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Nellore meat quality and genomics

Anirene Galvão Tavares Pereira

Thesis presented to obtain the degree of Doctor in Science. Area: Food Science and Technology

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Anirene Galvão Tavares Pereira
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Advisor:
Profª. Drª. **CARMEN JOSEFINA CONTRERAS CASTILLO**

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“I know how to be abased also be honored, all and in all circumstances, I have experience, I know what it is to need and I know what it is to walk with plenty. I have learned the secret of living happily everywhere and in every situation, whether well fed or hungry, having plenty, or through deprivation. I can do everything through Him who strengthens me”

Phillippians 4:12-13

“I have fought a good fight, I have finished the race, I have kept the faith”

2 Timothy 4:7

CONTENTS

RESUMO

Qualidade da carne Nelore e genômica

O presente trabalho foi desenvolvido com o objetivo de explorar regiões cromossômicas associadas à características de carcaça e carne em bovinos da raça Nelore, explorar suas funções em vias metabólicas e gênicas relacionadas às manifestações dessas características, assim como gerar novos fenótipos para futuros estudos de associação genômica, com vistas a descrever, de forma completa, as características relacionadas à qualidade do produto final. Para isso, 995 animais machos não castrados, genotipados para mais de 770.000 marcadores de polimorfismos de nucleotídeo único (SNP), foram avaliados quanto ao peso corporal ao nascimento, ganho de peso à desmama e ao sobre ano, conformação, precocidade de terminação e musculosidade à desmama e ao sobre ano. Como estas características são correlacionadas, foram aplicadas metodologias de mapeamento genômico com o objetivo de identificar regiões pleiotrópicas. Os resultados destacaram regiões do genoma bovino que contêm genes descritos por influenciarem em características de crescimento e ganho de peso nestes animais, com destaque para o gene *PLAG1*, pertencente à região do marcador mais significativo associado aos fenótipos, anteriormente associado ao peso, altura e precocidade sexual em animais dessa raça. Para acessar atributos de qualidade de carcaça e carne, 576 machos não castrados foram avaliados quanto ao peso de carcaça quente, área de lombo, espessura de gordura subcutânea, pH após 24 horas do abate, cor (L*, a*, b*) e perdas de peso por exsudação e cozimento e força de cisalhamento em diferentes tempos de maturação (7, 14 e 21 dias). Os animais foram genotipados em duas plataformas, Illumina® BovineHD BeadChip (HD) e GeneSeek® Genomic Profiler Bovine HD™ Illumina Infinium® (GGP), sendo os genótipos deste último imputados para o conjunto de maior densidade. As avaliações de perdas de peso por exsudação e cozimento e força de cisalhamento, utilizada para mensurar maciez, revelam a influencia da estrutura do citoesqueleto e da ação das enzimas proteolíticas, apontando o complexo enzimático serinas/serpinas como candidato na regulação do processo de proteólise e degradação da estrutura da fibra muscular. Foi realizada avaliação dos ácidos graxos no músculo *Longissimus thoracis et lumborum* de 148 animais com vistas à classificação das amostras quanto aos efeitos esperados no organismo humano (“benéfico”, “maléfico” ou “neutro”), assim como prover informação fenotípica para futuros estudos de associação genômica. A identificação de 42 ácidos graxos e 16 índices gerou informação detalhada sobre a gordura presente na carne destes animais, sendo observado, por análise de componentes principais (PCA), que a maior variação entre a composição das amostras avaliadas parece ser em decorrência da diferença de expressão das enzimas elongases e dessaturases. Dessa forma, espera-se que os dados, informações e conhecimento gerados por este trabalho, possam auxiliar os programas de melhoramento genético animal a aprimorar o rebanho brasileiro segundo características de interesse da cadeia produtiva de carne.

Palavras-chave: *Bos indicus*, GWAS; Peso corporal; Características corporais; Qualidade de carne; Perfil de ácidos graxos

ABSTRACT

Nellore meat quality and genomics

This study was developed in order to explore chromosomal regions associated with carcass and meat traits in Nellore cattle breed, identifying metabolic and genetic pathways related to its characteristics expression, as well as generate additional phenotypes for future genome association studies, in order to fully describe parameters related to final product quality. Thereunto, 995 bulls were genotyped for more than 770,000 single nucleotide polymorphisms (SNPs), were evaluated for body weight at birth, weight gain at weaning and yearling, conformation, finishing precocity and muscling at weaning and yearling. These traits are correlated, therefore, genomic mapping method were applied in order to identify pleiotropic regions. Results highlighted previously described genomic regions associated to beef cattle weight gain and growth traits, particularly *PLAG1* gene, sheltered by the most significantly associated marker region, which in other studies were associated to weight, height and sexual precocity in Nellore breed. To evaluate carcass and meat quality traits, 576 young bulls were evaluated for hot carcass weight, ribeye area, fat thickness, pH 24 hours after slaughter and color parameters (L*, a*, b*), for shearing force, dripping and cooking loss, evaluations were performed for different maturation times (7, 14 and 21 days). Animals were genotyped on two platforms, Illumina® BovineHD BeadChip (HD) and Bovine GeneSeek® Genomic Profiler ™ HD Illumina Infinium® (GGP). Animals genotyped at a lower density (GGP) were imputed to high density chip (HD). Shear force, dripping and cooking loss measures which relates to meat tenderness, were associated to cytoskeleton structure and proteolytic enzymes activity, pointing to serine/serpin enzyme complex as main candidates for regulate proteolysis and muscle fiber structure degradation. Were performed an evaluation of *Longissimus thoracis et lumborum* intramuscular fat content of 148 animals. It was approached by a human health perspective where samples received a classification regarding fatty acids effects on human organism ("beneficial", "evil" or "neutral"), as well as provided phenotypic information for future genome association studies. The identification of 42 fatty acids and 16 indexes, generated detailed information on these animals' meat fat composition. Principal component analysis (PCA) results showed that large variation proportion between samples fat composition occurs due to expression differences among desaturase and elongase enzymes. Thus, it is expected that generated data, information and knowledge hereby, can assist animal breeding programs to improve Brazilian herds according meat chain interests.

Keywords: *Bos indicus*; GWAS; Body live weight; Body traits; Meat quality; Fatty acid profile

1 INTRODUCTION

Cattle raising and meat production is of paramount importance for Brazilian agribusiness, ensuring a prominent position of the country on the world stage, with a herd that reached 212.3 million animals in 2014, maintaining Brazil as the second largest meat cattle producer in the world, behind only by United States of America (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA - IBGE, 2015; BRASIL, 2015). In 2014, 42.07 million animals were slaughtered producing over 10 million tons in carcass equivalent weight. Approximately 80% of this production was used to supply the domestic market and 20% were exported, leading Brazilian beef to more than 90 countries, generating revenues of nearly 6 billion dollars, 9% of increasing compared to 2013 exports (ASSOCIAÇÃO BRASILEIRA DAS INDÚSTRIAS EXPORTADORAS DE CARNE - ABIEC, 2015).

Brazilian cattle raising system is predominantly grass-feed, with approximately 167 million hectares of pastures, and Zebu cattle is highly performing in the activity, presenting proven hardiness and adaptation to Brazilian tropical environment (ABIEC, 2015). According to the Brazilian Association of Meat Exporters (ABIEC), about 80% of the herd is composed by Zebu breeds (*Bos indicus*), among which Nellore stands out with 90% of this percentage. Nellore cattle is widespread throughout the country due to some advantages over some other breeds such as, sexual precocity and high carcass yield, which meets meatpacking industry expectations, besides its environment adaptation (ASSOCIAÇÃO BRASILEIRA DOS CRIADORES DE ZEBU - ABCZ, 2015). Although, there are some controversies related to Nellore meat quality when compared to those from taurine breeds (*Bos taurus*), especially regarding meat tenderness (CROUSE et al., 1989; SHACKELFORD; WHEELER; KOOHMARAIE, 1995) and marbling (O'CONNOR et al., 1997; SHACKELFORD; WHEELER; KOOHMARAIE, 1995), which has been motivating new researches to better understand such differences (BOLORMAA et al., 2013; BRESSAN et al., 2011; HIGHFILL et al., 2012).

Ideally, meat quality is affected by technological, functional, sensorial, nutritional, toxicological, regulatory and ethical issues. Meat technical attributes such as water retention capacity and emulsification; sensorial, such as color and tenderness; and nutritional, especially related to protein content and fatty acid profile, are influenced by several factors, including environment, animal breed, age, gender, finishing weight, diet, stress during slaughter and *post-mortem* factors (ANDERSON

et al., 2012; CHENG; SUN, 2008; DEL CAMPO et al., 2008; KIM; LONERGAN; HUFF-LONERGAN, 2010; LAGERSTEDT; LUNDSTRÖM; LINDAHL, 2011; PESTANA et al., 2012). Currently, these attributes started to be assessed and evaluated without prior need for slaughter, allowing the selection and production of more efficient animals and reducing technological intervention needs during *post-mortem* period.

DNA analysis using molecular markers is one vented possibility to access technological and quality attributes in living animal. The first studies to use molecular markers to characterize and identify genetic resources, generating tools for animal breeding, date from the late 80s. In recent years, technology to generate molecular data went through several renovation cycles. The last wave of technological innovation represents a true revolution that brought methods to identify and characterize thousands of SNP markers (Single Nucleotide Polymorphism, defined as a punctual DNA sequence variation occurring in a single base) (CAETANO, 2009), enabling identification of large number of variants associated with biological functions and features of economic importance (PAREEK et al., 2011).

These technologies allow the selection of more efficient animals through the use of genetic/genomic information and is widespread and consolidated in taurine dairy cattle breeds. In these breeds, genomic information is used to increase accuracy of prediction of expected progeny differences (GEPDs) in breeding programs (HAYES et al., 2009). Another important application of genomic information is on the identification and mapping of genes involved in phenotype expression, in order to improve the genetic background regulating the expression understanding of complex traits. This approach, known as genome wide association study (GWAS), allows the identification of statistical associations between hundreds of genomic loci and the studied trait, increasing the understanding of important molecular and biological pathways (CORVIN; CRADDOCK; SULLIVAN, 2010). Housing direct measurements in cattle are difficult and costly to obtain, often requiring the animal to be slaughtered for meat quality evaluation. As the retail price of carcasses is essentially determined by its weight, beef cattle breeding programs have traditionally been focused on weight selection (GARRICK, 2011) and carcass visual scores (ARAÚJO et al., 2010) in living animals. Measurement of body weight as well as body conformation scores, carcass finishing precocity and musculature, are routinely used in beef cattle breeding programs to promote size and carcass yield improvement

(TORAL et al., 2011). Selection based on these features can lead to significant genetic changes (BOLIGON; MERCADANTE; ALBUQUERQUE, 2011). Performed by the main national breeding programs, the visual assessment known as CPMU (conformation, precocity, muscling and navel), seeks animals with functional biotype, comprising characteristics linked to productivity, and it has been of great value since carcass composition traits present heritability estimates ranging from medium to high (BOLIGON; MERCADANTE; ALBUQUERQUE, 2011).

Thus, efforts to identify QTLs (quantitative trait loci, chromosomal regions associated with phenotypic variations) explaining differences in weight and carcass characteristics of *Bos taurus* (PAUSCH et al., 2011), *Bos indicus* (UTSUNOMIYA et al., 2013) and cross breed populations (BOLORMAA et al., 2011), have been reported. More recently, associated genomic regions to visual scores of conformation, carcass finishing, precocity and musculature, were investigated in order to provide selection indexes for these traits (BOLIGON; MERCADANTE; ALBUQUERQUE, 2011; NEVES et al., 2014; SHIOTSUKI et al., 2009). Genomic studies for live weight in beef cattle, present some consensus regarding a QTL on chromosome 14, covering *PLAG1*, which explains part of the variation on this trait (FORTES; KEMPER; SASAZAKI, 2013; FORTES; REVERTER, 2012; UTSUNOMIYA et al., 2013), and also appears to be associated with animals height, growth rate, back fat thickness and body size (FORTES; REVERTER, 2012; LITTLEJOHN; GRALA; SANDERS, 2012). Moreover, live weight is closely related to hot carcass weight, which is a measure obtained after slaughter, evisceration and carcass cleaning. Hot carcass weight has positive genetic correlations with carcass yield and ribeye area (PARIACOTE; VAN VLECK; HUNSLEY, 1998), the main measures to evaluate meat industry gain. In addition to carcass, weight and performance traits, meat direct evaluation is crucial to assess consumers purchasing decisions and brand loyalty. Generally, *in natura* meat quality is commonly evaluated by its potential of Hydrogen (pH), subcutaneous fat thickness, marbling, color, water holding capacity and tenderness (KAMRUZZAMAN et al., 2012).

Potential of hydrogen is the most frequently measured parameter in fresh meat, not only for the analysis simplicity but also due to its relation with muscle to meat conversion biochemical processes. The pH variation rate during *post-mortem* period, is associated with meat sensory characteristics (DEL CAMPO et al., 2008), and have a strong effect on color, water-holding capacity and tenderness of the final

product (CONTRERAS-CASTILLO et al., 2016). Low final pH values adversely affect the functionality of muscle proteins, reducing the water retention capability and color stability, and may also alter μ-calpain activity and autolysis, thus affecting *post-mortem* proteolysis and meat tenderness (KIM; LONERGAN; HUFF-LONERGAN, 2010). On the other hand, higher pH values improves meat tenderness but lead to meat darkening (DFD – dark, firm and dry), resulting in market depreciation as consequence as shorter shelf life (CONTRERAS-CASTILLO et al., 2016; LOMIWES et al., 2014; WULF et al., 2002).

Meat color, though does not always indicate quality, is one of the few traits which consumers can evaluate by the purchase time, therefore, extremely important for their purchase decision (KAMRUZZAMAN et al., 2012; LAGERSTEDT; LUNDSTRÖM; LINDAHL, 2011). Consumers are attracted to red and bright color meat, and when this is not attractive, the product can be considered of poor quality (WU et al., 2012). Myoglobin, a sarcoplasmic heme protein, is the major responsible for meat color and its chemistry is specific to each specie. Interactions between this protein and multiple extrinsic (presence of oxygen, packaging in modified atmosphere) and intrinsic factors (pH, lipid oxidation, mitochondrial activity, muscle type, diet, management, genetics) determine the raw meat color (SUMAN; JOSEPH, 2013). Meat pigmentation occurs due to the iron atom associated with myoglobin porphyrin core, which main function is to store oxygen in mammalian muscle. The iron oxidative state can vary from Fe^{+2}, pigment known as deoxymyoglobin with a purplish red color, to its oxidized state Fe^{+3}, known as metmyoglobin showing pale brown color (SUMAN; JOSEPH, 2013).

Water holding capacity (WHC) is another important factor from an economic point of view, it is associated to product yield, affecting meat tenderness and juiciness. It expresses the meat capacity to retain the inherent or added water and is highly correlated to dripping, cooking and cooling losses (depending on the processing stage) color, and salinity. Moreover, it is also related to salable weight loss, which in addition to water, include proteins and other water soluble solids (CHENG; SUN, 2008; HUFF-LONERGAN; LONERGAN, 2005; REARDON et al., 2010). Classical hypotheses that explain meat water holding are based on electrostatic, osmotic and capillary forces, which entrap water and cause swelling of myofibrils, primarily affected by pH, salt concentration, actomyosin complex crosslinking and protein denaturation (PUOLANNE; HALONEN, 2010). These

authors also described the myofibrillar proteins composition and structure effect on meat water holding capacity, mentioning aspartic and glutamic amino acids, as those with higher ability to bind water molecules. Cheng and Sun (2008) also describe other factors that seem to influence WHC, such as animal feed, pre and post-slaughter treatment, cooling, maturation period, use of additives, massaging and genotypic profile. This latter associated with WHC through introduction of genotypes that increase connective tissue percent, intramuscular fat content and myofibrils development, those act as physical barriers to muscle water loss.

Meat tenderness is a sensory attribute that affect meat acceptability, especially beef, that has its final market price determined by tenderness degree, confirming researches that present this characteristic as the most important among consumer satisfaction (DERINGTON et al., 2011; KOOHMARAIE; GEESINK, 2006; MODZELEWSKA-KAPITUŁA et al., 2012; SCHENKEL et al., 2006; WU et al., 2012). This attribute is influenced by the combined effect of several factors, including connective tissue content, shortening length of sarcomeres, proteolysis of the myofibrillar proteins during meat maturation, pH and muscle fiber type (ANDERSON et al., 2012; KIM; LONERGAN; HUFF-LONERGAN, 2010). Recent studies have focused on meat tenderization process during *post-mortem* period and enzymatic systems potentially involved in muscle fiber structure degradation. It is believed that the extension rate of myofibrils proteolysis is largely responsible for tenderness (ANDERSON et al., 2012), and approximately 65 to 80% of tenderization occurs between the 3rd and 4th days after slaughtering, due to titin, nebulin, vinculin, desmin and dystrophin filaments degradation, that compose the cytoskeletal network with actin and myosin, linking these to the sarcomere structure and, in turn, this structure to the sarcolemma. The calpain enzyme complex is responsible for 90% of tenderization which occurs during this period. This enzymatic system is constituted by m, μ and calpain 3, and calpastatin, the main inhibitor of m and μ calpain (ANDERSON et al., 2012; GEESINK et al., 2006). Measurements of calpastatin activity 24 hours after slaughter, show high heritability and strong genetic correlation with shearing force strength (BURROW et al., 2001). While, actin and myosin filaments degradation, major sarcomere proteins, seems to occur around 7 to 10 days *post-mortem,* primarily by cathepsins action (ANDERSON et al., 2012; KOOHMARAIE; GEESINK, 2006; TAYLOR et al., 1995). These are proteolytic enzymes released from lysosomes during post-mortem membrane destruction, which

occurs due to decreasing pH and high temperatures, and are positively correlated to the increased beef tenderness (KEMP et al., 2010).

Additionally, caspase, proteasomes and small heat shock proteins enzymatic systems have received special attention because they seem to be related to meat tenderization process during *post-mortem* period, acting on muscle fiber structure proteins. Caspases, peptidases group of cysteine with apoptotic action, and proteasomes, a multicatalytic protease complex involved in the degradation of cytoplasmic and nuclear proteins, as well as calpains and cathepsins have been associated with the degradation of cytoskeleton constituents, i.e. myofibrillar proteins (HERRERA-MENDEZ et al., 2006; KEMP et al., 2010) promoting meat tenderization. Conversely, the small heat shock proteins (HSPs) due to anti-apoptotic and chaperone function in order to maintain homeostasis under stress conditions, has been associated with the maintenance of cell structure during *post-mortem* period, delaying the myofibrillar proteins degradation rate (LOMIWES et al., 2014), binding to caspases and inhibiting its activity (OUALI et al., 2013), with consequent increased shearing force (LOMIWES et al., 2013). Similarly, the serpin (serine protease inhibitor), exhibit inhibitory function of serine peptidases, some caspases and cathepsins, binding through irreversible covalent bonds with proteinase enzymes, altering its structure and making it unable to complete the catalytic process (BOUDIDA et al., 2014). As a result, their activity during *post-mortem* period affect maturation and tenderization processes (HULSMAN HANNA et al., 2014; SENTANDREU; COULIS; OUALI, 2002).

Similarly, carcass fat content and composition has been used in several bovine meat quality scoring systems as a palatability predictor, due to its association with tenderness, juiciness and taste, which affect consumer perception (DERINGTON et al., 2011). In general, meat with high intramuscular fat content receives higher quality scores (CHO et al., 2010). Jeremiah (1996) evaluated beef fat content regarding its acceptability by a trained panel of tasters and consumers, concluding that carcasses with a minimum subcutaneous fat thickness (8 mm) and low marbling levels presented acceptability rate of at least 90% without application of *post-mortem* techniques.

From a nutritional point of view, animal fat composition regarding its fatty acid profile is also of great importance and has become increasingly crucial in determining the meat quality components. Consumers are interested in knowing saturated fat

content, cholesterol (PADRE et al., 2006) and trans fatty acids (MCAFEE et al., 2010), especially because recent studies have linked meat consumption to increased risk of cardiovascular, cancer, inflammatory and auto-immune diseases (GANJI; KAMANNA; KASHYAP, 2003; MCAFEE et al., 2010; SIMOPOULOS, 2002). However, red meat contains various fatty acids associated with beneficial health effects, such as omega-3 family (ω3), particularly those of long carbon chain (EPA - eicosapentaenoic acid, and DHA - docosahexaenoic acid) and conjugated linoleic acids (CLA - mixture of geometric and positional isomers of linoleic acid C18:2-n6), which are associated to reduced risk of atherosclerosis, heart and inflammatory diseases and possible changes in behavior, such as depression (CONNOR, 2000; LIN; HUANG; SU, 2010).

Given the importance, a small universe of studies has associated genomic markers for most meat characteristics described above. Pinto et al. (2011) found a significant association between *E2FB* SNP (located in the leptin gene) and dripping loss, as well as identified a calpain gene (*CAPN1*) mutation located on *CAPN4751* SNP, which have additive effect on meat red and yellow color intensities. Another genome-wide association study in Nellore cattle, identified QTLs associated with shear force measures evaluated in different maturation times, at slaughter (without ageing) on BTA 23, BTA13 (7 days) and BTA2 (14 days), also a QTL on chromosome 11 that explains fat thickness variation and a major QTL in BTA8 explaining pH additive genetic variance for measures obtained after 24 hours of slaughter (TIZIOTO et al., 2013). In taurine breeds, *PKG3* gene, which encodes a regulatory protein of muscles glycogen content, had SNPs associated to cooking loss and color parameters H (color tone) and b* (intensity of blue to yellow), *GHR* gene, which affect growth, was associated to fat content and marbling, and *SCD* gene, which is a major protein complex for monounsaturated fatty acid synthesis, was associated to total fat content (REARDON et al., 2010). In Hanwoo Korean breed, fat synthesis was associated to three SNPs in *FABP4* gene region on chromosome 14 (SHIN; HEO; CHUNG, 2012). For the fatty acid profile, a genome association study in Nellore breed identified 23 associated genomic regions distributed among 12 chromosomes (2, 3, 6, 7, 8, 9, 10, 11, 12, 17, 26 and 27), which candidate genes seems to regulate fatty acids content in meat (CESAR et al., 2014).

Regarding meat tenderness, several studies reported associated markers in calpain/calpastatin complex, a known enzymatic system related to myofibrillar

proteolysis (ALLAIS et al., 2011; GANDOLFI et al., 2011; PAGE et al., 2002; SCHENKEL et al., 2006; TIZIOTO et al., 2013). Three SNP markers in the ankyrin gene (*ANK1*) region were also described to be associated to this trait. *ANK1* encodes structural muscle proteins that form a highly complex network of connections between myofibrils and between myofibrils and sarcolemma, contributing to meat texture and tenderness (ASLAN et al., 2010). Also, *MyoG* gene was associated to tenderness, due to its role on muscle fiber growth, and to water holding capacity (UJAN et al., 2013).

Lastly, particularly in Brazil, where indicine cattle play an important role in meat industry, it is expected that genomic predictions can improve production efficiency (GARCIA et al., 2012), and the application of GWAS studies for all characteristics described above, can lead to knowledge of genetic, biological and physiological factors related to qualitative and quantitative characteristics of interest for both producers and consumers.

Therefore, this study aimed to generate knowledge about the genetic mechanisms involved in the phenotypic expression of qualitative and quantitative traits related to weight, body conformation, carcass and meat quality in Nellore cattle through assessment of these traits (weight at birth, weight gain ate weaning and yearling, body conformation, finishing precocity and muscling at weaning and yearling, ribeye area, fat thickness, pH 24 hours *post-mortem*, color, weight loss by exudation and cooking and shear force) association with SNP molecular markers. In addition, further analysis was performed for significantly associated genomic regions with the aid of different databases, in order to propose biochemical and physiological mechanisms that explain the observed phenotypic variance.

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2 PLEIOTROPIC GENES AFFECTING CARCASS TRAITS IN *Bos Indicus* (NELLORE) CATTLE ARE MODULATORS OF GROWTH

Abstract

Two complementary methods, namely Multi-Trait Meta-Analysis and Versatile Gene-Based Test for Genome-wide Association Studies (VEGAS), were used to identify putative pleiotropic genes affecting carcass traits in *Bos indicus* (Nellore) cattle. The genotypic data comprised over 777,000 single-nucleotide polymorphism markers scored in 995 bulls, and the phenotypic data included deregressed breeding values (dEBV) for weight at birth, weight gain at weaning and yearling, as well visual scores taken at weaning and yearling for carcass finishing precocity, conformation and muscling. Both analyses pointed to the pleomorphic adenoma gene 1 (*PLAG1*) as a major pleiotropic gene. VEGAS analysis revealed 224 additional candidates. From these, 57 participated, together with *PLAG1*, in a network involved in the modulation of the function and expression of *IGF1* (insulin like growth factor 1), *IGF2* (insulin like growth factor 2), *GH1* (growth hormone 1), *IGF1R* (insulin like growth factor 1 receptor) and *GHR* (growth hormone receptor) genes, suggesting that those pleiotropic genes operate as satellite regulators of the growth pathway.

Keywords: Genetic correlations; Zebu; Insulin-like growth factor; Beef cattle

2.1 Introduction

Carcass yield plays a major economic role in beef cattle, as the carcass retail price is essentially determined by its weight. As differences in carcass yield between steers are partially heritable, selection and breeding are determinant operations in the beef cattle sector (GARRICK, 2011). However, direct carcass measurements are challenging as phenotype collection depends on animal slaughter. Therefore, the use of surrogate phenotypes such as body weight measurements and visual carcass evaluation in live animals is imperative for improving carcass yield (CANCIAN et al., 2014).

Weight measurements and visual scores of conformation, carcass finishing precocity and muscling (CPM) have been routinely employed in selection to improve carcass yield in Brazilian Nellore (*Bos indicus*) cattle. These traits are inexpensive and effortless to measure, and present moderate heritability in the breed (BOLIGON; MERCADANTE; ALBUQUERQUE, 2011). However, these different traits supposedly have distinct genetic architecture, and determining the extent of their genetic correlations, as well as identifying genes affecting multiple traits simultaneously (i.e. pleiotropic genes) would be beneficial to improve strategies for genetic selection.

Bolormaa et al. (2014) have recently described a method for mapping pleiotropic variants affecting traits of interest in beef cattle. The procedure consists in performing genome-wide association (GWA) scans for each trait separately, and then summarizing the effects of each genetic marker across traits with a meta-analytical approach. Additionally, other recently developed methods have aimed at increasing power and interpretability of association studies by combining single-marker results within functional elements (e.g. genes) or user-specified chromosomal windows (CAPOMACCIO et al., 2015; LIU et al., 2010). Combining these two approaches may be useful in the search for putative pleiotropic genes affecting traits of interest in animals and plants. Here, we attempted to apply these methods to a sample of 995 Nellore bulls genotyped for over 777,000 single-nucleotide polymorphism (SNP) markers, for which deregressed estimated breeding values (dEBV) were available for nine weight and CPM traits. More specifically, we aimed at identifying major pleiotropic genes underlying variation in traits that are predictive of carcass yield in *B. indicus* cattle.

2.2 Materials and Methods

2.2.1 Ethical statement

This study was exempt from the local ethical committee evaluation as DNA samples used for genotyping were obtained from industrialized semen straws.

2.2.2 Genotypes

A total of 995 Nellore bulls were genotyped with the Illumina® BovineHD Genotyping BeadChip assay, according to the manufacturer's protocol. The panel included 777,962 SNPs annotated in the UMD v3.1 bovine genome assembly. These bulls were part of the genomic selection reference population from a commercial breeding program, namely DeltaGen (http://www.deltagen.com.br/nelore.php). Data filtering was performed with PLINK v1.9 (CHANG et al., 2015; PURCELL et al., 2007). All genotyped samples had call rate greater than 90%. Only autosomal markers presenting a minimum call rate of 95% and a minor allele frequency of at least 2% were analyzed.

2.2.3 Estimated breeding values

Estimated breeding values (EBVs) for birth weight (BW), weaning gain (WG), conformation at weaning (CW), carcass finishing precocity at weaning (PW), muscling at weaning (MW), post-weaning gain (PG), conformation at yearling (CY), carcass finishing precocity at yearling (PY), and muscling at yearling (MY) were obtained from routine genetic evaluations. The single-trait animal models used to generate the EBVs were corrected for environmental and maternal effects, and included records from 1,278,057 animals born between 1985 and 2012, and raised in 315 grazing-based Brazilian herds. The variance ratios required to solve the mixed model equations were computed based on restricted maximum likelihood estimates of the variance components. The heritabilities obtained for BW, WG, CW, PW, MW, PG, CY, PY and MY were 0.37, 0.26, 0.25, 0.25, 0.26, 0.33, 0.31, 0.31 and 0.30, respectively. Prior to the association analysis, EBVs were deregressed following Garrick, Taylor and Fernando (2009), and only the bulls presenting deregressed EBVs with a minimum accuracy (based on prediction error variance) of 0.50 were analyzed.

Records for WG and PG were based on the weight gain from birth to weaning (adjusted for a period of 205 days) and from weaning to yearling (adjusted for a period of 550 days), respectively. Records for conformation, carcass finishing precocity and muscling were taken at weaning and yearling based on visual score evaluations relative to the animals of the same management group. Scores were assigned in a discrete ordered scale ranging from 1 to 5. The model used for BW included the fixed effects of contemporary group (defined as animals from the same herd, born in the same year and season, and belonging to the same birth management group) and age of dam at calving, as well as random maternal effects (maternal additive genetic effect and maternal permanent environmental effect). The model used for weaning traits included fixed effects of contemporary group (concatenation of BW contemporary group and herd-management group at weaning), Julian birth date within birth season, age at phenotype recording and age of dam at calving, in addition to the maternal effects described for BW. Post-weaning gain and the remaining yearling traits were corrected for the fixed effects of contemporary group (concatenation of WG contemporary group and herd-management group at yearling), age at phenotype recording and age of dam at calving.

2.2.4 Regression model

Prior to testing SNPs for association, the same single-trait regression model was applied across traits following eq. (1):

$$\mathbf{y} = \mathbf{1}_n\mu + \mathbf{g} + \mathbf{e} \quad (1)$$

Where **y** is the *n* x 1 vector of dEBVs, $\mathbf{1}_n$ is a *n* x 1 vector of 1s, μ is the overall mean, **g** is the *n* x 1 vector of random polygenic effects, and **e** is the *n* x 1 vector of random residual effects. Vector **g** was assumed a linear combination of additive marker effects as shown in eq. (2):

$$\mathbf{g} = \mathbf{Xb} \quad (2)$$

Where **X** is a *n* x *m* matrix of allele dosages at *m* markers (coded as 0, 1 or 2 copies of the minor allele), and **b** is a *m* x 1 vector of random additive marker effects. Vector **g** was assumed N(0, **G**), where $\mathbf{G} = \mathbf{K}\sigma_g^2$, **K** is the *n* x *n* matrix of additive relationships between individuals, and σ_g^2 is the variance due to genome-wide markers. Vector **e** was assumed N(0, **R**), where $\mathbf{R} = \mathbf{W}\sigma_e^2$, **W** is a diagonal matrix of weights accounting for heterogeneity of variance in dEBVs, and σ_e^2 is the residual variance. The resulting variance-covariance matrix of the model was **V** = **G** + **R**. Notice that this is essentially the polygenic (AMIN; VAN DUIJN; AULCHENKO, 2007; CHEN; ABECASIS, 2007) or the Genomic Best Linear Unbiased Predictor (GBLUP) model (TAYLOR, 2014) corrected for heteroscedastic residuals. Estimates of model parameters for each trait were obtained using the hglm v2.1-0 package in R v3.2.1 (RÖNNEGRARD; SHEN; ALAM, 2010).

2.2.5 Choice of residual weights

Following Garrick, Taylor and Fernando (2009), the diagonal elements of **W** were computed as $\mathbf{w} = \lambda^{-1}(\mathbf{d} + c)$, where $\lambda = (1-h^2)/h^2$, h^2 is the heritability of the trait before deregression, $\mathbf{d} = (1 - \mathbf{r^2})/\mathbf{r^2}$, $\mathbf{r^2}$ is the squared vector of accuracies (i.e., reliabilities) of the pseudo-phenotypes, and *c* is a parameter taking values between 0 and 1 controlling the relative contribution of pseudo-phenotypes on the basis of their reliabilities. As can be seen in the formula, the weights **w** used here are linearly related to the weights **d** used by Neves et al. (2014), except that they were scaled by the variance ratio λ and added by a constant *c*. In order to achieve a balanced contrast between dEBVs with high and low accuracy, we adopted $c = 0.5$.

2.2.6 GWAS

Conceptually, associations are tested by contrasting the null polygenic model against alternative models that include the fixed effect of one candidate marker at a time (CHEN; ABECASIS, 2007; KANG et al., 2010; LIPPERT et al., 2011). However, this contrast is redundant since the candidate marker was also included as a random effect in the null model through **g** = **Xb**. This introduces a bias known as 'proximal contamination' (LISTGARTEN et al., 2012), which can substantially reduce the power of the tests. In order to avoid it, we used the leave-one-chromosome-out approach described by Yang et al. (2014). Briefly, the method consists in partitioning the genome-wide scan procedure per chromosome. For each chromosome *j*, we fit a modified null model where matrix **K** is built excluding all markers on *j*, guaranteeing that the null model does not contain the marker being tested or any other marker in linkage disequilibrium (LD) with it. Then, each marker on chromosome *j* is contrasted against the modified null model by using the test statistic $t = b/SE(b)$, where $b = (\mathbf{x'V^{-1}x})^{-1}\ \mathbf{x'V^{-1}y^*}$ and $SE(b) = (\mathbf{x'V^{-1}x})^{-1}$ for $\mathbf{y^*} = \mathbf{y - 1\mu}$. In this way, *t* is conditional on the random effects of genome-wide markers, such that the model preserves power while correcting for relatedness and population substructure. Additionally, by incorporating matrix **W** on **V**, we accounted for heterogeneity of variance in dEBVs while estimating *b* and *SE(b)*. Estimates for *b* and *SE(b)* were obtained by providing **V** and **y*** to the mmscore function in GenABEL v1.8-0 (AULCHENKO et al., 2007). In summary, our single-trait GWA analysis was almost identical to the leave-one-chromosome-out (i.e. mlma-loco) procedure (YANG et al., 2014) in GCTA (YANG et al., 2011), except that our model accounted for heterogeneity in residual variance.

2.2.7 Detection of pleiotropic genes

In order to identify pleiotropic genes affecting CPM and weight traits, we combined two distinct but complementary strategies. The Multi-Trait Meta-Analysis method described by Bolormaa et al. (2014) was used to summarize single-marker statistics across all studied traits and detect major pleiotropic genes. Additionally, the Versatile Gene-Based Test for Genome-wide Association Studies (VEGAS) method (LIU et al., 2010) was applied to the single-trait associations to perform gene-set based analyses, and genes appearing in the significant list of at least four of the nine traits evaluated were considered as candidate pleiotropic genes. VEGAS and Multi-Trait Meta-Analysis were implemented in R v3.2.1 and are described below.

Multi-Trait Meta-Analysis. For each SNP, consider **t** as the *q* x 1 vector of signed *t*-values across *q* traits, and **C** the *q* x *q* matrix of *t*-values correlations across genome-wide markers. The test statistic $\mathbf{t'C^{-1}t}$ is distributed as χ^2 with *q* degrees of freedom (df) under the null hypothesis of no pleiotropic effect. One standing issue in this implementation is that assuming $\mathbf{C}_{i,i} = 1$ when some traits present higher average correlations than others may cause highly significant composite scores even when single-trait analyses collectively present poor evidence of association. This issue was corrected here by adding the average correlation of each trait to their respective diagonal elements. The expected proportion of false discoveries among the markers declared significant was computed as $f = \alpha m/s$, where *m* is the number of tests, α is the significance level threshold, and *s* is the number of tests with $p < \alpha$. In order to select a value of α resulting in a false discovery rate lower than 5%, we applied the procedure described by Benjamini and Hochberg (1995). Briefly, we manipulated the expression above to obtain $\alpha = fs/m$ and defined *s* as the largest *p*-value rank position *i* satisfying $p_i \leq fi/m$ for $f = 0.05$.

VEGAS. For each trait and for each gene, the joint VEGAS test was computed as the sum of squared *t*-values. The null distribution for the VEGAS test was derived from Monte Carlo simulations. Briefly, each random draw from the null distribution was obtained as $x^2 = z_1^2 + z_2^2 + \ldots + z_m^2$, where z_m^2 is one element of a *m* x 1 random vector $\mathbf{z} \sim \text{MVN}(\mathbf{0}_q, \mathbf{D})$ and **D** is a matrix of signed genotypic correlations among the *m* markers within the gene. Probability values were computed as the number of times the simulated x^2 values were greater than or equal to the observed VEGAS statistic, divided by the total number of simulations. The number of simulations was chosen adaptively: let $\boldsymbol{x^2}$ be the vector of random draws from the null distribution. At every iteration, if *p* was lower than the inverse of the current number of simulations *k*, *k* - length($\boldsymbol{x^2}$) extra samples were obtained, the new *p*-value was computed, and the new number of simulations was set to $10^{\log10(k)+1}$. We initialized the iterations using $k = 1{,}000$. In this way, the algorithm dynamically re-calibrated low *p*-values based on a sample precision of 10^3, 10^4, 10^5 and 10^6 simulations. Due to computational limitations, the process was interrupted at 10^6 simulations, and probabilities were bounded to $p < 10^{-6}$. Genes sheltering no SNPs had their *p*-values set to 1. In order to guarantee that **D** was positive definite, we used the maximum set of markers for which all pairwise squared correlations were lower than or equal to 0.5. In order to capture information from intergenic markers in LD with unobserved

variants lying within genes and their regulatory regions, we expanded gene boundaries in the UMDv3.1 assembly to ± 100 kb of the 5' and 3' UTRs. Finally, given the VEGAS test is highly conservative, the gene list for each trait comprised all genes with $p < 0.01$. Putative pleiotropic genes were defined as those appearing in the gene list of four or more traits.

2.2.8 Functional analysis

Interactions between protein-coding genes were predicted using the STRING database (SZKLARCZYK et al., 2015). Additionally, networks were graphed with gephi 0.8.2 (available at: http://gephi.github.io/).

2.3 Results and Discussion

2.3.1 Data filtering

A total of 516,740 SNPs and 995 individuals passed the filtering criteria and were retained in the dataset. After data filtering, the mean and median gap between any pair of consecutive markers on the same chromosome were approximately 5.26 kb and 3.15 kb, respectively. The resulting genotyping rate across markers and samples was approximately 0.99. The number of bulls with dEBV accuracy higher than 0.5 for BW, WG, CW, PW, MW, PG, CY, PY and MY were 837, 915, 875, 876, 875, 880, 844, 844 and 844, respectively.

2.3.2 Evidence of pleiotropic effect from additive genetic correlations

As dEBVs encapsulate individual additive genetic values and association *t*-values represent marked additive genetic effects, we considered both dEBVs and *t*-values correlations between traits as proxies for the additive genetic correlations between traits. On average (**Table 1**), dEBVs were moderately correlated across traits (r = 0.442), and strikingly similar results were found for *t*-values across traits (r = 0.423). An exception was BW, which was only mildly correlated with WG and moderately correlated with conformation traits. These noteworthy genetic correlations suggest that pleiotropic loci may contribute to the genetic variance of these traits.

Table 1 - Deregressed estimated breeding values (dEBV) and genome-wide SNP effects correlations (inside brackets) for weight and carcass traits in *Bos indicus* (Nellore) bulls

Trait	BW	WG	PG	CW	PW	MW	CY	PY	MY
BW	1.000 (1.000)	0.140 (0.202)	0.079 (0.062)	0.277 (0.321)	-0.059 (-0.063)	-0.056 (-0.045)	0.217 (0.272)	-0.051 (-0.078)	-0.052 (-0.070)
WG	0.140 (0.202)	1.000 (1.000)	0.501 (0.490)	0.776 (0.790)	0.493 (0.473)	0.507 (0.483)	0.734 (0.751)	0.435 (0.410)	0.430 (0.406)
PG	0.079 (0.062)	0.501 (0.490)	1.000 (1.000)	0.351 (0.360)	0.291(0.238)	0.234 (0.184)	0.571 (0.604)	0.456 (0.441)	0.429 (0.425)
CW	0.277 (0.321)	0.776 (0.800)	0.351 (0.360)	1.000 (1.000)	0.381 (0.325)	0.412 (0.359)	0.884 (0.877)	0.329 (0.265)	0.335 (0.274)
PW	-0.059 (-0.063)	0.493 (0.473)	0.291 (0.238)	0.381 (0.325)	1.000 (1.000)	0.884 (0.886)	0.393 (0.308)	0.917 (0.890)	0.833 (0.802)
MW	-0.056 (-0.044)	0.507 (0.483)	0.234 (0.185)	0.412 (0.359)	0.884 (0.886)	1.000 (1.000)	0.382 (0.303)	0.798 (0.770)	0.888 (0.855)
CY	0.217 (0.272)	0.734 (0.751)	0.571 (0.604)	0.884 (0.877)	0.393 (0.308)	0.382 (0.303)	1.000 (1.000)	0.451 (0.384)	0.445 (0.383)
PY	-0.051 (-0.078)	0.435 (0.410)	0.456 (0.441)	0.329 (0.265)	0.917 (0.890)	0.798 (0.770)	0.451 (0.384)	1.000 (1.000)	0.885 (0.887)
MY	-0.052 (-0.070)	0.430 (0.407)	0.429 (0.425)	0.335 (0.274)	0.833 (0.802)	0.888 (0.853)	0.445 (0.383)	0.885 (0.887)	1.000 (1.000)

BW = birth weight; WG = weaning gain; PG = post-weaning gain; CW = conformation at weaning; PW = carcass finishing precocity at weaning; MW = muscling at weaning; CY = conformation at yearling; PY = carcass finishing precocity at yearling; MY = muscling at yearling

2.3.3 Major pleiotropic effects map to the *PLAG1* region

After combining the results across the nine traits with the Multi-Trait Meta-Analysis method, a total of 983 markers were declared significant at an empirical threshold of $p < 9.20 \times 10^{-5}$ (**Figure 1-A**), resulting in a false discovery rate of approximately 5%. A single large, dominant signal mapping to chromosome 14:19.46-34.92 Mb was identified. The leading SNP, namely rs136543212 (probe BovineHD1400007373), mapped to position 25,502,915, in the vicinity of the well-known *PLAG1* (pleomorphic adenoma gene 1) chromosome domain.

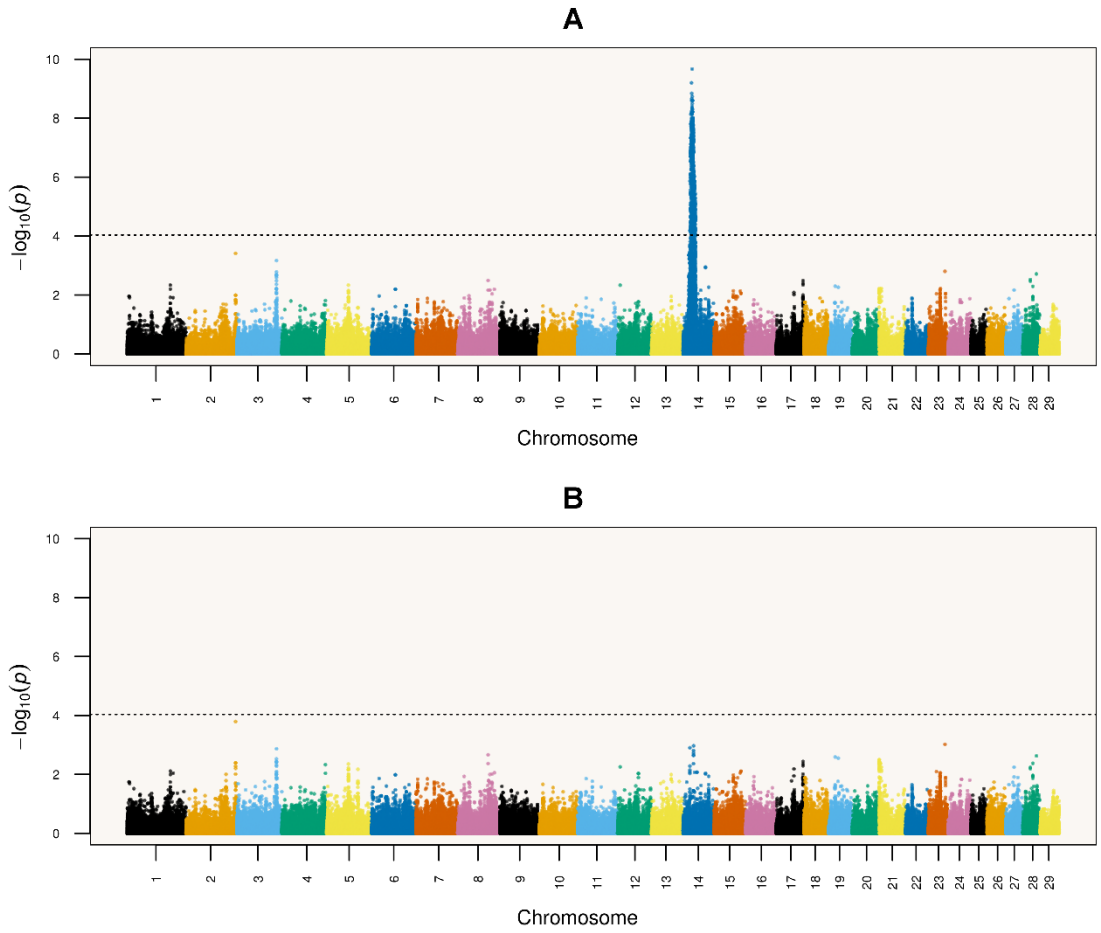

Figure 1 - Genome-wide Multi-Trait Meta-Analysis for loci affecting carcass traits in *Bos indicus* (Nellore) cattle. The dashed horizontal line represents the significance threshold ($p < 9.20 \times 10^{-5}$). Results are shown before (A) and after (B) the removal of the effect of marker rs136543212 (probe ID BovineHD1400007373)

This chromosomal segment has been implicated as a highly pleiotropic locus underlying genetic differences in growth, weight and reproductive traits in cattle (BOLORMAA et al., 2014; FORTES; KEMPER; SASAZAKI, 2013; FORTES et al.,

2012, 2013; KARIM et al., 2011; LITTLEJOHN; GRALA; SANDERS, 2012; UTSUNOMIYA et al., 2013, 2014). The functional candidacy of *PLAG1* is well supported by its regulation of the expression of insulin-like growth factors (IGF) (VOZ et al., 2000). These factors are major mediators of the growth pathway and the hypothalamic-pituitary-gonadal axis (VELAZQUEZ; SPICER; WATHES, 2008), and serum concentrations of IGF1 in cattle have been shown to be highly heritable, negatively correlated with weight and carcass traits (DAVIS; SIMMEN, 1997; DAVIS et al., 2003) and primarily determined by variants in the *PLAG1* chromosomal region (FORTES et al., 2013). The signal detected here suggested that the underlying causal variant has negative effects on carcass finishing precocity/muscling traits and positive effects on weight/conformation traits, consistent with a previous report of a candidate causal variant (KARIM et al., 2011) of *Bos taurus* origin (the C allele at SNP rs109231213, position 14:25003338) associated with decreased IGF1 serum concentrations and precocity, as well as with increased height and weight (FORTES; KEMPER; SASAZAKI, 2013).

To determine if this large segment comprised a single signal driven by a large LD block or it construed a mixture of signals, we re-analyzed our data using the same GWA model conditional on the fixed effect of the top scoring SNP. Correction for the effect of rs136543212 alone was able to remove most of the signal (**Figure 2-B**), which suggests that this large segment is a single LD block. However, as correcting for the leading SNP was not sufficient to completely eliminate the signal, it is hard to distinguish between the presence of more than one causal nucleotide within the LD block and residual effects captured by the remaining markers due to imperfect tagging of rs136543212.

2.3.4 Detection and interactions of additional candidate pleiotropic genes

We carried out the VEGAS analysis using single-trait GWA results. The top scoring gene appearing in the gene list of eight of the nine traits investigated was *PLAG1*, consistent with the findings of the Multi-Trait Meta-Analysis approach. Our analysis showed that the VEGAS and the Multi-Trait Meta-Analysis approaches are complementary, and that they can be used jointly to maximize the discovery of pleiotropic genes. As the *PLAG1* signal comprised a large LD block, we carried out further search for pleiotropic genes omitting results from chromosome 14. Additionally, we also omitted results from 25 olfactory receptor genes and a cluster of

32 histone genes, both mapping to the vicinity of other functional candidate genes. After applying these filters, we obtained a list of 224 candidate pleiotropic genes. These included 176 protein-coding genes, 12 pseudo-genes, 12 snoRNA, 11 snRNA, 11 miRNA and two misc_RNA. We then focused our functional annotation on protein-coding genes.

Besides *PLAG1*, we found a series of growth-related genes, including growth differentiation factors 2 (*GDF2*), 10 (*GDF10*) and 11 (*GDF11*), growth arrest-specific 2 like 3 (*GAS2L3*), fibroblast growth factor 22 (*FGF22*), PH domain and leucine rich repeat protein phosphatase 1 (*PHLPP1*), signal transducer and activator of transcription 2 (*STAT2*), SMAD family member 4 (*SMAD4*), and insulin-like growth factor binding protein 5 (*IGFBP5*). Genes involved in muscle development and function were also found, including methylmalonyl CoA mutase (*MUT*), troponin T type 1 (*TNNT1*), troponin I type 3 (*TNNI3*), and sarcoglycan delta (*SGCD*, also known as 35kDa dystrophin-associated glycoprotein).

We then used the STRING database to annotate protein-protein interactions among the candidate genes. From the initial list of 176 genes, 82 presented connections. From these, 54 genes were involved in a single network (**Figure 2-A**). The remaining 28 genes formed smaller networks ranging from two to six genes. Interestingly, *PLAG1* was not present in any network, in spite of being the major pleiotropic gene in our study.

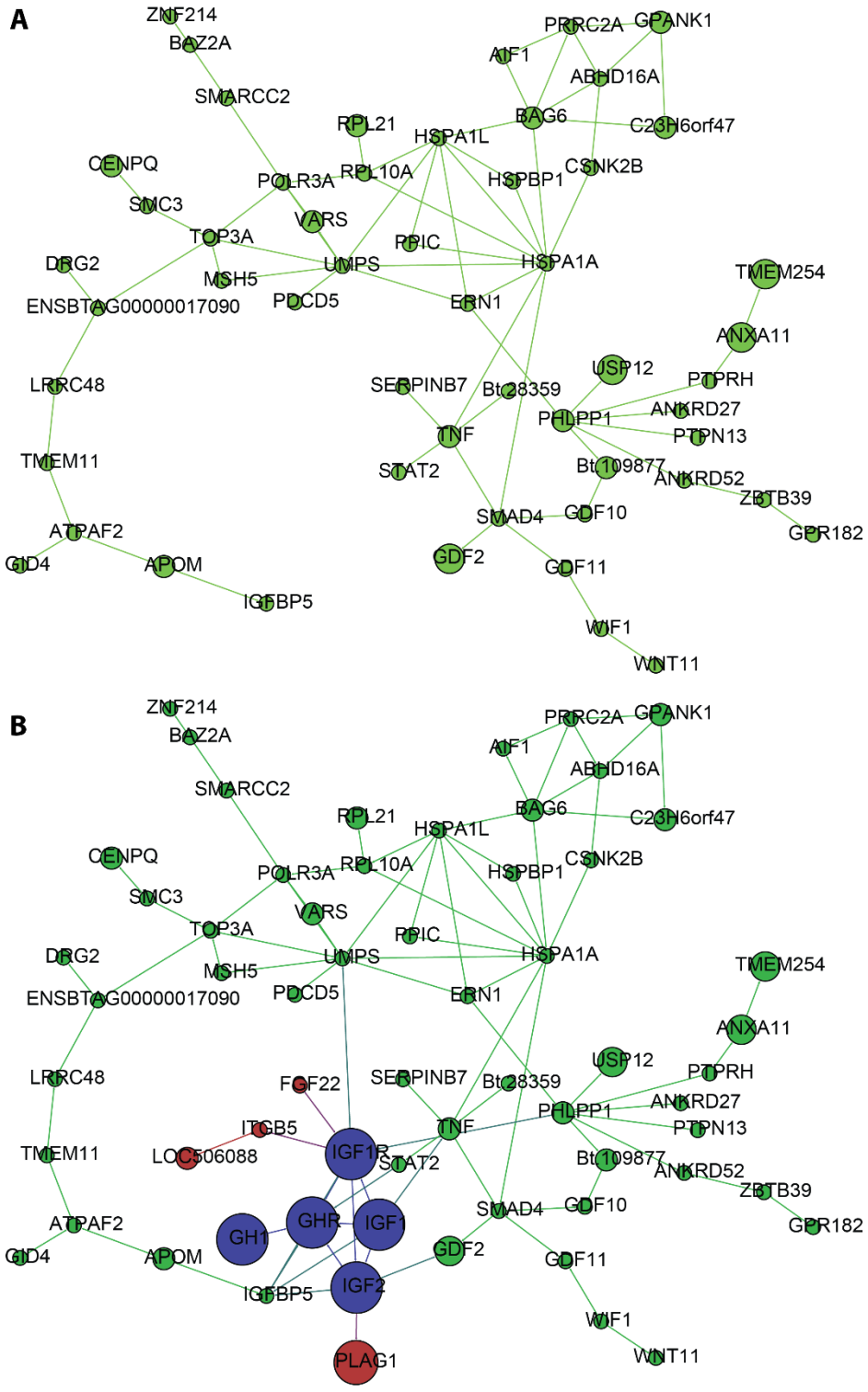

Figure 2 - Network of candidate pleiotropic genes for carcass traits in *Bos indicus* (Nellore) cattle. The network was built from known protein-protein interactions (edges) between gene products (nodes). The size of the node is proportional to the number of traits the gene is associated with. In A, the network is portrayed according to the list of genes obtained from the VEGAS analyses. In B, after the inclusion of five essential genes (in blue) form the growth pathway, the network presented itself as a satellite, and four more genes (in red) could be incorporated, including the major pleotropic gene *PLAG1*

Although key genes involved in growth, such as *IGF1* (insulin like growth factor 1), *IGF2* (insulin like growth factor 2), *GH1* (growth hormone 1), *IGF1R* (insulin like growth factor 1 receptor) and *GHR* (growth hormone receptor), were not found to shelter pleiotropic variants affecting carcass traits in the present study, we decided to add them to the list to reveal their interactions with the pleiotropic genes found here (**Figure 2-B**). The main motivation for this was the known function of *PLAG1*: by fine-tuning the expression of IGF family, the predicted downstream consequence of mutations in *PLAG1* is direct interference in the growth pathway. This could also be the case for other pleiotropic genes identified here. Another argument to support this strategy was that mutations directly occurring in the growth pathway are likely to produce extreme phenotypes, so common variation in carcass size is expected to be explained by variation in satellite genes modulating that pathway. For instance, mutations in *IGF1*, *IGF1R* and *GHR* have been implicated in extreme body size reduction across dog breeds (RIMBAULT et al., 2013), as opposed to common variation in human height being explained by a large number of variants in genes outside the growth pathway (LANGO ALLEN et al., 2010).

Surprisingly, when *IGF1*, *IGF2*, *GH1*, *IGF1R* and *GHR* were added to the gene list, the major network plotted as a satellite surrounding a central hub comprising the growth pathway. Additionally, *FGF22*, *ITGB5*, *LOC506088* and *PLAG1* were incorporated to the network when the genes mentioned above were included. This finding supported our hypothesis that the pleiotropic genes detected here are modulators of the growth pathway. It also opens the question whether genetic correlations among the weight and CPM traits encapsulate information about the growth pathway, in the sense that each one of these traits may be serving as a partial surrogate for the same underlying intermediate trait (i.e. growth). In this scenario, one would expect to find similar results in a direct GWA analysis on the intermediate phenotype.

2.4 Conclusions

Genes associated with multiple carcass traits in *Bos indicus* (Nellore) cattle were identified. These pleiotropic genes formed a network modulating *IGF1*, *IGF1R*, *IGF2*, *GH1* and *GHR*, which are well known major actors of the growth pathway. This finding suggests that common variation in carcass traits is not likely to be explained by mutations in essential genes controlling growth. Instead, the variation may lie in

accessory genes that regulate the function and expression of essential genes. Among these accessory genes, *PLAG1* seems to be the most influential. Moreover, we did not rule out the possibility that, at some extent, the genetic correlations among the nine traits studied here represent the indirect, partial measurement of a single underlying growth trait. In this case, the pleotropic genes identified here may simply represent genes affecting the intermediate phenotypes. The future characterization of causal variants in these genes may contribute to improved prediction of carcass yield and more informed mating decisions in *B. indicus* cattle.

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3 GENOME-WIDE SCANS FOR CARCASS AND MEAT TRAITS IN NELLORE CATTLE

Abstract

Genome-wide association study (GWAS) was performed in order to map genomic regions significantly associated with meat quality and carcass traits in Nellore cattle breed (*Bos indicus*). With this purpose, 407 animals were genotyped for over 770,000 SNP markers, using Illumina® BovineHD BeadChip assay (HD), and 169 for nearly 78,000 markers, using GeneSeek® Genomic Profiler™ HD (GGP), respectively. GGP genotyped animals were imputed for the HD markers set through FImpute v2.2 software. Recorded phenotypic data on hot carcass weight, ribeye area, back fat thickness, pH, meat color, shear force, cooking and drip loss association mapping were performed using a mixed model approach. Metabolic pathways between candidate genes enclosed in significantly associated genomic regions were elucidated by functional enrichment analyzes, revealing evidence for main pathways through phenotypic expression. Particularly, regarding meat tenderness, serine/serpin enzyme complex activity on proteolysis process and muscle fiber degradation showed to be a promising candidate responsible for this trait variability. Therefore, suggestive associated regions for mentioned traits would guide further investigations on gene relations and its phenotypic expression assisting breeding programs to improve bovine carcass and meat qualities.

Keywords: GWAS; *Bos indicus*: Meat quality

3.1 Introduction

Brazilian bovine meat has a prominent position on international market, present in over 90 countries besides the internal market (BRASIL, 2015). However, environment diversity and variety of production systems on such a vast territory, lead to diverse quality products (ABIEC, 2015). Coupled with these factors, genetic variability also exerts influence on meat quality, more expressively noticed when taurine and indicine animals are in comparison, revealing significant differences among genomic structures and resulted product quality (BRESSAN et al., 2011; CROUSE et al., 1989). Moreover, meat quality is affected by issues of technological, functional, sensory, nutritional, toxicological, regulatory and ethics order. Meat technological attributes, e.g., water holding and emulsifying capacity; sensory features, e.g., color and tenderness; and nutritional contents, e.g., proteins and fatty acids, are close related to several factors, including environment, breed, age, gender, finishing weight, diet, stress and *post-mortem* process (ANDERSON et al., 2012; CHENG; SUN, 2008; DEL CAMPO et al., 2008; KIM; LONERGAN; HUFF-LONERGAN, 2010; LAGERSTEDT; LUNDSTRÖM; LINDAHL, 2011; PESTANA et al., 2012). More recently, one propagated possibility to indirectly access these

attributes on living animals, still during breeding, calving, growing, fattening or finishing periods, consists in molecular markers use (DNA analysis), enabling identification of large number of variants associated with biological functions and economically important features (PAREEK et al., 2011).

Besides constant changes in animal production systems to improve food production, productivity drives the necessity to select more efficient animals (THORNTON et al., 2012). One of the most modern alternatives for herd improvement is through the assistance of genomic information acquired by genetic markers, namely single nucleotide polymorphisms (SNP). This technology is widespread and consolidated in taurine cattle of milk breeds, while indicine beef breeds still lacks of in-depth studies to determine effective methods for genomic assisted breeding programs. Although, a multitude of studies have been conducted to identify genomic regions associated with phenotype expression of desirable traits. Those related to bovine meat quality, such as for meat pH values after harvest (TIZIOTO et al., 2013), hot carcass weight (LU et al., 2013), meat color evaluation (PINTO et al., 2011; TIZIOTO et al., 2013), water holding capacity (PINTO et al., 2011; REARDON et al., 2010), tenderness (ASLAN et al., 2010; LEE et al., 2014; SCHENKEL et al., 2006; UJAN et al., 2013), intramuscular fat content (BUCHANAN et al., 2012; SHIN; HEO; CHUNG, 2011), subcutaneous fat thickness (MOKRY et al., 2013) and ribeye area (PINTO et al., 2011), are still sparse in indicine breeds. Specifically in Brazil, these breeds play an important role on meat production system and it is expected that genomic tools can improve production efficiency of these animals (GARCIA et al., 2012).

Therefore, this study main objective is to generate knowledge about genetic mechanisms that regulates Nellore cattle qualitative and quantitative carcass and meat traits.

3.2 Materials and Methods

3.2.1 Ethical Statement

Animals slaughtering were performed in accordance with the standards established by the Brazilian Ministry of Agriculture (Ministério da Agricultura, Pecuária e Abastecimento - MAPA) Instruction No. 3 of January 17, 2000 (BRASIL, 2000) and approved by the Ethics Committee of the Faculdade de Zootecnia e

Engenharia de Alimentos of Universidade de São Paulo, under the following record: MemoZAB/JBSF/06-22.

3.2.2 Animals, slaughter and phenotypic evaluations

Sixteen traits were evaluated for 576 genetically representative bulls, progenies of important breed founding sires of the Brazilian Nellore breed population. Animals were grass-feed from birth to 18 months of age and grain-fed fattening for 6 months. At the feedlot, animals received corn silage, average energy concentrate and mineral salt. Animals were slaughtered at a commercial processing plant in compliance with the Federal Inspection Service (SIF) regulations, located in Promissão city of the São Paulo Brazilian state. By the time of slaughter (24 months of age), registered mean live weight was 550 kg. After slaughter, carcasses were lengthwise sawed in half, weighted (hot carcass weight - HCW, given in kg) and headed to the cooling chamber at 2ºC (± 1°C). After 24 hours' carcasses reached an average temperature of 7ºC measured on round beef (*Semimembranosus muscle*). Next, potential of Hydrogen (pH24) was measured on *Longissimus thoracis et lumborum* (LTL) muscle by a digital pH meter (model HI8314, Hanna Instruments) equipped with a carcass penetration probe. Ribeye area (REA, given in cm^2), back fat thickness (BFT, given in mm) and color parameters (L*, a*, b*) were evaluated between 12^{th} and 13^{th} ribs. For REA and BFT measurements were used a clear graduated plastic grid and a metric ruler, respectively. Color parameters were recorded by direct reading of a XE MiniScan (HunterLab, USA) portable colorimeter with a D65 illuminant (day light) at an observation angle of 10° and a cell opening of 30 mm. Color measurements adopted the CIELab scale: L* (lightness between black and white), a* (intensity between green and red) and b* (intensity between blue and yellow). Following, samples were collected for physicochemical analyzes from the LTL muscle portions with a thickness of 2.5 cm, i.e. rib eye steak, of the left half carcass between the 10^{th} and 11^{th}, 11^{th} and 12^{th}, 12^{th} and 13^{th} ribs. After, samples were vacuum packed, stored at 2º C (+/-1º C) and ageing for 7, 14 and 21 days. For each ageing period, samples were evaluated regarding drip loss (DL7, DL14 and DL21, given in %), cooking loss (CL7, CL14 and CL21, given in %) and shearing force (WBSF7, WBSF14 and WBSF21, given in kg). Drip loss percentage was measured by the weight difference before and after pack opening and despised the exudate. Cooking loss percentage was determined by the weight difference before

and after cooking process. Steaks were cooked in a grill until they reached an internal temperature of 71º C. After CL measurements, eight steak sub-samples of 1.27 cm in diameter were collected with an electric drill (model Ferrari FG-13) for shearing force evaluation. These were assessed in a Warner-Bratzler mechanical device with a capacity of 25 kg and a shearing speed of 20 cm/min. Means and standard deviations of phenotypes are shown in Annex A, Table 1.

3.2.3 Genotyping, markers quality control and imputation

Extracted DNA from LTL muscle samples were genotyped by two different assays, BovineHD BeadChip (HD) (Illumina, USA) and GeneSeek® Genomic Profiler™ HD (GGP) (Illumina, USA) which interrogates the genome for more than 770,000 and nearly 78,000 SNP markers, respectively. HD (407 animals) and GGP (169 animals) genotyped samples were evaluated regarding markers and individuals' overall genotyping quality. For GWAS and imputation procedure, only the autosomal markers that met the inclusion criteria described below followed further analysis: 1) minor frequency allele (MAF) higher than 0:02; 2) Fischer's exact test p-value for Hardy-Weinberg Equilibrium (HWE) higher than $1*10^{-20}$; 3) SNP call rate of at least 95% and 4) individual call rate higher than 90%. Quality control steps were performed by SNP & Variation Suite (SVS) (Golden Helix, USA) and Plink (PURCELL et al., 2007) software. Missing alleles imputation of GGP samples to the HD SNP set, were performed in FImpute v2.2 software (SARGOLZAEI; CHESNAIS; SCHENKEL, 2014), which uses a sliding window approach to compare reference and target phased genotype data to impute markers allele. The reference data consisted of HD samples described above, summed to 2680 Zebu Genome Consortium (ZGC) (GARCIA et al., 2012), also genotyped in the HD assay. In order to assess imputation accuracy, imputation steps were repeatedly performed on 45 randomly selected individuals of the reference set, which genotypes were reduced to the GGP markers set. For each interaction these individuals imputed alleles were compared to genotyped alleles, resulting in a relation between the number of correct calls given the total number of alleles. Furthermore, GGP imputed samples were evaluated by principal component analysis in order to verify their distribution based on markers similarity among reference (HD + ZGC) samples.

3.2.4 Genome-wide association study

For GWAS, was adopted a linear mixed model: y ~ mean + fixed effects + SNP + animal + error. Fixed effects were defined based on meat biochemistry and physiology (Annex A, Table 2). Also, variables which Pearson correlation coefficient where greater than or equal to 0.6. Animal and error were modeled as random effects, assuming multivariate normal distribution. Errors were assumed to be independent and equally distributed, and the covariance of the animals' effects was modeled using an additive identity-by-state (IBS) kinship matrix from genotypic data (TAYLOR, 2014). Single loci association test was performed by EMMAX algorithm in SVS software (KANG et al., 2010). Markers that surpassed significance threshold (p-value < $1*10^{-4}$) were prioritized for investigation and followed further analysis.

3.2.5 Functional enrichment analysis

Chromosomic regions around significant markers were explored regarding contained genes and previously reported quantitative trait loci (QTL). These regions were determined by setting a 1 Mb window centered at significantly associated markers position. Overlapping windows or those that are separated by a maximum distance of 1 Mb between each other, were merged by "mergeBed" function in BEDtools v2.25.0 software (QUINLAN; HALL, 2010). Genes and QTLs within merged regions were obtained by intersecting them with customized bed format databases. Gene database comprehended all bed format exported genomic features of *Bos taurus* UMD 3.1 genome assembly through BioMart (Ensembl release 82) (KINSELLA; KÄHÄRI; HAIDER, 2011) data-mining tool. Exported bed format UMD 3.1 CattleQTLdb (HU et al., 2012) composed the QTL database. Intersection where performed by intersectBed function of BEDtools. Genes close to the most significant SNP within each region, were manually explored through GeneCards (http://www.genecards.org/) and Mouse Genome Informatics (EPPIG et al., 2015) databases. A functional enrichment analysis were performed for each trait gene set by DAVID v6.7 Functional Annotation Tools (HUANG; SHERMAN; LEMPICKI, 2009a) (HUANG; SHERMAN; LEMPICKI, 2009b) and GeneCodis Gene Annotations Co-occurrence Discovery (CARMONA-SAEZ et al., 2007; NOGALES-CADENAS et al., 2009; TABAS-MADRID; NOGALES-CADENAS; PASCUAL-MONTANO, 2012).

3.3 Results

Imputation accuracy from low (GGP) to high (HD) SNP densities was 98.6% and is visually assessed by a principal component analysis. PCA was performed on reference (ZGC + HD), imputed (GGP_Imputed) and non-imputed (GGP) genotypic information. **Figure 1** shows PCA results for the first two components which accounted for major proportion of the genotypic variation among samples and can be interpreted as a genetic distance between individuals, where samples close to each other are more similar than distant ones. Therefore, non-imputed and imputed samples showed negligible variation among them, also, their distribution follows those of HD and ZGC samples.

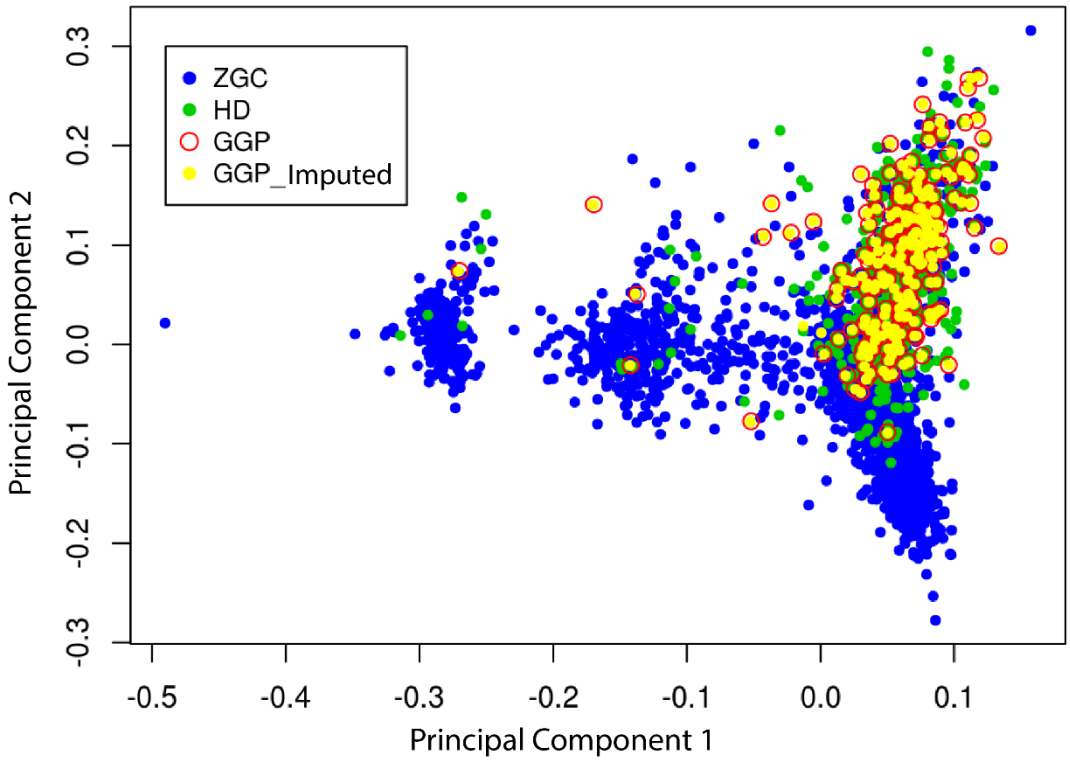

Figure 1 - PCA results for first two principal components

After quality control steps for both markers and individuals, 513,724 SNPs and 576 animals remained and were included in genomic association tests. Resulted manhattan plots, P-P plots, inflation levels showed significant genomic regions associated to each evaluated trait (Annex A, Table 3, Fig. 1-32). **Table 1** shows the total number of QTLs and genomic structures identified in those regions for each trait.

Table 1 - Number of regions, QTLs, genomic structures and associated chromosomes by trait

Trait	Number of associated regions	Number of QTLs	Number of genetic structures	Associated Chromosomes
HCW	28	116	322	2, 3, 5, 6, 8, 11, 14, 15, 18, 19, 21, 22, 27
RAE	15	195	229	5, 8, 11, 12, 14, 15, 16, 18, 20, 23, 25, 28
SFT	18	84	150	1, 5, 10, 11, 12, 13, 14, 16, 17, 22, 27, 29
pH24	11	98	196	7, 10, 14, 15, 19, 22, 27
*a**	26	76	289	1, 2, 7, 8, 10, 11, 16, 17, 18, 19, 22, 23, 24, 25, 26, 28
*b**	38	153	477	1, 2, 3, 4, 6, 7, 8, 10, 12, 13, 15, 17, 18, 19, 21, 23, 24, 25, 26, 27, 28, 29
*L**	39	175	528	1, 2, 3, 5, 6, 7, 10, 11, 14, 15, 17, 18, 19, 20, 21, 23, 24, 25, 29
DL7	19	142	194	1, 2, 3, 8, 9, 11, 13, 14, 15, 16, 17, 24, 25, 29
DL14	18	77	297	1, 3, 5, 13, 18, 19, 20, 21, 23, 25
DL21	19	191	181	1, 2, 4, 6, 7, 15, 17, 20, 22, 23,25
CL7	35	193	383	1, 2, 4, 6, 8, 9, 10, 11, 14, 16, 17, 18, 20, 21, 22, 23, 24
CL14	28	279	211	1, 2, 3, 5, 6, 7, 9, 10, 14, 15, 18, 19, 21, 25, 26, 27, 28, 29
CL21	32	222	302	2, 3, 5, 6, 9, 11, 12, 14, 15, 16, 17, 20, 21, 27, 29
SF7	23	81	200	1, 2, 3, 5, 7, 8, 10, 12, 14, 17, 19, 24, 25, 26, 28
SF14	19	42	233	1, 2, 4, 5, 9, 11, 13, 15, 20, 21, 22, 24
SF21	17	56	182	1, 2, 3, 5, 7, 8, 10, 12, 14, 17, 19, 24, 25, 26, 28

Table 2 details found genomic structures among chromosomal regions and provide additional information regarding most significant associated marker identification, position, association test p-value and closest gene to given position.

Table 2 - Identified genomic structures among associated regions and most significant associated marker description by trait

Trait	Number of genes	Number of novel genes	miRNA	miscRNA	Processed-pseudogene	Pseudogene	rRNA	snoRNA	snRNA	SNP ID	Position	P-value (10^{-X})	Gene
HCW	253	22	7	2	3	3	4	12	16	900003220	9:12513507	5.51	KCNQ5
REA	142	41	10	1	2	5	12	6	10	800004671	8: 14932451	5.46	-
SFT	116	12	4	3	0	4	0	4	7	100021374	1: 74568988	6.54	ATP13A4*
pH24	125	31	7	2	1	9	2	8	11	1900005565	19: 19610376	5.73	KSR1*
*a**	222	24	14	0	1	11	2	7	8	1700016699	17: 58806181	6.29	SUDS3
*b**	348	66	15	4	3	7	1	12	21	1000018402	10: 63637997	6.37	F1N717
*L**	428	37	15	2	3	8	4	7	24	2100005068	21: 18045417	6.22	KLHL25
DL7	153	12	6	0	0	4	1	11	7	1500006998	15: 26274793	5.83	CADM1*
DL14	141	22	72	1	2	2	1	45	11	1300023108	13: 79905684	5.36	NFATC2
DL21	124	12	10	3	1	7	2	1	21	2000001647	20: 5211562	6.98	BOD1
CL7	295	24	15	4	2	7	7	5	24	1000011582	10: 37443235	6.87	EHD4*
CL14	144	35	5	1	4	3	0	4	15	500019405	5: 69201758	5.35	C12orf75*
CL21	233	20	14	2	2	7	5	6	13	1200019376	12: 70512846	6.27	ABCC4
WBSF7	161	5	8	3	0	2	3	12	6	1400021859	14: 78047789	6.08	CNGB3
WBSF14	144	49	13	1	2	7	3	5	9	900018042	9: 65681959	6.58	TBX18
WBSF21	135	8	13	1	1	3	4	9	8	2100007753	21: 26891884	5.28	ARNT2*

HCW – hot carcass weight (kg); *REA* – ribeye area (cm^2); *BFT* – back fat thickness (mm); *pH24* – pH after 24 hours of slaughter; *a** - red intensity; *b** - yellow intensity; *L** - luminosity; *DL (7, 14, 21)* – drip loss (%) after 7, 14 and 21 days of ageing; *CL (7, 14, 21)* – cooking loss (%) after 7, 14 and 21 days of ageing; *WBSF (7, 14, 21)* – Warner Bratzler shear force (kg) after 7, 14 and 21 days of ageing; miRNA - micro RNA; miscRNA - miscellaneous small RNA; rRNA - ribosomal rRNA; snoRNA - small nucleolar RNA; snRNA - small nuclear RNA.
*intragenic marker
(-) no gene found within a 2 Mb window

3.4 Discussion

3.4.1 Hot carcass weight (HCW)

Brazilian meat industry earnings, basically is regulated by this measure, since retail price is essentially determined by how much weight a carcass has and how much of this weight is attributed to a premium beef. Hot carcass weight is obtained after slaughter, evisceration and carcass cleaning, presenting positive genetic correlation with carcass yield and ribeye area (PARIACOTE; VAN VLECK; HUNSLEY, 1998). Regions significantly associated with this trait contained 50 QTLs (**Table 1**), related to weight, average daily gain, carcass weight, ribeye area, meat yield, fat thickness and fat content (HU et al., 2012). These features are highly related to HCW. Also *KCNQ5* gene (Potassium Channel, Voltage Gated KQT-Like Subfamily Q, Member 5) enclosed by the most significant marker region (**Table 2**) and associated with ribeye area and fat deposition in pigs, pointed as a candidate gene to explain these parameters variability (GRAPES; ROTHSCHILD, 2006). *KCNQ5* gene family (KV7) expression is related to cell proliferation in skeletal muscle. Voltage-dependent potassium channels (Kv) seem to influence myogenesis due to its activity on myotube formation during multinucleated skeletal muscle fibers development. Although skeletal muscle cells are differentiated myotubes, Kv family seems to control membrane potential and cell volume during myotubes and myoblasts proliferation and differentiation, these are known muscle fibers precursors. Antagonists of these genes are also described as inhibitors of cell growth (ROURA-FERRER et al., 2008).

3.4.2 Ribeye area (REA)

Nine studies reported QTLs directly related to this trait and 26 are indirectly related, such as body weight, carcass and meat yield (HU et al., 2012). GeneCodis functional enrichment analysis clustered *GDF10* (growth differentiation factor 10), *INHBE* (Inhibin, Beta E), *GDF2* (growth differentiation factor 2) and *INHBC* (Inhibin, Beta C) genes as components of Transforming growth factor-beta, C-terminal (TGF-beta; InterPro: IPR001839) metabolic pathway. TGF-beta proteins family are characterized as multifunctional peptides that control different cell types proliferation and differentiation. Among numerous known proteins that belongs to this pathway, myostatin is defined as a key regulator of skeletal muscle mass, involved in muscle mass development and loss (ROTH; WALSH, 2004). The autosomal recessive locus

associated with muscle hypertrophy in Belgian Blue and Piedmontese cattle was mapped to chromosome 2 in the region of myostatin gene (*GDF8*) (CHARLIER et al., 1995), and associated polymorphisms were described a muscle growth regulators (KAMBADUR et al., 1997) potentially associated to bovine double muscling (SMITH et al., 1997).

SNP markers mapping to *GDF10* gene region were associated to depth and thoracic perimeter, hip height and width, rump length and withers height in chinese *B. indicus* (ADOLIGBE et al., 2012). Beyond *GDF10*, *NPBWR1* (Neuropeptides B/W Receptor 1, BTA14) gene is another potential candidate to affect growth, muscling and weight (EPPIG et al., 2015), which found highly associated regions to REA and HCW. This gene was also associated to growth traits in Creole and Colombian zebu cattle (MARTÍNEZ; GÓMEZ; ROCHA, 2014), probably due to its homeostatic regulatory function over feeding (TAKENOYA et al., 2010).

3.4.3 Back fat thickness (BFT)

The fat layer that covers body muscles is closely related to consumer satisfaction due to juiciness and flavor it provides. Furthermore, subcutaneous fat thickness is often related to other quality parameters such as increased tenderness, acting as a barrier against temperature fall during *post-mortem* cooling process, preventing *rigor mortis* state to be established at low temperatures and allowing proteolytic enzymes activity (AALHUS et al., 2001; JEREMIAH, 1996).

Chromosomal regions significantly associated with SFT contained seven QTLs associated with fatty acids profile and five related to intramuscular fat content. Among the most significant SNP region, was found the *ATP13A4* (ATPaseType 13A4) gene. It belongs to protein class that catalyze hydrolysis of esters, glycosides and peptides bonds, and show biological function in metabolism as a carrier for cations (MI et al., 2005; THOMAS et al., 2003). Additionally, DAVID results, using *Homo sapiens* genes as reference set, clustered *ATP2B4* (ATPase, Ca++ transporting, plasma membrane 4), *AVPR1A* (arginine vasopressin receptor 1A), *CAMK4* (calcium/calmodulin-dependent protein kinase IV), *CHRM5* (cholinergic receptor, muscarinic 5) and RYR3 (ryanodine receptor 3) genes as a calcium signaling metabolic pathway components (Kyoto Encyclopedia of Genes and Genomes - KEGG; http://www.genome.jp/kegg/). Taniguchi et al. (2008) observed increased expression of calcium transporter genes during differentiation of pre-

adipocytes to mature adipocytes in cattle perimuscular fat. Similar results were reported by Zhang et al. (2007), that found five genes of the same pathway (RACK1, WNT3A, VDAC3, S100A6, THBS4) which expression was regulated by a β-agonist used in pork production to obtain a lean meat (lower levels of fat and higher meat yield). The intracellular calcium modulates lipid metabolism and adipocyte differentiation by inhibiting early stages while promotes late differentiation stages (SHI et al., 2000). This action takes place through serine/threonine calcium dependent phosphatase enzyme control, which regulates many cellular pathways involved in cell proliferation, differentiation and apoptosis, being an important mediator of β-catenin-independent vial, integral component in the Wnt signaling pathway, associated with cell proliferation (KENNELL; MACDOUGALD, 2005) and adipogenesis regulation (CHRISTODOULIDES et al., 2009). Thus, genes encoding calcium carrier proteins, limiting calcium-dependent enzymes related to adipogenesis pathways are potential candidates for influencing subcutaneous fat deposition in Nellore cattle.

3.4.4 Potential of Hydrogen after 24 hours (pH24)

This is the most frequently measured fresh meat parameter, not only for the simplicity involved in data collection but also by its close relation to biochemical processes of muscle to meat conversion. This trait variability rate during post-mortem period is linked to meat sensory characteristics (DEL CAMPO et al., 2008), with major effects over color, dripping loss and tenderness of final product (CONTRERAS-CASTILLO et al., 2016).

No QTLs within significantly associated genomic regions were previously reported for this trait. Possibly, due to its susceptibility to extrinsic factors, like pre-slaughter stress, fasting short periods and water diet, hormonal changes and increased temperature, which causes pH abnormal changes due to partial or total consumption of muscle glycogen (GREGORY, 2010; WULF et al., 2002) diminishing the genetic effect on its variability, hampering genomic mapping. Still, most significant SNP is in the intragenic region of *KSR1* gene (kinase suppressor of ras 1) and about 150 kb of *NOS2* gene (Nitric Oxide Synthase 2, Inducible) which metabolic activity relates to homeostasis, blood glucose levels and glycogen storage by catalyzing NO (nitric oxide) synthesis (EPPIG et al., 2015), which expression is induced by low oxygen tensions (VARGIU et al., 2000). During biochemical process

of muscle to meat conversion, oxygen tension is close to zero (PÖSÖ; PUOLANNE, 2005), which favors inducible nitric oxide synthase activity and NO production, moreover, during *post-mortem*, acidosis follows tissues hypoxemia which physiological signaling is promoted by NO (LUNDBERG; WEITZBERG; GLADWIN, 2008). Additionally, NO production from L-arginine -NO synthase pathway is responsible for hypoxic vasodilatation and regulation of mitochondrial oxygen consumption (LUNDBERG; WEITZBERG; GLADWIN, 2008), in short, NO binds to certain enzymes that are involved in cellular respiration process, preventing them to properly work (QUEIROZ; BATISTA, 1999). By extension, *NOS2* gene expression relates to meat pH variation by regulating cellular oxygen availability. Since, during *post-mortem* period pH decrease results of cell anaerobic respiration due to oxygen depletion, which produces pyruvic acid molecules, later reduced to lactic acid that accumulates in muscle (BENDALL; TAYLOR, 1972).

Thus, how all these mechanisms interact among each other altering final meat pH are not yet clear, but *NOS2* appears to be a good candidate for further studies since its associated trait directly affect principal meat quality parameters.

3.4.5 Color parameters (L*, a* and b*)

Although meat color not necessarily relates to quality, is one of a few traits that consumers can visually evaluate at purchase, directly affecting their buying decision (KAMRUZZAMAN et al., 2012; LAGERSTEDT; LUNDSTRÖM; LINDAHL, 2011).

GWAS identified regions for these parameters harbor five QTLs for iron content (HU et al., 2012). Iron function in myoglobin porphyrin core is to store oxygen in mammalian muscle and also is responsible for meat pigmentation (SUMAN; JOSEPH, 2013). The most significant marker for lightness (L*), found on chromosome 21 (**Table 2**), was also reported by Tizioto et al. (2013) for Nellore cattle. As these authors, DAVID functional enrichment analysis revealed genes involved in cytoskeleton formation (Annex A, Table 4), such as structural and cell adhesion molecules that participates in muscle fibers development (BERTHIER; BLAINEAU, 1997). On the other hand, GeneCodis results for a* and b* color parameters, pointed to ferroxidin metabolic pathway genes, *AOX4* (aldehyde oxidase 1-like), *AOX2* (aldehyde oxidase 1-like) and *AOX1* (aldehyde oxidase 1), which comprises of small acidic proteins formed by hundreds of amino acids with four

conserved cysteine residues in which 2Fe-2S connects. These, act in redox biological systems transferring electrons (MITCHELL et al., 2014).

This pathway was not yet described associated to meat color, but a probable action is through myoglobin iron atom oxidative state, which may vary from Fe^{+2}, deoxymyoglobin pigment, to Fe^{+3} metmyoglobin, respectively conferring meat a purplish red and a brownish color. Transition between the different oxidation stages is maintained by the environment redox conditions (SUMAN; JOSEPH, 2013).

3.4.6 Dripping loss (DL)

Water retention capacity is an important factor economic associated to product yield, and also a quality factor associated to meat tenderness and juiciness, as it reflects meat capacity to retain inherent or added water. It highly correlates dripping, cooking and cooling losses (depending on measured processing stage), color and salinity. Besides, salable weight loss also entails the loss of proteins and other water soluble solids (CHENG; SUN, 2008; HUFF-LONERGAN; LONERGAN, 2005; REARDON et al., 2010). Additionally, dripping and cooking loss are the most commonly used tests to evaluate meat retention capacity.

Among associated chromosomal regions no directly related QTLs were found, although, shear force reported loci may indirectly relate to DL due to its close relation with meat tenderness. Most significant marker region of DL7 measures, shelter *CADM1* (cell adhesion molecule 1) gene, that act on cellular organization, adhesion and junction. Cytoskeletal proteins and adhesion molecules play an important role in cell muscle integrity, thus junction degradation leads to channels formation resulting in water loss increase during *post-mortem* (PEARCE et al., 2011). DAVID results also points to genes retaining similar function, such fibronectin type III, coding a cellular matrix glycoprotein that binds to cell membrane proteins known as integrin, related to adhesion, growth, migration and cellular differentiation (MITCHELL et al., 2014).

Closest gene to most significant SNP location associated with DL14 is *NFATc2* (nuclear factor ofactivated T-cells cytoplasmic, calcineurin-dependent 2), *NFAT* isoform necessary for muscle cell growth and nuclear accretion induced by prostaglandin F2 alpha (PGF2a) (HORSLEY; PAVLATH, 2003).

For 21 days of maturation (DL21), DAVID clustered *NKX2-5* (NK2 homeobox 5), *MAP3K13* (mitogen-activated protein kinase kinase kinase 13), *PVRL1* (Poliovirus Receptor-Related 1) and *UPK2* (uroplakin) genes, a protein homodimerization

activity pathway. These relates quaternary protein structure formation, in addition to compose cellular architecture may have greater or lesser water molecule affinity depending on their electron charges (HUFF-LONERGAN; LONERGAN, 2005). Since most of muscle water is trapped in cell structures, including myofibrillar spaces, any change to this organization can alter cells' ability to retain water (HUFF-LONERGAN; LONERGAN, 2005). Therefore, results obtained for these measures point to cell formation, organization and structuring genes as main candidates to explore meat capacity to retain water molecules.

3.4.7 Cooking loss (CL)

Like dripping loss, no chromosomal regions associated to different cooking loss maturation times sheltered known for this trait QTLs, probably due to database incompleteness regarding these parameters. However, chromosomes 5, 6, 8, 10, 14, 15, 16, 20, 21, 26 and 28 loci are indirectly related through CL correlation with tenderness (HU et al., 2012). *EHD4* gene, located in the most significant SNP region associated with CL7, belongs to calcium ion binding and nucleic acid binding pathways (http://www.genecards.org/), which function is similar to those reported by Tizioto et al., (2013) for WHC and WBSF gene clusters, suggesting that calcium ion dependent proteases, such as calpains, can affect cellular structures degradation altering meat water retention and tenderness (KRISTENSEN; PURSLOW, 2001). DAVID analysis for CL14, clustered *CPA6, PPP2CB*, *HSP90B1*, *ATG3* and *DDI1* genes, which function is linked to proteolysis. *HSP90B1* gene codes a protein (heat schock protein 90kDa beta (Grp94), member 1) which primary function is to prevent proteins deterioration and structural damage in apoptotic processes, it relates to various meat quality attributes such as texture, juiciness, flavor and color, and its expression is increased as a result of cellular stress suffered during *post-mortem* (GUILLEMIN et al., 2011; KEMP; PARR, 2012; LOMIWES et al., 2014).

The *ABCA4* gene (ATP-binding cassette, subfamily C (CFTR / MRP), member 4), located approximately at 350 kb from the most significant SNP associated to CL21, features ATPase activity (http://www.genecards.org/), releasing necessary energy for muscle fiber metabolism during *post-mortem* (HENCKEL et al., 2002). Functional enrichment analysis for this trait showed Keratin type II metabolic pathway components (Annex A, Table 5), which are essential for cytoskeleton and nuclear envelope structures (MITCHELL et al., 2014).

By before mentioned results, genetic components affecting cooking loss at different maturation times are close related to cytoskeleton formation and organization (CL7 and CL21) and proteolysis (CL14), which summed to before mentioned results for dripping loss and those described in literature (CHENG; SUN, 2008; HUFF-LONERGAN; LONERGAN, 2005; KRISTENSEN; PURSLOW, 2001; PEARCE et al., 2011), reiterate cellular architecture importance on meat water retention capacity.

3.4.8 Shearing force (SF)

Meat tenderness, a sensory qualitative attribute, is commonly measured by its strong correlation with shearing force. It mainly exerts influence on bovine meat market acceptability and their prices, ratifying researches that mentioned it as the most important characteristic in consumer satisfaction inquiries (DERINGTON et al., 2011; KOOHMARAIE; GEESINK, 2006; MODZELEWSKA-KAPITUŁA et al., 2012; SCHENKEL et al., 2006; WU et al., 2012). Genome mapping for this trait revealed 16 previously reported QTLs (HU et al., 2012). Functional enrichment analysis yielded similar results to those found for dripping and cooking loss, highlighting metabolic pathways related to cell structure and proteolytic enzymes.

Significantly associated regions associated to SF7, shelter seven genes clustered in cytoskeleton metabolic pathway. *ARPC2* (actin related protein 2/3 complex, subunit 2, 34kDa, BTA2), *MYO1C* (myosin IC, BTA19), *RPA1* (replicationprotein A1, 70kDa, BTA19), *TUBA4A* (tubulin, alpha 4a, BTA2), *PSRC1* (proline/serine-richcoiled-coil 1, BTA3), *FHL3* (four and a half LIM domains 3, BTA3) and *VIL1* (villin 1, BTA2) genes encode for proteins of filamentary elements that constitute the internal cell structure, consisting of intermediate filaments, microfilaments, microtubules, among other structures that are distributed throughout the cell (ASHBURNER et al., 2000; “Gene Ontology Consortium: going forward.”, 2014). Muscle myofibrillar structure, especially with regard to sarcomere length variations and fiber-type, important for animal growth and development, are directly related to the sensory perception of meat tenderness. In this aspect, growth and muscle fiber diameter, determined by the animal muscle type, sex and age, and oxidative-glycolytic fibers proportion, since fibers that readily rely on glycolytic mechanism also accelerate *rigor-mortis* state, have a significant effect shearing force measurements (HARPER, 1999).

Additionally, SF14 clustering results (*CELA3B* Chymotrypsin-Like Elastase Family, Member 3B; *PRSS2* Protease, Serine, 2 (Trypsin 2); ENSBTAG00000003633 uncharacterized protein; ENSBTAG00000024245 uncharacterized protein; *PRSS58* protease, serine, 58; *LOC615237* putative trypsin-6; *LOC780846* uncharacterized protein; *PRSS37* protease, serine, 37, *SEC11A* SEC11 homolog A, signal peptidase complex subunit), point to proteolysis activity, especially, serine-type peptidase activity protein family, responsible for catalyzing peptide bonds hydrolysis from serine amino acid present in the active site (ASHBURNER et al., 2000; “Gene Ontology Consortium: going forward.”, 2014).

Serine is part of cathepsin group enzymes, known by its activity during postmortem proteolysis and concomitant meat tenderness development, is associated with structural changes in cell organization and proteolytic degradation (GUILLEMIN et al., 2011), were reported as main candidates for bovine meat shearing force genetic variability (HULSMAN HANNA et al., 2014). This proteases action mechanism is not yet fully known, but it seems to actuate on neuromuscular junction catabolism (SENTANDREU; COULIS; OUALI, 2002).

Final meat tenderness depends on muscle structure modification degree and interrelated proteins, and during *post-mortem*, proteins that make up the cytoskeleton, including actin and myosin, are cleaved by proteolytic enzymes (KEMP et al., 2010).

Interestingly, DAVID results for SF21, clustered serpins family genes (*SERPINA11* serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 11, ENSBTAG00000007392 uncharacterized protein, *SERPINA10* member 10, *SERPINA1* member 1, *SERPINA12* member 12), that present inhibitory function over proteolysis and apoptotic processes, and mostly are inhibitors of serine (MITCHELL et al., 2014). Serpins, form irreversible covalent bonds with the target enzyme, altering proteinase structure and making it unable to complete the catalytic process (BOUDIDA et al., 2014), leading to low levels of beef tenderness (ZAMORA et al., 2005). More recently, bovSERPINA3-1 proteins were described as regulators of caspases 3 and 8 activity, which are aspartate cysteine proteases related to *post-mortem* muscle softening (OUALI et al., 2013), interacting with these enzymes as before described for serines, through covalent bonds formation (GAGAOUA et al., 2015). Hence, serines and serpins complex enzymes seems to regulate beef tenderness development during *post-mortem*.

3.4.9 Conclusion

This study identified QTLs and genes potentially related to Nellore carcass and meat quality traits. Results reiterated previously described genomic regions associated to these traits, as well as revealed novel regions sheltering genetic components that may explain part of the phenotypic variation. Especially with regard to meat tenderness, consumer favorite quality characteristic, pointing serines/serpins enzyme complex genes as candidate to explain shearing force variation. This complex seems to regulate meat softening through proteolytic processes and muscle fiber structure degradation. In short, presented results can assist future questions regarding phenotype and its genetic relation, and also, breeding programs through genomic prediction models improvement.

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4 NELLORE BEEF CATTLE LIPID PROFILE FROM THE HUMAN HEALTH PERSPECTIVE

Abstract

Given the attention dispended to beef lipids and the growing consumer interest in food nutritional value, this study aimed to assess 148 Nellore cattle *Longissimus thoracis et lumborum* (LTL) muscle samples fatty acids profile from the human health point of view. Fat content composition and quantification were obtained and results were evaluated regarding nutritional values, health indexes, enzymatic activity, individual fatty acids and health impact. A synthetic variable named Quality Index (QI), which scores interpretation is a sample overall effect on health, was created in order to facilitate PCA results interpretation and visualization. Results showed that among 42 identified fatty acids, samples variability was derived from total fat amount and enzymatic activity of Δ^5desaturase, Δ^6desaturase and Δ^9desaturase, directing their quality grade towards beneficial or harmful effects on health. Also observed for PUFA, n-6, HI, HH, TI, AI, and SFA variables. Information provided by this work pointed important targets for Nellore meat quality improvement regarding its effects on human health.

Keywords: Fatty acids; Meat; Nellore; Principal component analysis

4.1 Introduction

Besides being an important source of energy and essential fatty acids, food lipid content facilitates the absorption of dietary fat-soluble components such as vitamins and contribute to food texture, flavor and aroma (ARANCETA; PÉREZ-RODRIGO, 2012). More specifically, fresh meat and meat products technological and sensory properties, shelf life, oxidative stability, color, flavor and palatability are majorly affected by lipid fatty acids composition (HERDMANN et al., 2010; SHIROUCHI et al., 2014). Further, its consumption constitutes an important source of dietary protein, essential nutrients and highly bioavailable components such as iron, zinc, copper, selenium (DECKER; PARK, 2010) and B12, A and E vitamins (HOEHNE et al., 2012; MCAFEE et al., 2010). However, recent studies have suggested red meat consumption with increased risk of cancer, cardiovascular, inflammatory and autoimmune diseases (GANJI; KAMANNA; KASHYAP, 2003; MCAFEE et al., 2010; SIMOPOULOS, 2002), mainly related to cholesterol, saturated fat content, (PADRE et al., 2006) and *trans* fatty acids (MCAFEE et al., 2010). Thereby, the fatty acid type has more impact on human health than just the total amount of ingested fat (EKINE-DZIVENU et al., 2014), increasing consumers interest on lipid composition and respective food nutritional value (SIERRA et al., 2008).

Inversely, several studies have also associated some fatty acids present in red meat to beneficial health effects. For instance, the omega-3 fatty acids family (n-3), especially those with a long carbon chain length like eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), not only linked to risk reduction of disease occurrence such as atherosclerosis, inflammatory and heart diseases, but also related to behavior changes as in depression (CONNOR, 2000; LIN; HUANG; SU, 2010). Moreover, beef assumes role as major source of these fatty acids in human diet mainly due to its accessibility compared to other products like fish oil, becoming the only source of omega-3 (ENSER et al., 1996).

The conjugated linoleic acid (CLA) also highlights among the beef lipid profile. It is a set of geometric and positional isomers of linoleic acid (C18:2 n-6) which has two double bonds separated by a single bond where each pair can show *cis* or *trans* configuration. In beef fat, the main CLA isomer found is the *cis*-9, *trans*-11 CLA, also known as rumenic acid (PADRE et al., 2006). Biologically active isomers of conjugated linoleic acid have been associated with beneficial impact on human health by its anticarcinogenic activity on mammary, prostate, kidney, liver and skin tissues (DECKER; PARK, 2010). Besides, it acts on diabetes mellitus prevention, shows anti-obesity effect and the conjugated double bonds have antioxidant action (KALLAS; REALINI; GIL, 2014).

Several factors appear to influence the quantity, quality and lipid distribution in both animals and its byproducts, such as age, weight, sex, breed, genetics, nutritional status and castration, as well as external factors such as temperature, feeding (ALFAIA et al., 2006; KOUBA; MOUROT, 2011), animal handling and finishing systems (GAMA et al., 2013; RAES et al., 2003). Nevertheless, ruminants feed type has low effect over some fat components, since polyunsaturated fatty acids obtained through diet are partially or at some extent entirely, bio-hydrogenated in the rumen (ENSER et al., 1996; KOUBA; MOUROT, 2011; PADRE et al., 2006). Thus, breed and genetic effects gain significance on fat content composition and deposition in these animals. Therefore, accurate analysis of the relative and absolute concentration ratio of fatty acids is a basic requirement to get information on the total lipid composition, assess the functional value and characterize fatty acids concentration effect on metabolic pathways (HOEHNE et al., 2012).

Moreover, beef is the second most consumed meat protein source in Brazil, and by association, a large portion of this meat has its genetic roots on Nellore cattle

due to the importance attained by this breed in the Brazilian herd, which represents over 70% of all animals raised in the country (BRASIL, 2015). Hence, in order to obtain a meaningful evaluation of the Brazilian dietary quality, it's of paramount importance that the characteristics described before be evaluated in this breed beef byproducts.

The aim of this study was to evaluate Brazilian Nellore cattle (*Bos indicus*) *Longissimus thoracis et lumborum* (LTL) muscle fatty acids profile from the human health perspective.

4.2 Material and Methods

4.2.1 Ethical Statement

Animals slaughtering were performed in accordance with the standards established by the Brazilian Ministry of Agriculture (Ministério da Agricultura, Pecuária e Abastecimento - MAPA) Instruction No. 3 of January 17, 2000 (BRASIL, 2000) and approved by the Ethics Committee of the Faculdade de Zootecnia e Engenharia de Alimentos of Universidade de São Paulo, under the following record: MemoZAB/JBSF/06-22.

4.2.2 Sample collection and extraction of the lipid fraction

Samples were collected from 148 Nellore young bulls. Animals were grass-feed from birth to 18 months of age and grain-fed fattening for 6 months approximately. At the feedlot, animals received corn silage, average energy concentrate and mineral salt. Animals were slaughtered at a commercial processing plant located in Promissão city of the São Paulo Brazilian state that was in compliance with the Federal Inspection Service (SIF) regulations. By the time of slaughter (24-31 months of age), registered mean live weight was 550 kg. After slaughter, carcasses were lengthwise sawed in half and headed to the cooling chamber at 2ºC (± 1°C) for 24 hours until they reached an average temperature of 7ºC measured on round beef (*Semimembranosus muscle*). All samples were collected from LTL muscle portions between the 12th and 13th ribs for intramuscular fat extraction. The lipid fraction was extracted according to Bligh & Dyer (1959) method, weighted and diluted in hexane solvent for storage in amber vial under -20 °C for further analysis.

4.2.3 Fatty acids profile

The fatty acid methyl esters (FAMEs) were obtained according to the methodology described by Hartman and Lago (1973) with some adjustments based on the AOCS Ce-1b-89 method (AMERICAN OIL CHEMISTS SOCIETY, 1990). FAMEs were analyzed using gas chromatography (GC) (Shimadzu, Model GC 2010-Plus,Kyoto, Japan) high-resolution equipped with a fused-silica capillary column Supelco SP-2560 (100m x 0.25mm i.d., 0.20 µm df) and a flame ionization detector. Samples were split Injected at 1:30 ratio and carrier gas used was nitrogen at a flow rate of 1.5mL/min. Gas chromatograph oven program temperature was 100ºC by the time of injection and raised to 170°C at a rate of 2°C/minute, held at this temperature for 15 minutes then raised to 180°C at a rate of 0.5°C/min, increased from 180ºC to 200°C at a rate of 10°C/min and kept in this temperature for 10 minutes, then from 200 to 230°C at a rate of 2°C/min and maintained at this temperature (230ºC) for 13 minutes. The injector and detector temperatures were maintained at 240°C. Fatty acids identification was performed by comparison of samples retention times with commercial standard mixture of 37 fatty acids Supelco TM Component FAME Mix (Supelco, Bellefonte, PA). Fatty acids which its identification was unknown due to unavailability of the standard mix, followed a multiple comparison method using the equivalent chain length (KRISNANGKURA et al., 1997; MIWA et al., 1960), Kovat's retention index (KITTIRATANAPIBOON; JEYASHOKE; KRISNANGKURA, 1998; KOVATS, 1958) and the equivalent carbon number (KRISNANGKURA et al., 1997; WOODFORD; VAN GENT, 1960) extracted from literature and regressed against the samples respective index. Final identification was maintained if there was an agreement between those methods. Quantification was done by normalizing the area under the curve of methyl esters. Results were expressed as area percentage (fatty acid peak area/total peaks area) and as milligrams (mg) of fatty acid by 100 grams (g) of meat (fatty acid area percentage*total lipid*factor), where factor is equal 0.916 as described by Anderson, Kinsella and Watt (1975).

Based on the FAME results were calculated the atherogenic (AI) and thrombogenic indexes (TI) according to Ulbricht and Southgate (1991); hypocholesterolaemic and hypercholesterolaemic ratio (HH) (SANTOS-SILVA; BESSA; SANTOS-SILVA, 2002); C14, C16 and C18 desaturation indexes (ALDAI et al., 2006) and Health Index (HI) (ZHANG et al., 2008).

4.2.4 Multivariate analysis

All analyses were executed using R programing language (DEVELOPMENT CORE TEAM, 2011) with the aid of RStudio software (RACINE, 2012). In order to explore cryptic relations between a large number of variables (42 identified fatty acids and 16 indexes), a principal component analysis (PCA) were performed with the PCA function in the R package FactoMineR (LÊ; JOSSE; HUSSON, 2008). PCA was performed using the correlation matrix of standardized units where variables were in columns and observations in lines. To determine relevant data structures and produce interpretable evidence of those relations, a summary index of the overall meat quality regarding its impact on human health were developed. For that, all variables where investigated based on its beneficial or injurious effect on human organism, based on previous literature knowledge (ARANCETA; PÉREZ-RODRIGO, 2012; GERBER, 2012; MCAFEE et al., 2010; WILLIAMS, 2000). The Quality Index (QI) is the summation of each sample Z transformed beneficial variables minus the harmful ones (eq. (1)).

$$
\begin{aligned}
QI_i = {} & Z_{C18:2cis9,trans11_i} + Z_{C18:2cis9,12_i} + Z_{C18:3cis9,12,15_i} + Z_{C20:4cis5,8,11,14_i} + Z_{C20:5_i} \\
& + Z_{C22:6_i} + Z_{HH_i} + Z_{HI_i} + Z_{MUFA_i} + Z_{PUFA_i} + Z_{PUFA/SFA_i} + Z_{n3_i} \\
& + Z_{desaturationC14_i} + Z_{desaturationC16_i} + Z_{desaturationC18_i} - Z_{C12:0_i} - Z_{C14:0_i} \\
& - Z_{C16:0_i} - Z_{C18:1trans10_i} - Z_{C18:1trans9_i} - Z_{C18:2trans9,12_i} - Z_{AI_i} - Z_{SFA_i} \\
& - Z_{TI_i} - Z_{n6_i} - Z_{n6/n3_i}
\end{aligned}
$$

In Equation 1, QI_i is a sample Quality Index and Z_{x_i} is a standardized value for the i th observation. The obtained score is a synthetic variable used to distinguish samples according to its healthy quality grade, where “good”, “not so good”, “not so bad” and “bad” represent the quantiles interval of the QI distribution, (0.75,1], (0.5,0.75], (0.25,0.5] and [0,0.25] respectively. This procedure allowed a graphical visualization of the relations between samples quality grade and data variables on PCA without interference of the quality index on the components construction.

4.3 Results

Table 1 presents descriptive statistics for each identified fatty acid as mean concentration and respective standard deviation in both units, area percentage and mg of fatty acids per 100g of meat.

Table 1 - Fatty acids in Nellore *Longissimus thoracis et lumborum* (LTL) muscle

Fatty Acid common name	Formula	Area percentage		mg FAME/100g meat	
		Mean	*SD*	*Mean*	*SD*
Butyric	C4:0	0.007	0.004	0.154	0.069
Caproic	C6:0	0.014	0.010	0.259	0.131
Capric	C10:0	0.040	0.009	0.859	0.276
Lauric	C12:0	0.063	0.012	1.311	0.474
Myristic	C14:0	2.528	0.450	51.951	20.714
Myristoleic	C14:1 cis-9	0.705	0.181	14.659	6.886
Pentadecanoic	C15:0	0.264	0.042	5.384	2.027
Pentadecenoic	C15:1	0.080	0.030	1.414	0.372
Palmitic	C16:0	23.609	1.822	480.153	160.861
Palmitoleic	C16:1 cis-9	3.336	0.502	68.442	26.370
Margaric	C17:0	0.696	0.087	14.200	5.095
Heptadecenoic	C17:1	0.598	0.060	12.046	3.837
Stearic	C18:0	15.244	1.826	305.735	93.774
Elaidic	C18:1 trans-9	0.150	0.029	3.040	0.586
Trans-10-octadecenoic	C18:1 trans-10	0.174	0.033	3.760	1.258
Vaccenic	C18:1 trans-11	0.626	0.200	12.815	6.062
Oleic	C18:1 cis-9	39.125	2.950	794.533	263.144
Cis-vaccenic	C18:1 cis-11	1.407	0.181	28.147	8.958
Octadecenoic	C18:1 cis-12	0.123	0.028	2.589	0.772
Octadecenoic	C18:1 cis-13	0.337	0.095	7.123	3.419
Octadecenoic	C18:1 cis-15	0.120	0.026	2.728	0.974
Linolelaidic	C18:2 trans-9,12	0.096	0.028	2.133	0.944
Conjugated-linolenic (CLA)	C18:2 cis-9 trans-11	0.174	0.024	4.040	1.200
Linoleic (LA)	C18:2 cis-9,12	7.325	2.612	138.422	42.275
Arachidic	C20:0	0.099	0.019	2.024	0.624
Gamma-linolenic	C18:3 cis-6,9,12	0.054	0.021	1.035	0.274
Gondoic acid	C20:1	0.163	0.039	3.362	1.382
Alpha-linolenic (ALA)	C18:3 cis-9,12,15	0.496	0.172	9.497	3.239
Heneicosylic acid	C21:0	0.280	0.082	5.809	2.992
Eicosadienoic acid	C20:2	0.072	0.028	1.344	0.314
Behenic	C22:0	0.075	0.029	1.342	0.369
Dihomo-gamma-linolenic acid	C20:3 cis-8,11,14	0.422	0.173	7.949	2.637
Erucic	C22:1 cis-13	0.017	0.007	0.340	0.067
*Eicosatrienoic acid**	C20:3 cis-11,14,17	0.007	-	0.115	-
Arachidonic acid (AA)	C20:4 cis-5,8,11,14	1.810	0.667	33.948	10.207
Tricosylic acid	C23:0	0.111	0.166	2.094	2.709
Docosadienoic acid	C22:2	0.055	0.024	1.043	0.353
Lignoceric	C24:0	0.410	0.161	7.716	2.663
Eicosapentaenoic acid (EPA)	C20:5	0.038	0.054	1.160	1.850
Nervonic	C24:1 cis-15	0.013	0.004	0.227	0.037
Docosapentaenoic acid (DPA)	C22:5	0.187	0.067	3.507	0.987
Docosahexaenoic acid (DHA)	C22:6	0.130	0.053	2.445	0.844

*identified in one sample

Nutritional values and health indexes means and standard deviations are shown in **Table 2**, also expressed as area percentage and mg of FAME per 100g of meat.

Table 2 - Lipid profile indexes

Index	Area percentage		mg FAME/100g Meat	
	Mean	*SD*	*Mean*	*SD*
Atherogenic Index (AI)	0.5	0.08	0.5	0.08
Hypocholesterolaemic/ hypercholesterolaemic (HH)	2.0	0.26	2.0	0.26
Health Index (HI)	1.7	0.23	1.7	0.23
Thrombogenic Index (TI)	1.3	0.18	1.3	0.18
Desaturation C14	21.7	3.18	21.7	3.18
Desaturation C16	12.3	1.53	12.3	1.53
Desaturation C18	71.9	3.47	71.9	3.47
Sum of saturated fatty acids (SFA)	43.1	2.98	874.4	278.31
Sum of monounsaturated fatty acids (MUFA)	46.4	3.53	943.8	315.64
Sum of polyunsaturated fatty acids (PUFA)	10.3	3.59	195.9	56.86
PUFA/SFA	0.2	0.09	0.2	0.09
Sum of n-3 family fatty acids	0.8	0.22	16.0	4.46
Sum of n-6 family fatty acids	9.6	3.39	182.2	53.92
Sum of n-9 family fatty acis	39.2	2.98	797.7	264.45
n-6/n-3	11.6	2.82	11.6	2.82
*Total lipid**	-	-	2.2	0.64

**expressed as g/100g of meat.* PUFA/SFA: polyunsaturated and saturated fatty acids ratio; n-6/n-3: omega 6 and omega 3 family fatty acids ratio;
$AI = (C12{:}\,0 + 4C14{:}\,0 + C16{:}\,0)/(\sum(\text{n\_6} + \text{n\_3}) + \sum \text{MUFA})$;
$TI = (C14{:}\,0 + C16{:}\,0 + C18{:}\,0)/(0.5 * (C18{:}\,1\ cis\_9) + 0.5 * (\sum MUFA - oleic) + 0.5 * n\_6 + 3 * n\_3 + ((n\_3)/(n\_6)))$;
$HI = (\sum MUFA + \sum PUFA)/(4 * C14{:}\,0 + C16)$
$HH = (C18{:}\,1\ cis\_9 + C18{:}\,2n\_6 + C20{:}\,4n\_6 + C18{:}\,3n\_3 + C20{:}\,5n\_3 + C22{:}\,6\_n3)/(C14{:}\,0 + C16{:}\,0)$
$DI(14) = 100 * (C14{:}\,1cis\_9)/(C14{:}\,0 + C14{:}\,1cis\_9)$;
$DI(C16) = 100 * (C16{:}\,1cis\_9)/(C16{:}\,0 + C16{:}\,1cis\_9)$;
$DI(C18) = 100 * (C18{:}\,1cis\_9)/(C18{:}\,0 + C18{:}\,cis\_9)$.

The pattern of variation for fatty acids profile are shown in **Fig. 1**, which summarizes PCA results for the first two components (dimensions), these accounted for 41% of data variability (**Fig. 1-A**). Therefore, variables that are correlated with these components are greatly important in explaining referred variability. Representation quality ($\cos^2$), component correlation (arrow length) and variables relations (arrow direction) are expressed in **Fig. 1-B** (Annex B, Table 1). Those that highly contributed to these dimensions' construction where considered significant

(Annex B, Fig.1) and are characterized among samples distribution in the biplot (**Fig. 1-C**), where different colors indicate samples grouping factor based on QI scores ("good", "not so good", "not so bad" and "bad"). The hand drawn ellipses around variables indicate its group (1, 2 and 3), in order to facilitate figure comprehension and explanation. Its grouping factor was merely visual and evidenced variables clustering and main representation among the first (group 1 and 3) and second dimensions (group 2).

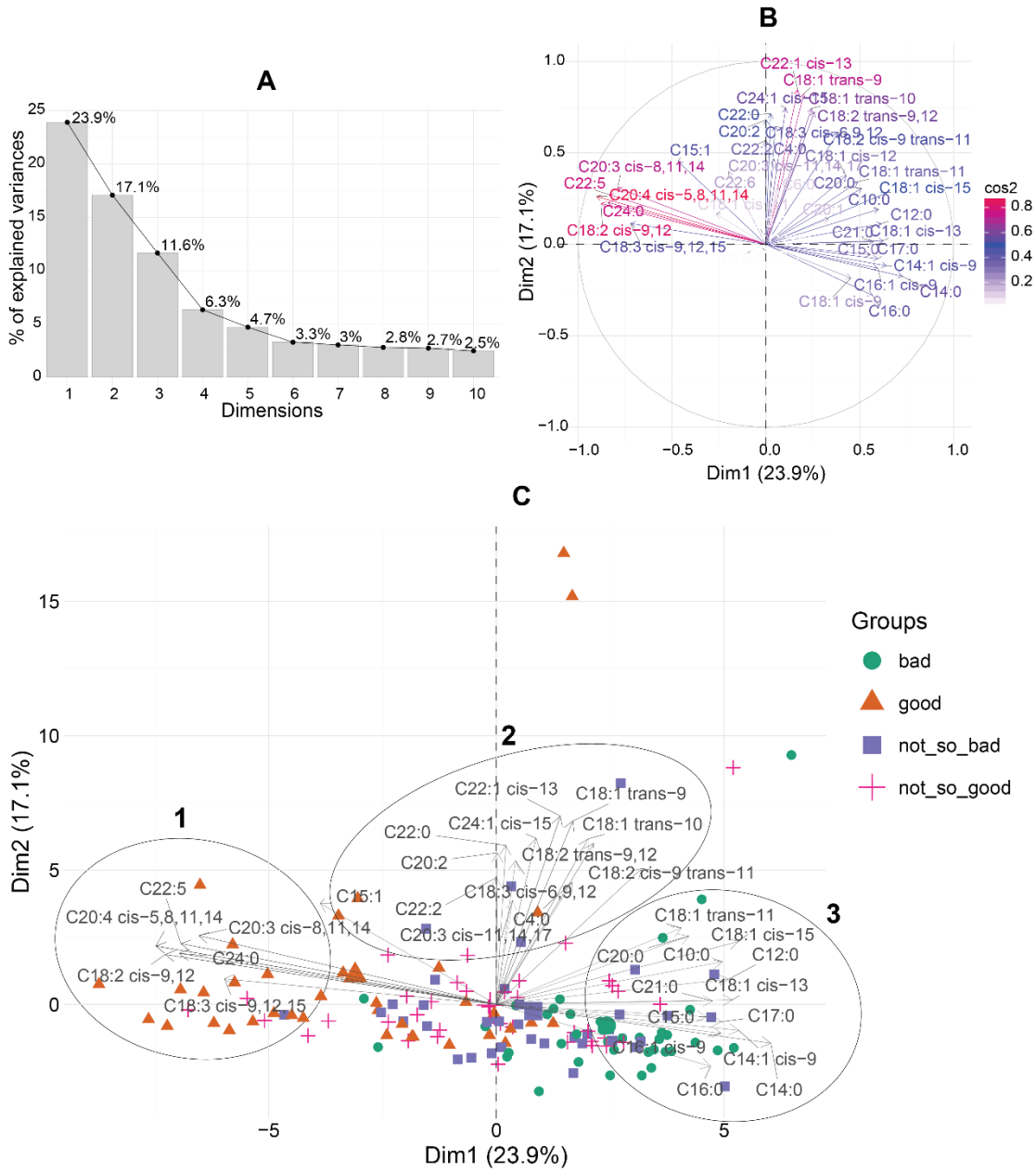

Figure 1 - Fatty acids profile PCA results

In **Fig. 1-C**, variables of group 1 and group 3 are highly associated with the first dimension consequently explains major part of samples variability. Group 2 is associated with the second dimension and at a lesser extent compose samples distribution pattern. Groups 1 and 3 are opposed to each other, negatively correlated, dividing samples grouping factors along its axis.

Fig. 2 summarizes PCA results for fat content indexes. The first two dimensions, accounted for 81.2% of data variability (**Fig. 2-A**). As described for **Fig. 1**, variables parameters are summarized in **Fig. 2-B** (Annex B, Table 2). In **Fig. 2-C**, only significant variables were taken into account (Annex B, Fig.2), therefore plotted among samples distribution, where different colors indicate samples group (“good”, “not so good”, “not so bad” and “bad”). Were added 95% confidence interval ellipses around samples of the same group in order to better visualize grouping factor distribution.

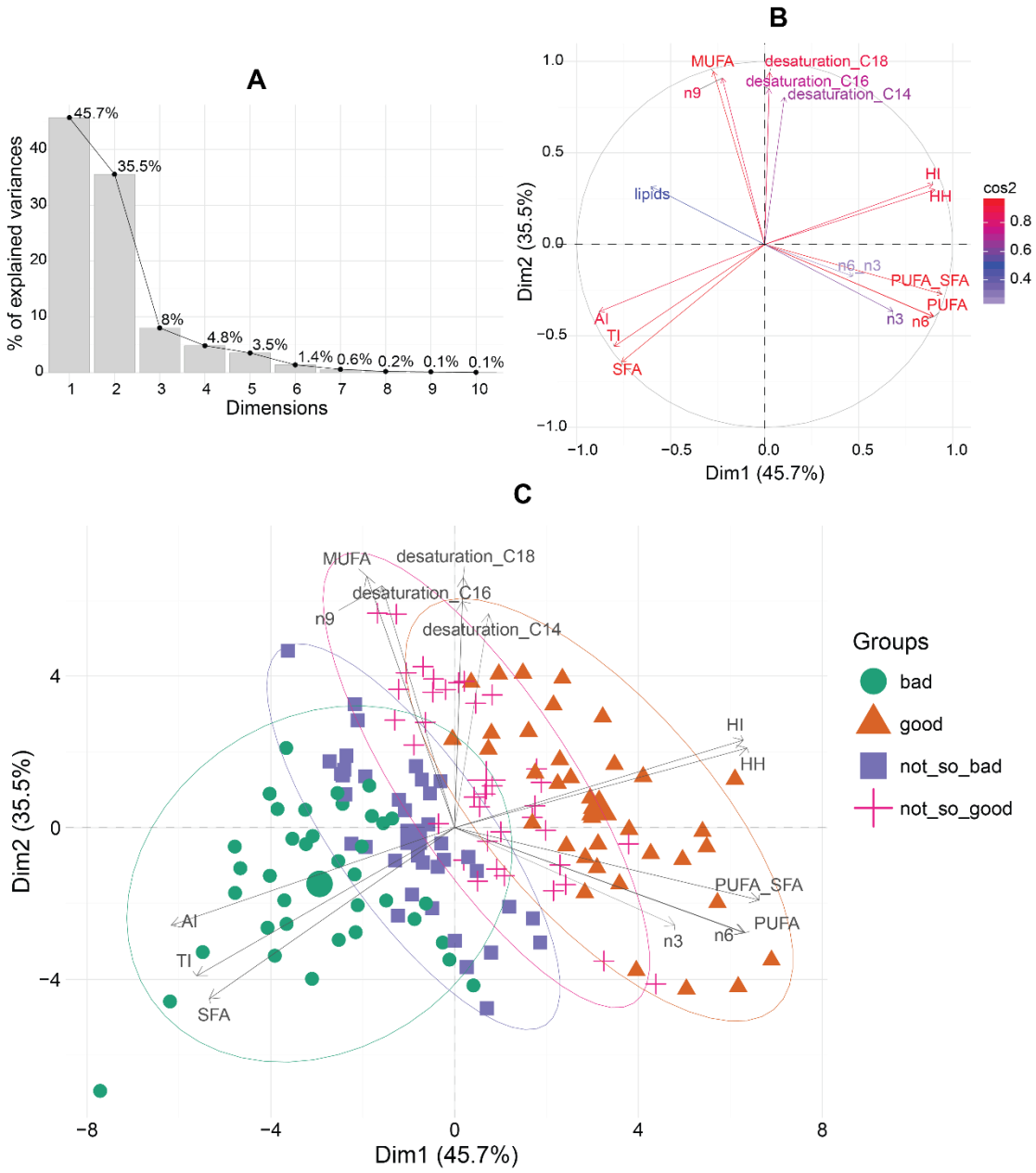

Figure 2 - Fat content indexes PCA results

In **Fig. 2-C**, AI, TI and SFA were strong negatively correlated with HI and HH indexes, majorly distributing samples group along their axis. PUFA/SFA ratio, n-6 and at a lower overall effect, n-3 family were positively correlated to HI and HH indexes. These indexes have strong effect on group differentiation, improving samples QI score, on the other hand, AI, TI and SFA clearly drives QI scores to lower values, “bad” and “not so bad” groups. MUFA, n-9 family and desaturase indexes are associated with the second dimension, thus, have lower effect over samples distribution.

4.4 Discussion

Identified fatty acids (**Table 1**) shows high variability concentration among those previously reported for Nellore breed or crossbreed animals (ANDRADE et al., 2014; GAMA et al., 2013; PADRE et al., 2006; SAN VITO et al., 2015). However, palmitic (C16:0), stearic (C18:0) and oleic (C18:1 cis-9) fatty acids, that account for more than 70% of all beef lipid composition, presented high degree of consistency among those results. Major variations were observed for linoleic (LA; C18:2 cis-9,12) and arachidonic acids (AA; C20:4 cis-5,8,11,14) concentrations compared to those presented by Andrade et al. (2014), Padre et al. (2006) and Fredriksson, Eriksson and Pickova (2007). Although these fatty acids showed higher amounts than observed by previous authors, Brugiapaglia et al. (2014) found equivalent concentrations in Friesian cattle. Omega 3 family alpha linolenic (ALA; C18:3 cis-9,12,15) and eicosapentaenoic acids (EPA, 20:5) presented lower concentrations than those observed by Kamihiro, Stergiadis, Leifert, Eyre, & Butler (2015), San Vito et al. (2015) and Herdmann et al. (2010), while docosahexaenoic (DHA; C22:6) showed similar results compared to those found by these authors and higher concentrations than reported by San Vito et al. (2015) and Andrade et al. (2014). Double conjugated bonds linolenic acid isomer (CLA; C18:2 cis-9 trans-11) concentrations was lower than those reported for Piedmontese, Limousin, Friesian (BRUGIAPAGLIA et al., 2014) and crude glycerin supplemented Nellore cattle (SAN VITO et al., 2015). The disparity among results implies that the lipid content is directly affected by a diversity of factors e.g. raising and finishing systems, feeding, age and breed, each differently evaluated among those studies.

Furthermore, Wood et al. (2008) reported that the total fat content deposited in muscle is one of the main factors affecting the fatty acid profile, since animals that have low lipid content have a high LA proportion, once bovines maintain long chain PUFA in muscle phospholipids, while the neutral fatty acids (SFA and MUFA) are deposited as triacylglycerols, showing proportionally lower concentrations. Phospholipids are essential components of cell membranes and its content is practically constant. In addition, LA has greater affinity for phospholipids insertion compared to ALA. This last fatty acid is supplied by grass-based diet, and is more susceptible to biohydrogenation due to prolonged retention period in the rumen.

Regarding fat content overall effect on human health, LA and AA lipid components have received special attention from scientific community due to its vascular, immune and anti-inflammatory activities (WILLIAMS, 2000). Researches shows inconsistent results on ALA significance over cardiovascular disease risk reduction (ARANCETA; PÉREZ-RODRIGO, 2012) even though it is a precursor of long chain eicosanoids such as EPA and DHA (WILLIAMS, 2000). These, have positive physiological activity on blood pressure, triglyceride, inflammatory process, endothelial and cardiac diastolic functions, presenting consistent evidence of its participation on occurrence reduction of fatal cardiovascular diseases (ARANCETA; PÉREZ-RODRIGO, 2012). Consumption recommendations from the World Health Organization for daily intake of ALA is between 0.5 to 2% of dietary total energy and for the EPA and DHA sum, between 0.25 to 2g for a healthy adult. Although, beef samples showed lower concentrations of these fatty acids (**Table 1**) than those recommended by healthy organizations, it is a complimentary source where daily fish consumption is negligible. As described before, CLA isomers have multiple beneficial effects, these are mainly formed by two processes in ruminant organisms, by incomplete bio hydrogenation of linoleic acid to stearic acid by rumen bacteria or by desaturation of the trans-11 vaccenic acid (C18: 1 trans-11), the primary intermediate of ruminant bio-hydrogenation (DECKER; PARK, 2010). De La Torre et al. (2006) mentioned that bovine meat CLA and its isomers (*cis-trans, cis-cis and trans-trans*) are highly modulated by dietary and animal related intrinsic factors such as animal's breed, age, sex and muscle type, pinpointing that this fatty acid is found in quantities below of that recommended for human's daily consumption, between 151 and 212 mg/day (RITZENTHALER et al., 2001). Beef is the main source of these fatty acids in Brazilian diet given its expressive consumption, among 32.8 kg/capita/year, compared to other ruminant's meat (ANUALPEC, 2012) and its negligible amounts in industrial hydrogenated fats (WANDERS et al., 2010).

In **Table 2**, SFA, MUFA and PUFA sums presented major variations when compared to those described in literature (ANDRADE et al., 2014; BRUGIAPAGLIA; LUSSIANA; DESTEFANIS, 2014; FREDRIKSSON ERIKSSON; PICKOVA, 2007; GAMA et al., 2013; PADRE et al., 2006; SAN VITO et al., 2015), as it reflects previously described disparities among identified fatty acids concentrations. LTL total intramuscular fat content (2.199 g/100g of meat) was lower than those observed in the Brazilian Food Composition Table (TACO) (http://www.unicamp.br/nepa/taco/)

which is 6 g/100g of meat, based on taurine animals. Beyond genetic differences, it worth to mention that 97% of all Brazilian herd is raised in grass-feed systems (ABIEC, 2015; LOBATO et al., 2014), which for meat industry, is a commercial strategy to increase its market, denominating its products as “green-beef”.

Recommended daily intake of omega 6 and omega 3 family fatty acids is expressed by its ratio (n-6/n-3), where no more than 5 times the n-3 amount of n-6 should be consumed in a per capita per day unit (FAO, 2008). This ratio is a quality index for oils and fats composition once the consumption of high amounts of n-6 is associated with reduction on anti-inflammatory mediators’ formation from n-3 acids due to the metabolic competition for elongase and desaturase enzymes between these two (ARANCETA; PÉREZ-RODRIGO, 2012). For the studied population, the n-6/n-3 ratio (11.685) is in agreement with Lage et al. (2014) results, still, it was inferior than the ratio mentioned by Brugiapaglia et al. (2014) and superior than those obtained by Andrade et al. (2014), Ekine-Dzivenu et al. (2014), San Vito et al. (2015) and Cesar et al. (2014) in different bovine breeds (**Table 2**). This variation is explained by the divergence between identified and quantified fatty acids among these works with consequent variations in the sum of n-3 and n-6 fatty acids. Additionally, Daley et al. (2010) described that the feeding system impact over the lipid content is clearly noticed when n-6 and n-3 family quantities are observed, pointing to increased amounts of n-3 fatty acids in grass-fed animals lowering the n-6/n-3 ratio. Thus, the higher mean values observed for this ratio in comparison to those obtained for the same breed (CESAR et al., 2014; SAN VITO et al., 2015), could be a result of plane of nutrition, also affecting n-6 family fatty acids, mainly linoleic fatty acid (FA).

The ratio between PUFA and SFA (PUFA/SFA) was higher when compared to those reported by Andrade et al. (2014) and Kamihiro et al. (2015), as a consequence of the expressive concentration of LA, which suggests its potential influence over PUFA/SFA and n-6/n-3 ratios (**Table 2**). PUFA and SFA concentrations are independent of diet composition (DALEY et al., 2010), implying that these parameters variability are mainly explained by genetic factors, as pointed by Cesar et al. (2014). Also, muscle fat content affects the fatty acid composition with proportional changes in concentrations of SFA and MUFA, but exerts no alteration on PUFA concentrations (DE SMET; RAES; DEMEYER, 2004). Therefore, besides the genetic component, observed higher PUFA/SFA ratio can also be explained by the

reduced SFA concentrations as result of low fat levels while PUFA and LA concentrations remained unaltered.

Hypocholesterolemic and hypercholesterolemic fatty acids ratio, expressed by HH index, is translated as a relation between beneficial (n-3 and n-6), neutral (oleic) and harmful (miristic and palmitic) effects promoted by its respective fatty acids on cholesterol blood levels (MCAFEE et al., 2010). Observed HH ratio values in **Table 2** where higher than those found for crossbred animals by Andrade et al. (2014). Similarly, atherogenic (AI) and thrombogenic (TI) indexes, which also relates fat content health effects, presented lower values than those presented by Cesar et al. (2014) and Vicenti et al. (2009) as well as those obtained for other species such as sheep (KUCHTÍK; ZAPLETAL; ŠUSTOVÁ, 2012) and pigs (SALVATORI et al., 2008). Such lower values can be explained by the desaturation indexes, higher than those observed for cattle (BRUGIAPAGLIA; LUSSIANA; DESTEFANIS, 2014; GAMA et al., 2013), as a result of increased expression of the stearoyl-CoA desaturase in these animals. The Health Index (HI), a modification of atherogenic index is positively correlated with beneficial fatty acids (ZHANG et al., 2008). Studied samples HI index were higher than those observed by Ekine-Dzivenu et al. (2014) and Zhang et al., (2008) for Angus and crossbred animals (Angus-Charolais). It reflects PUFA sums, higher than those obtained by previous authors as a direct consequence of higher LA concentrations described before.

In **Fig. 1-C** samples are clearly differentiated between group 1 and group 3 according to its QI score, where variables contained in these groups have major contribution towards samples healthiness score. The underlying meaning of those groups, seems to reside on enzymatic activity, modulating “beneficial” or “harmful” fatty acids concentrations. Group 1 fatty acids are predominantly regulated by Δ^5desaturase and Δ^6desaturase. Desaturase enzymes catalyze *cis* double bond insertion on a specific position along the fatty acid chain (WATERS et al., 2009). These enzymes activity is a proportion between the product fatty acid and respective substrate. LA FA is a precursor of all polyunsaturated fatty acids attained to this group, where its conversion to a long chain fatty acid occurs by a series of desaturation and elongation enzymatic reactions. Reduced Δ^6desaturase activity is given by high LA concentrations followed by low concentrations of AA (BOKOR et al., 2010; WILLIAMS, 2000). Genetic variations are also related to its activity as described by Bokor et al. (2010), that found associations between long chain

unsaturated fatty acids (PUFA) concentrations and Δ^5desaturase and Δ^6desaturase genes variations in humans. It is also of note that in mice hepatocyte cultures, myristic acid (C14:0) increased Δ^6desaturase activity (JAN et al., 2004), which might explain higher concentrations of these fatty acids to those previously observed on literature. Group 3 reflects the enzymatic activity of Stearoyl-CoA desaturase (Δ^9desaturase). This is a key enzyme in fatty acids metabolism, responsible of saturated to monounsaturated fatty acids conversion by double bonds formation (BARTON et al., 2010). Hoashi et al. (2008) found significant association between Δ^9desaturase gene (SDC) and fat composition in Japanese Black cattle. This enzyme gene expression is regulated by n-3 and n-6 family fatty acids concentrations, being negatively and positively correlated to n-3 and n-6 concentrations respectively (WATERS et al., 2009). In the present study, given the higher concentration of n-6 family linoleic acid, SDC gene expression could explain group 3 significance on samples variability.

Fig. 2-C composed a mapping for the main health indexes and nutritional values associated with samples healthiness quality. Where, AI, TI and SFA were responsible for most of the bad effects and n-6, HH, HI and PUFA/SFA ratio for the good effects on health. Those indexes express the general relation between “good” and “bad” fatty acids and basically are calculated taking into account PUFA and MUFA for the “good” side, and C12:0, C14:0 and C16:0 for the “bad” side of the equation. Thus, **Fig. 2-C** is a simplified extension of **Fig. 1-C**, where indexes major variation components are discriminated among its variables. While desaturase enzymes activity (C14, C16 and C18) along with n-9 and MUFA, seems to have a balancing effect over samples quality index, these are highly correlated among each other, explained by enzymatic activity products which are monounsaturate fatty acids and among them, oleic acid of the n-9 family is the most abundant in beef fat content.

4.5 Conclusions

This study presented a detailed description of the LTL muscle lipid profile of a sample of Nellore breed population, with the identification of 42 fatty acids. Additionally, animals’ Quality Index was able to evidence healthy quality grades relationship with evaluated variables. Resulted analysis showed that major part of the variability proportion among samples were explained by PUFA and MUFA fatty acids concentrations as consequence of total amount of fat and enzymatic activity of

Δ^5desaturase, Δ^6desaturase and Δ^9desaturase. Genetic factors related to desaturase and enlogase enzymes expression, combined with low concentrations of myristic and palmitic fatty acids proportionally to low fat content, improved health indexes AI, TI, HH, HI and PUFA/SFA. Thereby, generated information can elucidate consumer concerns on beef nutritional value and its health impact, as well as, generated an evaluation tool to be further applied in other bovine breeds.

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ANNEX

Annex A

Table 1 - Carcass and meat quality traits mean and standard deviation

Phenotype	**Mean**	**SD**
HCW	291.32	18.17
REA	73.64	7.17
BFT	4.36	1.92
pH24	5.96	0.26
*L**	37.74	3.36
*a**	15.83	1.78
*b**	13.35	1.96
DL7	2.76	1.23
DL14	3.77	1.61
DL21	4.6	2.17
CL7	12.51	5.09
CL14	10.62	3.21
CL21	10.81	3.92
WBSF7	5.89	1.45
WBSF14	4.9	1.23
WBSF21	4.35	1.1

Table 2 - Model fixed effects by trait

Trait	Fixed effect
HCW	age; slaughtering batch
REA	age; slaughtering batch
BFT	age; slaughtering batch
pH24	age; slaughtering batch
*L**	age; slaughtering batch; pH24; b*
*a**	age; slaughtering batch; pH24; b*
*b**	age; slaughtering batch; pH24; L*; a*
DL7	age; slaughtering batch; pH24
DL14	age; slaughtering batch; pH24
DL21	age; slaughtering batch; pH24
CL7	age; slaughtering batch; pH24; DL7
CL14	age; slaughtering batch; pH24; DL14
CL21	age; slaughtering batch; pH24; DL21
WBSF7	age; slaughtering batch; pH24; DL7; CL7; temperature
WBSF14	age; slaughtering batch; pH24; DL14; CL14; WBSF7 temperature
WBSF21	age; slaughtering batch; pH24; DL21; CL21; WBSF7; WBSF14; temperature

Table 3 - Genome-wide association study inflation factor (λ) by trait

Phenotype	**λ**
HCW	0.9933
REA	1.0114
BFT	1.0082
pH24	0.9381
*L**	1.0097
*a**	1.0015
*b**	1.0127
DL7	0.9942
DL14	0.9990
DL21	0.9851
CL7	1.0114
CL14	1.0101
CL21	1.0108
WBSF7	1.0055
WBSF14	1.0018
WBSF21	0.9895

Table 4 - DAVID gene clustering result for L* color parameter

Annotation cluster 1	Enrichment Score: 16.21
GO:0005856	Cytoskeleton
P-value	2.27E-12
Ensembl gene ID	*ENSBTAG00000030546*
	ENSBTAG00000030548
	ENSBTAG00000009327
	ENSBTAG00000030539
	ENSBTAG00000007958
	ENSBTAG00000030542
	ENSBTAG00000007794
	ENSBTAG00000038759
	ENSBTAG00000007520
	ENSBTAG00000024995
	ENSBTAG00000030555
	ENSBTAG00000013153
	ENSBTAG00000017408
	ENSBTAG00000020824
	ENSBTAG00000040460
	ENSBTAG00000008180
	ENSBTAG00000037665
	ENSBTAG00000014583
	ENSBTAG00000039644
	ENSBTAG00000002391
	ENSBTAG00000038386
	ENSBTAG00000030559

Table 5 - DAVID gene clustering result for CL21

Annotation cluster 1	Enrichment Score: 25.83
IPR003054	Type II keratin
P-value	5.066E-46
Ensembl gene ID	*ENSBTAG00000037791*
	ENSBTAG00000007204
	ENSBTAG00000037638
	ENSBTAG00000039891
	ENSBTAG00000000619
	ENSBTAG00000039425
	ENSBTAG00000012676
	ENSBTAG00000016121
	ENSBTAG00000006739
	ENSBTAG00000012034
	ENSBTAG00000016165
	ENSBTAG00000007145
	ENSBTAG00000007144
	ENSBTAG00000007904
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	ENSBTAG00000038033
	ENSBTAG00000038384
	ENSBTAG00000026880

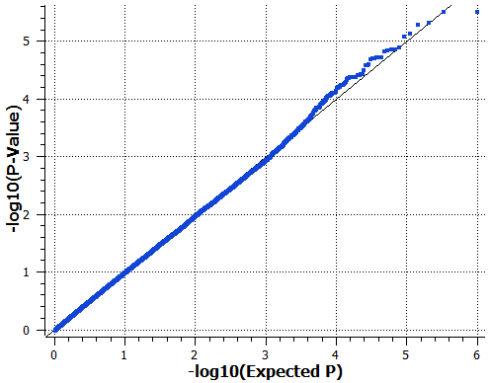

Figure 1 - HCW association analysis P-P plot for the test statistics (χ^2)

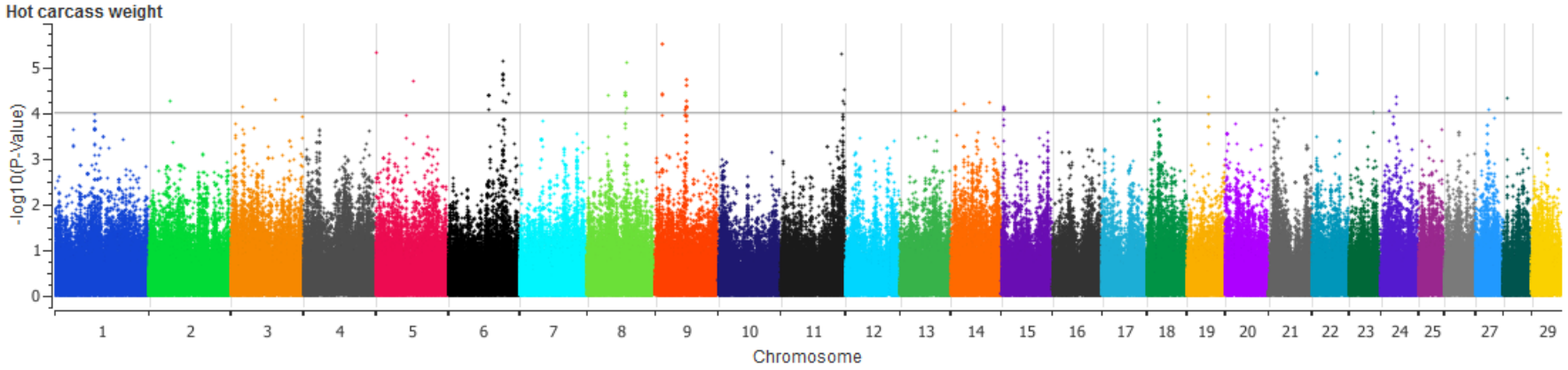

Figure 2 - Manhattan plot of genome-wide -log10(p-values) for HCW association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

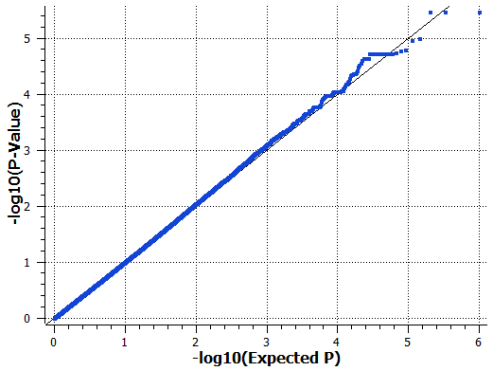

Figure 3 - REA association analysis P-P plot for the test statistics (χ^2)

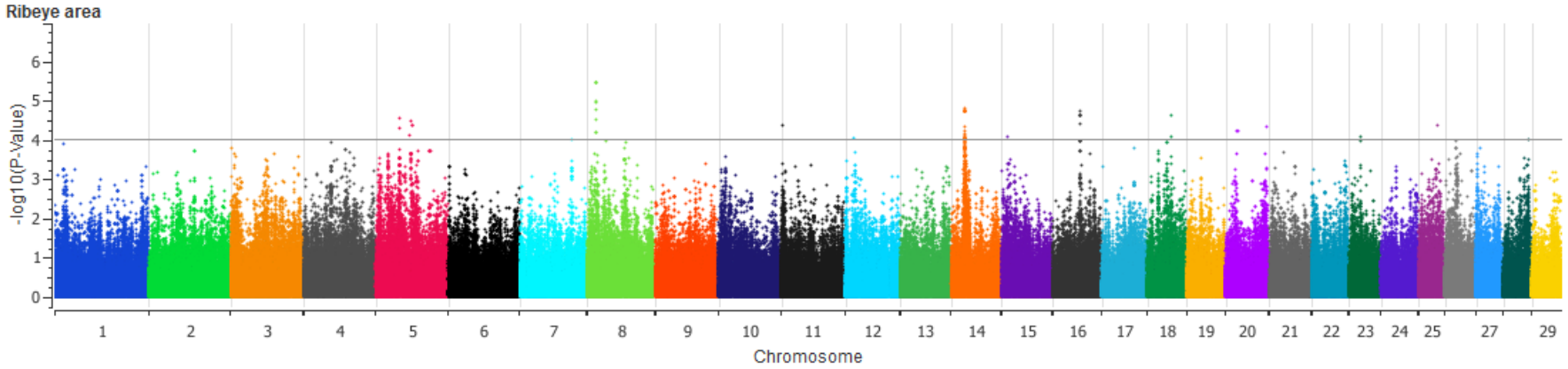

Figure 4 - Manhattan plot of genome-wide -log10(p-values) for REA association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

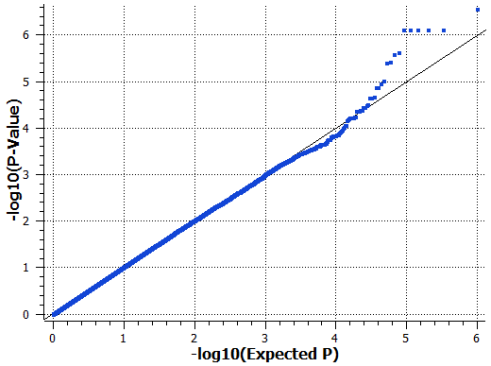

Figure 5 - BFT association analysis P-P plot for the test statistics (χ^2)

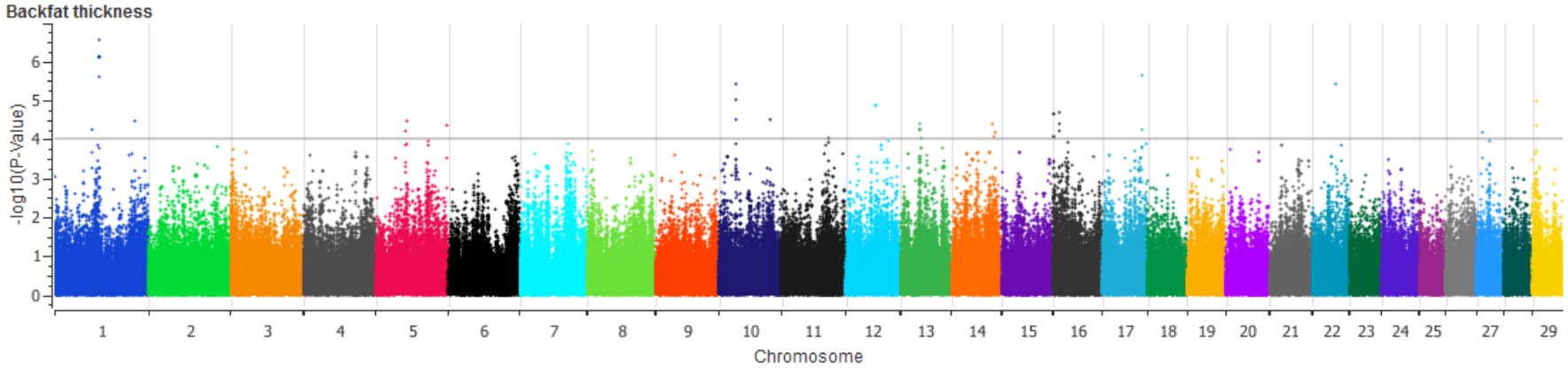

Figure 6 - Manhattan plot of genome-wide -log10(p-values) for BFT association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

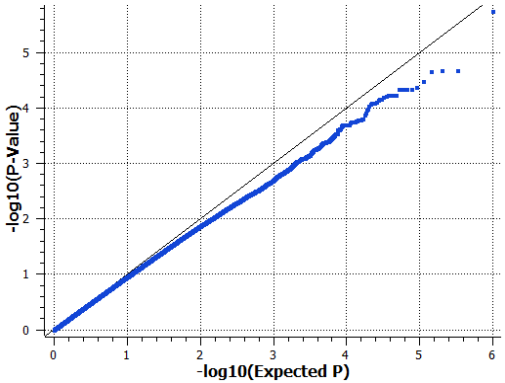

Figure 7 - pH24 association analysis P-P plot for the test statistics (χ^2)

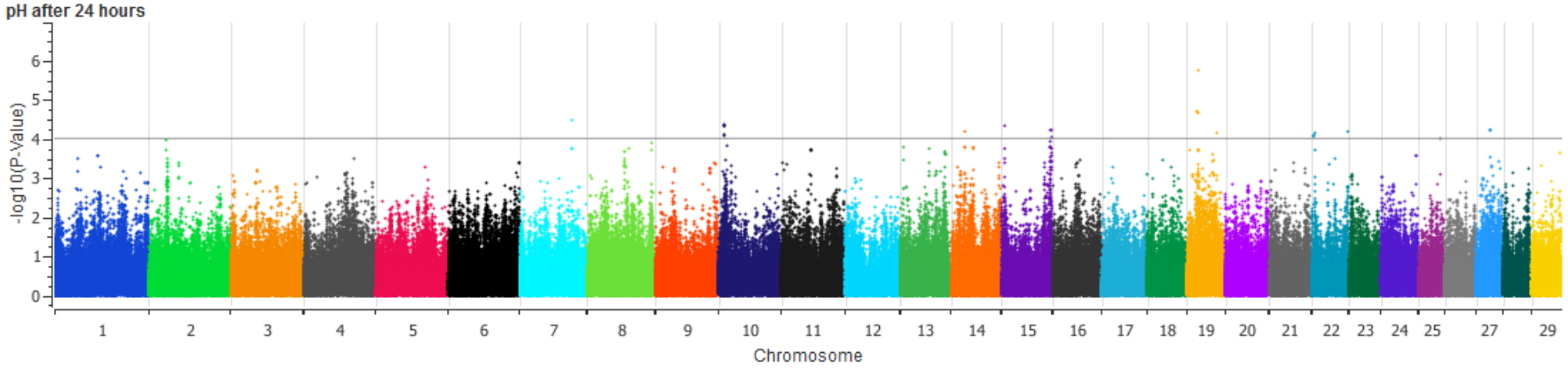

Figure 8 - Manhattan plot of genome-wide -log10(p-values) for pH24 association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

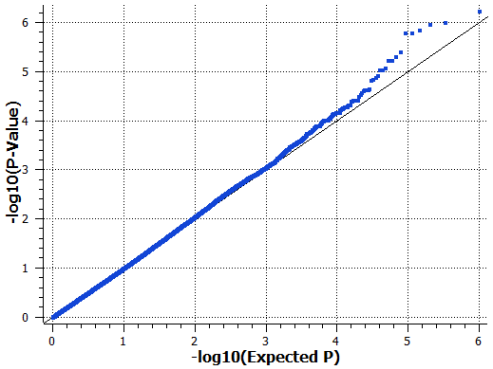

Figure 9 - L* color parameter association analysis P-P plot for the test statistics (χ^2)

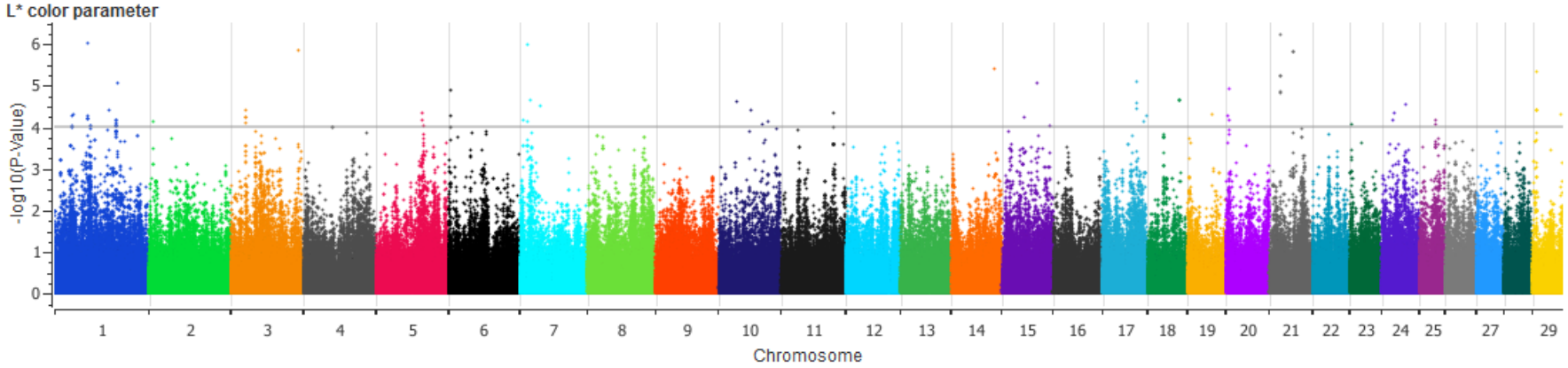

Figure 10 - Manhattan plot of genome-wide -log10(p-values) for L* association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

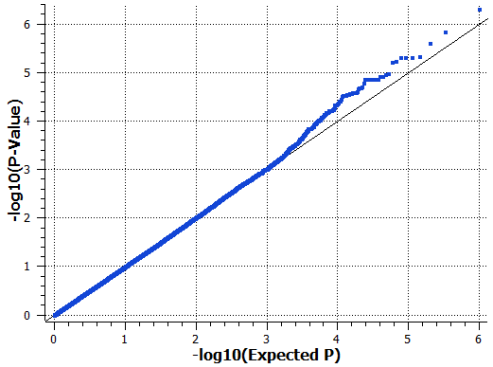

Figure 11 - a* color parameter association analysis P-P plot for the test statistics (χ^2)

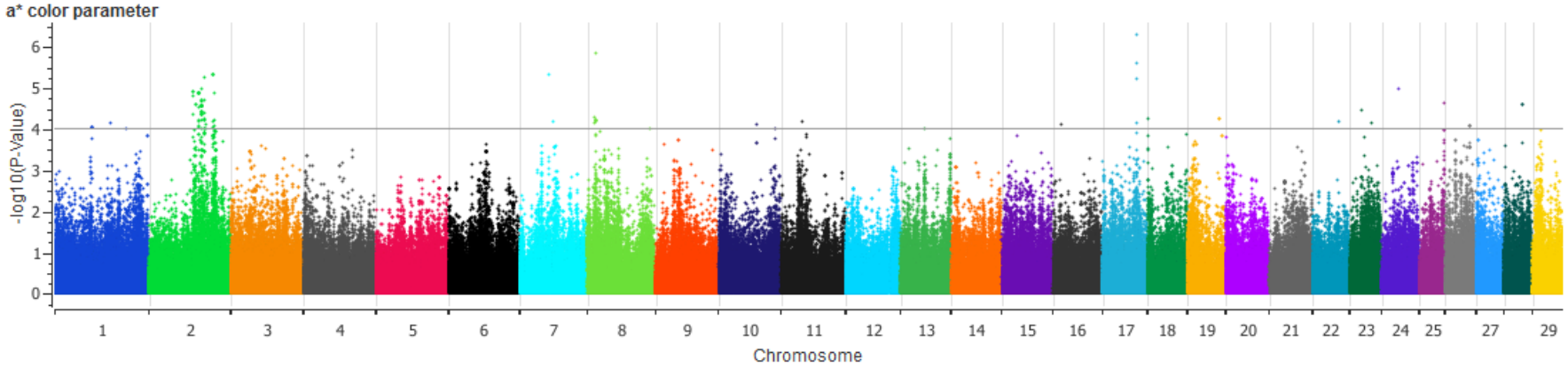

Figure 12 - Manhattan plot of genome-wide -log10(p-values) for a* color parameter association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

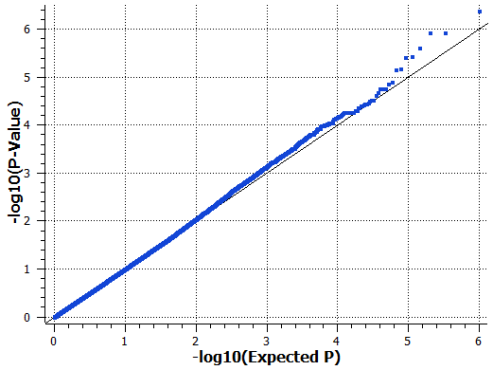

Figure 13 - b* color parameter association analysis P-P plot for the test statistics (χ^2)

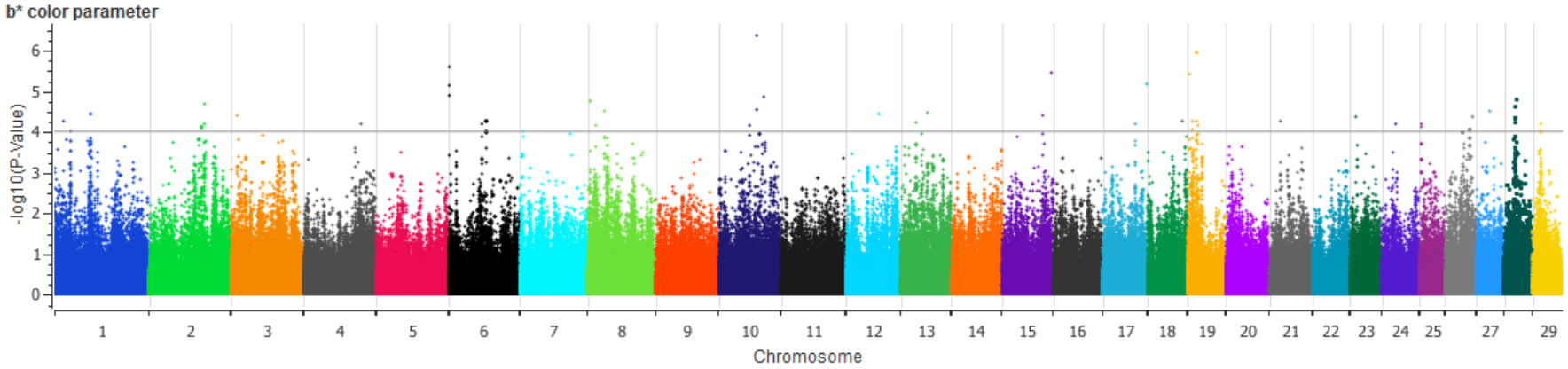

Figure 14 - Manhattan plot of genome-wide -log10(p-values) for b* color parameter association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

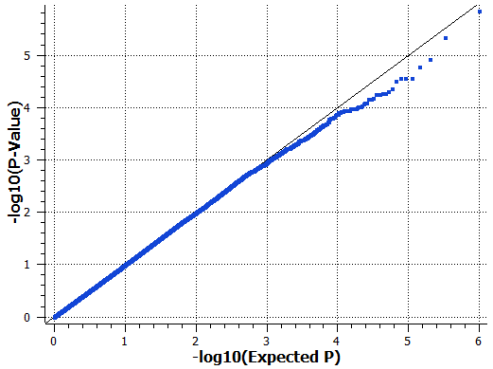

Figure 15 - DL7 association analysis P-P plot for the test statistics (χ^2)

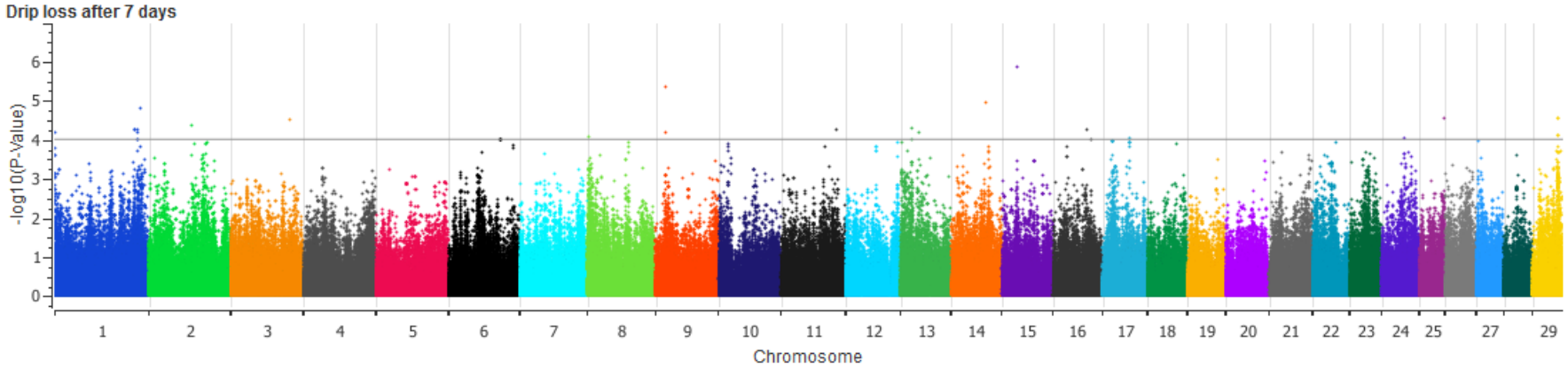

Figure 16 - Manhattan plot of genome-wide -log10(p-values) for DL7 association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

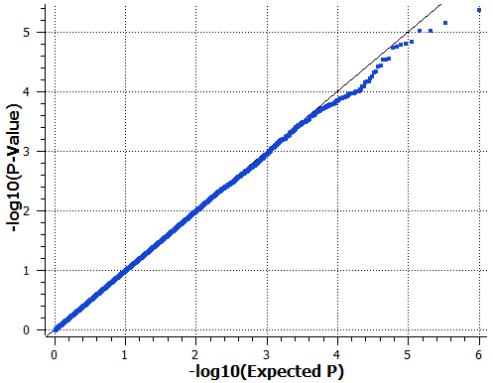

Figure 17 - DL14 association analysis P-P plot for the test statistics (χ^2)

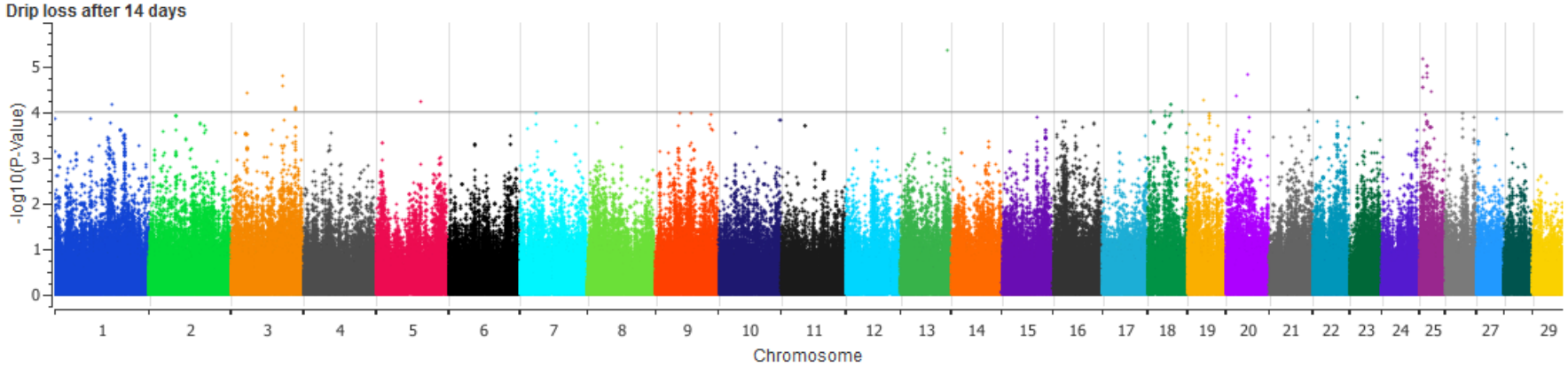

Figure 18 - Manhattan plot of genome-wide -log10(p-values) for DL14 association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

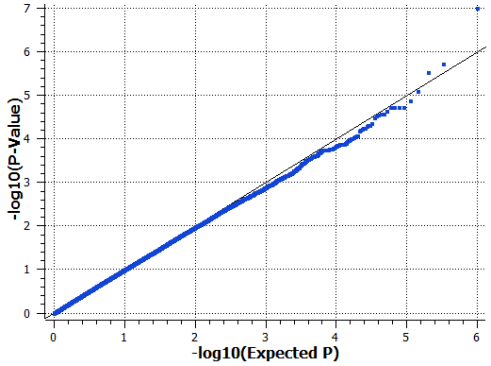

Figure 19 - DL21 association analysis P-P plot for the test statistics (χ^2)

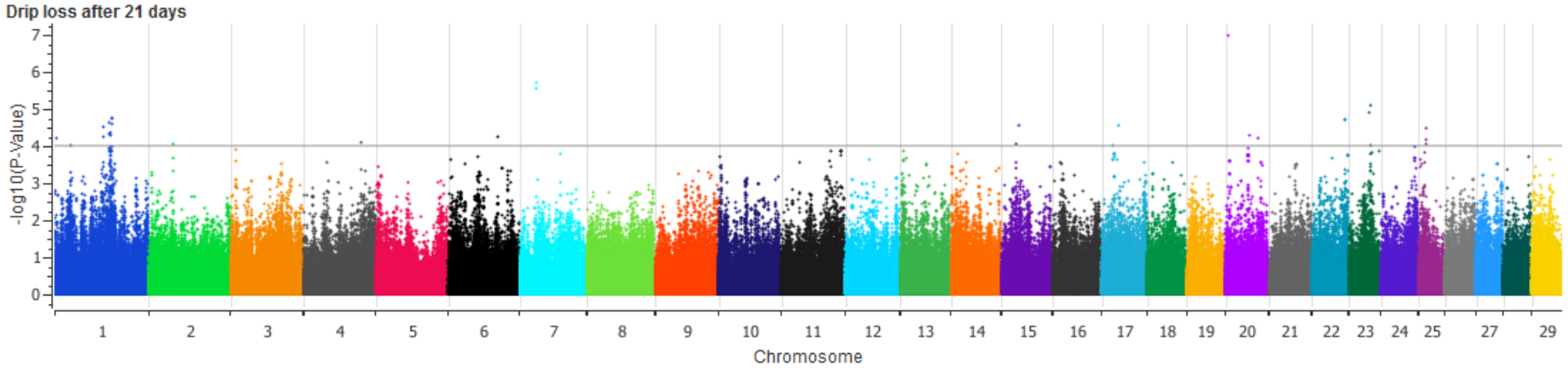

Figure 20 - Manhattan plot of genome-wide -log10(p-values) for DL21 association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

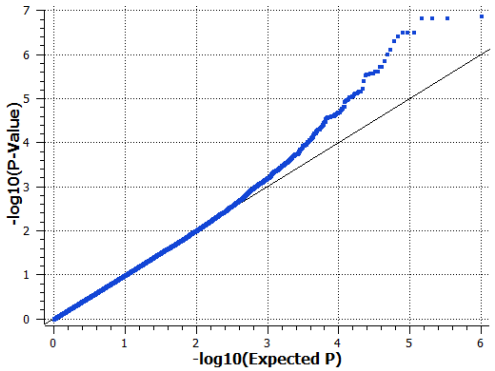

Figure 21 - CL7 association analysis P-P plot for the test statistics (χ^2)

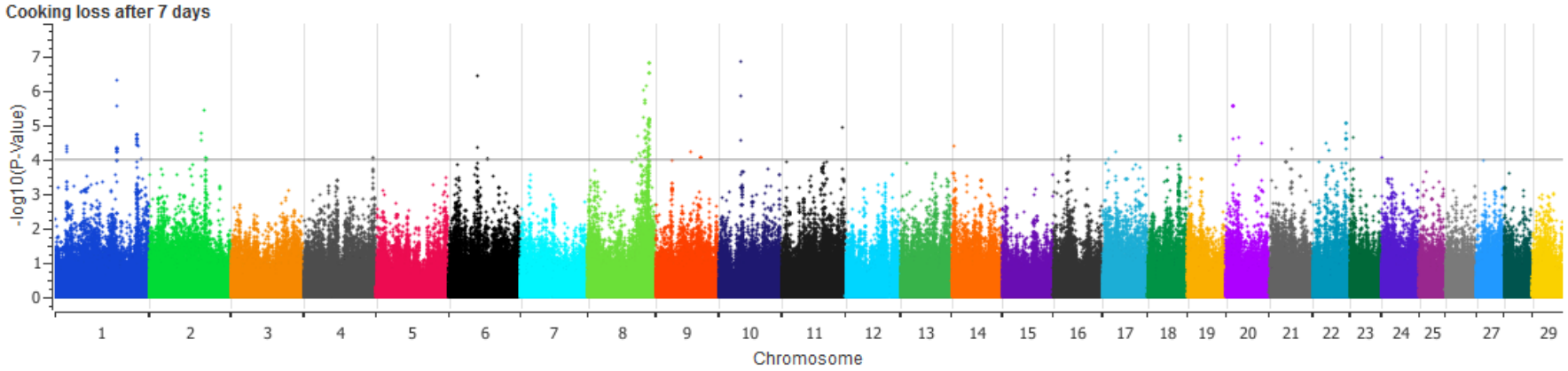

Figure 22 - Manhattan plot of genome-wide -log10(p-values) for CL7 association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

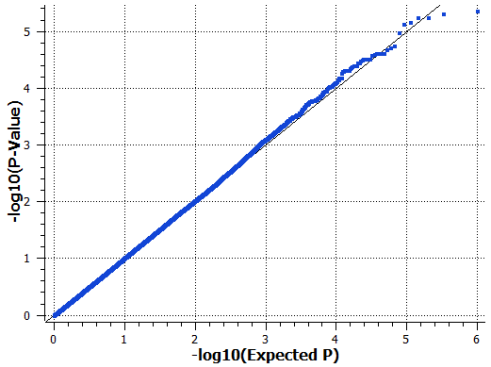

Figure 23 - CL14 association analysis P-P plot for the test statistics (χ^2)

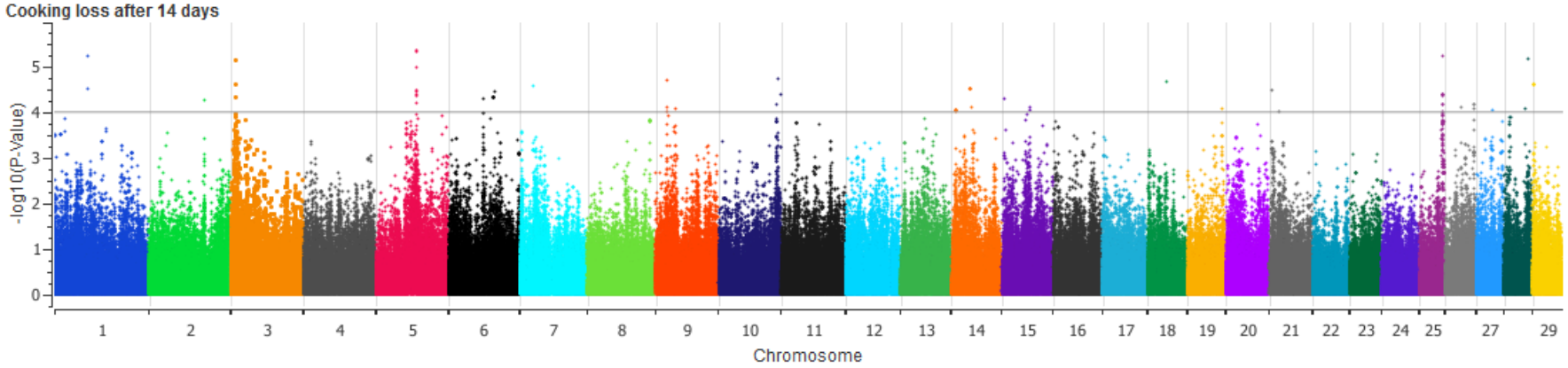

Figure 24 - Manhattan plot of genome-wide -log10(p-values) for DL26 association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

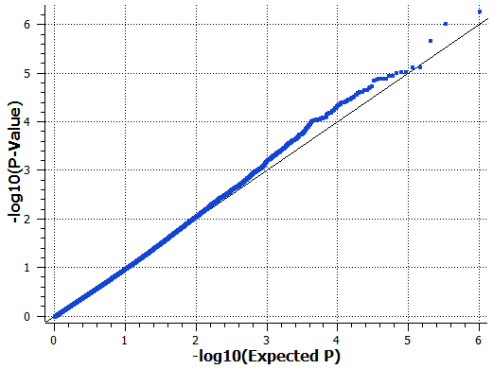

Figure 25 - DL21 association analysis P-P plot for the test statistics (χ^2)

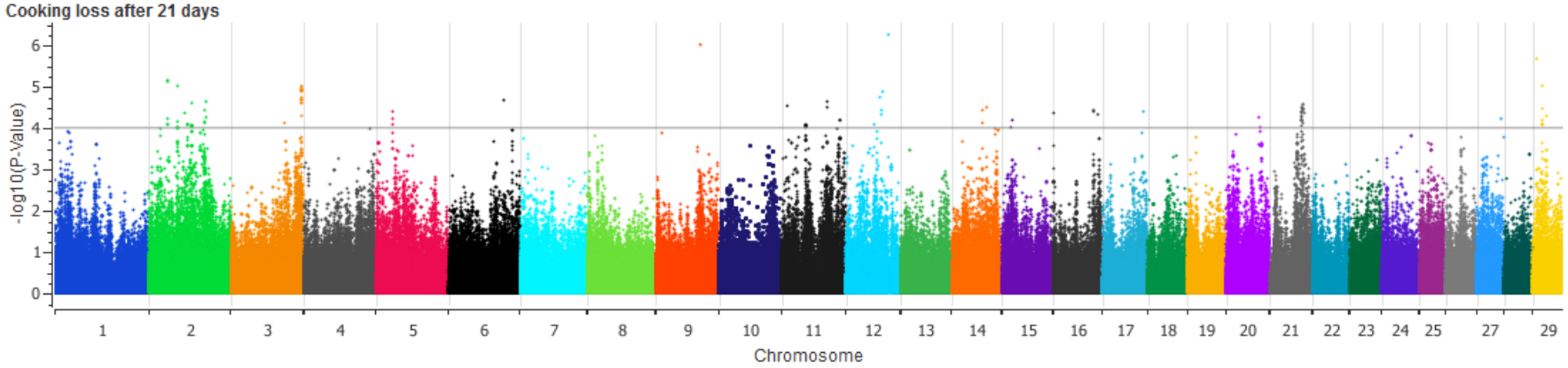

Figure 26 - Manhattan plot of genome-wide -log10(p-values) for DL21 association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

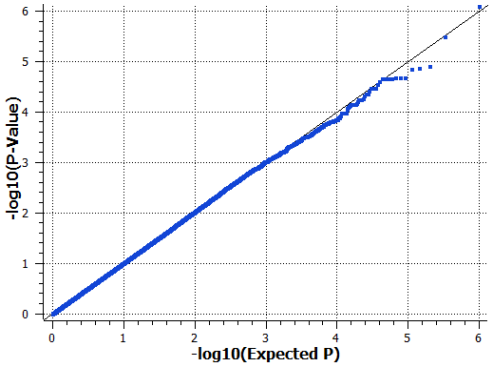

Figure 27 - WBSF7 association analysis P-P plot for the test statistics (χ^2)

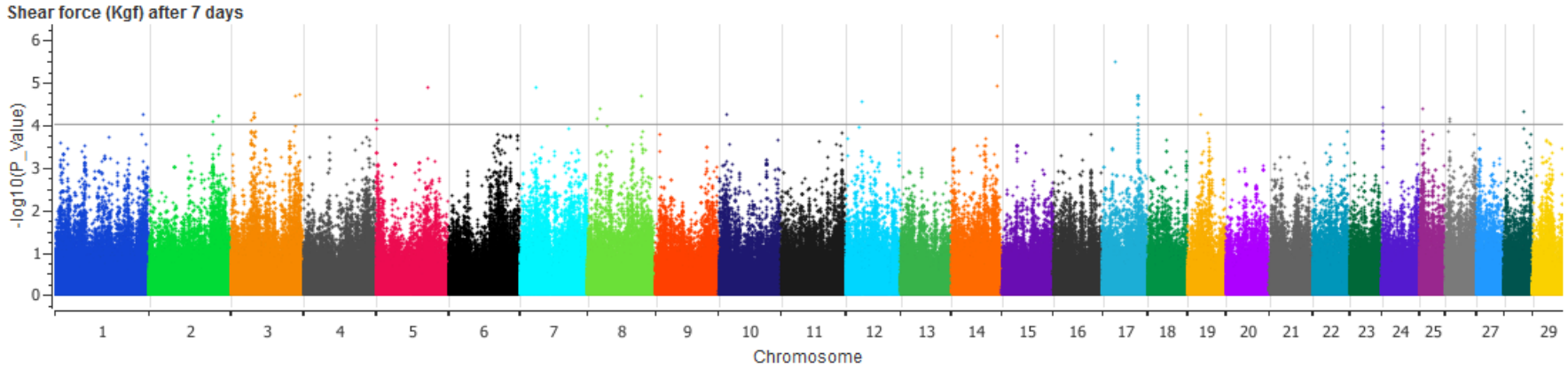

Figure 28 - Manhattan plot of genome-wide -log10(p-values) for WBSF7 association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

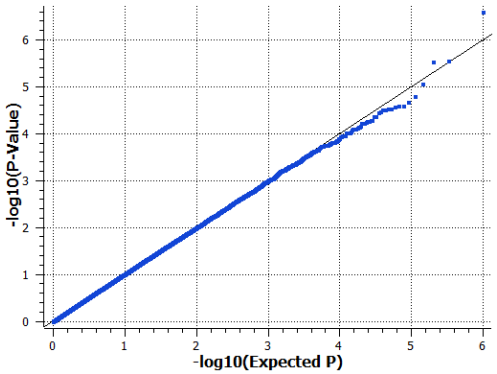

Figure 29 - WBSF14 association analysis P-P plot for the test statistics (χ^2)

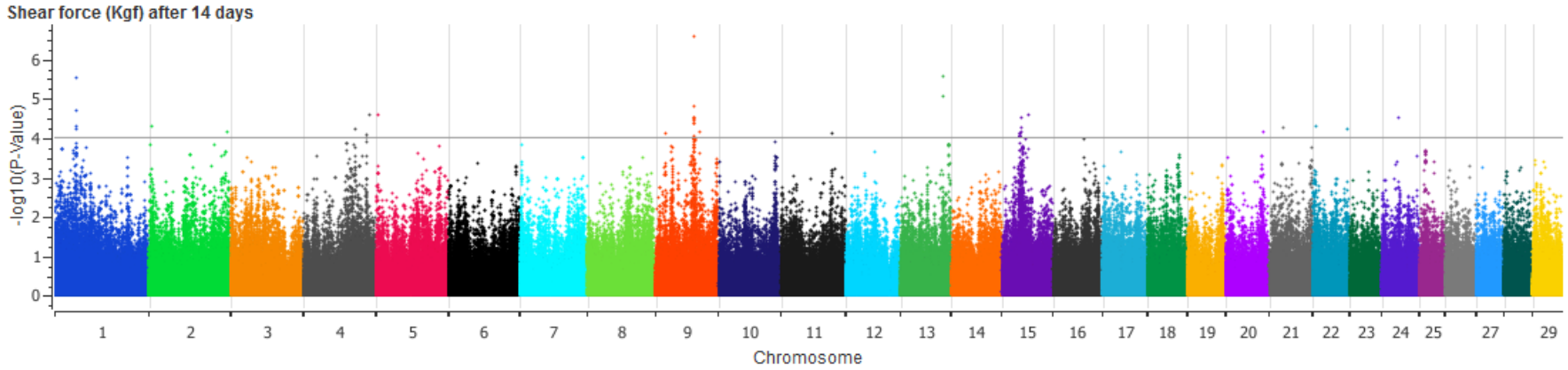

Figure 30 - Manhattan plot of genome-wide -log10(p-values) for WBSF14 association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

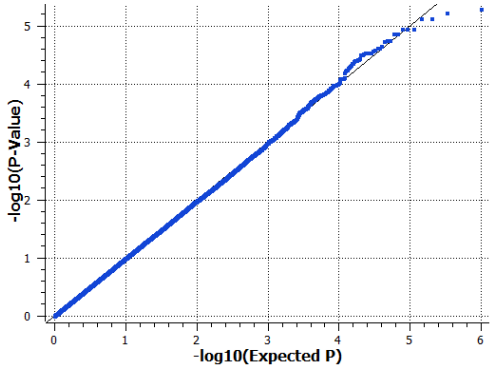

Figure 31 - WBSF21 association analysis P-P plot for the test statistics (χ^2)

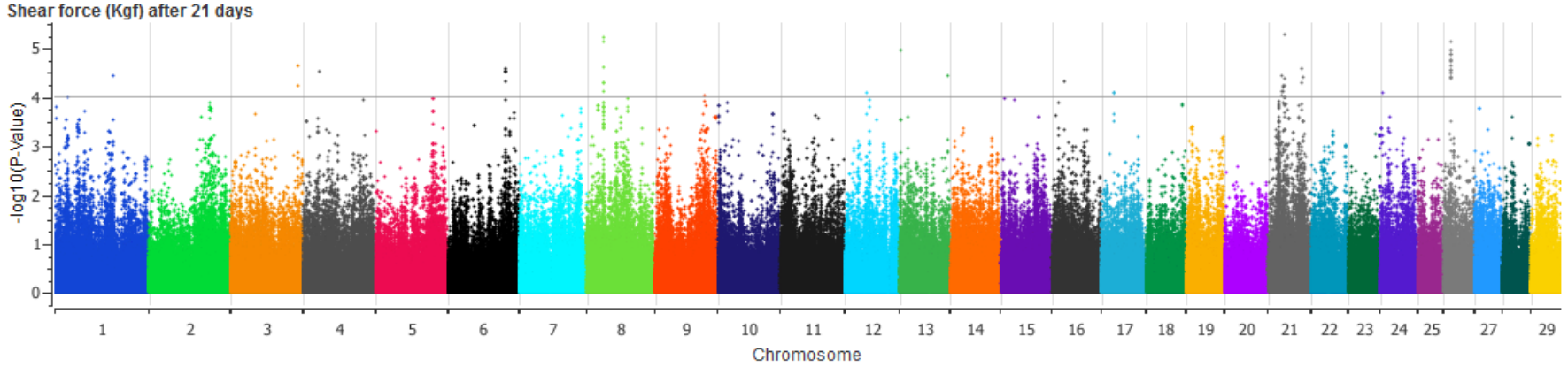

Figure 32 - Manhattan plot of genome-wide -log10(p-values) for WBSF21 association analysis. The horizontal solid line represents the significance level adopted ($\alpha = 1x10^{-4}$)

Annex B

Table 1 - PCA results for fatty acids correlation matrix

Variable	Group	Contribution		Loading		Cos2	
		PC1	*PC2*	*PC1*	*PC2*	*PC1*	*PC2*
C18:2 cis-9,12	1	7.68	0.699	-0.878	0.224	0.771	0.05
C18:3 cis-9,12,15	1	5.176	0.182	-0.721	0.114	0.52	0.013
C20:3 cis-8,11,14	1	6.203	1.331	-0.789	0.309	0.623	0.095
C20:4 cis-5,8,11,14	1	8.123	0.958	-0.903	0.262	0.815	0.069
C22:5	1	6.971	1.038	-0.836	0.273	0.7	0.074
C24:0	1	7.047	0.733	-0.841	0.229	0.707	0.053
C15:1	2	2.174	2.87	-0.467	0.454	0.218	0.206
C18:1 trans-10	2	0.589	7.708	0.243	0.744	0.059	0.553
C18:1 trans-9	2	0.41	9.469	0.203	0.824	0.041	0.679
C18:2 cis-9 trans-11	2	1.484	5.204	0.386	0.611	0.149	0.373
C18:2 trans-9,12	2	0.666	7.323	0.259	0.725	0.067	0.525
C18:3 cis-6,9,12	2	0.026	5.809	0.051	0.645	0.003	0.417
C20:2	2	0	6.474	0.003	0.681	0	0.464
C20:3 cis-11,14,17	2	0.019	3.053	0.043	0.468	0.002	0.219
C22:0	2	0.006	7.119	0.025	0.715	0.001	0.511
C22:1 cis-13	2	0.287	9.98	0.17	0.846	0.029	0.716
C22:2	2	0	4.526	0	0.57	0	0.325
C24:1 cis-15	2	0.111	7.769	0.106	0.746	0.011	0.557
C4:0	2	0.405	4.201	0.202	0.549	0.041	0.301
C10:0	3	3.581	0.512	0.6	0.192	0.359	0.037
C12:0	3	3.779	0.179	0.616	0.113	0.379	0.013
C14:0	3	5.279	0.427	0.728	-0.175	0.53	0.031
C14:1 cis-9	3	4.122	0.191	0.643	-0.117	0.414	0.014
C15:0	3	3.096	0.044	0.557	-0.056	0.311	0.003
C16:0	3	3.215	1.084	0.568	-0.279	0.323	0.078
C16:1 cis-9	3	3.544	0.237	0.596	-0.13	0.356	0.017
C17:0	3	3.552	0.085	0.597	-0.078	0.357	0.006
C18:1 cis-13	3	3.906	0.006	0.626	0.02	0.392	0
C18:1 cis-15	3	4.105	1.111	0.642	0.282	0.412	0.08
C18:1 trans-11	3	2.399	1.64	0.491	0.343	0.241	0.118
C20:0	3	2.601	1.311	0.511	0.307	0.261	0.094
C21:0	3	3.275	0.003	0.573	0.015	0.329	0

Table 2 – PCA results for indexes correlation matrix

Variable	Contribution		Loading		Cos2	
	PC1	*PC2*	*PC1*	*PC2*	*PC1*	*PC2*
AI	10.573	2.371	-0.879	-0.367	0.773	0.135
HH	11.232	1.577	0.906	0.299	0.821	0.09
HI	10.956	1.906	0.895	0.329	0.801	0.108
TI	8.745	5.46	-0.8	-0.557	0.64	0.311
Desaturation C14	0.151	11.387	0.105	0.805	0.011	0.648
Desaturation C16	0.008	12.726	0.024	0.851	0.001	0.724
Desaturation C18	0.01	15.57	0.027	0.941	0.001	0.885
SFA	7.917	7.276	-0.761	-0.643	0.579	0.414
MUFA	1.018	15.737	-0.273	0.946	0.074	0.895
PUFA	11.092	2.742	0.901	-0.395	0.811	0.156
PUFA/SFA	12.191	1.298	0.944	-0.272	0.892	0.074
n-3	6.385	2.362	0.683	-0.367	0.467	0.134
n-6	11.016	2.745	0.898	-0.395	0.806	0.156
n-9	0.694	14.542	-0.225	0.909	0.051	0.827

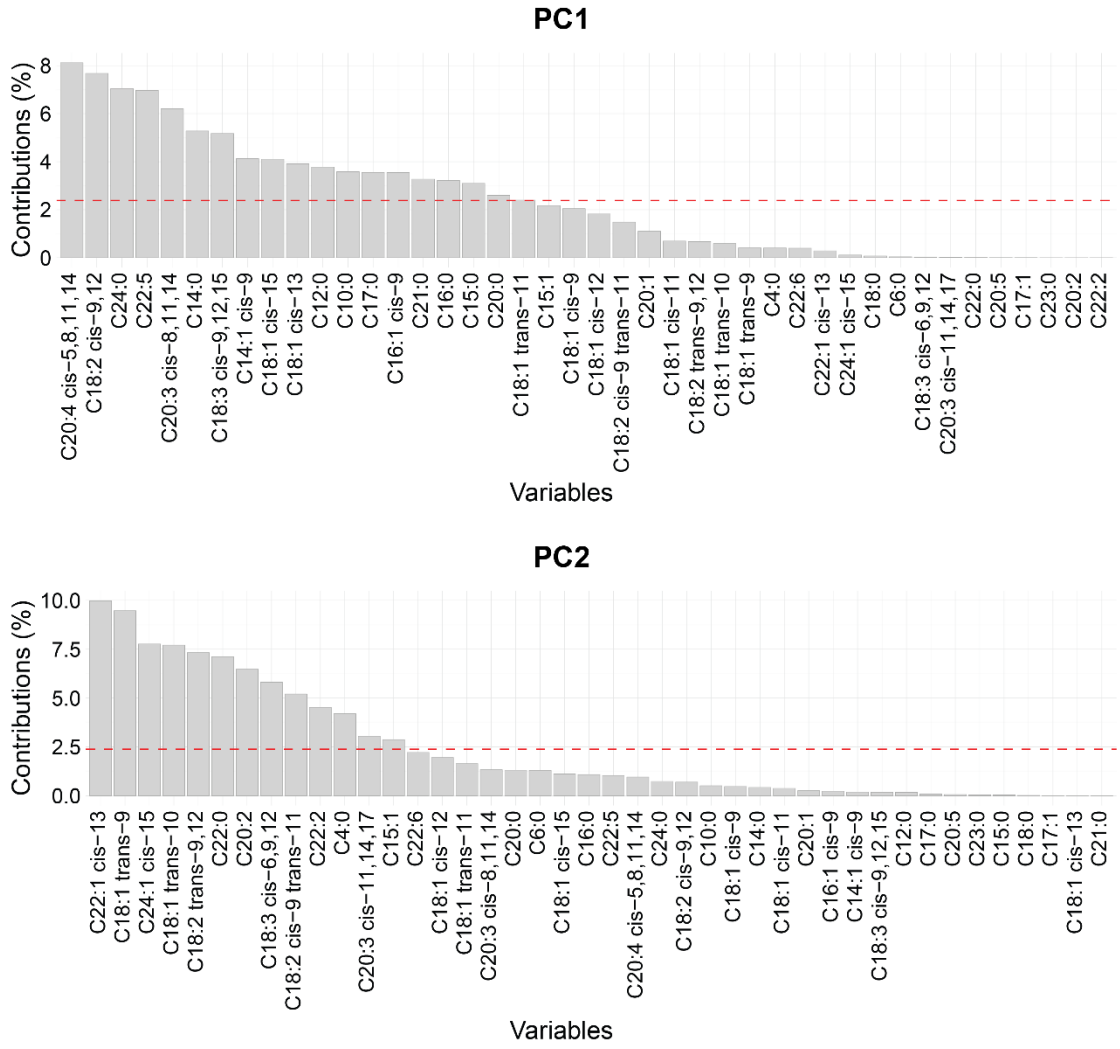

Figure 1 - Fatty acids that majorly contributed for components construction

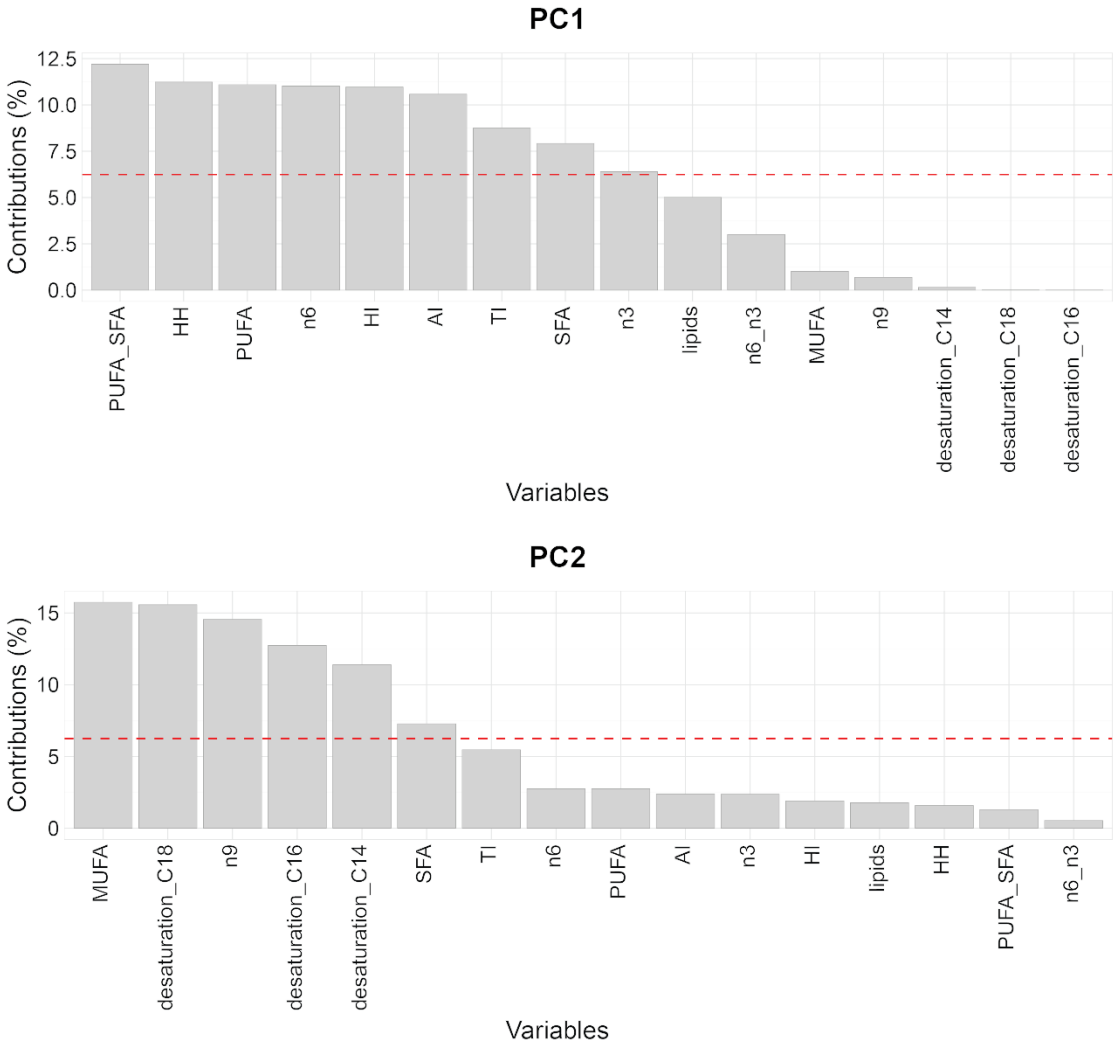

Figure 2 - Indexes that majorly contributed for components construction