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Reverse phase protein arrays for the identification/validation of biomarkers of beef texture and their use for early classification of carcasses

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Running title: Protein biomarkers as discriminators of beef texture

Abstract

The validation of biomarkers and tools for the prediction of beef texture remains a challenging task. In this study, reverse phase protein arrays (RPPA) quantified 29 protein biomarkers in the m. *Longissimus thoracis* of Charolais cattle sampled early *post-mortem.* Myosin heavy chain 1 (MHC1, slow-oxidative fibers) and Retinal dehydrogenase 1 (ALDH1A1, oxidative enzyme) discriminated between tender and juicy *vs.* tough meat with residues classes and are validated as prime biomarkers of beef texture. Several proteins belonging to energy metabolism, heat shock and oxidative stress, cytoskeletal, cell signaling and apoptosis were related with tenderness. Among the unusual proteins, four and a half LIM domains 1 (FHL1) and Tripartite motif protein 72 (TRIM72) correlated respectively negatively and positively with beef tenderness. Principal component regression was used for the first time to explain beef texture traits using biomarkers. The results are very promising as they revealed sophisticated mechanisms behind the tenderizing process.

Keywords: Biomarkers; RPPA; Meat texture; young bulls; Relationships; Clustering

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1. Introduction

The meat industry has been increasingly requested by consumers to guarantee both high-quality products as well as stable sensory quality. The factors that determine the quality of cooked muscle foods such as beef are the intersection of tenderness and juiciness, thereby of texture desirability, and also flavor and color attributes (Grunert *et al.* 2004). To obtain high-quality products, it is necessary to understand the mechanisms, which underlie the conversion of muscle into meat, and hence those of beef texture determinism (Ouali *et al.* 2013; Gagaoua *et al.* 2015a). Over the last 15 years, there has been a growing interest in the relationship between proteins and related genes, and meat quality traits (Gagaoua *et al.* 2015b; Picard *et al.* 2015). Functional proteomics aim to elucidate the biological function of proteins by combining two-dimensional gel electrophoresis (2DE) with mass spectrometry (MS). Thus, protein biomarkers were identified (Picard *et al.* 2017b) and used for tenderness prediction (Zapata *et al.* 2009; Picard *et al.* 2014; Picard & Gagaoua 2017) as this later is the primary sensory quality trait that consumers consider at re-purchase of meat (Grunert *et al.* 2004).

The identified beef tenderness biomarkers belong to myriad biological pathways grouping proteins implicated in apoptosis, oxidative stress and autophagy (Picard & Gagaoua 2017). Biomarkers were developed since the earlier methods of tenderness evaluation namely sensory panels as well as shear force methods are destructive, time consuming and ill-suited in routine as they require removing a piece of steak from the carcass to perform the measurement, hence leading to carcass depreciation. The first publication using proteomics to study *post-mortem* changes in meat was published by (Lametsch & Bendixen 2001). However, during the past fifteen years, the number of studies and reports has increased significantly (Picard *et al.* 2017b). This was accompanied by great improvements in methods and instrumentation. In our laboratory, new molecular tools were developed for the simultaneous analysis of these biomarkers on a large number of meat samples. For example, a DNA chip with specific genes involved in muscle biology or beef quality was developed (Hocquette *et al.* 2012). At the protein level, a Dot-Blot tool was implemented to measure the relative abundance of proteins and linked them with tenderness (Guillemin *et al.* 2009) and other technological beef traits (Gagaoua *et al.* 2015c; Gagaoua *et al.* 2017a). The Dot-Blot tool allows simultaneous measurement of the relative abundance of only one protein for a maximum of 21 muscle samples. In this study, we report for the first time the use of reverse phase protein arrays (RPPA), a promising quantitative microformat Dot-Blot approach, for the quantification of the proposed biomarkers in hundred samples. Therefore, this makes RPPA

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very attractive for the analysis of meat and biomarker discovery/validation. Despite the recent studies, the knowledge of large-scale proteomics analysis for understanding meat texture is scarce and techniques such as RPPA are widely welcomed for better management of carcass and meat quality potential. This study aims also to help produce a list of biomarkers and validate them on beef texture traits. The validation of the biomarkers would be used to explain and then predict meat texture evaluated by both sensory and instrumental protocols.

2. Materials and methods

2.1. Animals, handling and slaughtering

A total of 43 young Charolais bulls of an average of 530 days at slaughter were used. These animals were a dataset from the study of (Mialon *et al.* 2015) conducted at the INRA research center (Theix, France). The animals were all from a group without any stressful conditions and chosen accordingly to avoid stress effects and use an homogenate group. These animals were fattened for a minimum of 228 days with a high-concentrate diet composed of a concentrate mixture and barley straw fed *ad libitum*. During the experiment, bulls were housed in pens (7m^2/bull) bedded with barley straw. Before slaughter, all animals were food deprived for 24 h to limit the risk of carcass contamination by microbes in the digestive tract during evisceration, but had free access to water. At a live weight around 732±65 kg, the animals were all slaughtered in the same condition at the experimental slaughterhouse of INRA research center, stunned using captive-bolt pistol prior to exsanguination and dressed according to standard commercial practice. Slaughtering was performed in compliance with French welfare regulations and respecting EU regulations (Council Regulation (EC) No. 1099/2009).

2.2. Muscle sampling

Carcasses were not electrically stimulated. Immediately after slaughter (at ~45 min *post-mortem*), muscle samples from *Longissimus thoracis* (LT, mixed fast oxido-glycolytic muscle) were excised from the right side of the carcass of each animal and were subsequently frozen in liquid nitrogen and kept at –80°C until analyzed for protein extraction and RPPA analysis. For meat texture assessment (sensory and instrumental), the muscle was excised from the 7th rib of the same side of each carcass 24 h after slaughter. The samples were cut into steaks (5 cm thick) and placed in 80-micron sealed plastic bags (40 nylon/60 polyethylene with permeability specifications of 50 cm^3 O_2/m^2/d, 10 cm^3 N_2/m^2/d, 150 cm^3 CO_2/m^2/d and 2.4 g H_2O/d at 23 °C and 75% RH (Terinex, Bedford, England)) in a Multivac

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A300/42 vacuum packager (Multivac UK, Swindon, UK) to – 980 mbar and kept between 2 and 4 °C for 14 days for ageing. Each loin sample was then frozen and stored at – 20 °C until sensory and instrumental assessment.

2.3. Meat texture assessment

2.3.1. Sensory traits evaluation

For sensory beef assessment the protocol recently described by (Gagaoua *et al.* 2016a) was used. Briefly, steaks were thawed, without stacking or overlapping, at 5°C in vacuum packs for 48 h before cooking and sensory assessment at 55 °C. One hour before sensory assessment, the meat samples were cut into approximately 1.50 cm thick steaks and grilled on a double grooved plate griddle (SOFRACA, Morangis, France) heated to 300°C for 30 min before cooking. Steaks were heated for 2 min until the end-points temperature of 55°C in the geometric center of the steak was reached (measured using a temperature probe (Type K, HANNA HI 98704, Newark, USA)). After grilling, each steak was cut into 20 mm cubes that were immediately served to 12 panelists chosen according to the criteria described by (Gagaoua *et al.* 2016a). The panelists rated the steaks on a 10-cm unstructured line scale (from 0 to 10) measured in mm for the following texture attributes:

- ***Global tenderness*** defined as the ease of chewing the sample between teeth: from extremely tough (0) to extremely tender (10).

- ***Juiciness*** defined as the amount of moisture released in the mouth: from extremely dry (0) to extremely juicy (10).

- ***Perceptible residues*** defined as the amount of connective tissue remaining after most of the sample has been masticated: from none (0) to abundant (10).

The sessions were carried out in a sensory analysis room equipped with individual booths under artificial red light to reduce the influence of the appearance of the samples. At each session, a monadic presentation of 6 samples was done, each sample being selected in random order. Each tasting booth was equipped with computer terminals linked to a fileserver running a sensory software program (Fizz v 2.20 h, Biosystemes, Couternon, France) that facilitated the direct entry of assessor ratings, which were later formatted in Excel.

2.3.2. Warner-Bratzler shear force measurement

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For objective tenderness, the Warner-Bratzler shear force (WBSF) was evaluated according to (Lepetit & Culioli 1994). Briefly, from the sample cuts cooked as previously described, two to five 1 x 1 x 4 cm cores per steak sample were removed parallel to the longitudinal orientation of the muscle fiber of LT muscle. WBSF was assessed using an Instron Universal Testing Machine (Model 5944, Instron Corp., USA) equipped with a Warner–Bratzler shearing device. The measurement was done two or three times per core in order to obtain around 10 repetitions per sample. The load capacity was 10 kN with cross-head speed 200 mm min– 1. Force at rupture during shear compression testing was expressed in N/cm^2.

2.4. Protein extraction and quantification

Proteins were extracted from frozen muscle samples by homogenizing the samples in the "Precellys 24" tissue homogenizer (Bertin technologies, Saint Quentin-en-Yvelines, France). Briefly, an approximate of 80 mg of frozen muscle for each animal sample was mixed in a buffer containing 50 mM Tris (pH 6.8), 2% SDS, 5% glycerol, 2 mM DTT, 2.5 mM EDTA, 2.5 mM EGTA, 1x HALT Phosphatase inhibitor (Perbio 78420), Protease inhibitor cocktail complete MINI EDTA-free (Roche 1836170, 1 tablet/10 mL), 2 mM Na_3VO4 and 10 mM NaF. The extracts were then boiled for 10 min at 100°C, sonicated to reduce viscosity and centrifuged 10 min at 15000 rpm. The supernatants were collected and stored at –80°C until further use. Protein concentrations were determined with a commercial protein assay (Pierce BCA reducing agent compatible kit, ref 23252) with BSA as standard.

2.5. RPPA quantification of the biomarkers

2.5.1. Antibodies validation

The relative abundances of the 29 protein biomarkers of tenderness and/or muscle adiposity chosen and validation according to earlier investigations by our group were determined using specific antibodies. These proteins corresponded to seven biological functions (**Table 1**): ***energy metabolism***: Malate dehydrogenase, α-enolase 1, β-enolase 3, Retinal dehydrogenase 1, Triosephosphate isomerase, cGMP-dependent protein kinase 1, Fructose-bisphosphate aldolase and Glycogen phosphorylase; ***heat shock proteins***: αB-crystallin, Hsp20, Hsp27, Hsp40, Hsp70-1A, and Hsp70-8); ***oxidative resistance***: DJ-1, Prdx6 and SOD1; ***muscle fibre structure***: α-actin, α-actinin 2, α-actinin 3, MLC-1F, Myosin heavy chain-I, Myosin heavy chain-IIx, Troponin T, Titin and α-Tubulin; ***Cell death, protein***

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binding and proteolysis: Tripartite motif protein 72, Four and a half LIM domains 1 and μ-calpain.

An antibody was considered specific against the studied protein when only one band at the expected molecular weight was detected by western blot (Gagaoua *et al.* 2015b). Optimal dilution ratios for each of the 29 antibodies were determined at the same time, using routine procedures of validation following the conditions indicated by the supplier of the reactant and adapted to bovine muscle samples. Details concerning the proteins and the antibodies used are summarized in **Table 1**.

2.5.2. Reverse Phase Protein Arrays (RPPA) procedure

After antibodies validation, the RPPA technique was adapted for animal muscle samples for the quantification of the biomarkers from the protocol described by (Akbani *et al.* 2014). RPPA technology constitutes a miniaturized immunoassay that uses a sandwich system allowing multiplexed protein analysis (Akbani *et al.* 2014). The extracted muscle proteins are immobilized on a solid phase with high protein binding capacity per unit area, and are revealed with specific antibody. Hundreds of samples can be measured at the same time with very high sensitivity and precision from a starting sample volume of only 20 µl. Briefly, the meat extract samples of all the animals were deposited onto nitrocellulose covered slides (Supernova, Grace Biolabs) using a dedicated arrayer (Aushon Biosystems 2470). Four serial dilutions, ranging from 2000 to 250 µg/ml, and two technical replicates per dilution were printed for each sample. Arrays were labeled with 29 specific antibodies (see **Table 1** for a complete list of antibodies references and their Uniprot IDs) or without primary antibody (negative control), using an Autostainer Plus (Dako). The slides were incubated with avidin, biotin and peroxydase blocking reagents (Dako) before saturation with TBS containing 0.1% Tween-20 and 5% BSA (TBST-BSA). Slides were then probed overnight at 4°C with primary antibodies diluted in TBST-BSA. After washes with TBST, arrays were probed with horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch Laboratories, Newmarket, UK) diluted in TBST-BSA for 1 h at room temperature. To amplify the signal, slides were incubated with Bio-Rad Amplification Reagent for 15 min at room temperature. The arrays were washed with TBST, probed with Alexa647-Streptavidin (Molecular Probes) diluted in TBST-BSA for 1 h and washed again in TBST. For staining of total protein, arrays were incubated 15 min in 7% acetic acid and 10% methanol, rinsed twice in water, incubated 10 min in Sypro Ruby (Invitrogen) and rinsed again. The processed slides were dried by centrifugation and scanned using a GenePix 4000B microarray scanner

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(Molecular Devices). Spot intensity was determined with MicroVigene software (VigeneTechInc).

2.5.3. Protein intensities calculation and normalization

The relative abundances of proteins were determined according to the following procedure. First, raw data were normalized using NormaCurve (Troncale *et al.* 2012), a SuperCurve-based method that simultaneously quantifies and normalizes Reverse Phase Protein Array Data for fluorescent background per spot, a total protein stain and potential spatial bias on the slide. Next, each RPPA slide was median centered and scaled (divided by median absolute deviation). We then corrected for remaining sample loadings effects individually for each array by correcting the dependency of the data for individual arrays on the median value of each sample over all the arrays using a linear regression.

2.6. Statistical analyses

A preliminary principal component analysis (PCA) was performed using all the variables (meat texture traits and biomarkers) with XLSTAT 2017.19.3 (AddinSoft, Paris, France). After that, a PCA combined with a *k*-means cluster analysis ($k = 2$) was undertaken to create two meat texture classes using the sensory and instrumental meat quality traits [tenderness, juiciness, residues and WBSF], that were named respectively "tender and juicy class" *vs.* "tough with residues class". The categorization of beef cuts was performed as recently described (Gagaoua *et al.* 2017a; Gagaoua *et al.* 2017b). After that, the classes were compared for both texture traits and protein biomarkers relative abundances, using the PROC GLM procedure of SAS (SAS 9.4, SAS Institute INC, Cary, NC, USA).

Pearson correlation coefficients were evaluated using the PROC CORR procedure of SAS to determine the relationships among meat texture traits and protein biomarkers.

Further PCAs were carried out using **i**) the dependent variables and the differential proteins to obtain a more complete picture of the differences according to the barycenter's of the classes; **ii**) the correlated biomarkers with each meat texture trait by the projection of the animals belonging to each texture class as supplementary variables. They aimed to visually illustrate the related biomarkers with each texture trait according to the classes barycenter's.

Principal component regression (PCR) analysis on standardized data was conducted per texture trait with the 29 biomarkers to generate predictive models using the optimal number of components in each case. This is an appropriate tool to manage the multicollinearity observed

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between in this study between the biomarkers. The basic idea behind PCR is to calculate the principal components and then use some of these components as predictors in a linear regression model fitted using the typical least squares procedure (Rougoor *et al.* 2000).

3. Results and discussion

3.1. Beef muscle cuts clustering and use of biomarkers as discriminators of texture

The projection of the texture traits (q = 4) and protein biomarkers (q = 29) using PCA allowed us to visualize the links that may occur (**Fig. 1A**). After that and thanks to the innovative "*k*-means–PCA" statistical-based approach (Gagaoua *et al.* 2017b), the ribeye steaks of the Charolais animals were clustered into tender and juicy group *vs.* tough with residues group, which are shown on the same bi-plot (**Fig. 1A**). The PCA characterized namely by the first two axes with eigenvalues of respectively 4.82 and 3.58, explained 45.5% of the variability. Multivariate statistical analyses are broadly used for meat quality clustering, using namely muscle proteome variables (Jia *et al.* 2006; Kwasiborski *et al.* 2009; Gagaoua *et al.* 2017a; Gagaoua *et al.* 2017c). WBSF and residues traits, *i.e,* tough with residues group, were loaded on the positive side of the first axis together with Hsp70-1A, MLC1F, α-actinin 3, MDH1, DJ1 and TRIM72 that had eigenvectors > 0.5. On the negative side of the first axis, tenderness and juiciness, *i.e,* tender and juicy group, are projected together with ALDH1A1, PRDX6, α-actinin 2, TTN, MHC1 and PYGB. The first axis highlights that tenderness and juiciness are exclusively loaded within proteins reflecting oxidative properties by proteins of oxidative stress, oxidative metabolism and slow oxidative fibers. These agree with previous studies suggesting positive link between oxidative properties of muscle and tender beef (Chriki *et al.* 2012; Chaze *et al.* 2013; Picard *et al.* 2014). For example, an earlier proteomic study on the same muscle in Blond d'Aquitaine bulls, (Morzel *et al.* 2008) found succinate dehydrogenase, an oxidative enzyme, to be a good marker of tenderness. Moreover, (Jia *et al.* 2009) reported PRDX6, a bifunctional protein with both glutathione peroxidase and phospholipase A2 activities, as a potential biomarker for beef tenderness in LT muscle. PRDX6 is expressed in nearly all tissues and protects cells against oxidative stress by playing great role in tenderness determinism (Ouali *et al.* 2013; Gagaoua *et al.* 2015b).

Variance analyses applied between the two texture classes revealed significant differences (P < 0.01) between the 4 texture traits (**Table 2**). Tenderness and juiciness scores were the highest in tender and juicy group (P < 0.001) and had low WBSF values and perceptible residue scores. Two proteins were significantly different (P < 0.05) between classes,

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ALDH1A1 and MHC1, which were the highest in tender and juicy group (**Table 2**). Both proteins discriminated further the two texture classes and loaded within tenderness and juiciness of the second PCA (**Fig. 1B**). In addition to their energy metabolism role, mitochondrial Aldehyde dehydrogenase are proposed to protect *post-mortem* cells from oxidative stress through the oxidation of cytotoxic aldehyde derivate (Ouali *et al.* 2013). Overall, the proteins involved in oxidative stress were already identified by proteomics investigations of meat tenderness (Picard & Gagaoua 2017). So, our results agree with the statements discussed above and with the earlier proteomic studies. Among recent studies, (Grabez *et al.* 2015) reported that Aldehyde dehydrogenase and Pyruvate dehydrogenase, two oxidative enzymes, to be negatively liked with WBSF values of beef *Semimembranosus* muscle. This latter muscle was reported by our group to share common properties than LT muscle (Picard *et al.* 2017a). Similarly, (Zapata *et al.* 2009) described positive correlation between tenderness of LT muscle and MHC1. The link with myosin heavy chains may be partly explained by the proteolytic effects known to induce degradation of myofilaments and thereby modify the texture of muscle cuts. For example, the occurrence of MHC degradation in the early *post-mortem* period has been observed in beef (Wu *et al.* 2014), although the underlying mechanisms are not yet fully understood. A meta-analysis approach on individual data from more than 500 animals of 7 cattle breeds, our group found an association between improved beef tenderness, smaller cross-sectional areas of fibers, and an overall fiber type composition that display more oxidative fibers than glycolytic fibers in LT muscle (Chriki *et al.* 2012). Recent comprehensive reviews support the underlying mechanisms described above (Picard & Gagaoua 2017). In conclusion to this part and in agreement to our hypothesis, the findings support the earlier works proposing apoptosis *via* mitochondria (oxidative conditions) and other pathways, likely autophagy, to play pivotal roles during the first hours of texture development (Ouali *et al.* 2013; Picard & Gagaoua 2017).

3.2. Relationships between meat texture traits and protein biomarkers

The correlation analyses between texture traits and biomarkers are summarized in **Table 3**. The tenderizing process finely orchestrated is strongly comforted by the findings of this study using several texture traits. These latter were as expected interrelated between them (**Table 3**). Tenderness was weakly correlated (positively) with juiciness. The weak correlation agree to the findings reported for French panelists (Gagaoua *et al.* 2016a). However, tenderness was correlated negatively with both residues scores and WBSF values, irrespective of texture class (data not shown). These findings are in line with the different studies reported in the large

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literature and cited above (Morzel *et al.* 2008; Zapata *et al.* 2009; Chaze *et al.* 2013; Picard *et al.* 2014; Grabez *et al.* 2015).

Different proteins were correlated with texture traits, mainly tenderness (**Table 3**). However, we assume that not only one protein is involved in the conversion of muscle into meat but several proteins, that belong to different but partly related biological pathways and might act in concert under fine and sophisticated processes (Ouali *et al.* 2013; Picard & Gagaoua 2017). Muscles do not suddenly terminate all their living functions and become meat, but a great number of physical and chemical changes take place over a period of several hours that dictate the final texture. The quantification of dozen proteins from muscle cuts sampled early *post-mortem* by RPPA allowed in this study to validate candidate biomarkers from the list identified in previous works that differ in muscle, animal, breed and rearing factors as well as the omics technique used. In this study, tenderness scores were directly linked with 6 proteins, positively for 5 of them (HSP27, Hsp70-8, MHC1, ALDH1A1 and TPI1) and negatively for one, TRIM72. These 6 proteins were then all projected on a new PCA (**Fig. 1C**). TRIM72 seems to load exclusively alone and in the opposite axis to that of tenderness scores. Tripartite motif-containing 72 (TRIM72), a signaling protein that is expressed in skeletal muscle, acts as a sensor of oxidation on membrane damage (Cai *et al.* 2009). The negative relationship with tenderness would be explained by its implication in the clearance of harmful agents accumulated under the apoptotic process. Thus, one might suppose that a reduced apoptotic phase in tough meat occurred. In line with our findings, this protein was recently reported more abundant in tough beef and to play a great role in the apoptotic pathway (Grabez *et al.* 2015).

The positive link with the 5 remaining proteins have been already reported in LT muscle of beef: Hsp27 by (Morzel *et al.* 2008; Picard *et al.* 2014); Hsp70-8 by (Grabez *et al.* 2015); MHC1 and ALDH1A1 by (Zapata *et al.* 2009; Grabez *et al.* 2015; Picard & Gagaoua 2017) and TPI1 by (Grabez *et al.* 2015). The quantification of these biomarkers by RPPA technique allow their validation as robust biomarkers since they are quantified in a large population of animals compared to the proteomic studies using generally very few samples that come from extreme groups population (tough *vs.* tender) (Picard & Gagaoua 2017). Accordingly, these proteins have several biological pathways, which are further related with other beef quality traits. For example, TPI1 was proposed as a potential biomarker of intramuscular fat (Kim *et al.* 2008) which may explain its link with tender and juicy group as lipids are know to play a great role. On another hand, the relation with Hsp70-8 agrees with the role that HSPs would

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play in meat texture determinism (Picard & Gagaoua 2017). Accordingly, Hsp70 proteins were proposed as master regulators in protein degradation as they have an essential role in substrate degradation through the ubiquitin-proteasome system, as well as through different autophagy pathways (Fernandez-Fernandez *et al.* 2017).

Other proteins were specific with the other texture traits. Among them, FHL1 correlated negatively with WBSF. Four and a half LIM domains 1 (FHL1) regulates gene transcription, cell proliferation, metabolism and apoptosis (Shathasivam *et al.* 2010). This protein is confined to the Z-line of skeletal muscle and its proteolysis is linked to the release of intact α-actinin from bovine myofibrils and contributes to the weakening of the Z-line during meat tenderizing (Morzel *et al.* 2004). FHL1 may further interact with other biological pathways, namely metabolic enzymes in response to both hypoxia and oxidative stress (Shathasivam *et al.* 2010), which may explain its loading within the tender and juicy group characterized by low glycolytic properties and high levels of ALDH1A1, MHC1, HSP27 and HSP70-8 (**Fig. 1C**). In contrast to tenderness scores, HSP27 was the only protein negatively correlated with juiciness (**Table 3**). The few proteins directly linked with juiciness maybe explained by the first validation of the used list of proteins for beef tenderness. However, the result with HSP27 agrees with the findings by (Bernard *et al.* 2007). HSP27 is part of the small HSP chaperone network that plays a canonical role in the cellular response to stress including heat shock, oxidative stress and chemical stress. HSP27 has been reported to be affected early *post-mortem* in beef (Jia *et al.* 2006) and to be involved in the central underlying mechanisms of the tenderizing process (Gagaoua *et al.* 2015b), including an effect on calcium homeostasis that would impact the drip loss of muscle, thereby juiciness appreciation.

3.3. Protein biomarkers for the prediction of beef texture traits using principal component regression (PCR)

The objective of this part is to use the 29 biomarkers in a same predictive model of each texture trait to understand the potential biological pathways that would be involved in beef texture traits. To our knowledge, principal component regression (PCR) has still not been applied to describe the relationship between protein biomarkers and meat qualities. PCR combines linear regression and principal component analysis (Rougoor *et al.* 2000). It establishes a relationship between the dependent variable and the selected principal components of the independent variables, which allows the transformation of a set of correlated x-variables into an equal number of uncorrelated variables. As a result, PCR may help to solve the problem of multicollinearity (Rougoor *et al.* 2000). In this report, PCR

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allowed us to identify the similarities and differences in the biological pathways that would intervene soon after animal bleeding (**Fig. 2A-D**). The prediction models explained between 55 and 60% of the variability, hence validating 17 proteins from the 29 for tenderness scores and WBSF values and 16 proteins for juiciness and residues scores (**Fig. 2A-D**). The best model was found with tenderness, explaining 60% of the variability. The models highlighted that the recorded individual proteins are explicative of texture quality. However, several biomarkers need to be included in the prediction equation to achieve a high accuracy and reflect the entire biological mechanism that may occur in *post-mortem* muscle. From the 29 proteins, TPI1, HSP20, α-actin, α-actinin 2 and TTN entered in the 4 texture traits models but differed in their direction (**Fig. 2E**). The entrance of α-actin, α-actinin 2 and TTN structural proteins in the 4 models of texture traits although the direction differ, agree with the pivotal role of proteolysis and protein degradation during ageing (Ouali *et al.* 2013). TTN is a giant filamentous protein, which connects Z-discs and M-lines in the sarcomeres of striated muscles. This protein was proposed to play a role in the tenderization process of meat (Taylor *et al.* 1995). TTN orchestrates the ordered assembly of hundreds of protein subunits into regular sarcomeres and acts as a receptor and transmitter of mechanical signals to muscle regulatory systems (Gregorio *et al.* 1999). According to these authors, some regions of the TTN molecule interact with thin (actin) filaments in the I-zone of the sarcomere (Gregorio *et al.* 1999). Earlier studies by (Taylor *et al.* 1995), found that TTN underwent partial degradation directly after slaughter and further degradation takes place during the first 24 h *post-mortem*. We think that this degradation would affect the texture and final aspect of meat quality, hence justifying its entrance in the models. On another hand, because TTN is bound to thick filaments in the A-band and to thin filaments in the Z-disk, rotation of thin filaments by the cross-bridges must inevitably lead to winding of TTN upon them, leading to the production of a torque in α-actinin also linked herein with meat texture.

When only the regression models of tenderness scores and WBSF traits are compared, 10 proteins were common but differed in their direction (sign). TPI1, PYGB, HSP70-1A, SOD1, MHC-I, MHC-IIx and FHL1 were positive in tenderness model and Hsp20, α-actinin 2 and TRIM72 were negative (the inverse is verified for WBSF). This study is the first to show in a large dataset the links and biological pathways that may occur during the *post-mortem* period to achieve the final texture of Charolais beef. In addition, the identified biomarkers have the potential to predict accurately and at very early *post-mortem* the tenderness of ribeye steaks. The findings confirm the trait-dependency of the relationships as earlier postulated in several

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studies by our group for muscles, meat quality traits, breeds and rearing practices (Picard *et al.* 2014; Gagaoua *et al.* 2016b; Gagaoua *et al.* 2017c). For example, PGK1, ALDOA and DJ1 were in this study specific for WBSF only. The three proteins are involved in both oxidative stress and energy metabolism of *post-mortem* muscle. However, other proteins failed to enter in none of the models, namely MDH1, HSP40, PRDX6, MLC1F, TNNT1 and α-Tubulin. This highlight that the biological mechanisms, when all proteins are taken together, depend on highly regulated mechanisms remaining unknown.

4. Conclusion

This study is the first to highlight the validation of at least 17 proteins of beef texture from the primary list of 29 proteins. The relationships were texture-trait dependent, thus there is a dependency in validation that may be related to the type of the evaluation protocol of texture traits. Among the validated biomarkers, some of them are very important, likely ALDH1A1 and MHC1 that were able to categorize texture traits into classes. From the biomarkers, TPI1, HSP20, α-actin, α-actinin 2 and TTN were already retained in the 4 texture traits models but in a specific-association manner. In addition, a combination of measurement techniques of beef texture reveal the most relevant relationships and the proteins that would be considered for future validations. The future investigations would take in account those aspects linked with the statistical and tenderness assessment approaches. Overall, the refinement of the PCR models would be implemented in beef industry for tenderness categorization with high accuracy, into tough, intermediate and tender groups.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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Figure captions

Fig. 1. Principal components analysis (PCA) and meat texture classes.

A) Projection of the biomarkers and meat texture traits (both sensory and instrumental) categorized by the iterative procedure *k*-means. The lozenges highlighted the barycenter's of tender and juicy class (green lozenge) and tough with residues class (red lozenge). **B**) PCA obtained using textural traits and significantly different protein biomarkers between classes (tender and juicy class in green *vs.* tough with residues class in red circles). **C**) PCA obtained using textural traits and correlated (Pearson) protein biomarkers. The distribution of the animals according to the two textural classes is shown as in **Fig. 1B**.

Fig. 2. Analysis of principal component regression (PCR) for **A)** tenderness, **B)** juiciness, **C)** WBSF and **D)** residues texture traits of the meat cuts. The regression coefficients for all the proteins are given from the highest and significant to the lowest and non-significant. Thus, green and red colors highlight the positive and negative coefficients in the PC regressions. The non-significant proteins were in blank. **E)** Summary of the retained proteins in the PCR for the four texture traits.

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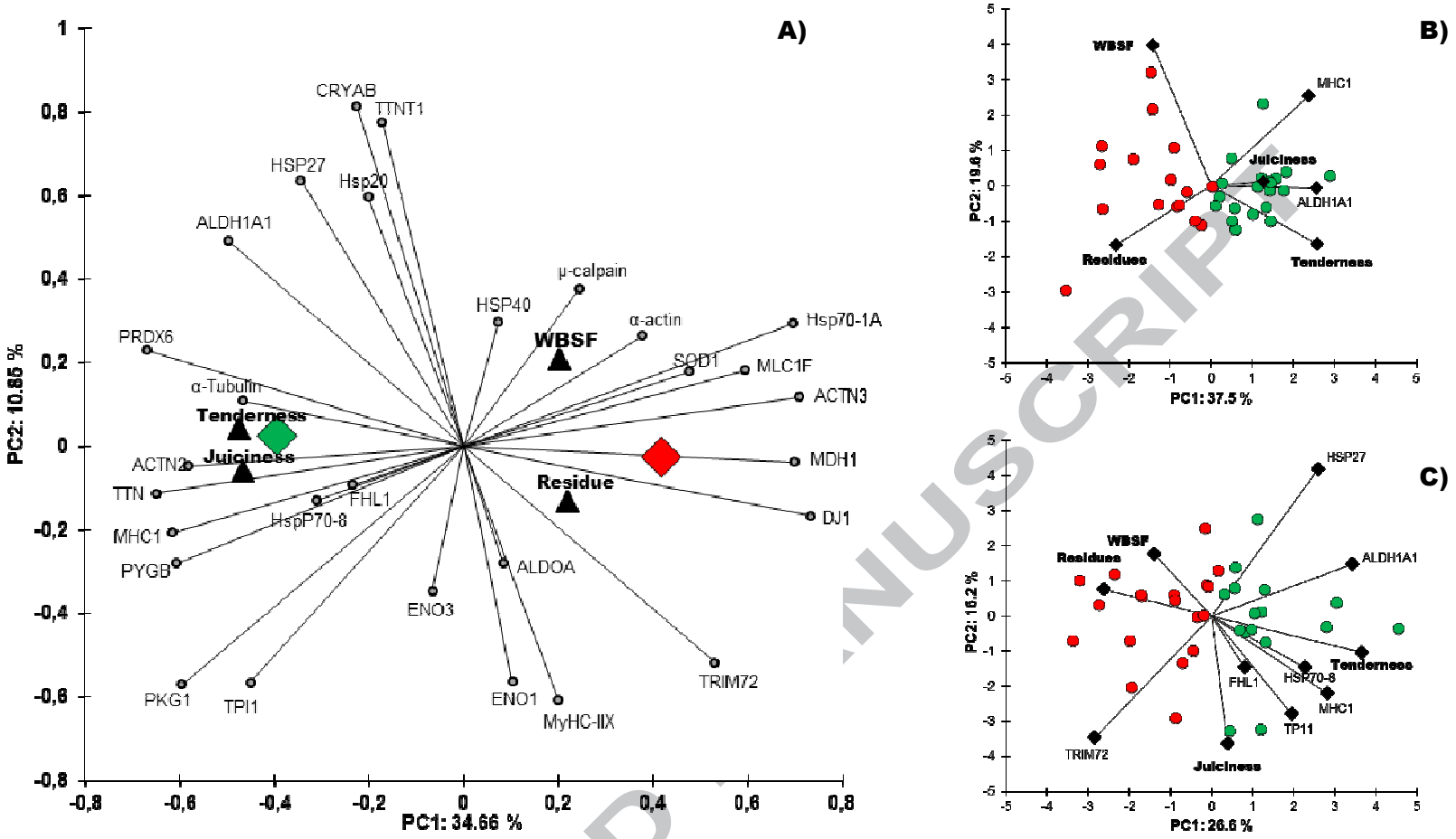

Fig. 1.

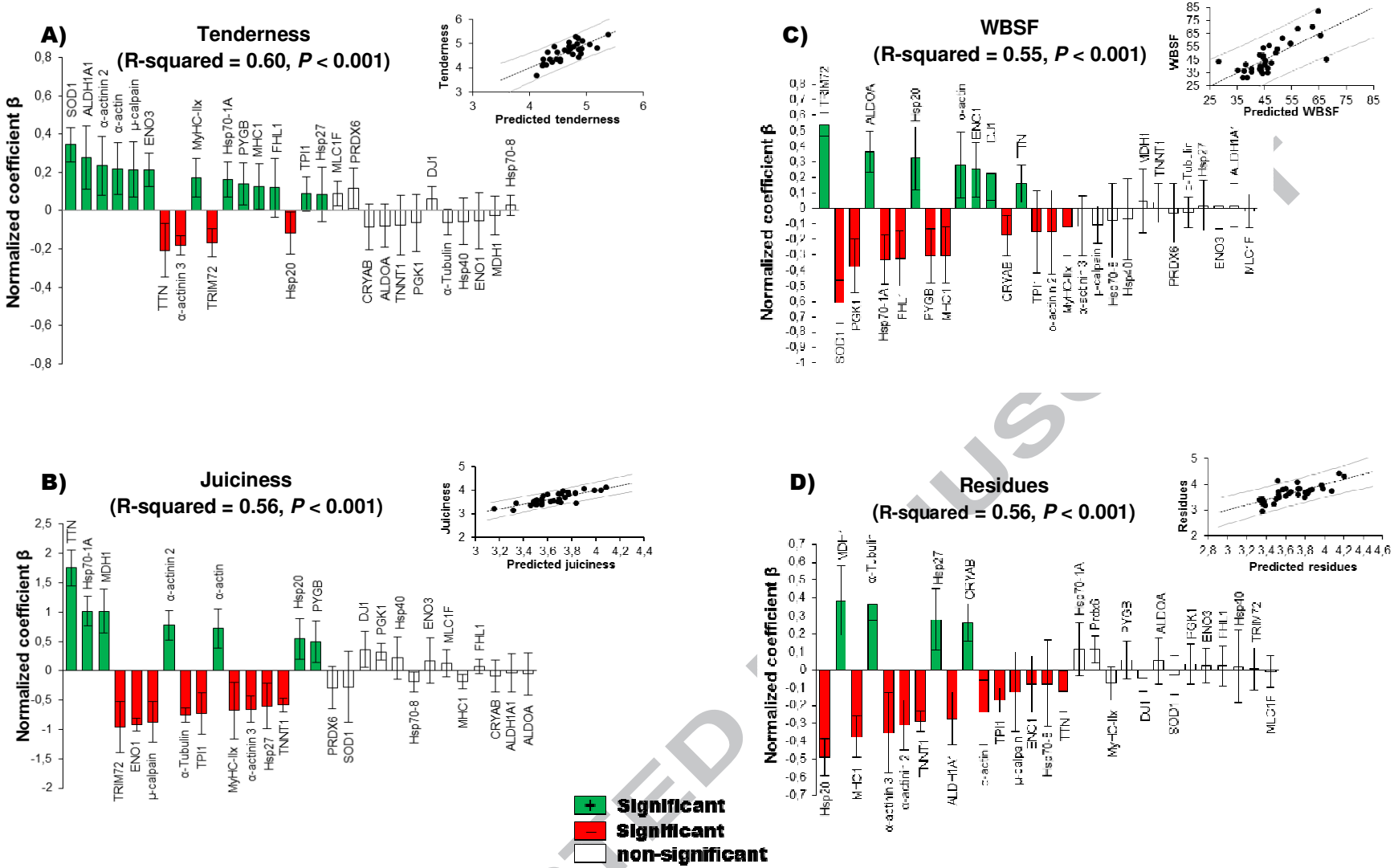
A)
Tenderness
(R-squared = 0.60, P < 0.001)
Normalized coefficient β
Predicted tenderness
B)
Juiciness
(R-squared = 0.56, P < 0.001)
Predicted juiciness
C)
WBSF
(R-squared = 0.55, P < 0.001)
Predicted WBSF
D)
Residues
(R-squared = 0.56, P < 0.001)
Predicted residues
Significant
Significant
non-significant
E)
Protein Biomarkers
Tenderness
Juiciness
Residues
WBSF
MDH1
ENO1
ENO3
ALDH1A1
TPI1
PGK1
ALDOA
PYGB
αB-crystallin
Hsp20
Hsp27
Hsp40
Hsp70-1A
Hsp70-8
PRDX6
DJ1
SOD1
α-actin
α-actinin 2
α-actinin 3
MLC-1F
MHC-I
MHC-IIX
TNNT1
TTN
α-Tubulin
Trim72
FHL1
μ-calpain

Fig. 2.

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Table 1. List of the 29 protein biomarkers quantified and validated using the Reverse Phase Protein Array (RPPA) technique. The suppliers and conditions for each primary antibody used in this study after western blotting validation are given.

Protein biomarkers name (*gene*)	Uniprot ID	Monoclonal (Mo) or Polyclonal (Po) antibodies references	Antibody dilutions
Metabolic enzymes			
Malate dehydrogenase (*MDH1*)	**P40925**	Mo. anti-pig Rockland 100-601-145	1/1000
α-enolase 1 (*ENO1*)	**Q9XSJ4**	Po. anti-humanAcris BP07	1/20 000
β-enolase 3 (*ENO3*)	**P13929**	Mo. anti-human Abnova Eno3 (M01), clone 5D1	1/30 000
Retinal dehydrogenase 1 (*ALDH1A1*)	**P48644**	Po. anti-bovine Abcam ab23375	1/500
Triosephosphate isomerase (*TPI1*)	**Q5E956**	Po. anti-human Novus NBP1-31470	1/50 000
Phosphoglycerate kinase 1 (*PGK1*)	**Q3T0P6**	Po. anti-human Abcam ab90787	1/5000
Fructose-bisphosphate aldolase (*ALDOA*)	**A6QLL8**	Po. anti-human Sigma AV48130	1/4000
Glycogen phosphorylase (*PYGB*)	**Q3B7M9**	Po. anti-human Santa Cruz SC-46347	1/250
Heat shock proteins			
αB-crystallin (*CRYAB*)	**P02511**	Mo. anti-bovine Assay Designs SPA-222	1/1000
Hsp20 (*HSPB6*)	**O14558**	Mo. anti-human Santa Cruz HSP20-11:SC51955	1/500
Hsp27 (*HSPB1*)	**P04792**	Mo. anti-human Santa Cruz HSP27 (F-4):SC13132	1/3000
Hsp40 (*DNAJA1*)	**P31689**	Mo. anti-human Santa Cruz HSP40-4 (SPM251):SC-56400	1/250
Hsp70-1A (*HSPA1A*)	**Q27975**	Mo. anti-human RD Systems MAB1663	1/1000
Hsp70-8 (*HSPA8*)	**P11142**	Mo. anti-bovine Santa Cruz HSC70 (BRM22):SC-59572	1/250
Oxidative proteins			
Peroxiredoxin6 (*PRDX6*)	**P30041**	Mo. anti-human Abnova PRDX6 (M01), clone 3A10-2A11	1/500
Protein deglycase DJ-1(*PARK7*)	**Q99497**	Po. anti-human Santa Cruz DJ-1 (FL-189):SC-32874	1/4000
Superoxide dismutase [Cu-Zn]*(SOD1)*	**P00441**	Po. anti-rat Acris SOD1 APO3021PU-N	1/1000
Structural proteins			
α-actin (*ACTA1*)	**P68133**	Mo. anti-Rabbit Santa Cruz α-actin (5C5):SC-58670	1/1000
α-actinin 2 (*ACTN2*)	**P35609**	Po. anti-human Sigma SAB2100039	1/10 000
α-actinin 3 (*ACTN3*)	**Q0III9**	Po. anti-human Sigma SAB2100040	1/10 000
MLC-1F (*MYL1*)	**P05976**	Po. anti-human Abnova MYL1 (A01)	1/1000
Myosin heavy chain-I (*MYH7*)	**P12883**	Mo anti-bovine Biocytex 5B9	1/1000
Myosin heavy chain-IIx (*MYH1*)	**P12882**	Mo anti-bovine Biocytex 8F4	1/500
Troponin T, slow skeletal muscle (*TNNT1*)	**Q8MKH6**	Po. anti-human Sigma SAB2102501	1/4000
Titin (*TTN*)	**Q8WZ42**	Mo. anti-human Novocastra NCL-TITIN	1/100
Tubulin alpha-4A chain (*TUBA4A*)	**P81948**	Mo anti-human Sigma T6074	1/1000
Cell death, protein binding and proteolysis			
Tripartite motif protein 72 (*TRIM72*)	**E1BE77**	Po. anti-human Sigma SAB2102571	1/2000
Four and a half LIM domains 1 (*FHL1*)	**Q3T173**	Po. anti-human Sigma AV34378	1/5000
μ-calpain (*CAPN1*)	**P07384**	Mo. anti-bovine Alexis μ-calpain 9A4H8D3	1/500

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Table 2. Least square of the means (± SD) of the texture traits and biomarkers (protein concentration values of the samples in log2) for *Longissimus thoracis* muscle that are significantly different between the two texture classes.

Variables	*Tender and juicy class*	*Tough with residues class*	SEM [a]	*p*-value [b]
Tenderness (0 – 10)	5.0±0.24	4.0±0.31	0.07	***
Juiciness (0 – 10)	3.8±0.22	3.5±0.15	0.05	***
Residues (0 – 10)	3.5±0.24	3.8±0.28	0.06	***
WBSF (N/cm²)	41±7.32	49±13.0	2.50	**
MHC1	0.04±0.60	-0.53±0.69	0.16	*
ALDH1A1	-0.36±0.54	-0.83±0.51	0.13	*

[a] Standard error of mean
[b] Significance levels: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Table 3. Pearson correlation analysis between sensory and instrumental beef texture traits with the biomarkers quantified and validated by RPPA.

	Texture traits			
Variables	Tenderness	Juiciness	Residue	WBSF
Juiciness	0.29[t]			
Residue	–0.37*	–0.33*		
WBSF	–0.45**			
	Protein biomarkers			
Hsp27	0.32t	-0.28[t]		
Hsp70-8	0.34*			
TRIM72	–0.31[t]			
FHL1				–0.43**
MHC1	0.35*		–0.42*	
ALDH1A1	0.42*		–0.35*	
TPI1	0.34*			

Significance levels: t $P = 0.06$; *$P < 0.05$; **$P < 0.01$

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Highlights

- First use of Reverse phase protein array for meat texture biomarkers quantification/validation
- PCA – *k*-means as a powerful tool to discriminate meat texture into classes
- First link of 29 protein biomarkers with beef tenderness from dozens Charolais bulls
- MHC1 and ALDH1A1 as prime biomarkers to discriminate between beef texture traits
- First use of principal component regression (PCR) for beef texture prediction using biomarkers

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