genotype			FABP4 Genotype		
	GG (84)	GA(13)	AA (0)	GG (36)	GC (49)	CC (10)
ALD				-6.048	-2.112	0
Ld				-1.654	-0.828	0
LCS	-2.672	0		1.005	1.601	0
LC				-1.388	-1.676	0
AC				0.351	-0.526	0
BC				-1.144	-1.129	0
FW				0.750	-0.806	0
CLP				-0.082	-3.712	0
wbSF				-0.880	1.066	0

Table 5.2 - Genotypes at the DGAT and FABP4 genes for physical analysis.

Trait	Gene					
	DGAT1 Genotype			FABP4 Genotype		
	GG (84)	GA(13)	AA (0)	GG (36)	GC (49)	CC (10)
SFAP	2.120	0		0.630	0.525	0
MUFAP				1.683	0.743	0
C16-0	1.451	0		0.941	0.394	0
C18-0				-0.488	0.002	0
C18-2 N6	-3.083	0		-1.345	-0.554	0
C18-1 N9				1.164	0.521	0
n6/n3	-2.632	0		-0.058	0.315	0
PUFAP	-3.741	0		-1.853	-0.846	0
C18:1				0.995	0.363	0

Table 5.3 - Genotypes at the DGAT and FABP4 genes for chemical analysis.

CAST282 has the same effect for the most of the traits analyzed. In particular the use of pedigree data for the **MUFAP**, **C18-2 n6**, **C18-1 n9**, and **C18:1** has shown the same trend and emphasizing the distance between homozygote and heterozygote genotypes.

The **AC** shows the same trend for the two models but in the pedigree model the CC genotype has more lower value than the CG.

CAST282 showed different trend for **BC** and **CLP** traits; In fact the CC combination showed lower effect in pedigree model for **BC** and **CLP** traits compared to GLM output. On the contrary CAST282 seems to show an overdominance for **FW**.

These conflicting data in CAST282 may result from a poorly genotypic distribution of forms within the population studied.

Trait	Gene					
	CAST282 Genotype			**LEP** Genotype		
	CC (44)	CG (44)	GG (1)	CC (47)	CT (43)	TT (7)
ALD	-10.369	-6.065	0	5.013	0.283	0
Ld	-2.272	-1.606	0	1.586	0.511	0
LCS	0.496	-1.171	0			
LC	1.300	1.284	0			
AC	-1.090	-0.453	0			
BC	-0.055	0.269	0			
FW	-0.280	-0.226	0			
CLP	0.117	-0.381	0			
wbSF	-3.751	3.534	0			

Table 5.4 - Genotypes at the CAST282 and LEP genes for physical analysis.

Trait	Gene					
	CAST282 Genotype			LEP Genotype		
	CC (44)	CG (44)	GG (1)	CC (47)	CT (43)	TT (7)
SFAP	-2.353	-1.166	0			
MUFAP	1.002	0.678	0			
C16-0	-2.248	-1.598	0			
C18-0	0.707	0.801	0			
C18-2 N6	0.436	0.030	0			
C18-1 N9	1.050	0.609	0			
n6/n3	-0.762	-0.082	0			
PUFAP	0.951	0.273	0			
C18:1	1.184	0.689	0			

Table 5.5 - Genotypes at the CAST282 and LEP genes for chemical analysis.

CAPN316 SNP shows the same trend for all the physical traits confirming thus the strong effect of this mutation. Whereas for the chemical traits CAPN316 shows contrasting results with the output data of GLM using SNPs like a fixed effects. **C18-0** and **n6/n3** present lower values for the homozygote GG form, whereas **C18-2 n6** and **PUFAP** have lower values for the heterozygote GC genotype.

CAPN530 shows an opposite situation to the CAPN316. For the chemical traits CAPN530 presents the same trend except for the **C18-2 n6.** This trait shows an opposite trend because the pedigree data shows lower values for the two genotypes while the GLM model has more high values. So in this case the genotype AA does not influence the linoleic acid deposition.

Furthermore CAPN530 shows some contrasting data for the physical parameters. **ALD** has an opposite trend in pedigree data, in fact in this output the two genotypes have low values for this trait, so it seems that genotype AA is not involved in the process to have a major area of *Longissimus dorsi*, that is in contrast with GLM data.

Always comparing the results from GLM or pedigree models, marker 4751 shows different trend for the **C16-0** and **C18-0** fatty acids. The **n6/n3** presents higher values for TT and TC forms in pedigree output; it seems that CAPN4751 has a negative effect on this trait because it increases the value of the n6/n3 ratio. For the other traits it is confirmed the effect of CAPN4751.

In conclusion, the incorporation of the additive matrix relationship within the statistical model confirms the effect of SNPs on both chemical and physical traits studied, with the exception of those cases specified above.

Indeed, in many cases, the input of these data led to a better balance between the genotypes studied.

Trait	Gene								
	CAPN316 Genotype			**CAPN530** Genotype			**CAPN4751** Genotype		
	GG (65)	GC (29)	CC (1)	AA (15) (37)	AG (45)	GG	TT (24) (28)	TC (44)	CC
ALD	6.111	7.493	0	-0.365	-2.298	0	-2.400	-2.381	0
Ld	0.053	-0.642	0	-0.571	-0.631	0	0.374	-0.340	0
LCS	4.288	4.449	0	1.172	1.448	0	-0.310	0.396	0
LC	-4.735	-4.108	0	0.769	0.699	0	-0.626	0.416	0
AC	-1.688	-2.175	0	-0.559	-0.328	0	0.810	0.314	0
BC	-1.781	-1.966	0	-0.194	0.325	0	-0.170	0.497	0
FW	3.351	2.867	0	-0.940	1.376	0	1.433	1.250	0
CLP	12.754	18.036	0	-2.236	-3.333	0	-0.059	-1.706	0
wbSF	13.035	11.438	0	3.411	-0.004	0	-5.365	-1.331	0

Table 5.6 - Genotype at the calpain gene for chemical analysis.

Trait	Gene								
	CAPN316 Genotype			**CAPN530** Genotype			**CAPN4751** Genotype		
	GG (65)	GC (29)	CC (1)	AA (15) (37)	AG (45)	GG	TT (24) (28)	TC (44)	CC
SFAP	-2.128	-2.625	0	0.951	1.059	0	-0.517	-0.048	0
MUFAP	1.385	2.231	0	0.204	-0.353	0	-1.067	-1.455	0
C16-0	-1.072	-1.144	0	0.628	0.757	0	-0.242	0.042	0
C18-0	-0.110	-0.463	0	0.035	0.103	0	-0.222	0.036	0
C18-2 N6	0.020	-0.345	0	-0.527	-0.474	0	1.067	1.013	0
C18-1 N9	1.907	2.697	0	-0.438	-0.574	0	-0.649	-1.032	0
n6/n3	-0.717	-0.874	0	-0.241	-0.481	0	0.153	0.090	0
PUFAP	0.362	-0.039	0	-0.758	-0.658	0	1.386	1.331	0
C18:1	2.011	2.808	0	-0.492	-0.623	0	-0.587	-1.020	0

Table 5.7 - Genotype at the calpain gene for chemical analysis.

SNP EFFECT							
Traits							
	CAPN316	**CAPN530**	**CAPN4751**	**CAST282**	**DGAT1**	**LEP**	**FABP4**
ALD	2,510	0,016	0,719	10,343		2,639	4,214
Ld	0,000	0,039	0,017	0,497		0,264	0,315
LCS	1,236	0,163	0,012	0,024	0,232		0,116
LC	1,507	0,070	0,049	0,163			0,222
AC	0,191	0,037	0,082	0,114			0,014
BC	0,213	0,004	0,004	0,000			0,151
FW	0,755	0,105	0,256	0,008			0,065
CLP	10,931	0,595	0,000	0,001			0,001
wbSF	11,418	1,384	3,592	1,354			0,089
SFAP	0,304	0,061	0,032	0,533	0,146		0,046
MUFAP	0,129	0,003	0,135	0,097			0,326
C16-0	0,077	0,027	0,007	0,486	0,069		0,102
C18-0	0,001	0,000	0,006	0,048			0,027
C18-2 N6	0,000	0,019	0,135	0,018	0,309		0,208
C18-1 N9	0,244	0,013	0,050	0,106			0,156
n6/n3	0,035	0,004	0,003	0,056	0,225		0,000
PUFAP	0,009	0,039	0,229	0,087	0,456		0,396
C18:1	0,272	0,016	0,041	0,135	0,146		0,114

Table 5.8 - SNPs effects on traits analyzed.

The table 5.8 shows SNP effects estimated for the parameters analyzed.

FABP4 shows a high additive variance component for the chemical traits (**SFAP**, **C16-0**, **C18-2 N6**, **C18-1 N9**, **PUFAP**, and **C18:1**) where it is a functional gene for the fat deposition (*Michal et al., 2006; Avilès et al., 2013*).

Also DGAT1 show an important high additive variance component for **C18-2 N6, PUFAP**, and **C18:1.**

LC, AC, and **BC** seem determined for a high quota of environmental effect rather than additive. It emphasizes the CANP316 effect on the **LC** and **AC** and CAST282 only for **AC.**

CAPN316 explains a large part of additive variance for the traits **SFAP**, **C18-1 N9**, **C18:1.** Also the CAST282 plays the same role for the **SFAP**, **C16-0**, and **C18:1.**

In conclusion it is possible to assert that the FABP4 and DGAT1 are involved in the fat deposition process, as stated by the effect found in some traits and these data confirmed the results found in literature. Also the CAPN316 and CAST282 are involved in this biological process, this information is a new information because none authors have studied this possible association between calpain and calpastatin genes and fat deposition.

In order to compare the effect of the different SNPs on selection goals figures 5.1, 5.2, 5.3, and 5.4 report the effect of one of the homozygous forms for four different traits, namely **FW**, **wbSF**, **C18-2 n6**, and **n6/n3**. Comparison was made possible standardizing the results of the analyses for the different traits (i.e. value of the SNP effect/phenotypic standard deviation of the trait).

It is noteworthy to point out that for **FW** and **wbSF** traits the selection will be greatly enhanced with the inclusion of the CAPN316 and CAPN530 SNPs; namely C and G allele respectively, because they decrease the loss of free water content and produce a meat that is more tender as showed by the figure xx. The other SNPs analysed, even if with positive effects, have less impact on selection goals.

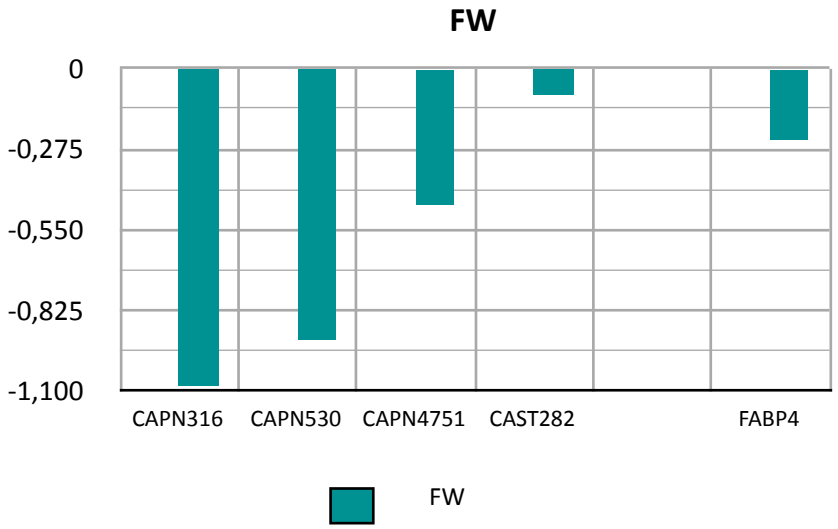

Figure 5.1 -Free water content trait.

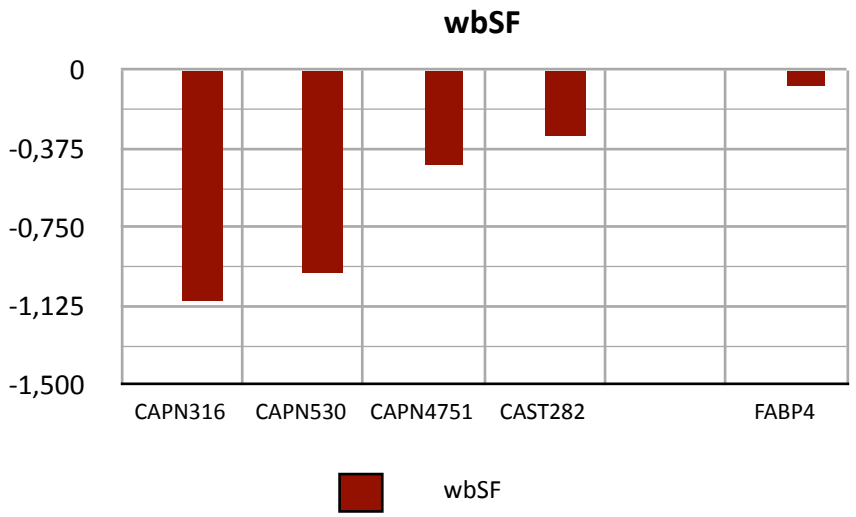

Figure 5.2 - Shear force of raw meat.

Considering the percentage of **C18-2 n6**, the A allele for the DGAT1 and the C allele for the FABP4 seems to improve the level of this fatty acid. The situation observed to **n6/n3** trait shows that the two SNPs decrease the n6/n3 ratio, in this case the favourbale alleles are the G allele for both two SNPs. Also the CAPN316 and CAST282 seem to decrease this parameter.

In conclusion, trying to design a set of SNPs more useful for selection, the figures suggest to address the choice for the C allele of CAPN316 for **FW**, **wbSF**, and **n6/n3** traits, as well as for the G allele of CAPN530 that it seems a favourable allele for the tenderness of the meat. Furthermore, considering the fatty acids, the A allele of DGAT1 and the C allele of FABP4 could be favourable for the **C18-2 n6** levels but not for the n6/n3 ratio.

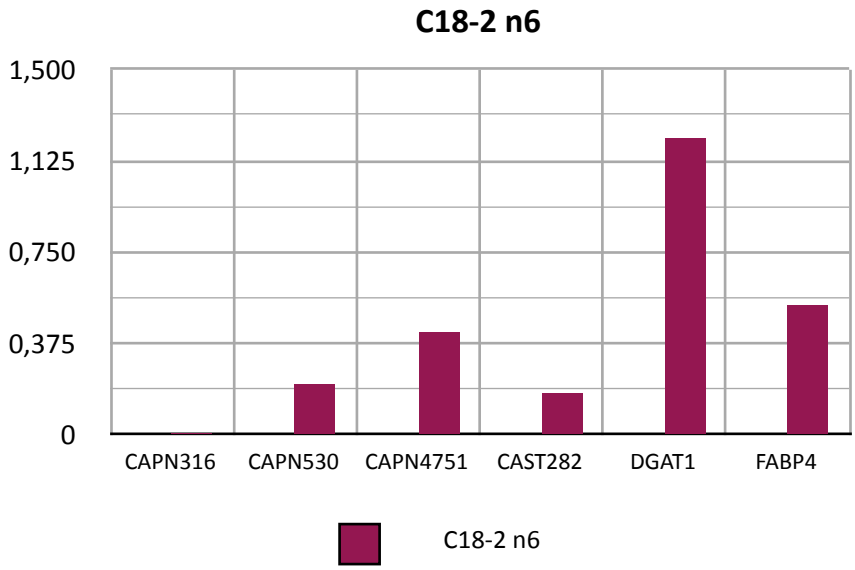

Figure 5.3 - C18- 2 n6 trait.

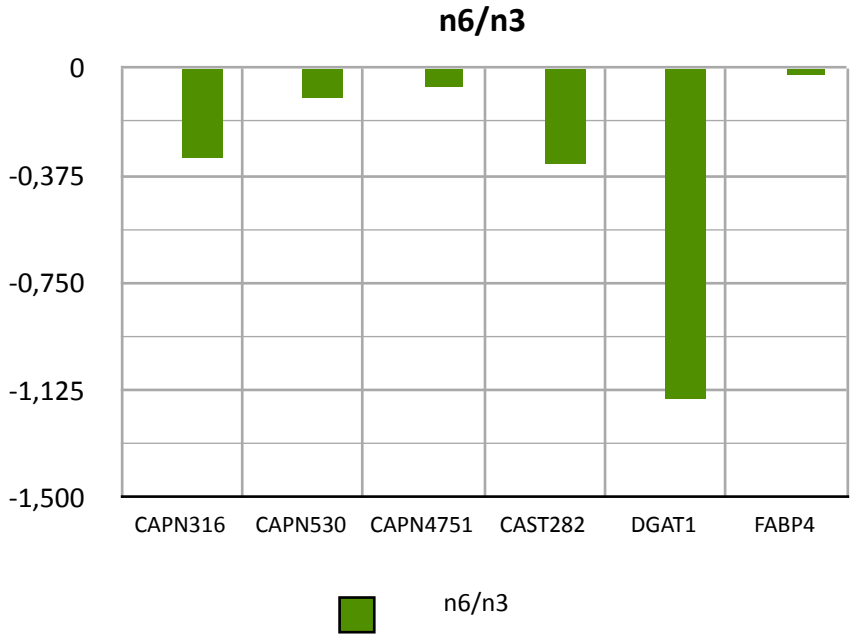

Figure 5.2 - n6/n3 ratio trait.

Literature cited

- Avilés C., Polvillo O., Peña F., Juárez M., Martínez A. L., Molina A., 2013. Association between DGAT1, FABP4, LEP, RORC, and SCD1 gene polymorphisms and fat deposition in Spanish commercial beef. Journal of Animal Science, vol. 91, 4571-4577.
- Falconer D. S. and Mackay T. F. C.,1996. Introduction of Quantitative genetics. Fourth Edition, Addison Wesley Longman (Pearson education).
- Michal J. J., Zhang Z. W., Gaskins C. T., Jiang Z., 2006. The bovine fatty acid binding protein 4 gene is significantly associated with marbling and subcutaneous far depth in Wagyu x Limousine F_2 crosses. Animal Genetics, short communication, vol 37, 400-402.

General Conclusion

The study investigated the variability of 7 genes in the Limousine breed and the association of SNPs with different traits linked with meat tenderness, one of the most important quality trait for consumer.

The results presented here confirm some of the previously documented association, like the association between CAPN316 and CAPN530 SNPs and meat tenderness, while the CAPN4751 shows contrasting results for this trait.

For CAST282 the C allele is associated with low shear force for meat and a reduction of *Longissimus dorsi* area, lean yield and free water content.

Furthermore, novel association have been identified between fatty acids and calpain and calpastatin genes, where further validation studies in other populations it might be important to develop in future.

The power analysis indicated that DGAT1 allele association was detected only for few fatty acids. Also FABP4 SNP confirms its important role in lipid metabolism of intramuscular fat but its negative correlation with area and weight of *Longissismus dorsi*.

Mutation in the bovine LEP gene has not always shown associations with physical and fatty acid traits in beef cattle. It has to be pointed out that fat content is a complex trait that is influenced by multiple environmental factors that could confound results leading to non-significant findings.

The results for the SNPs abovementioned are also confirmed by the association analysis where the effect of the pedigree was incorporated.

F94L, Q204X, and TG were fixed in our population so it was impossible to investigate their effects on the traits analyzed.

Moreover a lack of true association between traits and SNPs could be caused by the difference in SNP frequencies, genotype and environment

interactions or dominance effect but also it is possibly due to the quite moderate size of population.

These results expand the possibilities for using markers to improve meat tenderness in many commercial herds, but before that it might be advisable to study these new associations also in other commercial breeds.

This first study on Limousine breed suggests to incorporate markers information in the breeding programme of the breed at the aim of increasing the rate of genetic gain.

Appendix 1

Forty-three variables analyzed

Ald	SS_TQ	C18_1-n7_P
ld_p	PG_TQ	Somma C18_1n9&n7
am_p	EE_TQ	C18_2-n6cis_P
gs_p	CEN_TQ	C20_1-n9_P
gp_p	n6_n3	C20_3-n6_P
oss_p	SFA_TOT_P	C20_4-n6_P
pH	MUFA_TOT_P	Somma C22_1n9&n11
L_Media	Somma PUFA n6+n3	C22_4-n6_P
a_Media	iso-C14_0_P	C22_5-n3_P
b_Media	C14_0_P	
Croma_M	Somma C14_0	
Tinta_M	C16_0_P	
H2O L Media	C16_1n9_P	
calo_p	C16_1-n7_P	
Wbcru Media	Somma C16_1n9&n7	
Tagcru Media	C18_0_P	
Tagcott Media	C18_1-n9ct_P	

Appendix 2

Sire's Genotypes

Genotypes									
Sire Name	**CAPN 316**	**CAPN 4751**	**CAPN 530**	**CAST 282**	**DGAT1**	**F94L**	**FABP4**	**LEP**	**Q204X**
ASTERIX	?	?	?	G:C	?	A:A	G:C	?	C:C
ARAMIS	G:G	C:C	G:G	G:G	G:G	A:A	G:G	C:C	C:C
APOLINAIRE	G:G	T:C	A:G	G:C	G:G	?	G:C	C:C	C:C
APACHE	*G:C*	*T:C*	*A:G*	*G:C*	*G:G*	*A:A*	*G:C*	*T:C*	*C:C*
BAMBOU	G:G	T:C	A:G	G:C	G:G	A:A	G:G	C:C	C:C
ALEJANDRO	G:G	T:C	A:G	C:C	G:A	A:A	G:C	T:C	C:C
VAURIEN	G:G	T:T	A:G	C:C	G:G	A:A	G:C	T:C	C:C
CHARMEIRE	G:C	T:C	A:G	C:C	G:G	A:A	G:G	C:C	C:C
DIOCLEZIANO	G:C	C:C	G:G	G:C	G:G	A:A	G:C	C:C	C:C

In italic the possible genotype reconstruction for the Sire Apache.