Beef tenderness and intramuscular fat proteomic biomarkers: Effect of gender and rearing practices

Brigitte Picard, Mohammed Gagaoua, Marwa Al Jammas, Muriel Bonnet

To cite this version:

Brigitte Picard, Mohammed Gagaoua, Marwa Al Jammas, Muriel Bonnet. Beef tenderness and intramuscular fat proteomic biomarkers: Effect of gender and rearing practices. Journal of Proteomics, 2019, 200, pp.1-10. 10.1016/j.jprot.2019.03.010 . hal-02627558

Beef tenderness and intramuscular fat proteomic biomarkers: effect of gender and rearing practices

Brigitte Picard*, Mohammed Gagaoua**, Marwa Al Jammas, Muriel Bonnet

Université Clermont Auvergne, INRA, VetAgro Sup, UMR Herbivores, Saint-Genès-Champanelle, France

Correspondence:
* Dr. Brigitte Picard: brigitte.picard@inra.fr
ORCID: 0000-0002-8075-671

ABSTRACT

This study analyzed the effect of gender on the abundances of 20 protein biomarkers of tenderness and/or intramuscular fat content in five muscles: *Longissimus thoracis*, *Semimembranosus*, *Rectus abdominis*, *Triceps brachii* and *Semitendinosus*, from cows and steers of the Protected Designation Origin Maine Anjou. The protein abundances were quantified using Reverse Phase Protein Array with specific validated antibodies. Among the 20 studied proteins, the abundance of 8 biomarkers involved in energetic metabolism, contraction and cellular stress, was different according to gender. The gender effect was different depending on the muscle type with greater abundances in *Semitendinosus*, *Rectus abdominis* and *Longissimus thoracis* muscles. On the basis of animal characteristics and rearing factors, three rearing practices classes were identified for cows. Among the factors, fattening duration modified the abundance of 12 proteins mainly in *Triceps brachii* muscle. A positive correlation between the abundance of the small HSP20 and slaughter age was observed in the 5 muscles. Two proteins, Four and a half LIM domains 1 (FHL1) and Glycogen phosphorylase (PYGB) appeared to be muscle, gender and rearing practices independent. These results constitute valuable data to understand how to manage beef quality by controlling these different factors.

Keywords: Beef proteome, Gender, Skeletal muscles, Rearing factors, Proteomics, Reverse Phase Protein Array

SIGNIFICANCE

This study is the first to compare the relative abundance of 20 proteins previously identified as biomarkers of tenderness and/or intramuscular fat (IMF) content of beef meat between cows and steers among 5 different muscles. Its originality is in the use of Reverse Phase Protein Array for fast quantification of the proteins and the integration of data from rearing factors, carcass characteristics and biomarkers of meat qualities. The findings provide evidence for modulating biomarker levels by controlling the choice of animal type and rearing factors according to the type of muscle that would produce animals with the desired meat qualities.

1. Introduction

The control of meat qualities is a societal issue that concerns all the meat sectors. Meat qualities are defined by a set of intrinsic and extrinsic properties where the former correspond to safety, health, convenience, nutritional and sensorial qualities; and the later are associated with the product and production system characteristics from the farm-to-fork, including animal welfare, carbon footprint and marketing variables (for review: [1-3]). For beef meat, the most crucial quality traits are tenderness and marbling associated with intramuscular fat (IMF) content. Tenderness defined as the ease with which meat can be sliced or chewed, is a multifactorial quality criterion the most variable and therefore the most difficult to control or predict. The appreciation of beef tenderness is generally positively associated with IMF content, the decrease in IMF content can also reduce tenderness [4]. Indeed, a minimum amount of IMF is needed for the expression of beef flavor as well as better tenderness [5]. IMF also plays an important role in beef juiciness, meat with high IMF content is always less dry than lean meat. Despite industry efforts to control the eating quality of beef, a high level of variability remains in these quality traits, which is one reason for consumer dissatisfaction. Thus, for producers and consumers, the control and management of beef tenderness and IMF content constitute a challenging task for better sustainability of the beef sector.

The large literature reported that those beef qualities are the result of complex biological mechanisms involved in muscle biochemistry in the live animals and after slaughtering during the aging period [6, 7]. Over the last decades, numerous studies have analyzed the factors affecting these traits. The effect of factors related to the animal and its production systems such as muscle type, breed, age, sex, physiological stage of animals, nutritional diet, physical activity and fattening duration has been investigated [2, 8]. The earlier results reported that early maturing Anglo-Saxon breeds such as Aberdeen Angus or Japanese black cattle, are characterized with high degree of fatness, on the contrary late maturing breeds such as French beef breeds or double muscled cattle, have high muscle yield and low fatness scores [9]. The development of adipose tissues in some specific muscles appears to disorganize the muscle structure and contributes to tenderization of highly marbled beef during the late fattening period [10]. Increasing age seems to be favorable for juiciness and flavor (due to more intramuscular fat), but unfavorable for tenderness due to connective tissue characteristics despite an attenuation of this effect by high amounts of IMF [11]. Furthermore, gender plays an important role. For example, at the same age, females provide more flavorful, tenderer and intense color beef than steers or bulls[12]. Compared to beef from young bulls, meat from steers contains more IMF [12, 13]. In the large literature, many controversies have been reported regarding the relationships between rearing factors and quality traits, with many conflicting results [8, 12, 14, 15] and today there is still no reliable online tools to predict these quality traits and deliver consistent quality beef for consumers. In this context, researches were conducted during the last 15 years to better understand the biological mechanisms underpinning tenderness and IMF variability to propose indicators or biomarkers which could be used for their prediction and/or management soon after slaughter of the animals [16, 17]. "Omics" approaches which allow a large number of genes, proteins or metabolites to be simultaneously studied without any *a priori*, have been extensively applied (for review [16, 18]). These approaches had revealed that large amount of macromolecules may be potential molecular indicators of muscle mass and growth performance [19], sensory attributes [20-23] or marbling of meat [24, 25]. The question is now how to modulate them in order to control and manage beef quality. The expression or abundance of these biomarkers could be modulated through rearing factors. As the control of the zootechnical performance of animals and the quality of their products is of major economic importance in the context of beef sustainability, the aim of this study was to analyze the gender effect by comparing cows *vs.* steers and link with the rearing factors on the relative abundance of 20 biomarkers of tenderness and/or IMF content in 5 muscles. The proteins were quantified using the Reverse Phase Protein Array (RPPA) on 101 Protected Designation Origin (PDO) Maine-Anjou cattle [8, 21, 26]. A classification based on rearing factors was applied as described by Gagaoua *et al* [8, 22] to identify rearing practices classes. Then, carcass properties and relative abundances of the biomarkers were analyzed for each class among 5 muscles. The results revealed new insights that could be applied for a better understanding of the biological pathways involved in meat quality according to gender and rearing practices.

2. Materials and Methods

2.1. Animals, handling and slaughtering

A total of 101 cattle including 86 cows and 15 steers from the French PDO (Protected Designation of Origin) Maine-Anjou, using "Rouge des Prés" breed [21], were collected [26]. The PDO Maine-Anjou animals originated in the northwestern part of France from a cooperative of livestock farmers located in the department of Maine-et-Loire. This breed was the second (since 2004) among the four breeds allowed to be used in France for PDO meat production. It is composed of around 80% of cows (justifying the high number of animals in this study), younger than 10 years of age, having calved at least once and a minimal carcass weight of 380 kg. Steers over 30 months of age with a carcass weight of 400 kg minimum can also be found (20%). PDOs are of special importance for the valorization of local breeds, and the specifications of animal products under PDO are paid increasing attention [21]. The rearing practices of each animal were surveyed by a questionnaire as detailed in Gagaoua *et al.* [8] based on the study by Couvreur *et al*. Briefly, the questionnaire included variables about (i) the finishing period [part of hay, haylage, and/or grass in the finishing diet (% w/w); total amount of concentrate (kg); fattening duration (days); physical activity of the animals (% days out)] and (ii) the animal characteristics by the age at slaughter in months. Those variables were used to identify rearing practices as detailed in the statistical section of this manuscript.

Before slaughter, all animals were food deprived for 24 h and had free access to water. The slaughtering was performed in the same industrial abattoir (Charal, Sablé sur Sarthes, France). The animals were stunned using captive-bolt pistol prior to exsanguination and dressed according to standard commercial practices. The slaughtering was also performed in compliance with the French welfare regulations and respecting EU regulations (Council Regulation (EC) No. 1099/2009).

After slaughter, the carcasses were characterized and graded according to the European beef grading system (CE 1249/2008). Thus, information for each carcass were measured, namely hot carcass weight (HCW, kg), EUROP conformation score (EUROP grid), carcass fat weight and fat to muscle ratio (% w/w) as described by Gagaoua *et al.* [11, 27].

2.2. Muscle sampling

The carcasses were not electrically stimulated and they were chilled at 3 to 4°C until 24 h *post-mortem*. The right half carcass was used for muscles measurements. Then, aliquots of five muscles: *Longissimus thoracis* (LT), *Semimembranosus* (SM), *Rectus abdominis* (RA), *Triceps brachii* (TB) and *Semitendinosus* (ST), from each carcass of the 101 PDO Maine Anjou cattle were sampled. These heterogeneous muscles were chosen according to their differences in contractile and metabolic type [26]. The LT muscle was excised from the 6th rib as detailed by Gagaoua *et al.* [27]. As the samples were for omics biomarkers analysis, the muscles (an approximate of 2 g) trimmed of connective and superficial fat tissue were immediately and carefully frozen in liquid nitrogen and stored at -80°C until analysis following the protocol previously described by Picard *et al.* [26].

2.3. Protein biomarkers quantification by Reverse Phase Protein Array

The relative abundance of 20 protein biomarkers of tenderness and/or IMF content was measured in the 5 muscles by the Reverse Phase Protein Array (RPPA) recently described by our group [20, 22, 26]. The specificity of the 20 antibodies on bovine muscle and their conditions of use have been previously defined by western blotting which uses the same technical principle as the RPPA method [26]. Briefly, the samples were firstly disrupted in a Laemmli 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_3VO_4 and 10 mM NaF, using a Precellys (Bertin). Extracts were then boiled for 10 min at 100°C, sonicated to reduce viscosity and centrifuged 10 min at 15000 rpm. The supernatant was harvested and stored at -80°C. Protein concentration was determined using the Pierce BCA reducing agent compatible kit (ref 23252).

The sample extracts were then deposited onto nitrocellulose covered slides (Supernova, Grace Biolabs) using a dedicated arrayer (2470 arrayer, Aushon Biosystems). 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 each of the 20 specific antibodies or without primary antibody (negative control), using an Autostainer Plus (Dako) as detailed in our previous papers [20, 22, 26]. After protein quantification by RPPA, the raw data were normalized using Normacurve following the procedure described by [28], which normalizes 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 20 arrays using a linear regression.

2.5. Statistical analysis

The statistical analyses were performed using SAS statistical software (SAS 9.1, SAS Institute INC, Cary, NC, USA) and XLSTAT 2017.19.4 (AddinSoft, Paris, France). Before analysis, raw data means were scrutinized for data entry errors and outliers. Normal distribution and homogeneity of the dataset was first tested by the Shapiro-Wilk test ($P > 0.05$). The PROC GLM procedure of SAS was then used to study the muscle type (5 muscles), gender (cows *vs.* steers) and interactions effects on the relative abundances of the proteins. Significant differences among muscles were performed using Tukey's test at a significance level of $P < 0.05$. Similarly, the protein abundances were further compared between the two genders within each muscle separately and the effect of rearing practices on the abundances of the 20 proteins was analyzed for both cows and steers.

For the 86 cows only, rearing practices classes were created using the statistical approach described by Gagaoua *et al.* [8, 20] based on principal component analysis (PCA) combined to *k*-means clustering. For that, the fattening period data (part of hay, haylage and/or grass in the finishing diet (% w/w)); total amount of concentrate (kg); duration (days) and physical activity (% days out) of the animals at the farm were used [8]. Two factors with eigenvalues >1.0 were extracted on the basis of the scree plot and evaluation of the factor loading matrix after orthogonal rotation. These allowed us to identify using Z-scores on the two axis 3 rearing practices that were named to simplify the discussion as follow: Class 1= "Hay class"; Class 2 = "Grass class", and Class 3 = "Haylage class", respectively (Table 3). Z-scores represent the deviation of each observation relative to the mean of the corresponding individual in each rearing practice and were calculated using PROC STANDARD of SAS that standardizes data to a mean of 0 and standard deviation of 1. These normalized data were used to build PCAs to depict the relationships between the rearing practices of the 86 PDO Maine-Anjou cows with i) animal, rearing factors and carcass characteristics, and with ii) the 20 protein biomarkers from the 5 muscles quantified by RPPA technique within the rearing factors. The Kaiser-Meyer-Olkin (KMO) measure, known also as Kaiser's Measure of Sampling Adequacy (MSA) was applied to test the validity of the sampling [29]. Subsequently, unsupervised hierarchical clustering heatmap was generated using the same data to assess the differences among the 5 muscles based on the normalized data for each rearing practice. For the 15 steers, only two rearing practices were identified (grass (n = 5) and haylage (n = 10)) and were considered in the analyses in same manner than cows.

Finally, the PROC CORR of SAS after Z-scores calculation was used to compute the Pearson's correlations of coefficients between the 20 proteins and the animal, rearing factors and carcass characteristics of the whole data of the 86 cows. Correlation coefficients were considered significant at $P< 0.05$.

3. Results and discussion

3.1. Gender effect

The gender (cows *vs.* steers) had a highly significant effect on the relative abundance of 8 proteins among the 20 analyzed: HSP20, PGK1 (P<0.001), PRDX6, ALDOA (P<0.01), MDH1, TPI1, MyHC-IIX, TNNT1 (P<0.05) (Table 2). All muscle combined, the cows comparatively to steers had significantly (P< 0.01) higher abundance of HSP20, ALDOA, MDH1, MyHC-IIX, and lower abundance of PGK1, PRDX6, TPI1 and TNNT1 (Table 2).

Of the 20 proteins analyzed, only HSP20 had an abundance that differed between steers and cows irrespective of the considered muscle. An interaction of muscle x gender was observed for this protein (Table 2) which was more abundant in cows for LT, SM, ST muscles, and was not different in RA and TB. Figure 1 illustrates higher differences between muscles in steers than in cows. In the two genders, the abundance of HSP20 was the highest in RA muscle. On another hand, the abundances of CRYAB, HSP27, HSP40, HSP70-1, FHL1, TRIM72, PYGB, ALDH1A1, ENO3, TTN, MLC1F and α-tubulin were not different between steers and cows.

Our results showed that the muscles of cows comparatively to steers differed by the abundance of 8 proteins among the 20 analyzed. Thus, gender affects less proteins than muscle type reported to modify the abundance of 16 of 20 proteins, only 4 proteins namely HSP40 (Heat shock protein), FHL1 (Four and a half LIM domains protein 1), PYGB (Glycogen phosphorylase B) and MDH1 (Malate dehydrogenase), were found to do not differ among the 5 muscles [26]. Thus, according to these two studies, HSP40, FHL1 and PYGB were not modified either by gender or muscle type while HSP20, PRDX6, PGK1, ALDOA, MyHC-IIX, TNNT1 and TPI1 showed both muscle and gender effects.

The analysis of gender effect in each of the 5 muscles showed that it was most important for ST, RA, and LT muscles. It is particularly significant for HSP20 which abundance between cows and steers was not modified in TB and RA muscles and was significantly different between the two genders for the three other muscles. In TB muscle, the abundances of 19 of 20 proteins were not different between cows and steers. This indicates that this muscle is insensitive to the sex or gender effect (Table 2). This result is coherent with previous data of our group showing no effect of castration on contractile and metabolic properties of TB muscle while the effect of castration was the greatest in ST and LT muscles [30], in accordance with the results of the present study. Indeed, in the present study, the most important differences between the two genders were observed in ST muscle as the abundances of 8 proteins were different between cows and steers, whereas 6 were different in LT and RA and 5 in SM muscle. To our knowledge, very few studies in the literature have compared the muscle proteome properties of cows comparatively to steers in different muscle types. Previous results of our group showed that RA muscle of heifers comparatively to steers, was more oxidative with greatest ICDH and COX activities and less glycolytic with a lowest LDH activity [21, 31]. These data are coherent with the present results showing modifications of contractile [MyHC-IIX (fast glycolytic isoform), TNNT1 (slow isoform)] and metabolic [ALDOA (glycolytic enzyme involved in glycogen storage), MDH1 (involved in tricarboxylic acid cycle), PGK1 (glycolytic enzyme) and TPI1 (involved in gluconeogenesis and carbohydrate biosynthesis)] properties of the muscles between cows and steers. This effect could be explained mainly by differences in sex hormones between the two genders. The effect of estrogens on skeletal muscle properties has been largely studied in different species [32, 33]. Indeed, estrogens and their receptors play key roles in the regulation of energy metabolism pathways, including glucose transport, glycolysis, tricarboxylic acid cycle, mitochondrial respiratory chain, adenosine nucleotide translocator and fatty acid β-oxidation and synthesis [34]. A higher insulin sensitivity was also reported in female, and the ratio of glycolytic/oxidative enzyme activities within skeletal muscle correlated negatively. These modifications in muscle physiology induced by estrogens are in accordance with the modifications in protein abundances observed in this study.

Among the differential proteins, HSP20 and PGK1 showed and all muscles confounded the highest differences between the two genders (Table 2). To the best of our knowledge, only one publication reported a higher abundance of HSP20 (*HSPB6* gene) and a lower abundance of PGK1 in muscle from women than men as observed for cattle in this study [35]. Few data are available in the literature about the effect of castration or estrogens on *HSPB6* gene expression (HSP20). In line to this scarcity of studies in the large literature, a recent review by Gianazza *et al.* [36] reported that the first proteomic survey on the proteome of male *vs* female serum in humans is also as recent as 2010 [37]. Therefore, it is difficult to compare the findings of this study to the literature.

The findings of HSP20 protein may be partly linked to its binding to structural proteins such as TNNT1 [38]. These data are coherent with the differences observed between cows and steers for both HSP20 and TNNT1. Moreover, earlier studies demonstrated that HSP20 is phosphorylated in response to insulin in skeletal muscle [39] and the authors proposed HSP20 as a potential modulator of insulin's functions. The differences in TNNT1 abundance between cows and steers could be the consequence of insulin sensitivity induced by estrogens. The action of estrogen is also through circulating adipokines as adiponectin and leptin which levels are higher in females [40]. These adipokines are involved in muscle metabolism and fat deposition.

The main effect of gender in the present study was observed for PGK1 as it is the only protein among the 20 analyzed which was more abundant in steers comparatively to cows in each of the 5 muscles. This protein is involved in glycolysis as it is the first ATP-generating enzyme in the glycolytic pathway, catalyzing the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate. It has been recently shown that PGK1 translocates to the mitochondria where it specifically phosphorylates pyruvate dehydrogenase kinase [41]. These data are in accordance with a high effect of sex hormone on glucose metabolism [42] that would also be linked to IMF deposition within steers [43]. Several data of the literature indicated that castrated cattle have higher fast-twitch glycolytic fiber proportion and lower slow-twitch oxidative fiber than intact males.

3.2. Effect of rearing practices

The variance analysis showed that the abundance of very few proteins was modified by rearing practices (Table 3). In cow muscles, only 3 proteins were significantly different (P<0.05): PRDX6, PGK1, ALDOA, and 3 others showed tendencies (P< 0.1): HSP20, ENO3, MDH1. In steer muscles, we observed no significant differences between the two rearing practices for 18 proteins and only 2 tended to be different: ALDOA and ALDH1A1. Only the abundance of ALDOA was affected by rearing practices in both cows and steers. It is worthwhile to note that the abundance of this protein was also different among the 5 muscles in cows and in steers. An effect of gender was observed only in LT muscle with a lower abundance in LT of steers comparatively to cows. The results demonstrated that the effect of rearing practices on the abundance of the 20 biomarkers is weak, and lower than the effect of gender which is weaker than muscle type effect.

The analysis of animal and rearing factors on cows allowed to distinguish 3 rearing practices classes that differed by 9 factors (Table 4). The most discriminating factors were animal activity, percentage of grass, haylage or hay in the diet during the fattening period (*P*<0.001) (Table 4). Accordingly, these 3 classes were called “grass”, “hay” and “haylage” [8]. For steers, we have identified “grass” and “haylage” rearing practices only (data not shown) and they were not different for any of the studied biomarkers, therefore the results are not discussed in the following sections (Table 3).

Comparatively to the “hay” and “haylage” classes, the “grass” class was characterized by higher animal activity, longer fattening period duration and the carcasses of the animals had a lower conformation score (Table 4 and Figure 2a). The haylage class was characterized by a higher carcass weight than the two other classes.

For the effect of rearing practices on the studied protein biomarkers, the “grass” class had an impact mainly on the properties of the SM and ST muscles known as fast glycolytic muscles (Figure 2b). This class was characterized by high relative abundance of MLC1F (fast isoform), PRDX6 (an antioxidant enzyme) and of three glycolytic enzymes (PGK1, TPI1 and ENO3). Hay finishing practices affected the properties of RA muscle known as slow oxidative muscle. This class was characterized by high abundance of small Heat Shock Proteins (HSP20, 27 and CRYAB) as well as HSP70-1A, TNNT1 (slow structural protein isoforms) and ALDH1A, and by a low abundance of MyHC-IIX (fast glycolytic). Furthermore, the results revealed that LT and TB muscles, known as mixed oxido-glycolytic muscles, were less impacted by rearing practices than the 3 other muscles. Interestingly, the abundance of 3 proteins FHL1, MDH1 and PYGB was not different among the 3 rearing practices classes whatever the muscle (Figure 3). Abundance of HSP40 and α-tubulin was modified in the Hay class only.

One of the main results of the present study is to show that rearing practices classes are different according to the studied muscle. Grass class is composed mainly of SM and ST muscles (fast glycolytic muscles); haylage class groups LT and TB muscles (mixed oxydo-glycolytic muscles) and hay class contains only RA muscle. These data indicate that the impact of rearing practices is muscle type dependent. In this study, the fast glycolytic muscles were the most impacted by grass finishing diet. These modifications are interesting in term of beef tenderness as well as other sensory qualities [44]. Indeed, we have recently showed that ST muscle is more tender when it is more fast glycolytic [45]. A recent study of our group showed that the LT muscle of Rouge de Prés cows with grass diet had lower proportions of IIX fibres (fast glycolytic and higher proportion of IIA fibres fast oxydo-glycolytic) [8]. An opposite effect of rearing practices on LT and ST muscles has already been observed. However, despite an opposite response, the effect of a grass finishing diet has a positive impact on tenderness in both muscles, since for LT, unlike ST, the less glycolytic are the most tender [45].

3.3. Correlations between biomarkers and the carcass and rearing factors

The correlation analyses, although they are weak but coherent, showed that among the 9 factors discriminating the 3 rearing practices classes of cows, fattening duration and age at slaughter had an influence on the protein abundances in the 5 muscles (Figure 4). Fattening duration modified the abundance of 12 among the 20 studied proteins (Figure 4). This effect was the most important in TB muscle as the abundance of 6 proteins was modified. For TB muscle, the abundances of MLC1F, PYGB, PRDX6 and FHL1 decreased when fattening duration increased whereas abundance of HSP70-1A and TTN increased. The abundance of PYGB was also modified in LT and ST muscles (with a negative correlation between fattening duration and PYGB abundance) but not in RA and SM muscles. HSP70-1A was modified also in RA but inversely in comparison with TB muscle. We observed also that the abundance of ENO3 was inversely correlated with fattening duration in LT (positively) and SM (negatively). The present abundance variations seem to be related to the composition of the fibrous part of the diet and/or animal activity that was independent of the slaughter weight and age. These are consistent with previous observations by our group highlighting that fattening duration is the most influencing rearing factors for meat quality, particularly tenderness [2, 8, 11].

For slaughter age, the main effect observed was a positive correlation with the abundance of HSP20 in the 5 muscles (Figure 4). It is the only protein which abundance was modified in the same way in the 5 muscles with an increase with age at slaughter of the animals. Interestingly, HSP20 discussed above to be affected by gender was the only protein which abundance was modified in the same way in the 5 muscles. HSP20 belongs to a family of at least 10 different small HSPs [17]. HSP20 is expressed in multiple tissues but it is more abundant in muscle [46]. In human and rat, an increase of its expression with age has been reported in accordance with the present results [47, 48]. This increase is considered in the literature as an essential cellular response to fiber aging; according to our results this response seems to be muscle type independent. The modifications of HSP20 abundances with slaughter age are in accordance with the modification of contractile and metabolic properties observed in aged muscles in cows and steers toward a shift from fast glycolytic to slow oxidative [8, 16, 45]. The main effect of slaughter age was observed for RA muscle with a correlation with the abundance of 5 proteins: positively with HSP20, FHL1, ALDH1A1, TNNT1 and negatively with MyHC-IIX. EUROP conformation and carcass weight were linked to the studied proteins in 4 muscles unless TB muscle which was not influenced as any correlation with proteins abundances were observed (Figure 4). The EUROP conformation had an impact mainly in SM muscle in which it was correlated with 4 proteins: positively with TTN, MDH1, TRIM72 and negatively with PGK1.

Factors associated with diet composition had weak effects on protein abundances. Grass % was correlated with 4 proteins in LT: positively with TPI1, negatively with HSP70-1A, MDH1, PYGB. Total concentrate (in kg) was correlated with proteins abundances in 4 muscles and no correlations were observed in LT muscle. It was negatively correlated with MDH1 abundance in RA, SM and ST muscles, but not for LT and TB. The abundance of this protein in LT and

RA was negatively correlated with animal activity, no correlations were observed for the 3 other muscles. It was also negatively correlated with animal slaughter age in LT and SM. We observed that in LT muscle, the abundance of this protein was correlated negatively with 4 rearing factors: animal activity, % grass in the diet, carcass weight and fattening duration. On another hand, animal activity showed no correlations with the protein abundances of ST and TB muscles. In each of the three other muscles, animal activity was correlated with the abundances of 3 proteins.

Of the 5 muscles, the proteins in TB muscle were the least sensitive to variations in rearing practices. No correlations were observed with any proteins irrespective of rearing practices with EUROP conformation and carcass weight. Only one protein was correlated with the activity of the animals at the farm, mainly MLC-1F as well as with total concentrate for FHL1. However, TB muscle was the most modified muscle by fattening duration. On the contrary, RA and SM muscles were the most sensible to rearing practices as correlations with all rearing factors except grass% for RA and haylage % for SM, were observed.

3.4. Proteins that did not discriminate the rearing practices classes with no difference among muscles and genders

The abundances of FHL1 (Four and a half LIM domains protein 1) and PYGB (Glycogen phosphorylase B) were not different between the three rearing practices classes. Interestingly, the abundances of these proteins were not significantly different among the two genders and among the5 muscles in cows and in steers. This indicates that the abundances of these proteins are muscle, gender and rearing practices independent.

FHL1 also named SLIM1 or KyoT1, belongs to the FHL protein family composed of four and a half Lin-11, Isl-1, and Mec-3 (LIM) domains. FHL LIM domains mediate protein – protein interactions, scaffolding signaling proteins in the cytoplasm, and transcription factors in the nucleus. FHL1 as mentioned above is considered as a regulator of skeletal muscle mass, and strength enhancement by binding with the calcineurin-regulated transcription factor NFATc1 [49]. 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 [50]. FHL1 may also interact with other biological pathways, namely metabolic enzymes [26, 51] in response to both hypoxia, apoptosis and oxidative stress [52]. This protein seems to play a fundamental role in muscle mass and muscular strength which could explain why its expression is relatively stable according to muscle, gender or rearing practices. For example, FHL1 increased the myostatin activity on a SMAD reporter and increased myostatin dependent myotube wasting [53]. According to these authors, FHL1 is expressed at higher levels in type II than in type I fibers raising the possibility that it contributes to the greater sensitivity of type II fibers to myostatin. However, these differences in fiber types expression were not observed among our 5 muscles as previously reported by our group [26]. On another hand, PYGB is a Glycogen Phosphorylase which catalyzes the glycogen degradation. Its activity is positively regulated by AMP and negatively regulated by ATP, ADP, and glucose-6-phosphate [6].The non-variation on this protein abundance would be due to a lack of an enhanced glycogen degradation by the factors considered in this publication.

4. Conclusion

This study is the first to consider the effect of gender and rearing practices on the abundances of biomarkers of tenderness and IMF content in five different muscles in cattle. The main results showed a higher effect of muscle type than gender or rearing practices. Moreover, factors associated with diet composition had few effects on proteins abundances. This knowledge constitutes important information to understand how to manage the expression of biomarkers of tenderness and IMF content according to gender and rearing practices.

Author contributions

BP and MB defined the experiment design, managed the experiment, co-wrote the paper, and approved the final draft of the manuscript. MG managed the database, analyzed the data, prepared figures and/or tables, co-wrote the paper and approved the final draft of the manuscript. MEJ participated in the database preparation. All authors collaborated with interpretation and discussion of the results. All authors have given approval to the final versions of the manuscript.

Conflict of interest

The authors declare no competing financial interest

Funding Sources

This experiment was conducted with funding from the regional council of Pays de Loire and SICA Rouge des Prés (France).

Acknowledgements

The authors thank Albéric Valais, Ghislain Aminot and Marlène Pécot from SICA Rouge des Prés for muscle sampling and data on animal, rearing factors and carcass properties. They thank Leanne De Koning, Aurélie Cartier and Bérengere Ouine from Institut Curie, RPPA Plateform, Paris France, for the quantification of the biomarkers.

References

[1] Hocquette JF, Van Wezemael L, Chriki S, Legrand I, Verbeke W, Farmer L, et al. Modelling of beef sensory quality for a better prediction of palatability. Meat science. 2014;97:316-22.

[2] Gagaoua M, Monteils V, Picard B. Data from the farmgate-to-meat continuum including omics-based biomarkers to better understand the variability of beef tenderness: An integromics approach. J Agric Food Chem. 2018;66:13552–63.

[3] Gagaoua M, Picard B, Monteils V. Assessment of cattle inter-individual cluster variability: the potential of continuum data from the farm-to-fork for ultimate beef tenderness management. Journal of the Science of Food and Agriculture. 2019;In press.

[4] Dransfield E, Martin J-F, Bauchart D, Abouelkaram S, Lepetit J, Culioli J, et al. Meat quality and composition of three muscles from French cull cows and young bulls. Animal Science. 2003;76:387-99.

[5] Wood JD, Richardson RI, Nute GR, Fisher AV, Campo MM, Kasapidou E, et al. Effects of fatty acids on meat quality: a review. Meat science. 2004;66:21-32.

[6] Ouali A, Gagaoua M, Boudida Y, Becila S, Boudjellal A, Herrera-Mendez CH, et al. Biomarkers of meat tenderness: present knowledge and perspectives in regards to our current understanding of the mechanisms involved. Meat science. 2013;95:854-70.

[7] Hocquette JF, Botreau R, Legrand I, Polkinghorne R, Pethick DW, Lherm M, et al. Win–win strategies for high beef quality, consumer satisfaction, and farm efficiency, low environmental impacts and improved animal welfare. Anim Prod Sci. 2014;54:1537-48.

[8] Gagaoua M, Monteils V, Couvreur S, Picard B. Identification of Biomarkers Associated with the Rearing Practices, Carcass Characteristics, and Beef Quality: An Integrative Approach. Journal of Agricultural and Food Chemistry. 2017;65:8264-78.

[9] Gotoh T, Albrecht E, Teuscher F, Kawabata K, Sakashita K, Iwamoto H, et al. Differences in muscle and fat accretion in Japanese Black and European cattle. Meat science. 2009;82:300-8.

[10] Nishimura T, Hattori A, Takahashi K. Structural changes in intramuscular connective tissue during the fattening of Japanese black cattle: effect of marbling on beef tenderization. J Anim Sci. 1999;77:93-104.

[11] Gagaoua M, Picard B, Soulat J, Monteils V. Clustering of sensory eating qualities of beef: Consistencies and differences within carcass, muscle, animal characteristics and rearing factors. Livestock Science. 2018;214:245-58.

[12] Gagaoua M, Terlouw EMC, Micol D, Hocquette JF, Moloney AP, Nuernberg K, et al. Sensory quality of meat from eight different types of cattle in relation with their biochemical characteristics. Journal of Integrative Agriculture. 2016;15:1550-63.

[13] Pogorzelska-Przybyłek P, Nogalski Z, Sobczuk-Szul M, Purwin C, Kubiak D. Carcass characteristics and meat quality of Holstein-Friesian × Hereford cattle of different sex categories and slaughter ages. Arch Anim Breed. 2018;61:253-61.

[14] Maltin CA, Balcerzak D, Tilley R, Delday M. Determinants of meat quality: tenderness. Proc Nutr Soc. 2003;62:337-47.

[15] Ellies-Oury M-P, Bonnet M, Gagaoua M, Mialon M-M, Durand D, Gruffat D, et al. Clustering of fatty acids composition, sensory quality and proteomic biomarkers of young Charolais bulls. In: Troy D, McDonnell C, Hinds L, Kerry J, editors. Proceedings of the 63rd International Congress of Meat Science and Technology. First edition ed. Cork, Ireland: Wageningen Academic Publishers; 2017. p. 838-9.

[16] Picard B, Gagaoua M, Hollung K. Chapter 12 - Gene and Protein Expression as a Tool to Explain/Predict Meat (and Fish) Quality In: Purslow P, editor. New Aspects of Meat Quality : From Genes to Ethics. United Kingdom: Woodhead Publishing; 2017. p. 321-54.

[17] Picard B, Gagaoua M. Chapter 11 - Proteomic Investigations of Beef Tenderness. In: Colgrave ML, editor. Proteomics in Food Science: from farm to fork. London: Academic Press; 2017. p. 177-97.

[18] Picard B, Lebret B, Cassar-Malek I, Liaubet L, Berri C, Le Bihan-Duval E, et al. Recent advances in omic technologies for meat quality management. Meat science. 2015;109:18-26.

[19] Cao X-K, Cheng J, Huang Y-Z, Wang X-G, Ma Y-L, Peng S-J, et al. Growth Performance and Meat Quality Evaluations in Three-Way Cross Cattle Developed for the Tibetan Plateau and their Molecular Understanding by Integrative Omics Analysis. Journal of Agricultural and Food Chemistry. 2019;67:541-50.

[20] Gagaoua M, Bonnet M, De Koning L, Picard B. Reverse Phase Protein array for the quantification and validation of protein biomarkers of beef qualities: The case of meat color from Charolais breed. Meat science. 2018;145:308-19.

[21] Gagaoua M, Couvreur S, Le Bec G, Aminot G, Picard B. Associations among Protein Biomarkers and pH and Color Traits in Longissimus thoracis and Rectus abdominis Muscles in Protected Designation of Origin Maine-Anjou Cull Cows. J Agric Food Chem. 2017;65:3569-80.

[22] Gagaoua M, Bonnet M, Ellies-Oury MP, De Koning L, Picard B. Reverse phase protein arrays for the identification/validation of biomarkers of beef texture and their use for early classification of carcasses. Food Chemistry. 2018;250:245-52.

[23] Gagaoua M, Terlouw EM, Boudjellal A, Picard B. Coherent correlation networks among protein biomarkers of beef tenderness: What they reveal. J Proteomics. 2015;128:365-74.

[24] Ceciliani F, Lecchi C, Bazile J, Bonnet M. Proteomics Research in the Adipose Tissue. In: de Almeida AM, Eckersall D, Miller I, editors. Proteomics in Domestic Animals: from Farm to Systems Biology. Cham: Springer International Publishing; 2018. p. 233-54.

[25] Zhang Q, Lee HG, Han JA, Kim EB, Kang SK, Yin J, et al. Differentially expressed proteins during fat accumulation in bovine skeletal muscle. Meat science. 2010;86:814-20.

[26] Picard B, Gagaoua M, Al-Jammas M, De Koning L, Valais A, Bonnet M. Beef tenderness and intramuscular fat proteomic biomarkers: muscle type effect. PeerJ. 2018;6:e4891.

[27] Gagaoua M, Picard B, Monteils V. Associations among animal, carcass, muscle characteristics, and fresh meat color traits in Charolais cattle. Meat science. 2018;140:145-56.

[28] Troncale S, Barbet A, Coulibaly L, Henry E, He B, Barillot E, et al. NormaCurve: a SuperCurve-based method that simultaneously quantifies and normalizes reverse phase protein array data. PLoS One. 2012;7:e38686.

[29] Gagaoua M, Terlouw EM, Micol D, Boudjellal A, Hocquette JF, Picard B. Understanding Early Post-Mortem Biochemical Processes Underlying Meat Color and pH Decline in the Longissimus thoracis Muscle of Young Blond d'Aquitaine Bulls Using Protein Biomarkers. J Agric Food Chem. 2015;63:6799-809.

[30] Brandstetter AM, Picard B, Geay Y. Muscle fibre characteristics in four muscles of growing bulls: I. Postnatal differentiation. Livestock Production Science. 1998;53:15-23.

[31] Oury MP, Dumont R, Jurie C, Hocquette JF, Picard B. Specific fibre composition and metabolism of the rectus abdominis muscle of bovine Charolais cattle. BMC Biochem. 2010;11:12.

[32] Enns DL, Tiidus PM. The Influence of Estrogen on Skeletal Muscle. Sports Medicine. 2010;40:41-58.

[33] Sauerwein H, Meyer HHD. Androgen and Estrogen Receptors in Bovine Skeletal Muscle: Relation to Steroid-Induced Allometric Muscle Growth. Journal of Animal Science. 1989;67:206-12.

[34] Xu Y, López M. Central regulation of energy metabolism by estrogens. Molecular Metabolism. 2018;15:104-15.

[35] Welle S, Tawil R, Thornton CA. Sex-Related Differences in Gene Expression in Human Skeletal Muscle. PLOS ONE. 2008;3:e1385.

[36] Gianazza E, Miller I, Guerrini U, Palazzolo L, Parravicini C, Eberini I. Gender proteomics I. Which proteins in non-sexual organs. Journal of Proteomics. 2018;178:7-17.

[37] Miike K, Aoki M, Yamashita R, Takegawa Y, Saya H, Miike T, et al. Proteome profiling reveals gender differences in the composition of human serum. PROTEOMICS. 2010;10:2678-91.

[38] Rembold CM, Foster DB, Strauss JD, Wingard CJ, Van Eyk JE. cGMP-mediated phosphorylation of heat shock protein 20 may cause smooth muscle relaxation without myosin light chain dephosphorylation in swine carotid artery. The Journal of Physiology. 2000;524:865-78.

[39] Wang Y, Xu AM, Cooper GJS. Phosphorylation of P20 is associated with the actions of insulin in rat skeletal and smooth muscle. Biochemical Journal. 1999;344:971-6.

[40] Wyskida K, Franik G, Wikarek T, Owczarek A, Delroba A, Chudek J, et al. The levels of adipokines in relation to hormonal changes during the menstrual cycle in young, normal-weight women. 2017;6:892.

[41] Li X, Zheng Y, Lu Z. PGK1 is a new member of the protein kinome. Cell cycle (Georgetown, Tex). 2016;15:1803-4.

[42] Lundsgaard A-M, Kiens B. Gender Differences in Skeletal Muscle Substrate Metabolism – Molecular Mechanisms and Insulin Sensitivity. Frontiers in Endocrinology. 2014;5.

[43] Jeong J, Bong J, Kim GD, Joo ST, Lee HJ, Baik M. Transcriptome changes favoring intramuscular fat deposition in the longissimus muscle following castration of bulls. J Anim Sci. 2013;91:4692-704.

[44] Gagaoua M, Terlouw EMC, Picard B. The study of protein biomarkers to understand the biochemical processes underlying beef color development in young bulls. Meat Science. 2017;134:18-27.

[45] Picard B, Gagaoua M, Micol D, Cassar-Malek I, Hocquette JF, Terlouw CE. Inverse relationships between biomarkers and beef tenderness according to contractile and metabolic properties of the muscle. J Agric Food Chem. 2014;62:9808-18.

[46] Lomiwes D, Farouk MM, Wiklund E, Young OA. Small heat shock proteins and their role in meat tenderness: a review. Meat science. 2014;96:26-40.

[47] Charmpilas N, Kyriakakis E, Tavernarakis N. Small heat shock proteins in ageing and age-related diseases. Cell Stress and Chaperones. 2017;22:481-92.

[48] Doran P, Gannon J, O'Connell K, Ohlendieck K. Aging skeletal muscle shows a drastic increase in the small heat shock proteins αB-crystallin/HspB5 and cvHsp/HspB7. European Journal of Cell Biology. 2007;86:629-40.

[49] Cowling BS, McGrath MJ, Nguyen M-A, Cottle DL, Kee AJ, Brown S, et al. Identification of FHL1 as a regulator of skeletal muscle mass: implications for human myopathy. The Journal of Cell Biology. 2008;183:1033-48.

[50] Morzel M, Chambon C, Hamelin M, Sante-Lhoutellier V, Sayd T, Monin G. Proteome changes during pork meat ageing following use of two different pre-slaughter handling procedures. Meat science. 2004;67:689-96.

[51] Lange S, Auerbach D, McLoughlin P, Perriard E, Schäfer BW, Perriard J-C, et al. Subcellular targeting of metabolic enzymes to titin in heart muscle may be mediated by DRAL/FHL-2. Journal of cell science. 2002;115:4925-36.

[52] Gagaoua M, Hafid K, Boudida Y, Becila S, Ouali A, Picard B, et al. Caspases and Thrombin Activity Regulation by Specific Serpin Inhibitors in Bovine Skeletal Muscle. Appl Biochem Biotechnol. 2015;177:279-303.

[53] Lee JY, Lori D, Wells DJ, Kemp PR. FHL1 activates myostatin signalling in skeletal muscle and promotes atrophy. FEBS open bio. 2015;5:753-62.

Tables and Figures

Figure captions

Figure 1. Interaction between muscle x gender for HSP20 protein.

Figure 2. Principal component analysis (PCA) depicting the relationships between the rearing practices of the 86 PDO Maine-Anjou cows identified following the procedure by Gagaoua *et al.* [8] with **A)** animal, rearing factors and carcass characteristics, and with **B)** the 20 protein biomarkers from the 5 muscles quantified by RPPA technique within the rearing factors. The projection of the individuals of haylage class (red), hay class (bleu) and grass class (green) are encircled in ellipses (x,y-means ± x,y-standard deviation (SD)) using the corresponding schematic colors. Furthermore, the barycenter of each muscle with the corresponding color are given.

Figure 3. Unsupervised hierarchical classification heatmap highlighting the differences in the quantified proteins in the five muscles and among the three rearing practices for cows. The proteins that were not affected by rearing practices or muscle type are shown by "*". Colors correspond to the z-scores of the standardized values of protein fold-change between the muscles according to the 3 rearing factors.

Figure 4. Significant correlations ($P < 0.05$) between the 20 protein biomarkers and animal, rearing factors and carcass characteristics by muscle type. The negative correlations are given in red and the positive in green. The summary of the number of the correlations by muscle with the animal, rearing factors and carcass characteristics are given in a gradient-blue dependent color legend at the right down of the graph from 1 to 6 correlations in each muscle and with the same factor. For example, for TB muscle 6 significant correlations (intense bleu color) were found with fattening duration compared to animal activity where only one correlation was found (light bleu color).

Table 1. List of the 20 protein biomarkers quantified 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 as in Picard *et al.* [26] and Gagaoua *et al.* [11, 27].

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
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
Oxidative proteins			
Peroxiredoxin6 (*PRDX6*)	**P30041**	Mo. anti-human Abnova PRDX6 (M01), clone 3A10-2A11	1/500
Structural proteins			
MLC-1F (*MYL1*)	**P05976**	Po. anti-human Abnova MYL1 (A01)	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

Table 2. Muscle, gender and muscle x gender interaction effects on the 20 beef tenderness and intramuscular fat proteomic biomarkers.

Proteins[1]	G	Muscle (M)[2]					Gender (G)		P-values[3]		
		TB	ST	RA	SM	LT	Cows (C)	Steers (S)	M	G	M*G
CRYAB	C	-0.15^{bc}	-0.62^{d}	1.03^{a}	-0.21^{c}	-0.02^{b}	0.03	-0.09	***	ns	ns
	S	-0.18^{b}	-0.67^{c}	0.57^{a}	-0.35^{b}	-0.06^{b}			***		
Sign.[4]		ns	ns	**	ns	ns					
HSP20	C	-0.23^{c}	-0.25^{c}	0.29^{a}	0.01^{b}	0.17^{a}	0.05^{a}	-0.33^{b}	***	***	**
	S	-0.34^{b}	-0.78^{b}	0.16^{a}	-0.46^{b}	-0.42^{b}			**		
Sign.		ns	***	ns	***	***					
HSP27	C	-0.06^{b}	-0.08^{b}	0.61^{a}	-0.44^{c}	-0.04^{b}	0.03	-0.14	***	ns	ns
	S	-0.19^{b}	-0.27^{b}	0.27^{a}	-0.65^{c}	-0.05^{b}			***		
Sign.		ns	*	*	*	ns					
HSP70-1A	C	-0.20^{c}	-0.36^{c}	0.28^{a}	0.17^{ab}	0.08^{b}	-0.02	0.05	***	ns	ns
	S	-0.02	-0.17	0.17	0.01	0.15			ns		
Sign.		ns	ns	ns	ns	ns					
HSP40	C	-0.11	0.02	-0.05	0.06	-0.11	-0.05	0.03	ns	ns	ns
	S	-0.03	0.30	0.09	0.12	-0.01			ns		
Sign.		ns	*	ns	ns	ns					
FHL1	C	0.12	-0.16	0.04	-0.03	0.01	-0.01	0.07	ns	ns	ns
	S	0.13	0.03	0.14	-0.05	0.13			ns		
Sign.		ns	ns	ns	ns	ns					
TRIM72	C	0.41^{a}	-0.08^{b}	0.01^{b}	-0.11^{b}	0.32^{a}	0.13	0.04	***	ns	ns
	S	0.34^{a}	-0.20^{c}	-0.04^{bc}	-0.07^{bc}	0.11^{b}			***		
Sign.		ns	*	ns	ns	**					
PRDX6	C	0.16^{a}	0.12^{ab}	-0.03^{b}	0.26^{a}	-0.33^{c}	0.00^{b}	0.23^{a}	***	**	ns
	S	0.37^{a}	0.35^{a}	0.33^{a}	0.38^{a}	-0.07^{b}			*		
Sign.		ns	ns	**	ns	*					
MDH1	C	0.09	0.04	0.01	-0.11	0.07	0.04^{a}	-0.09^{b}	ns	*	ns
	S	-0.01	-0.08	-0.15	-0.14	-0.09			ns		
Sign.		ns	ns	ns	ns	ns					
PYGB	C	0.08	0.11	-0.02	0.05	0.01	0.04	0.07	ns	ns	ns
	S	0.06	0.08	0.07	0.18	-0.08			ns		
Sign.		ns	ns	ns	ns	ns					
PGK1	C	0.11^{b}	0.39^{a}	-0.95^{c}	0.35^{a}	0.11^{b}	-0.06^{b}	0.30^{a}	***	***	ns
	S	0.33^{b}	0.83^{a}	-0.52^{c}	0.59^{ab}	0.39^{b}			***		
Sign.		*	***	*	*	*					
ALDOA	C	-0.04^{b}	0.26^{a}	-0.24^{c}	0.16^{a}	-0.02^{b}	0.04^{a}	-0.11^{b}	***	**	ns
	S	-0.03^{ab}	0.25^{a}	-0.25^{b}	0.02^{ab}	-0.27^{b}			**		
Sign.		ns	ns	ns	ns	*					
ALDH1A1	C	-0.16^{bc}	-0.07^{b}	0.73^{a}	-0.28^{c}	-0.15^{bc}	0.00	0.14	***	ns	ns
	S	-0.06^{b}	0.11^{b}	0.67^{a}	-0.03^{b}	0.05^{b}			***		
Sign.		ns	ns	ns	*	ns					
ENO3	C	0.22^{bc}	0.58^{a}	-1.22^{d}	0.33^{b}	0.10^{c}	-0.03	0.14	***	ns	ns
	S	0.17^{b}	0.76^{a}	-0.70^{c}	0.31^{b}	0.27^{b}			***		
Sign.		ns	*	**	ns	ns					
TPI1	C	0.04^{c}	0.55^{a}	-1.02^{d}	0.31^{b}	-0.03^{c}	-0.08^{b}	0.18^{a}	***	*	ns
	S	0.11^{b}	0.86^{a}	-0.56^{c}	0.45^{b}	0.24^{b}			***		
Sign.		ns	**	*	ns	ns					

[1] Least-square means in the same row with different superscript letters are significantly different ($P<0.05$).

[2] Muscle abbreviation:

TB: *Triceps brachii*; ST: *Semitendinosus* ; RA: *Rectus abdominis* ; SM: *Semimembranosus* ; LT: *Longissimus thoracis*

[3] Significances: ns: not significant; * $P<0.05$; ** $P<0.01$; *** $P<0.001$

[4] Gender effect significance on the proteins by muscle.

Table 2. *Continued*

Proteins[1]	G	Muscle (M)[2]					Gender (G)		P-values[3]		
		TB	ST	RA	SM	LT	Cows (C)	Steers (S)	M	G	M*G
TTN	C	0.30^{a}	-0.33^{c}	-0.05^{b}	-0.31^{c}	0.34^{a}	-0.01	0.04	***	ns	ns
	S	0.34^{a}	-0.32^{b}	-0.20^{b}	-0.05^{b}	0.22^{a}			**		
Sign.		ns	ns	ns	ns	ns					
MHC-IIX	C	0.27^{b}	0.75^{a}	-0.91^{d}	0.06^{b}	-0.21^{c}	0.03^{a}	-0.24^{b}	***	*	ns
	S	0.08^{a}	0.30^{a}	-0.83^{b}	-0.07^{a}	-0.54^{b}			***		
Sign.		ns	**	ns	ns	ns					
MLC1F	C	0.26^{ab}	0.39^{a}	-0.56^{c}	0.08^{b}	0.09^{b}	0.06	-0.02	***	ns	ns
	S	0.20^{a}	0.24^{a}	-0.54^{b}	0.09^{a}	0.09^{a}			***		
Sign.		ns	ns	ns	ns	ns					
TNNT1	C	0.09^{b}	-0.97^{d}	0.88^{a}	-0.13^{c}	0.08^{b}	-0.02^{b}	0.19^{a}	***	*	ns
	S	0.27^{b}	-0.76^{c}	0.87^{a}	0.05^{b}	0.28^{b}			***		
Sign.		ns	ns	ns	ns	*					
α-Tubulin	C	0.05^{a}	-0.03^{ab}	0.10^{a}	-0.02^{ab}	-0.13^{b}	0.01	-0.08	**	ns	ns
	S	0.05	0.03	-0.01	-0.16	-0.27			ns		
Sign.		ns	ns	ns	*	ns					

[1] Least-square means in the same row with different superscript letters are significantly different ($P<0.05$).

[2] Muscle abbreviation:

TB: *Triceps brachii*; ST: *Semitendinosus* ; RA: *Rectus abdominis* ; SM: *Semimembranosus* ; LT: *Longissimus thoracis*

[3] Significances: ns: not significant; * $P<0.05$; ** $P<0.01$; *** $P<0.001$

[4] Gender effect significance on the proteins by muscle.

Table 3. Variance analyses of the rearing practices and muscle x rearing practices interaction effects on the 20 beef tenderness and intramuscular fat proteomic biomarkers for cows and steers.

Proteins[1]	Gender	Effects[3]	
		Rearing practices	Rearing practices x muscle
CRYAB	Cows (C)	ns	ns
	Steers (S)[2]	ns	ns
HSP20	C	0.073	ns
	S	ns	ns
HSP27	C	ns	ns
	S	ns	ns
HSP70-1A	C	ns	ns
	S	ns	0.093
HSP40	C	ns	ns
	S	ns	ns
FHL1	C	ns	ns
	S	ns	ns
TRIM72	C	ns	ns
	S	ns	ns
PRDX6	C	0.019	ns
	S	ns	ns
MDH1	C	0.088	ns
	S	ns	ns
PYGB	C	ns	0.087
	S	ns	ns
PGK1	C	0.038	ns
	S	ns	ns
ALDOA	C	0.035	ns
	S	0.098	ns
ALDH1A1	C	ns	ns
	S	0.056	ns
ENO3	C	0.056	ns
	S	ns	ns
TPI1	C	ns	ns
	S	ns	ns
TTN	C	ns	ns
	S	ns	ns
MHC-IIX	C	ns	ns
	S	ns	ns
MLC1F	C	ns	ns
	S	ns	ns
TNNT1	C	ns	ns
	S	ns	ns
α-Tubulin	C	ns	ns
	S	ns	ns

[1] Least-square means in the same row with different superscript letters are significantly different ($P<0.05$).

[2] Only two rearing factors were identified for steers (Grass class (n = 5) and Haylage class (n= 10)).

[3] ns: not significant ($P > 0.1$).

Table 4. Differences in animal, rearing factors and carcass characteristics among the three identified rearing practices.

Variables	Grass class (n = 24)	Haylage class (n = 21)	Hay class (n = 41)	P-values[1]
Animal activity, %	78.79^{a}	2.81^{b}	5.29^{b}	***
Grass, %	19.10^{a}	0.80^{b}	0.53^{b}	***
Haylage, %	59.71^{b}	81.99^{a}	4.70^{c}	***
Hay, %	21.15^{b}	17.21^{b}	94.77^{a}	***
Total concentrate, kg	857	741	788	ns
Fattening duration, days	120.3^{a}	100.3^{b}	99.5^{b}	*
Age, months	64.50^{b}	65.19^{b}	71.22^{a}	t
Carcass weight, kg	461.33^{a}	434.10^{b}	462.24^{a}	*
Conformation score	3.54^{b}	4.10^{a}	3.85^{ab}	t

[1] Significances: ns: not significant; t $P < 0.1$; * $P<0.05$; *** $P<0.001$

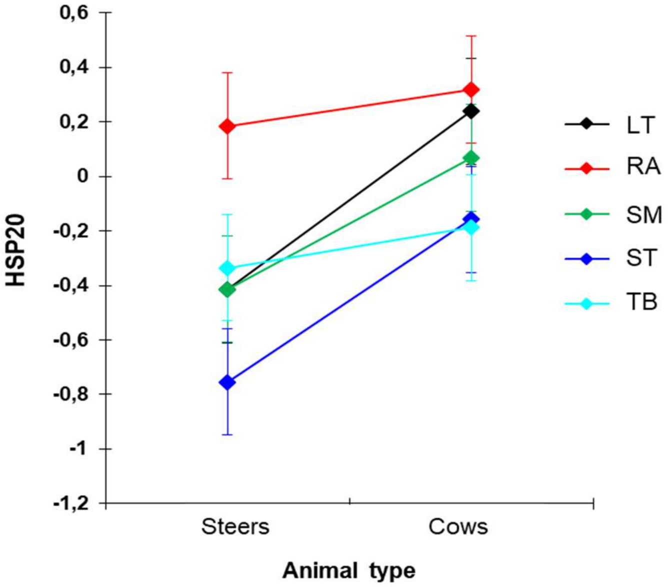

0,6
0,4
0,2
0
-0,2
-0,4
-0,6
-0,8
-1
-1,2
HSP20
Steers
Cows
Animal type
LT
RA
SM
ST
TB

Figure 1.

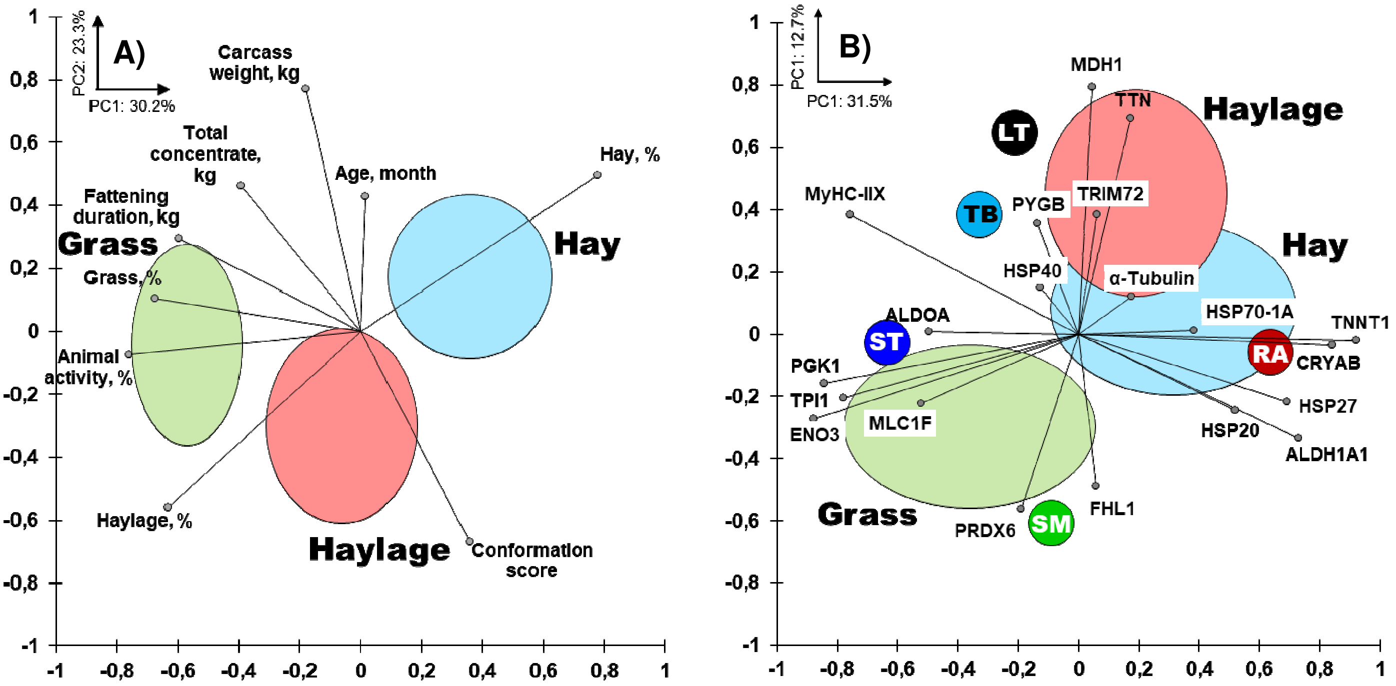

A)
PC2: 23.3%
PC1: 30.2%
Carcass weight, kg
Total concentrate, kg
Fattening duration, kg
Age, month
Hay, %
Grass
Grass, %
Hay
Animal activity, %
Haylage, %
Haylage
Conformation score
1
0,8
0,6
0,4
0,2
0
-0,2
-0,4
-0,6
-0,8
-1
-1
-0,8
-0,6
-0,4
-0,2
0
0,2
0,4
0,6
0,8
1
B)
PC1: 12.7%
PC1: 31.5%
MDH1
TTN
Haylage
LT
MyHC-IIX
TB
PYGB
TRIM72
Hay
HSP40
α-Tubulin
ALDOA
HSP70-1A
TNNT1
ST
RA
CRYAB
PGK1
TPI1
HSP27
MLC1F
HSP20
ENO3
ALDH1A1
Grass
FHL1
PRDX6
SM
1
0,8
0,6
0,4
0,2
0
-0,2
-0,4
-0,6
-0,8
-1
-1
-0,8
-0,6
-0,4
-0,2
0
0,2
0,4
0,6
0,8
1

Figure 2.

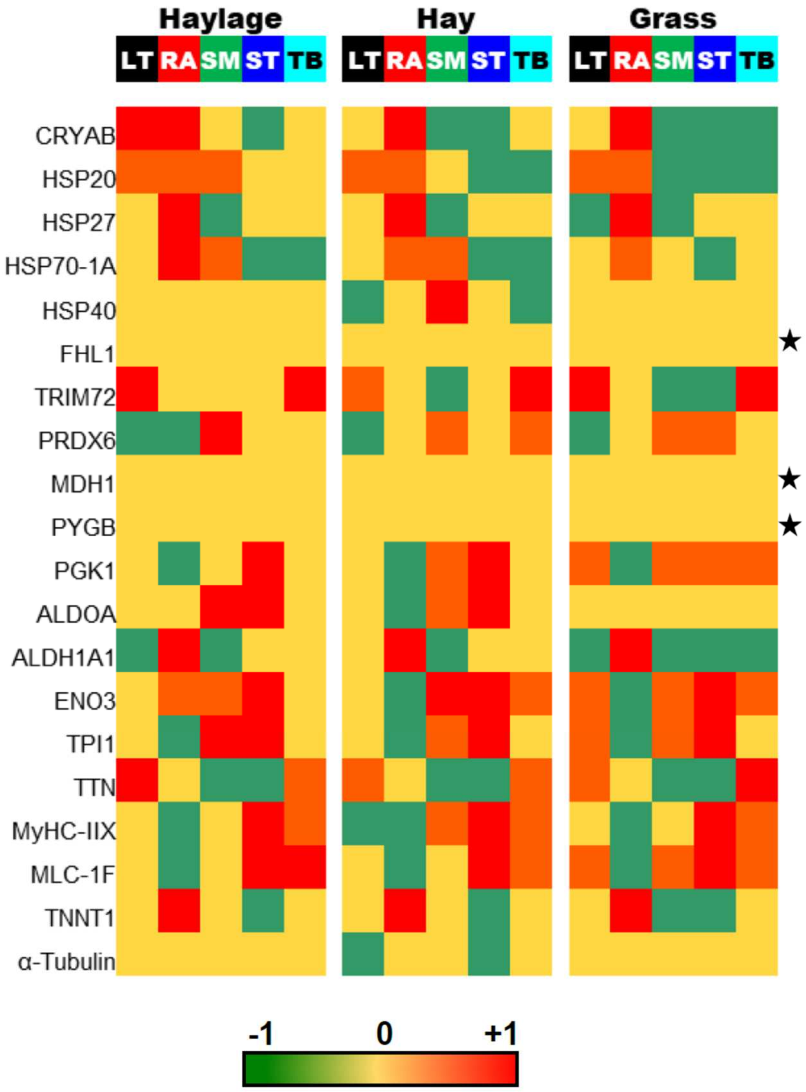

Haylage
Hay
Grass
LT
RA
SM
ST
TB
CRYAB
HSP20
HSP27
HSP70-1A
HSP40
FHL1
TRIM72
PRDX6
MDH1
PYGB
PGK1
ALDOA
ALDH1A1
ENO3
TPI1
TTN
MyHC-IIX
MLC-1F
TNNT1
α-Tubulin
★
★
★
-1
0
+1

Figure 3.

Figure 4.

	Animal activity, %				
	LT	RA	SM	ST	TB
CRYAB					
HSP20			-0,30		
HSP27					
HSP70-1A					
HSP40					
FHL1			0,32		
TRIM72		0,25			
PRDX6					
MDH1	-0,23	-0,28			
PYGB	-0,25				
PGK1					
ALDOA					
ALDH1A1					
ENO3					
TPI1	0,28				
TTN			-0,24		
MyHC-IIX		-0,24			
MLC-1F					-0,29
TNNT1					
α-Tubulin					

	Grass, %				
	LT	RA	SM	ST	TB
CRYAB					
HSP20				-0,24	
HSP27					
HSP70-1A	-0,25				
HSP40					
FHL1			0,37		
TRIM72					
PRDX6					
MDH1	-0,32				
PYGB	-0,24				
PGK1					
ALDOA				-0,23	
ALDH1A1			0,26		
ENO3					
TPI1	0,23				
TTN			-0,29		
MyHC-IIX					
MLC-1F					
TNNT1					
α-Tubulin					

	Haylage, %				
	LT	RA	SM	ST	TB
CRYAB					
HSP20					
HSP27					
HSP70-1A					
HSP40					
FHL1					
TRIM72		0,28			
PRDX6					
MDH1					
PYGB					
PGK1					
ALDOA	0,26	0,23			
ALDH1A1					
ENO3					
TPI1					
TTN					
MyHC-IIX					
MLC-1F					
TNNT1					
α-Tubulin					

	Hay, %				
	LT	RA	SM	ST	TB
CRYAB					
HSP20					
HSP27					
HSP70-1A					
HSP40					
FHL1			-0,23		
TRIM72		-0,24			
PRDX6					
MDH1					
PYGB					
PGK1					
ALDOA	-0,27	-0,25			
ALDH1A1					
ENO3					
TPI1					
TTN		0,24	0,25		
MyHC-IIX					
MLC-1F					
TNNT1					
α-Tubulin					

	Total concentrate, kg				
	LT	RA	SM	ST	TB
CRYAB					
HSP20					
HSP27					
HSP70-1A			-0,27		
HSP40					
FHL1					-0,31
TRIM72					
PRDX6					
MDH1		-0,28	-0,25	-0,29	
PYGB					
PGK1					
ALDOA					
ALDH1A1					
ENO3		0,27			
TPI1		0,22			
TTN				-0,29	
MyHC-IIX					
MLC-1F					
TNNT1					
α-Tubulin					

	EUROP conformation				
	LT	RA	SM	ST	TB
CRYAB					
HSP20					
HSP27	0,22				
HSP70-1A					
HSP40				-0,29	
FHL1					
TRIM72			0,23		
PRDX6					
MDH1			0,38		
PYGB					
PGK1			-0,34		
ALDOA					
ALDH1A1					
ENO3					
TPI1					
TTN			0,32		
MyHC-IIX					
MLC-1F		-0,23			
TNNT1					
α-Tubulin					

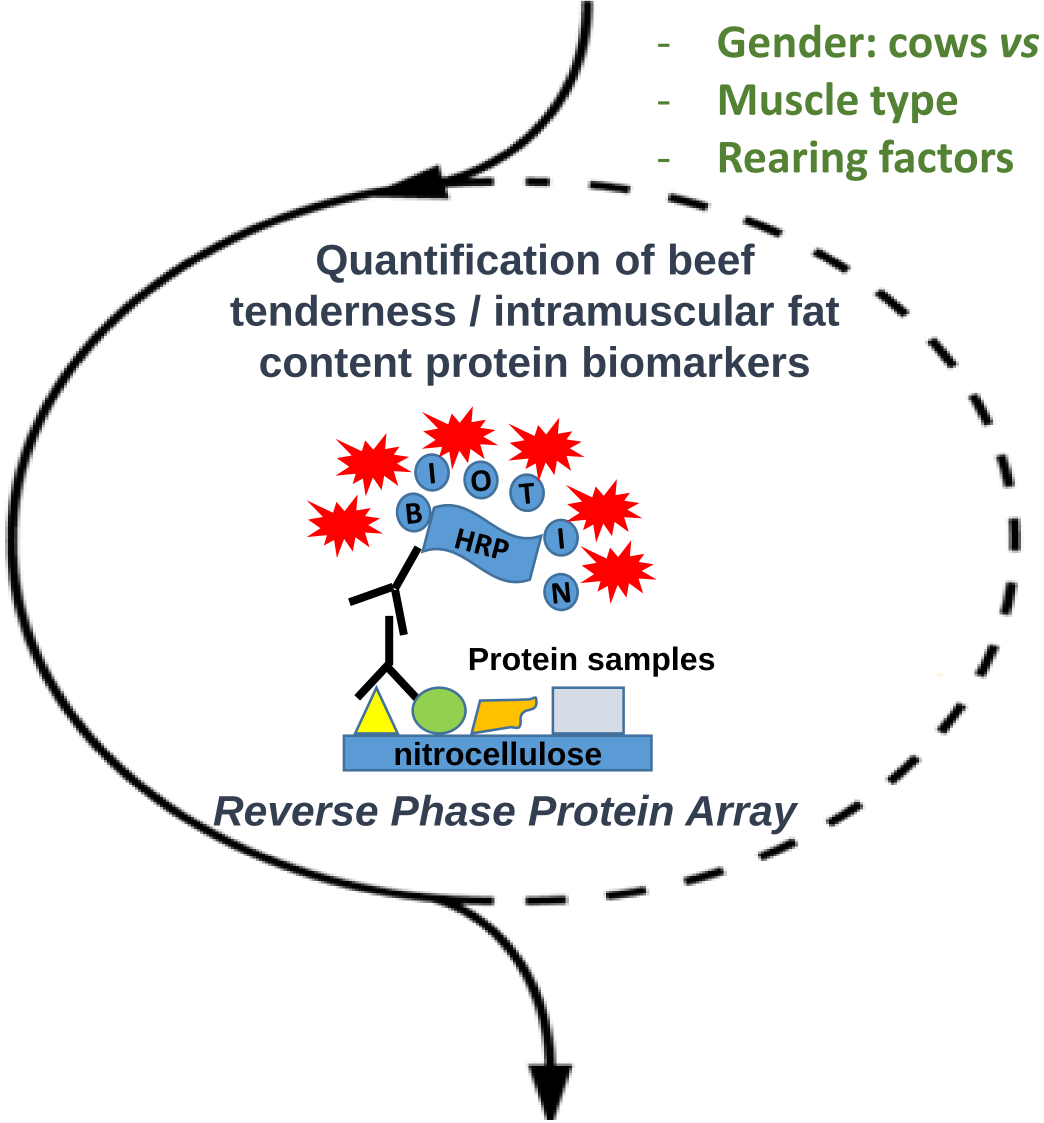
	Age at slaugher, months				
	LT	RA	SM	ST	TB
CRYAB					
HSP20	0,44	0,23	0,31	0,22	0,23
HSP27					
HSP70-1A					
HSP40					
FHL1		0,33			
TRIM72					
PRDX6				0,27	
MDH1					
PYGB					
PGK1					
ALDOA					
ALDH1A1		0,26		0,32	
ENO3					
TPI1					
TTN					
MyHC-IIX		-0,25			
MLC-1F					
TNNT1		0,26			
α-Tubulin					

	Carcass weight, kg				
	LT	RA	SM	ST	TB
CRYAB					
HSP20					
HSP27					
HSP70-1A	-0,24				
HSP40					
FHL1		0,26			
TRIM72			-0,25		
PRDX6				0,23	
MDH1	-0,27		-0,28		
PYGB					
PGK1					
ALDOA					
ALDH1A1				0,23	
ENO3					
TPI1					
TTN			-0,29		
MyHC-IIX					
MLC-1F					
TNNT1					
α-Tubulin					

	Fattening duration, days				
	LT	RA	SM	ST	TB
CRYAB					
HSP20					
HSP27					
HSP70-1A		-0,25			0,24
HSP40		0,24			
FHL1					-0,36
TRIM72				-0,29	
PRDX6					-0,26
MDH1	-0,37				
PYGB	-0,26			-0,22	-0,32
PGK1					
ALDOA					
ALDH1A1			0,39		
ENO3	0,28		-0,24		
TPI1					
TTN					0,29
MyHC-IIX					
MLC-1F					-0,29
TNNT1		-0,24			
α-Tubulin					

Effect of

- Gender: cows *vs* steers
- Muscle type
- Rearing factors



Quantification of beef tenderness / intramuscular fat content protein biomarkers

Reverse Phase Protein Array

Management of beef meat qualities by rearing practices