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Different Phenotypic and Proteomic Markers Explain Variability of Beef Tenderness across Muscles

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Abstract

This study analyzed the abundance of tenderness biomarkers: 24 proteins and 11 phenotypic carcass characteristics and muscle properties. This was done on 111 samples of two muscles, *Longissimus thoracis* (LT) and *Semitendinosus* (ST) from the Charolais cattle breed. The strategy was to constitute and explain three tenderness classes on the two muscles separately, on the shear-force data (Warner-Bratzler). Results showed that ST classes were explained by 12 proteins and 6 phenotypic characteristics. LT classes could only be explained by 7 phenotypic characteristics. This demonstrates that in ST and LT the tenderness variability is explained by different factors. In ST, the main results demonstrated the importance of Heat Shock Proteins such as Hsp27 ($P = 0.002$) and the oxidative stress protein: PRDX6 ($P = 0.003$). We also confirmed the role of the glycolytic enzyme Enolase 3 ($P = 0.003$), and contractile protein such as MyHC IIx ($P = 0.028$).

Keywords: Beef, Muscle, Biomarkers, Phenotypic, Proteomic, Tenderness classes

1. Introduction

Tenderness is an important criterion in consumer's meat appreciation, and so a determining factor in purchase (Geay, Bauchart, Hocquette, & Culioli, 2001). Beef represents an important economic sector in some countries such as the United States, Brazil, Australia and France (Data from French Agriculture Ministry and Food and United Nations Agriculture Organization). However, uncontrolled tenderness variability is the number one reason for consumer's dissatisfaction.

This tenderness variability is due to the muscle nature, which is a complex biological structure (Dransfield et al., 2003), consisting of fibers, adipocytes and connective tissue with different properties, each partially responsible for the tenderness variability.

For years, functional genomics programs were developed to explain this tenderness variability and to identify tenderness biomarkers, at DNA (Hocquette, Lehnert, Barendse, Cassar-Malek, & Picard, 2007), RNA (Bernard et al., 2007) and protein levels (Picard et al., 2010). Some markers have been identified in different contexts of countries, breeds, muscles, etc, but these markers and their effects are not identical according to the contexts. Commercialized genetic tests, based on utilization of SNP (Single Nucleotide Polymorphism) markers of genes encoding μ -calpain and calpastatine, can predict the muscle tenderness class (Barendse, 2002; Page et al., 2004). However, these tests are not efficient on French breeds (Van Eenennaam et al., 2007). So the French cattle industry is looking for specific tenderness prediction tools, based on biomarkers. The French industry supports functional genomics programs to identify potential tenderness biomarkers.

The objective of this study was to confirm or not the predictive value of 24 proteins with respect to tenderness. These proteins were identified as biomarkers for beef tenderness by previous works (Bernard, et al., 2007; Bouley, Chambon, & Picard, 2004; Morzel, Terlouw, Chambon, Micol, & Picard, 2008) by comparing extreme tenderness groups. They belong to different families: heat shock proteins, metabolism, structure, oxidative resistance and proteolysis (Picard, et al., 2010).

2. Materials and Methods

2.1 Animals and samples

This study took place over a two years period with 67 Charolais young bulls and 44 Charolais steers (castrated at 3 months of age) from the French MUGENE program (Genanimal-APISGENE 2005-2009). The young bulls were slaughtered at 17 months of age on average and the steers at 30 months of age on average at the INRA experimental slaughterhouse in compliance with current INRA ethical guidelines for animal welfare (Guillemin et al., 2011). Muscle samples from the *Longissimus thoracis* (LT, 6th rib, fast oxido-glycolytic with 25 % of MyHC-I, 62 % of MyHC-IIA and 13 % of MyHC-IIx in our experiment by electrophoresis (Picard et al., 1999), and in accordance with Jurie *et al.* (2007; 1995) in bovine) and *Semitendinosus* (ST, middle of muscle, fast glycolytic with 11 % of MyHC-I, 24 % of MyHC-IIA and 66 % of MyHC-IIx in our experiment, and in accordance with Schreurs *et al.* (2008) in bovine) were excised within 15 minutes after slaughter. Muscle samples (N = 111 for each muscle) were immediately frozen in liquid nitrogen and stored at -80°C until protein extraction. Steaks for each muscle were matured at 4°C for 21 days, and then frozen at -20°C. After thawing,

they were cooked to an internal temperature of 55°C. Shear-force measurements of each sample were carried out with a Warner-Braztler (WB) apparatus (Wheeler et al., 1997).

2.2 Immunological protein quantification

Total protein extractions were performed according to Bouley et al. (2004) in a denaturation/extraction buffer (8.3M urea, 2M thiourea, 1% DTT, 2% CHAPS). All chemical reagents were from Sigma (St Louis, MO, USA). The protein concentration was determined by spectrophotometry with the Bradford assay (Bradford, 1976). Protein extractions were stored at -20°C until use.

The conditions for use and specificities of primary antibodies against the 24 proteins analyzed in bovine muscle were assessed according to the methodology described by Guillemain et al. (2009). Briefly, western blots were used in order to check the specificity of all the antibodies. An antibody was considered specific against the studied protein when one band at the expected molecular weight was detected by western blot (Duffy, Scofield, Rodgers, Patton, & Bowyer, 1999). BLAST analyses (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) were done between the 24 studied bovine protein sequences against human protein databases, to assure that proteins share more than 90 % of identity. Western blots with the 24 primary antibodies show that all the antibodies bind specifically to a bovine protein with the theoretical molecular weight of the attempted protein (data not shown). Optimal dilution ratios were determined in the same time, from suppliers' conditions and adapted to bovine muscle samples. Conditions retained and suppliers for all primary antibodies are reported in Table 1.

Secondary fluorescent-conjugated IRDye 800CW antibodies (anti-mouse, anti-sheep, anti-rabbit) were supplied by LI-COR Biosciences (Lincoln, Nebraska, USA) and used at 1/20000. Protein quantifications with the validated antibodies were carried out by Dot blot, with the protocol described by Guillemain et al. (2009). Dot blot is a technique 15 times faster than western blot, characterized by the same technical variations (10%). This characteristic allowed us to analyze these 24 protein markers in less time than with western blot (6 months for Dot blot versus 53 for western blot). Briefly, protein samples of each of the 222 muscle samples (111 for LT and 111 for ST) were spotted (4 replications per muscle sample) on a nitrocellulose membrane with the Minifold I Dot blot from Schleicher&Schuell Biosciences (Germany) and hybridized with the specific antibody of each protein, with conditions defined by western blot. Then membranes were scanned by the Odyssey scanner (LI-COR Biosciences) at 800 nm. Protein abundance for each sample, given in arbitrary units, was normalized according to a mix of different samples, used as reference.

2.3 Phenotypic data

Phenotypic data used in this study was provided by the MUGENE program. The methods for analyzing carcass composition: carcass fat weight in kg (CFW) and percentage of muscle in the carcass (PMC) were described in Renand et al. (1995); enzyme activities in $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ (Cytochrome Oxidase (COX), Citrate Synthase (CS), Isocitrate Dehydrogenase (ICDH), Lactate Dehydrogenase LDH) were described in Jurie et al. (2007) and Piot et al. (1998). Methods used for measurements of total collagen content (Totcol), insoluble collagen (Inscol) in μg of OH-proline. mg^{-1} and its percentage of solubility (Solubility) in dry muscle, were described by Listrat and Hocquette (2004), and were described in Schreurs et al. (2008) for total lipids, triglycerides in $\text{mg}\cdot\text{g}^{-1}$ in fresh muscle, and fiber mean cross-section area in μm^2 . Data for fiber mean cross-section area were available only for LT muscle.

2.4 Statistical analysis

A disjoint cluster analysis was made, using the R software (2.12.1), with the package classInt (0.1-14, Roger Bivand), to produce three quality classes from shear-force measurement (Warner-Braztler) of all samples, previously adjusted for the year effect. This cluster analysis was made per muscle with the Fisher's method.

The three classes' values of all proteins (Dot-Blot quantification) and phenotypic data, adjusted for the year effect, were compared by ANOVA for the two muscles LT and ST separately. For each proteins and phenotypic data, the statistically significant differences between classes were determined with the Tukey's Honestly Significant Difference test.

3. Results

3.1 Tenderness classes

Three tenderness classes were produced from the shear-force measurement, for LT and ST (Table 2): inferior tenderness (Inf), medium tenderness (Med), and superior tenderness (Sup). For LT muscle, Inf-class (N = 13) had an average of 58.2 $\text{N}\cdot\text{kg}^{-1}$ (± 2.51). The Med-class (N = 64) had an average of 40.8 $\text{N}\cdot\text{kg}^{-1}$ (± 0.46). The Sup-class (N = 34) had an average of 30.0 $\text{N}\cdot\text{kg}^{-1}$ (± 0.61).

For ST muscle, Inf-class (N = 21) had an average of 82.7 N.kg⁻¹ (\pm 2.01). The Med-class (N = 36) had an average of 63.0 N.kg⁻¹ (\pm 0.72). The Sup-class (N = 54) had an average of 46.7 N.kg⁻¹ (\pm 0.76).

We can observe that for LT muscle the majority of samples (58%) were classified in the Med-class, whereas in ST they were classified in the Sup-class (49%). However, the shear-force values were higher in ST muscle for each class which agrees with the data in the literature generally indicating reduced tenderness of ST compared to LT muscle (Renand, Picard, Touraille, Berge, & Lepetit, 2001).

3.2 Proteins differential abundance per tenderness class in ST muscle

In ST muscle, 11 proteins significantly described tenderness classes (Table 3). Some proteins were more specific to Inf-class or Sup-class than others, and some described the Inf-class comparatively to the Sup-class, but not the Med-class. No protein discriminated the Med-class from the two others.

Abundances of Hsp27, Hsp70-8, MLC-1F, M-calpain, PRDX6 and My HC IIX were significantly different ($P = 0.002$, $P = 0.037$, $P = 0.006$, $P = 0.015$, $P = 0.003$, $P = 0.028$ respectively) in Inf-class compared to the others. The toughest ST class was characterized by higher abundance of Hsp70-8, MLC-1F, M-calpain, PRDX6, MyHC IIX and lower abundance of Hsp27.

Abundances of Hsp20 and Eno3 were significantly different ($P = 0.01$, $P = 0.003$ respectively) in Sup-class compared to the others. The ST Sup-class was characterized by lower abundance of Hsp20 and higher abundance of Enolase 3 involved in glycolytic metabolism.

Abundances of α B-crystallin, μ -calpain and Hsp70-GRP75 were significantly different ($P = 0.027$, $P = 0.006$ respectively) between Inf-class and Sup-class, but not with Med-class.

3.3 Proteins differential abundance per tenderness class in LT muscle

In LT muscle, only one protein significantly described tenderness classes (Table 4). The abundance of M-calpain was significantly different between the Inf-class and Med-class ($P = 0.042$), but not between these two classes and the Sup-class. There were no significant differences for the 23 other proteins. Only the abundance of PRDX6 had a tendency to discriminate the Med-class from the Sup-class ($P = 0.079$), but not between these two classes and Inf-class. In spite of a trend for μ -calpain ($P = 0.086$), this protein does not discriminate any class. Consequently, none of the 24 quantified proteins explained the classification according to the shear-force measurement.

3.4 Differential phenotypic characteristics per tenderness class in ST muscle

In ST muscle, 5 phenotypic characteristics significantly described tenderness classes (Table 5). Some characteristics discriminated Inf-class or Sup-class from the others, and some discriminated the Inf-class from the Sup-class, but not the Med-class. No protein discriminated the Med-class from the two others as previously observed for protein abundances.

Only one phenotypic characteristic significantly discriminated the Inf-class from the two others: total lipids content ($P = 0.001$). The tenderest ST muscle contained the highest lipid content (values for Inf-class, Med-class and Sup-class respectively: 22.6, 16.9, and 15.6 mg.g⁻¹ in fresh muscle). The carcass composition (CFW, PMC) and the collagen solubility significantly discriminated the Sup-class from the others ($P = 0.029$, $P = 0.014$, $P = 0.031$ respectively). The less tender ST muscles were characterized by lower carcass adiposity and higher muscle mass and solubility of collagen.

Triglycerides were significantly different ($P = 0.022$) between Inf-class and Sup-class, but not between these two classes and Med-class.

There were no significant differences for the 6 other phenotypic characteristics. Only insoluble collagen tended to discriminate the Inf-class from the Sup-class ($P = 0.051$), but not between these two classes and Med-class.

3.5 Differential phenotypic characteristics per tenderness class in LT muscle

In LT muscle, 6 phenotypic characteristics significantly described tenderness classes (Table 6). Some characteristics discriminated the Inf-class or Sup-class from the others. No characteristics discriminated the Med-class from the two others, as observed for ST muscle.

CFW, collagen solubility, fiber mean cross-section area, triglycerides and lipids discriminated the Inf-class from others ($P = 0.031$, $P = 0.023$, $P = 0.019$, $P = 0.018$ and $P = 0.016$ respectively). The least tender LT muscles were characterized by lower adiposity of carcass, lower intra muscular fat content, lower solubility of collagen and higher mean cross-section areas.

INSCOL discriminated the Sup-class from the others ($P = 0.018$).

There were no significant difference for the 6 other phenotypic characteristics. Only PMC tended to discriminate the Inf-class from the two others ($P = 0.075$).

4. Discussion

Our results clearly demonstrated that the LT and ST muscles' tenderness was differentially discriminated. The quantified proteins considered as potential tenderness markers did not allowed a discrimination of LT tenderness. Among the 24 studied proteins, only 11 explained tenderness classes in ST muscle. No protein in either muscle allowed discrimination with respect to the medium class of tenderness.

4.1 Discrimination of ST tenderness classes

In the ST muscle, most of the proteins such as Hsp70-8, MCL-1F, M-calpain, PRDX6 and MyHC IIx were more expressed in the Inf-class, so they could be considered as toughness markers. On the contrary, Hsp27 discriminated the least tender ST by a lower abundance in coherence, with a higher abundance of Hsp20 and α B-crystallin observed in the tenderest ST. These three HSP could be considered as tenderness markers as they allowed the identification of the tender group.

These three Heat Shock Proteins explaining tenderness classes in the same way belong to the same family called small HSPs or the HSP20 family (Sun & MacRae, 2005). The expression of these proteins is highly correlated, as observed in human and mice (Golenhofen, Perng, Quinlan, & Drenckhahn, 2004). They are particularly involved in the protection of structural proteins like desmin, titin, and actin (Bullard et al., 2004; Ghosh, Houck, & Clark, 2007). The HSP20 proteins inhibit the protein aggregates formation, to maintain protein accessibility (Melkani, Cammarato, & Bernstein, 2006). So, HSP20 proteins could enhance structural proteins proteolysis, especially during meat tenderization, and so meat tenderness.

Two others Heat Shock Proteins, Hsp70-8 and Hsp70/GRP75 belonging to the family of HSP70 appeared to be toughness markers in this study. These HSP70s are chaperone proteins. Unlike HSP20, their activity could inhibit meat tenderization by blocking protein-protein interactions of an important number of target proteins (Houry, Frishman, Eckerskorn, Lottspeich, & Hartl, 1999).

So HSP20s are tenderness markers, whereas HSP70s are toughness markers. We can hypothesize that tenderization efficiency is dependent on the HSP20s/HSP70s ratio. The HSP20s and HSP70s are also involved in oxidative resistance of the cell (Fink, 1999; Laufen et al., 1999). Oxidative stress could be important in meat tenderization, by enhancing HSP expression and thus modifying the HSP20s / HSP70s ratio. The overall cellular abundance of HSPs could influence meat tenderization, by another important cellular function of HSPs. HSP20s and HSP70s inhibit apoptosis by sequestering pro-apoptotic factors such as Bax (Beere & Green, 2001; Concannon, Gorman, & Samalli, 2003). Apoptosis have been identified as a tenderization enhancer just after slaughtering by Ouali et al. (2006), notably by protein proteolysis done by caspases, enzymes able to degrade structural proteins (Chen, Chang, Trivedi, Capetanaki, & Cryns, 2003; Nakanishi, Maruyama, Shibata, & Morishima, 2001). So, this is in contradiction with the positive role of HSP20s with tenderness. But the ST muscle is characterized by a less abundance of HSP20s and HSP70s contrary to LT muscle (Guillemin, et al., 2011). So, in this ST muscle, there is a strong competition for the pro-apoptotic factor Bax sequestration between HSP20s and HSP70s. We hypothesize that HSP20s mainly assure their function of chaperone against structural proteins aggregates, and HSP70s in Bax sequestration. This is the reason why in ST muscle, HSP20s are associated with tenderness, by protecting structural proteins and maintain proteolysis, contrary to HSP70s, associated with toughness, as these proteins inhibit apoptosis. In LT muscle, HSPs content is higher than in ST, and so these proteins are not related to tenderness classes.

Another marker related to toughness in our study was PRDX6. This enzyme, involved in redox reduction of the cell notably during oxidative stress, was validated as a tenderness marker in the LT muscle by the work of Jia et al. (2009). Our results showed a reverse role of PRDX6, e.g. as a toughness marker in ST muscle, not in LT. This result is surprising in the light of the work by Jia et al. (2009) on LT muscle. However, it is well-known that there are muscle-type effects on protein implication in tenderness (Hocquette, et al., 2007; Maltin, Balcerzak, Tilley, & Delday, 2003). The role of PRDX6 on tenderness could depend on muscle type. This could be due to the differential tolerance for reactive oxygen species, between oxido-glycolytic muscle like LT and glycolytic muscle like ST (Guillemin, et al., 2011). This result confirms the implication of oxidative stress in tenderization suggested by HSPs.

So, oxidative stress could be a pertinent cellular mechanism to explain tenderness classes. In fact this cellular mechanism could control *in fine* the functions of HSPs, notably to protect structural proteins against oxidative stress and proteolysis, essential for tenderness (Sentandreu, Coulis, & Ouali, 2002).

The role of calpains (M- and μ -calpain) in proteolysis is well-known (Koohmaraie, Whipple, Kretchmar, Crouse, & Mersmann, 1991). So, their implication in the tenderness classes discrimination was not a surprise. However, we found that the protein M-calpain acts as a toughness marker since it was more abundant in the less tender ST, which is surprising. However, Picard et al. (2007) also observed that the levels of M-calpain and micro-calpain, measured only in the LT muscle, were positively correlated with the maximum shear-forces (which correspond to the Inf-class) in meat from young bulls. This result must be interpreted carefully as here we measured only the abundance of protein and not its activity. Moreover, it was well-demonstrated that tenderness depends on the ratio between the activity of calpastatine (calpains inhibitor) and calpain (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010). In our study, we have no information about calpastatine because the tested specified antibody was not functional in our samples.

According to our data, we can hypothesize from the implication of HSPs and calpains that in ST muscle, tenderness is higher when protein proteolysis by calpains is low, protein proteolysis by caspases and aggregates formation inhibition by HSPs are high. However, this hypothesis must be validated.

Three structural and contractile proteins have been identified as toughness markers: MLC-1F, CapZ- β and MyHC IIX. These proteins are involved in the structure of fast-twitch fiber myofilaments (Clark, McElhinny, Beckerle, & Gregorio, 2002). According to these results, we can hypothesize that proteins of fast-twitch fibers are toughness markers because they are more resistant to mechanical efforts during muscle contraction, and so are characterized by a resistance to proteolysis during meat tenderization.

As the fast-twitch fiber is associated with a glycolytic metabolism, it was not surprising to find lower abundance of Enolase 3, a glycolytic enzyme in the tenderest ST.

Among the 5 metabolic proteins quantified, only Enolase 3 could explain the tenderness classes. This demonstrated that ST muscle metabolic properties are not the most discriminating parameters for tenderness.

4.2 Opposition between LT and ST tenderness discrimination

Previous studies have proved that LT and ST muscles exhibit different metabolic and contractile properties. ST muscle has a lower oxidative and a higher glycolytic activities than LT muscle (Jurie, et al., 2007; Jurie, et al., 1995). LT muscle is has a higher amount of slow-twitch myosins and fibre, and ST has a higher amount of fast-twitch myosins and fibre (Guillemin, et al., 2011; Schreurs, et al., 2008).

In our study, the tenderest LT muscles were characterized by lower carcass adiposity and intramuscular fat, less soluble collagen content and higher mean cross-section areas of fibers. These relationships between these characteristics and tenderness are coherent with the numerous data in the literature concerning this muscle (Crouse, Koohmaraie, & Seideman, 1991; Jurie, et al., 2007; Picard, et al., 2007; Renand, et al., 2001). However for ST muscle the contrary was observed, except for collagen content, because the least tender ST was characterized by higher intramuscular fat. Picard et al (2007) also found a positive correlation between lipid content and measurement of shear-force in ST muscle from young bulls in coherence with our results. In accordance with this, in our study the tenderest ST had lower carcass adiposity and intramuscular fat, while on the contrary they had higher muscle mass. This relationship between muscle mass and tenderness is in coherence with the data obtained on double-muscled cattle (Allais et al., 2010). They have higher muscle mass and higher meat tenderness, particularly for ST muscle which is hypertrophied in double-muscled cattle (Bouley et al., 2005). The contradiction between ST and LT muscles has been already observed. For example, Picard et al (2007) demonstrated on young bulls and cows muscles that in the LT, tenderness was correlated positively with the slow oxidative state while for the ST, it was correlated positively with the glycolytic properties. Inverse relationships between protein markers and tenderness have been revealed also by proteomic analysis of these two muscles. For example, in Blonde d'Aquitaine young bulls, Hsp27 and α B-crystallin were less abundant in the most tender LT muscles, while on the contrary, in the most tender ST from the same animal, these 2 proteins were less abundant (for review Picard et al, 2010).

It is interesting to observe that carcass adiposity and intramuscular lipid content explained tenderness in the same way. In LT, both were less abundant in the least tender meat. On the contrary, in ST, they were both in lower abundance in the most tender meat. They could constitute good tenderness predictors but in an opposite way for the two muscles.

Only the data concerning collagen solubility discriminated for tenderness in the same way for both ST and LT muscles. The insoluble collagen concentration was a toughness marker, whereas solubility was a tenderness marker. So, the less abundant the collagen and the more soluble it is, more tender is the meat. This type of result is in accordance with the numerous works on collagen and tenderness (McCormick, 1999; Renand, et al., 2001).

It confirms that collagen defines a basal level of tenderness, with the same effect on tenderness in both ST and LT muscles.

In conclusion, this study clearly demonstrates that tenderness is not discriminated by the same parameters in LT and ST muscles. The proteins considered as phenotypic markers of tenderness according to previous genomic functional programs allowed a good discrimination of tenderness in ST but not in LT muscle. So genomics studies can improve and promote tenderness of poor quality meat. Only collagen properties discriminate the tenderness classes in the same way for both muscles.

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Table 1. Suppliers and conditions for each primary antibody used in this study

Target protein	Antibody references	Dilution
Heat Shock Proteins		
α B-crystallin	Monoclonal anti-bovine Assay Designs SPA-222	1/500
Hsp20	Monoclonal anti-human Santa Cruz HSP20-11:sc51955	1/200
Hsp27	Monoclonal anti-human Santa Cruz HSP27 (F-4):sc13132	1/3,000
Hsp40	Monoclonal anti-human Santa Cruz HSP40-4 (SPM251):sc-56400	1/250
Hsp70-1A	Monoclonal anti-human Abnova HSPA1B (M02), clone 3B7	1/2,000
Hsp70-8	Monoclonal anti-bovine Santa Cruz HSC70 (BRM22):sc-59572	1/250
Hsp70/GRP75	Monoclonal anti-human RD Systems Clone 419612	1/250
Metabolism		
Eno1	Polyclonal anti-human Acris BP087	1/2,000
Eno3	Monoclonal anti-human Abnova Eno3 (M01), clone 5D1	1/45,000
LDHB	Monoclonal anti-human Novus LDHB NB110-57160	1/50,000
MDH1	Monoclonal anti-pig Rockland 100-601-145	1/1,000
PGM1	Monoclonal anti-human Abnova PGM1 (M01), clone 3B8-H4	1/8,000
Structure		
CapZ- β	Monoclonal anti-human Abnova CAPZB (M03), clone 4H8	1/250
Desmin	Monoclonal anti-human DAKO clone D33, M0760	1/250
MLC-1F	Polyclonal anti-human Abnova MYL1 (A01)	1/1,000
MyBP-H	Monoclonal anti-human Abnova MYBPH (M01), clone 1F11	1/4,000
MyHC-I	Monoclonal anti-human Biocytex 5B9	1/2,000
MyHC-II	Monoclonal anti-human Biocytex 15F4	1/4,000
MyHC-IIx	Monoclonal anti-human Biocytex 8F4	1/500
Oxidative resistance		
DJ-1	Polyclonal anti-human Santa Cruz DJ-1 (FL-189):sc-32874	1/250
PRDX6	Monoclonal anti-human Abnova PRDX6 (M01), clone 3A10-2A11	1/500
SOD1	Polyclonal anti-rat Acris SOD1 APO3021PU-N	1/1,000
Proteolysis		
M-calpain	Monoclonal anti-bovine ABR M-calpain MA3-942	1/1,000
μ -calpain	Monoclonal anti-bovine Alexis μ -calpain 9A4H8D3	1/1,000

Hsp: Heat Shock Protein. Eno: Enolase. LDHB: Lactate DeHydrogenase-B. MDH1: Malate DeHydrogenase-1. PGM: PhosphoGlucoMutase-1. MLC: Myosin Light Chain. MyHC: Myosin Heavy Chain. PRDX: Peroxiredoxin. SOD: Super Oxide Dismutase.

Table 2. Warner-Bratzler (WB) mean values among the three tenderness classes for each of the two muscles: *Longissimus Thoracis* (LT) and *Semitendinosus* (ST)

LT						ST					
Inf (N=13)		Med (N=64)		Sup (N=34)		Inf (N=21)		Med (N=36)		Sup (N=54)	
Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
58.2	2.5	40.8	0.5	30.0	0.6	82.6	2.0	63.0	0.7	46.6	0.8

Table 3. Protein means (arbitrary units) in the three tenderness classes for ST muscle

	Inf-class		Med-class		Sup-class		P-values
	Mean	SE	Mean	SE	Mean	SE	
Heat Shock Proteins							
α B-crystallin	8.0b	0.6	10.4ab	0.7	11.4a	0.8	0.027
Hsp20	10.5b	0.4	10.8b	0.4	12.1a	0.3	0.01
Hsp27	9.6b	0.6	13.0a	0.9	14.4a	0.7	0.002
Hsp40	12.9	0.6	12.9	0.5	13.4	0.4	0.69
Hsp70-1B	10.7	0.5	11.1	0.5	10.7	0.4	0.84
Hsp70-8	15.4a	0.5	14.0b	0.4	13.9b	0.3	0.037
Hsp70-GRP75	15.8a	0.4	14.7ab	0.4	13.8b	0.4	0.006
Metabolism							
Enolase 1	16.4	0.7	14.8	0.4	14.9	0.4	0.121
Enolase 3	15.5a	0.6	14.4a	0.4	13.4b	0.3	0.003
LDHB	15.1	0.7	13.6	0.7	15.2	0.5	0.12
MDH1	16.5	0.6	15.6	0.4	15.6	0.3	0.32
PGM	13.9	0.4	12.8	0.4	13.1	0.4	0.256
Structure							
CapZ- β	16.2a	0.5	14.8b	0.4	15.2ab	0.3	0.079
Desmin	14.3	0.6	14.0	0.4	14.5	0.3	0.597
MLC-1F	16.1a	0.7	14.0b	0.5	13.8b	0.3	0.006
MyBP-H	12.6	1.1	13.5	0.7	14.1	0.7	0.453
MyHC-I	14.3	0.6	14.7	0.5	14.1	0.5	0.695
MyHC-II	16.1	0.4	15.3	0.4	16.0	0.3	0.261
MyHC-IIx	26.0a	2.5	19.4b	1.2	21.4b	1.2	0.028
Oxidative resistance							
DJ-1	15.6	0.4	15.0	0.4	15.0	0.3	0.533
PRDX6	18.3a	0.7	16.4b	0.5	15.6b	0.4	0.003
SOD1	15.5	0.5	14.6	0.3	15.1	1.1	0.844
Proteolysis							
M-calpain	15.4a	0.6	13.9b	0.4	13.5b	0.4	0.015
μ -calpain	16.0a	0.5	15.1ab	0.4	14.4b	0.4	0.049

Hsp: Heat Shock Protein. LDHB: Lactate DeHydrogenase-B. MDH1: Malate DeHydrogenase-1. PGM: PhosphoGlucoMutase-1. MLC: Myosin Light Chain. MyHC: Myosin Heavy Chain. PRDX: Peroxiredoxin. SOD: Super Oxide Dismutase

Table 4. Protein means (arbitrary units) in the three tenderness classes for LT muscle

	Inf-class		Med-class		Sup-class		<i>P</i> -values
	Mean	SE	Mean	SE	Mean	SE	
Heat Shock Proteins							
α B-crystallin	18.2	1.5	21.4	1.0	23.3	1.3	0.396
Hsp20	20.2	1.4	18.4	0.6	17.4	0.8	0.206
Hsp27	13.8	1.2	13.8	0.5	13.3	0.6	0.82
Hsp40	15.5	0.7	15.8	0.3	15.9	0.4	0.831
Hsp70-1B	16.7	1.0	15.8	0.5	15.8	0.7	0.767
Hsp70-8	15.3	0.7	15.9	0.3	15.9	0.4	0.718
Hsp70-GRP75	13.7	0.6	15.2	0.3	15.5	0.4	0.193
Metabolism							
Enolase 1	12.5	0.4	13.5	0.3	14.1	0.5	0.152
Enolase 3	13.8	0.5	15.0	0.3	14.5	0.3	0.191
LDHB	15.1	1.2	14.8	0.5	15.8	0.6	0.505
MDH1	15.1	0.7	15.6	0.3	15.7	0.4	0.699
PGM	14.5	0.7	14.5	0.3	14.8	0.4	0.806
Structure							
CapZ- β	14.4	0.5	15.0	0.3	15.7	0.4	0.128
Desmin	13.3	0.6	15.0	0.4	14.1	0.5	0.119
MLC-1F	13.3	0.8	14.3	0.4	14.1	0.3	0.503
MyBP-H	18.8	2.1	16.0	0.5	16.7	0.9	0.191
MyHC-I	16.0	0.5	16.3	0.3	16.3	0.4	0.906
MyHC-II	14.1	0.3	14.4	0.2	14.8	0.3	0.33
MyHC-IIx	7.8	0.7	9.6	0.7	10.6	1.2	0.359
Oxidative resistance							
DJ-1	14.8	0.4	15.5	0.3	15.7	0.3	0.355
PRDX6	15.3	0.6	16.3	0.3	15.1	0.4	0.079
SOD1	14.6	1.0	15.5	0.5	15.2	0.8	0.808
Proteolysis							
M-calpain	12.8b	0.7	14.6a	0.3	14.1ab	0.3	0.042
μ -calpain	14.5	0.6	15.6	0.3	14.9	0.3	0.086

Hsp: Heat Shock Protein. LDHB: Lactate DeHydrogenase-B. MDH1: Malate DeHydrogenase-1. PGM: PhosphoGlucoMutase-1. MLC: Myosin Light Chain. MyHC: Myosin Heavy Chain. PRDX: Peroxiredoxin. SOD: Super Oxide Dismutase.

Table 5. Phenotypic measurement means in the three tenderness classes for ST muscle

	Inf-class		Med-class		Sup-class		<i>P</i> -values
	Mean	SE	Mean	SE	Mean	SE	
COX ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	12.5	1.2	11.4	0.6	10.7	0.6	0.264
CS ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	5.9	0.4	5.6	0.3	5.3	0.2	0.362
CFW (kg)	66.6a	3.5	64.3a	2.1	58.8b	1.5	0.029
ICDH ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	1.4	0.1	1.5	0.1	1.3	0.1	0.771
Inscol (μg of OH-proline. mg^{-1})	4.1	0.1	4.0	0.1	3.7	0.1	0.051
LDH ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	917.2	24.2	868.9	17.2	893.7	14.4	0.243
Lipids ($\text{mg}\cdot\text{g}^{-1}$)	22.6a	2.4	16.9b	1.0	15.6b	0.8	0.001
Mean area fiber (μm^2)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PMC (%)	69.4b	0.7	69.9b	0.4	71.2a	0.4	0.014
Solubility (%)	20.1b	2.5	21.5b	2.0	27.9a	2.1	0.031
Totcol (μg of OH-proline. mg^{-1})	5.2	0.2	5.2	0.1	5.2	0.1	0.962
Triglycerides ($\text{mg}\cdot\text{g}^{-1}$)	10.7a	1.8	8.0ab	0.9	6.6b	0.7	0.022

n.a: non available data

COX: Cytochrome Oxidase. CS: Citrate Synthase. CFW: Carcasse Fat Weight. ICDH : Isocitrate Dehydrogenase. Inscol: Insoluble Collagen. LDH: Lactate Dehydrogenase. PMC: Percentage of muscle in the carcass. Totcol: Total collagen content.

Table 6. Phenotypic measurement means in the three tenderness classes for LT muscle

	Inf-class		Med-class		Sup-class		<i>P</i> -values
	Mean	SE	Mean	SE	Mean	SE	
COX ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	12.0	1.0	12.5	0.5	12.6	0.7	0.902
CS ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	4.1	0.3	4.5	0.1	4.7	0.2	0.282
CFW (kg)	53.4b	3.7	63.6a	1.4	62.5a	2.5	0.031
ICDH ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	1.3	0.1	1.3	0.0	1.3	0.1	0.777
Inscol (μg of OH-proline. mg^{-1})	2.9a	0.1	2.7a	0.1	2.5b	0.1	0.018
LDH ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	991.0	48.3	982.8	14.7	969.2	19.4	0.822
Lipids ($\text{mg}\cdot\text{g}^{-1}$)	18.3b	1.3	27.7a	1.4	26.7a	1.8	0.016
Mean area fiber (μm^2)	3350.9a	152.7	2925.2b	63.6	2916.6b	82.6	0.019
PMC (%)	72.0	0.8	70.2	0.3	70.2	0.5	0.075
Solubility (%)	14.5b	1.8	24.3a	1.8	27.0a	2.4	0.023
Totcol (μg of OH-proline. mg^{-1})	3.4	0.2	3.7	0.1	3.5	0.1	0.284
Triglycerides ($\text{mg}\cdot\text{g}^{-1}$)	9.4b	1.4	18.9a	1.5	18.1a	1.9	0.018

COX: Cytochrome Oxidase. CS: Citrate Synthase. CFW: Carcasse Fat Weight. ICDH : Isocitrate Dehydrogenase. Inscol: Insoluble Collagen. LDH: Lactate Dehydrogenase. PMC: Percentage of muscle in the carcass. Totcol: Total collagen content.