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# Contribution of connective tissue components, muscle fibres and marbling to beef tenderness variability in *Longissimus thoracis*, *Rectus abdominis*, *Semimembranosus* and *Semitendinosus* muscles

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**Abstract:**

**Background:** This study aimed to identify relationships between components of intramuscular connective tissue, the proportions of the different fiber types, intramuscular fat and sensory tenderness of beef cooked at 55°C. For that, four muscles differing in their metabolic and contractile properties as well as in their collagen content and butcher value, from dairy and beef cattle of several ages and sexes were used in order to create variability.

**Results:** Correlation analyses and/or stepwise regressions were applied on Z-scores to identify the existing and robust associations. Tenderness scores were further categorized into tender, medium and tough classes using unsupervised learning methods. The findings revealed a muscle-dependant role on tenderness of total and insoluble collagen, cross-links and of type IIB+X and IIA muscle fibers. The *Longissimus thoracis* and *Semitendinosus* muscles that, in this study, were extreme in their tenderness potential were very different from each other and of the *Rectus abdominis* (RA) and *Semimembranosus* (SM). RA and SM muscles were very similar for their relationship for muscle components and tenderness. A relationship was present between marbling and tenderness only when the results were analysed irrespective of all the factors of variations of experimental model that are muscle and animal type.

**Conclusion:** The statistical approaches applied in this trial performed using the z-scores allowed to identify the robust associations between muscle components and sensory beef tenderness and also to found discriminatory variables of beef tenderness classes.

**Keywords:** Collagen; Cross-links; Proteoglycans; Lipids; Sensory beef quality; Meat.

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## 1. Introduction

Beef tenderness is one of the most important quality attributes for consumers. However, it is often described as inconsistent, therefore affecting consumer satisfaction. It is assumed that variations in this quality trait result partly from the differences in muscle characteristics[1]. Among these characteristics, proportion of different types of muscle fibres, intramuscular connective tissue (IMCT) composition in total (TCol), insoluble (ICol) and soluble collagen (SCol) have been extensively investigated[1, 2]. Different chemical cross-links (CLs) stabilize the molecule of collagen and consequently are involved in collagen solubilization and its mechanical properties after meat cooking[3]. The main CLs in skeletal muscle are the pyridinolines. Total amount of these CLs present per volume of cooked meat was approximately proportional to the elastic modulus of collagenous fractions of connective tissue, suggesting the contribution of collagen CLs to meat toughness[4]. However, the role of CLs in beef tenderness is still controversial and not fully elucidated[5, 6]. The other main components of IMCT (after collagen) are the proteoglycans (PGs)[1]. PGs have an important role in tissue architecture and function by interacting with several collagen and non-collagen components and with water molecules to create a water compartment[7]. From these properties, few authors have suggested that PGs could contribute to meat texture[8-11] but only two studies have investigated their relation with beef tenderness[6, 12].

Marbling, sill called intramuscular fat content (IMF) develops inside IMCT during the fattening period of the animals. Accordingly, earlier studies reported that in highly marbled beef (>10% of IMF), the structure of IMCT was modified and that meat was judged very tender[5]. However, in less marbled beef, the role of IMF on beef tenderness is unclear and the relationships were mostly curvilinear[13] and weak[2].

Muscle fibers, IMCT composition and IMF role on beef tenderness has been studied by several authors but often in an independent manner of each other or two by two factors but never all together. From those studies, we can retain that the relations between the components of the muscle and tenderness are muscle dependent[12], strong and consistent in their direction, weak or insignificant and furthermore do not depend on a single component only but would rather be multifactorial[3, 14, 15].

The aim of the present study was thus to determine the relationships between sensory tenderness and IMCT components (TCol, ICol and Scol as classical measurements) and others less often studied such as CLs and PGs, the proportions of fiber types and IMF. In an attempt

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of creating variability representative of French cattle breeding, the experimental design of this trial consisted of four muscles differing in their metabolic and contractile properties as well as in their collagen content and butcher value from to animal categories that are dairy and beef cattle of different ages and sexes.

## 2. Material and methods

### *2.1. Experimental design: beef production and muscle sampling*

The experiment was performed on four muscles that were sampled from the same animals [*Longissimus thoracis* (LT), n = 48; *Rectus abdominis* (RA), n = 48; *Semimembranosus* (SM), n = 36 and *Semitendinosus* (ST), n = 36] of dairy (Holstein breed) and beef (Charolais breed) animals. The dairy animals were cull cows (66%), steers (27%) and young bulls (8%); and the beef animals were cull cows (35%), young bulls (26%) and heifers (39%). The 48 animals came from 16 different farms but were all slaughtered in the same abattoir (Le Lion d'Angers, France, license number #49 176 001) following the same conditions for slaughter and carcasses management.

The muscles were removed from carcasses 48h *post-mortem* using the same protocol, between the 5$^{th}$ and 10$^{th}$ rib for LT and from the middle part of the muscle for ST, SM and RA [16]. Each muscle was divided into two parts. The first one was stored under vacuum for 7 days for ageing, and then stored at -20°C until sensory analysis. The second part (about 150 g) was carefully cut into pieces of 1 cm cross-section avoiding any contamination, sealed under vacuum in plastic bags and stored at -20°C until preparation for biochemical analyses.

### *2.2. Biochemical characteristics of intramuscular connective tissue*

For total collagen (TCol) and insoluble collagen (ICol) contents, crosslinks (CL) and proteoglycans (PG) measurements, frozen muscles were homogenized in a household cutter, freeze-dried for 48 h, pulverized in a horizontal blade mill and stored at +4°C in stopper plastic flasks until analyses.

#### *2.2.1. Total, insoluble collagen and cross-link measurements*

For TCol and CLs, about 200 mg of muscle powder were weighed in duplicate, acid hydrolysed with 10 mL of 6 N HCl overnight at 110°C in a screw-capped glass tube. The acid hydrolysate was diluted 5 times in 6 N HCl and the subsequent procedure used was that previously described and updated[17]. For CLs, 1 mL of the 6N acid hydrolysate was centrifuged

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at 16,000 x g for 5 min at + 4°C. For ICol, muscle powder (250 mg) was weighted in duplicate and rehydrated for one hour with 1X solubilization buffer containing 0.23 M NaCl, 25 mM Tris–HCl, pH 7.4 and heated in a water bath at 75°C for one hour. The soluble fraction was separated from the insoluble fraction by filtration (pleated filters in cotton cellulose, VWR 512–0206) and discarded. Insoluble fraction was hydrolysed according to the same method as for TCol. For TCol and ICol data were expressed in mg of hydroxyproline per g of dry matter (mg OH-pro $g^{-1}$ dry matter (DM). The soluble collagen was determined as following: Soluble Collagen (SCol) = (TCol-ICol)/TCol)* 100.

For CLs, 600 µL 6 N NaOH and 600 µL 1 M Tris were added to 600 µL acid supernatant. Final pH was adjusted between 7 and 8 by adding some drops of 6 N HCl or NaOH. Pyridinoline cross-links (pyridinoline + deoxypyridinoline) were determined by the enzyme-linked immunoassay Metra Pyd EIA kit (Quidel Corporation, USA) according to the manufacturer and adapted by our group to the muscular tissue[17]. The results were then expressed in nM of pyridinoline per g of dry matter (nM pyr $g^{-1}$ DM).

### *2.2.2. Total proteoglycan (PGs) content*

For total PGs, the procedure used was that previously described and updated[17]. Briefly, muscle powder was incubated 24 h at +4°C in the extraction buffer containing 6 M Urea, 1 M NaCl, 2% CHAPS and protease inhibitor cocktail (Complete, Roche Diagnostics GmbH, ref. 11 836 145 001). The solid to liquid ratio was 100 mg of muscle powder to 1 mL of extraction buffer. The following day, the samples were centrifuged 40 min at +4°C, 15 000 xg. The supernatant (muscle extract) was recovered and used to determine PGs content. This assay was based on the ability of sulphated glycosaminoglycans (GAGs) to bind the cationic dye 1,9-dimethylmethylene blue (DMMB). Thus, 1 mL of DMMB solution was added in excess to 100 µL of muscle extract and shaken 30 min at room temperature. After centrifugation for 15 min at 12 000 x g, the supernatant (DMMB excess) was removed. One mL of 50 mM sodium acetate buffer solution was then added to the residue and shaken for 30 min. Subsequently, the absorbance was measured at 656 nm using a micro-plate reader (TECAN Infinite® M200). The concentrations were determined by comparison to a determined standard curve by chondroitine-4-sulfate, ranging from 0 to 2.5 µg of C4S. Each sample was measured twice and data were expressed in µg of GAGs per g of dry matter (µg GAGs $g^{-1}$ DM).

### *2.2.3. Myosin heavy chains isoforms quantification by electrophoresis*

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Myosin heavy chain isoforms were separated with an adapted SDS-PAGE electrophoresis[18]. Briefly, 100 mg of frozen muscle was ground using a Polytron in 5 mL of extraction buffer solution (0.5 M NaCl, 20 mM Na Pyrophosphate, 50 mM Tris, 1 mM EDTA and 1 mM Dithiothreitol). The samples were kept 10 min at 4 °C on ice, and then centrifuged for 5 min at 5,000xg. Following centrifugation, the supernatant was diluted 1:1 (v/w) with glycerol at 87% and stored at −20 °C until used. The samples were then mixed with an equal volume of loading buffer (4% SDS (w/v), 125 mM Tris, pH 6.8, 20% glycerol (v/v), 10% β mercaptoethanol (v/v), 0.02% pyronin Y (w/v)) incubated at room temperature 10 min and then heated (70 °C) 10 min. The proteins were separated using 9.2% polyacrylamide gels (the lower running buffer consisted : 50 mM Tris (base), 75 mM glycine and 0.05% w/v SDS; the upper running buffer 2× the concentration of the lower running buffer added of β-mercaptoethanol (0.07% v/v)) [18]. Ten micrograms of protein extracts were loaded per well onto 0.75-mm-thick gels mounted on a Mini-Protean II Dual Slab Cell electrophoretic system (Bio-Rad). The migration was carried out at 4 °C at a constant voltage of 70 V for 30 h. After migration, the gels were fixed in 30% (v/v) ethanol and 5% acetic acid (v/v) and then stained with colloidal Coomassie Blue R250 for 24 h. Gels were destained in a 30% ethanol (v/v) and 5% acetic acid (v/v) solution until the background was sufficiently cleared. After staining, the gels were scanned and the proportions of the different MyHC bands were quantified by densitometry with ImageQuant Software5500 (Amersham Biosciences/GE Healthcare). MyHC-IIB isoform was found in only 2 animals, thus, MyHC-IIB percentage was totalised with those of MyHC-IIX creating a new variable "MyHC-IIX+B".

#### *2.2.4. Intramuscular fat content*

Total lipid content of LT, RA, SM and ST was estimated by near infrared spectroscopy (NIRS) according to the procedure previously described by Guy *et al.*[19]. Briefly, muscle samples (about 5 g of muscle lyophilized powder) were scanned in a circular cup (diameter 50 mm, depth 10 mm) (Part number IH – 0307, NIRSystems, Infrasoft International, South Atherton St. State College, PA 16801, USA), compressed and sealed with a disposable paper-backed wrap. Samples were scanned in reflectance mode (400 – 2500 nm) in a NIRS 6500 scanning monochromator (NIRSystems, Silver Spring, MD, USA) using ISI software, version 3.01 (Infrasoft International, South Atherton St. State College, PA 16801, USA) equipped with a spinning module. Reflectance data were recorded at 2 nm intervals and stored as log (1/reflectance). Then the reflectance data were exported into WinISI II version 1.60 (Infrasoft International, South Atherton St. State College, PA 16801, USA) which was used to estimate

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IMF values. A NIRS model was used to analyse the data based on spectra and biochemical analyses performed on 48 samples used in the current study. For the biochemical analyses, muscle dry matter was assayed gravimetrically after drying at 80°C for 48 h. Then, total lipids were extracted by mixing 6 g of muscle powder with chloroform-methanol[20]. Each sample was measured in triplicate and data were expressed in g per 100 g of dry matter (g/100 g DM). The statistical parameters (coefficient of determination and standard error of prediction) of the prediction model were 0.92 and 1.19 g/100 g of fresh matter respectively.

### *2.3. Sensory analysis*

After thawing at 2 to 5 °C in vacuum packs for at least 24 h before cooking, muscles were cut into pieces of 3 cm cross-section and cooked in an oven at 250°C. They were removed at an internal temperature of 55°C, a medium rare cooking usually used in France[21]. The samples were presented in sequential monadic sessions involving 12 panellists. The expert panellists were trained in accordance with the ISO standards ISO/TC 34 as described by Gagaoua *et al*.[22]. Thus, the panellists rated global tenderness of the grilled meat on a 10 cm unstructured line scale (from 0 to 10), where 0 refers to extremely tough and 10 to extremely tender meat. 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. Each tasting booth was equipped with computer terminals linked to a fileserver running a sensory software (Fizz v 2.20 h, Biosystemes, Couternon, France) that facilitated the direct entry of assessor ratings.

### *2.4. Statistical analysis*

The statistical analyses were performed using XLSTAT 2017.19.4 software (AddinSoft, Paris, France). First, normal distribution and homogeneity of the dataset was tested by Shapiro-Wilk test ($P > 0.05$). It is worthwhile to note that there is scarcity in the publications studying the muscle components and sensory quality traits of dairy animals. So, although experimental design was not developed to compare dairy and beef, the differences in sensory tenderness scores among muscles (M) and animal types (AT) (dairy and beef) were investigated using a GLM model at a significance level of 5%. The differences in biochemical composition between the M and AT were presented elsewhere[16]. For any comparisons including interactions, Student–Newman–Keuls (SNK) was used to separate the least-squares means (LSmeans). Differences were considered to be statistically significant if $P < 0.05$. The distribution of the variables of interest to investigate the trend of differences among M and AT was illustrated by a principal component analysis (PCA), performed on raw data, by considering all the factors

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together including tenderness as a supplementary variable. Two factors with eigenvalues >1.0 were considered in this PCA on the basis of the scree plot and evaluation of the factor loading matrix after orthogonal rotation following the procedure described by Gagaoua *et al.*[23]. An eigenvalue represents the amount of variance that is captured by a given component. Eigenvalue criterion is the main criteria used for solving the number of components problem, also known as the Kaiser-Guttman criterion (Kaiser, 1974). Accordingly, to check the suitability of the factorial model, the Kaiser-Meyer-Olkin (KMO) measure, known also as Kaiser's Measure of Sampling Adequacy (MSA) was applied to test sampling adequacy was used. The overall KMO value of the PCA was 0.72, which is acceptable [24].

Z-scores were computed in a stepwise manner using multivariate regression analyses to consider the fixed effects of M and to avoid the effects due to the other factors of variations present in the database by taking into account the differences in the number of animals for LT and RA muscle compared to SM and ST. Z-scores represent the deviation of each trait observation relative to the mean of the corresponding animal irrespective of the breed, experiment and other confounded effects and were calculated using PROC STANDARD of SAS, which standardizes data to a mean of 0 and standard deviation of 1. Subsequently and using the Z-scores, Pearson correlations were performed to investigate simple relationships between muscle characteristics and sensory tenderness. Also, multiple regression analyses were applied to propose regression equations explaining the overall tenderness among the investigated factors. The relationships were studied **i)** irrespective of the M and of other factors of variation; **ii)** for each M, irrespective of the other factors of variation. For regression analysis, the "optimal model" explaining maximal variability option was used[25]. The percentage of variability in meat tenderness explained by the explanatory variables was based on the $r^2$ value of the regression analysis x 100.

To identify the variables most discriminating potential tenderness of the steaks, a clustering analysis was performed on the whole data[14]. For that and according to the work by Gagaoua *et al.* [14], several unsupervised learning methods: *k*-means, partitioning around medoids (PAM) and hierarchical clustering analysis (HCA) were tested. *K*-means was the method retained, because it gave the best results based on the homogeneity of the classes determined by the average silhouette width (*Si*) criterion[26, 27]. Silhouette width refers to a succinct graphical representation method for interpretation and validation of consistency within clusters of data. It is a measure of how similar an object is to its own cluster (cohesion) compared to others[26]. The value of silhouette width ranges from −1 to +1, and can be interpreted as follow: i)

observations with a large *Si* (almost 1) are very well clustered; ii) a small *Si* (around 0) means that the observation lies between two clusters and iii) observations with a negative *Si* are probably placed in the wrong cluster. After clustering, the tenderness classes were compared for the whole variables described above using variance analysis at a significant level of 5%. This allowed identifying the main splitters of tenderness groups.

In this trial, relationships between muscle components and tenderness were considered robust when they were present in at least 3 of 4 muscles and the two AT or that parameters in relationships with tenderness were found by both correlation and regression analyses and those variables distinguishing the three tenderness classes.

## 3. Results

### *3.1. Description of structural, biochemical and quality attributes*

Table 1 summarized the means, standard deviations, coefficient of variation and range of the variables. Table 2 showed a high M and AT effects ($P<0.001$) on LSmeans of sensory tenderness. The average scores of tenderness ranged from 4.5 ± 1.4 for ST muscle to 6.4 ± 1.0 for LT muscle ($P<0.001$), with a difference of +29%. The tenderness score of SM muscle (4.6 ± 0.8) was equivalent to that of ST. The tenderness of RA muscle was intermediate (5.6 ± 1.1) and significantly different of that of LT and of SM and ST scores ($P<0.001$). The general trend of tenderness among muscles was: LT>RA>SM=ST. The muscles of beef cattle were on average +11% more tender ($P<0.001$) with a mean value score of 5.6 ± 0.2 than those of dairy cattle (mean value of 4.9 ± 1.4, Table 2). An interaction M x AT ($P<0.05$) was observed. This interaction was mainly explained by the differences of tenderness of RA, SM and ST muscles of dairy and beef animals. Tenderness of ST and SM muscles were scored higher for beef than for dairy animals (Fig. 1) while LT muscle tenderness was equivalent.

The PCA, performed on raw data, illustrated the relationships between muscle components and tenderness (Fig. 2A) as well as the general trend of differences between M (Fig. 2B) and AT (Fig. 2C). Together, the two first axis of the PCA explained 55.77% of the variability. First PC accounting for 29.91% of variability was positively related with TCol and ICol and their CLs and negatively with tenderness. The PC2 accounting for 25.86% of variability was positively related with IMF, type I and IIA muscle fibers and negatively with TPGs and type IIX+B muscle fibers. The bi-plot highlighted the separation in the first two PC of the four muscles (Fig. 2B). ST samples were characterized by their content in TCol, ICol and CLs; RA samples by their proportion in oxidative muscle fibers (type I and IIA) and IMF content; LT

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samples by their content in IMF and SCol; SM samples by their content in TPGs and glycolytic (IIX+B) muscle fibers. Individual scores of first and second axes averaged by muscles showed that the LT and ST muscles were very different from each other while the RA and SM muscles had similar characteristics.

The bi-plot highlighted the separation in the first two PC of the two categories (Fig. 2C). The samples of dairy animals were mostly characterized by greater collagen (TCol, ICol and their CLs), IMF and proportions of oxidative muscle fibers (I and IIA). Samples from beef animals were mainly located in the left part of the bi-plot, characterised by greater TPGs content and proportions of glycolytic muscle fibers (IIX+B). For individual scores averaged by animal type, none of the axes discriminated efficiently the beef from dairy animals. The distribution on the first axis highlighted that variability of measured muscle characteristics was higher for dairy than beef animals.

### *3.2. Associations between sensory tenderness scores and muscle characteristics*

#### *3.2.1. Correlation analyses*

Irrespective of the M and other factors of variation, TCol, ICol and CLs were negatively and significantly correlated with tenderness ($P$>0.001) while SCol was positively correlated (Table 3). The negative correlation with tenderness remained unaffected for ICol, in the LT, RA and SM muscles. For TCol and CLs, the negative correlation with tenderness remained unaffected for RA, SM and ST muscles. Positive correlation with tenderness remained unaffected for SCol in ST muscle. TPGs were positively correlated with tenderness but only in ST muscle. Type I muscle fibers were positively correlated with tenderness irrespective of the M and other factors. This correlation was absent when analysed by muscle irrespective of the other factors. Type IIX+B muscle fibers were positively correlated with tenderness in LT, RA and SM muscles. Type IIA muscle fibers were negatively correlated with tenderness in RA, SM and ST muscles. IMF was positively correlated with tenderness irrespective of muscle and the other factors but this correlation was absent when analysed by muscle irrespective of the other factors. The muscle characteristics that most often correlated with tenderness (Table 3) were IMCT parameters (TCol, ICol and CLs, 4 times) then type IIA and IIX+B muscle fibers (3 times).

#### *3.2.2. Regression analyses*

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Irrespective of muscle and other factors, the regression equations explained 39% of tenderness variability ($P$<0.001) and retained 5 variables: ICol and CLs (negative impact on tenderness) and SCol, type I muscle fibers and IMF (positive impact on tenderness). When muscles were individually considered irrespective of the other factors, the best models explained respectively 18%, 17%, 31% and 57% of tenderness variability of LT, RA, SM and ST muscles. Among all the regression equations, ICol was retained 4 times. The other variables (except TCol and TPGs retained in any of the models) were retained once or twice (Table 4).

### *3.3. Clustering of tenderness into classes*

The *k*-means clustering method allowed the identification of 3 tenderness classes that significantly differ ($P$<0.001): class 1 (tender), class 2 (medium) and class 3 (tough) (Table 5). The tender class (1) grouped 39.88% of the steaks and had an average score of 6.61 ± 0.12. The medium tenderness class (2) grouped 40.47% of the steaks with an average score of 5.05 ± 0.09. The tough class (3) grouped the lowest number of the steaks (19.64%) and had an average score of 3.67 ± 0.09.

The tender class was mainly composed of 76 % LT muscle, 37 % RA and of equivalent proportion of SM and ST muscles (16 %), and the whole from 33 % of dairy and 43 % of beef cattle. The medium class was composed of 23% LT, 52 % of RA, 55 % SM and 33 % ST across animal types from 35% dairy and 45 % beef animals. Finally, the tough class was composed of 50% ST, 27 % SM, 10 % RA from 31% of dairy and 10% of beef animals (Fig. 3A,B).

The 3 tenderness classes were compared for the muscle characteristics (Table 5). Among the muscle characteristics, the CLs was the only variable to differ significantly ($P$<0.001) among the tenderness classes. The lowest values of CLs were found in the tender meat class. For the other variables, namely TCol and the TPGs, they were the lowest in tender class and equal for medium and tough classes. IMF content was the highest in tender class and similar for the two other classes. SCol distinguished tough samples from the others and was the lowest for tough class. Finally, the muscle fibres were not different among the classes.

## 4. Discussion

The experimental design of this study was realized to create variability in sensory tenderness among the steaks. Also, we selected the muscles based on their tenderizing profile, their contractile and metabolic properties and their collagen content. This goal was achieved

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since on a scale of 0 to 10, the samples used had a tenderness ranging from 1.91 to 8.45. The muscle characteristics measured presented also a large range of variability.

### *4.1. Differences of tenderness among the muscles*

In this trial, LT and RA muscle were mostly classified as tender and intermediate meat while SM and ST were equivalent in tenderness and likely classified as tough muscles. For the LT and RA muscles, these differences were in agreement with earlier studies[28, 29]. For the SM and ST, some studies described that ST was more tender than SM [28, 30, 31] and others, described the opposite[29, 32]. The findings of this study confirmed that meat of dairy was on average tougher than meat of beef cattle. In contract to our results, an earlier study on cull cows of dairy and beef, the overall meat tenderness was found not different when the animals were slaughtered at the same age, fattening duration and fatness score[32]. On the contrary, other studies have shown that shear force of raw meat from Holstein, a dairy breed, was higher than that of young Charolais or Limousin (two beef breeds) bulls[33]. It is worthy to note that after 10 days ageing and cooking at an end-point temperature of 75°C internal temperature, the shear force of meat of these breeds was similar. However, there are very few data in the literature that addressed this point. The findings of the present trial further showed that meat of dairy types presented a greater variability of tenderness than beef types for a period of ageing of 7 days and a cooking temperature of 55°C. This is in line to the literature describing a muscle-dependency[34]. Accordingly, the cluster analysis of tenderness revealed that 31% of meat cuts of dairy animals were considered tough by the sensory panel against only 11% for beef category. Within muscles, the meat of dairy animals could be, as least in this study, as tender as meat of beef animals. These results need further validation on a larger number of animals and on a model that have less variability than this trial.

### *4.2. Relationships between muscle components and meat tenderness*

The findings of this trial highlighted the impact of different components of IMCT (TCol, ICol, SCol, CLs and PGs), of muscle fibre types (I, IIA, IIX+B) and IMF on beef tenderness. Overall, TCol, ICol and their CLs were negatively correlated with tenderness, this result remaining unaffected in at least three of the four studied muscles. The two statistical approaches that are correlation and regression analyses used to investigate the relationships between the muscle components and tenderness focused on a leading role of TCol, ICol and CLs, in RA and SM muscles. For the LT muscle, only ICol was correlated with tenderness and retained in the regression equations. For ST muscle, a more glycolytic muscle, the PGs were

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involved in tenderness including TCol, CLs and SCol. In addition to be involved in tenderness of RA, SM and ST muscles, CLs were the only components of IMCT to be significantly different between the three tenderness classes. This result was coherent, since we previously found in the experimental same design, that ICol and CLs were highly correlated irrespective of muscle[16]. CLs are known to be involved in collagen solubility determinism[35]. The only muscle for which the CLs were not correlated with tenderness was LT muscle, result that we expect related to the fact that this muscle had the lowest CLs content. On the contrary, an earlier study revealed in LT muscle a negative correlation between CLs and tenderness[17].

Although many groups investigated the relationship between the composition of connective tissue in TCol, ICol, CLs and meat tenderness, the conclusions have not been clearly established[4, 36, 37]. This can be due to several reasons including the large variability of tenderness within muscle, time of ageing, cooking method, as well as to rearing practices of the animals[13, 38]. Overall, the authors did not always specify the location of sampling within the muscle, that is very important[39] nor the exact conditions of ageing and method or conditions of cooking. When they do, the cooking and ageing conditions vary so much from one study to another that it is difficult to compare the results. It is worthwhile to cite that tenderness evaluation by sensory panels depend also on the consumer habits and preferences[21, 22]. Moreover, it is rare that the authors work on standardized data as we did in this experiment. This approach allowed to test the robustness and accuracy of the associations by investigating if they exist independently of the fixed effects of factors of variation of the model[40].

Unlike TCol, ICol and CLs, few authors investigated the relationships between TPGs and tenderness. To our knowledge, only two studies evaluated TPGs in the context of meat quality[6, 12]. In the present study, the correlation between TPGs and tenderness was muscle-dependent. However, in agreement to earlier studies, PGs were retained in the proposed regression equations of beef tenderness of young bulls[12]. The non-involvement of TPGs is not surprising considering their low proportion in muscle and their low elastic modulus[3]. On contrary, in LT muscle of Qinchuan steers[6], the authors reported a positive correlation with shear force. It is worthwhile to note that the authors expressed their data in mg of GAG per g of total collagen that would partly explain their findings. We think that if they expressed their data in mg of GAG per mg of dry matter, they probably would have found any relationship or inverse relationship as we did in ST muscle.

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In cattle, the relationships between muscle fiber characteristics and tenderness have been extensively studied[1, 2]. They are complex and vary according to muscle, sex, age, breed and cooking temperature and method[1, 21, 41]. IIA and IIX+B muscle fibers were involved in tenderness of three of four muscles.

As for IMCT components, the same relationships were present between muscle fiber types and tenderness for RA and SM muscles, IIA being negatively correlated and IIX+B, positively. ST and LT muscles were very different from the point of view of type of fibers involved in their tenderness. For LT muscles, tenderness was rather related to IIX+B, while for ST muscles, the three types seemed involved (shown both with correlations and regression analyses). The facts that our results show marked differences between the LT and ST muscles were in agreement with the previous studies[42]. These authors identified that LT and ST muscles were different in the relationship between their metabolic properties and tenderness. For ST muscle, higher degrees of fast glycolytic properties were associated with tenderness whatever the cooking temperature and country origin of the panellists[21]. For LT muscle, higher degrees of fast glycolytic properties were associated with lower tenderness. In the present study, the muscle fiber types were less frequently involved in correlation and regression analyses than IMCT components and not able to separate the three tenderness classes. Then, in this model, muscle fibers were less involved in tenderness variability than IMCT.

Adipocytes of IMF develop inside IMCT and cause remodelling of extra cellular matrix and reduce the mechanical strength of IMCT, contributing to the tenderization of beef[5]. In our model, IMF was generally positively correlated with tenderness, and was only retained in the regression equation for LT muscle, the marbled muscle. As in this study, most of the other studies on continental beef breeds have shown that IMF plays a positive (but weak) role in meat tenderness[5]. This result was interpreted as a decrease of the perception of chewing residues and an increase of the sensation of juiciness[5]. According to Nishimura and co-workers, the adipocytes, during their development, cause a disorganization of the perimysial connective tissue leading to its weakening and dilution of the perimysial fibers[43]. This would have, consequently, a decrease of the compressive strength. Also according to the same authors, this phenomenon is only noticeable when meat has at least 8% of IMF[43]. In the present study, LT muscles contained on average 6% of IMF, a value near the threshold given by Nishimura and co-workers[43] and greater to that of beef tenderness acceptability[13]. This average high IMF content was due to the high levels of IMF in LT muscle of dairy animals.

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## 5. Conclusion

The different statistical approaches applied in this trial to analyse the data highlighted a relationship between TCol, ICol and CLs and tenderness and a much less robust association with muscle fiber types. From the two muscle fiber types, the most variables that are related to tenderness were the type IIA and IIX+B. The LT and ST muscles that, in this study, were extreme in tenderness, but very different from each other and of two other muscles for the components involved in tenderness while the RA and SM muscles were very similar. IMF content was globally a positive driver of tenderness. This study confirmed also under representative variation of French cattle, that LT and RA muscles were mostly classified as tender and intermediate meat while SM and ST were equivalent in tenderness and likely classified as tough muscles.

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**Conflicts of Interest:** The authors declare no conflict of interest

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## Figure captions

**Figure 1.** Differences in tenderness scores among the 4 muscles (*Longissimus thoracis* (LT), *Rectus abdominis* (RA), *Semimembranosus* (SM) and *Semitendinosus* (ST)) whatever the animal type. The results are presented as LSmeans ± SEM of individual determinations (NS: non significant; *: $P < 0.05$; **: $P < 0.01$).

**Figure 2.** Principal component analysis (PCA) based on raw data showing **A)** the loading of muscle characteristics (variables of intramuscular connective tissue (TCol: total collagen, ICol: insoluble collagen, SCol: soluble collagen, CLs: cross-links, TPGs: total proteoglycans), the muscle fibre types (I, IIA, IIX+B), intramuscular fat (IMF) correlated with tenderness scores in the two first axis and **B)** bi-plot of the individual scores averaged per muscle (LT: *Longissimus thoracis*, RA: *Rectus abdominis*, SM: *Semimembranosus* and ST: *Semitendinosus*) and **C)** bi-plot of the individual scores per animal type (Dairy and Beef). The width and height of each ellipse (x,y-means ± x,y-standard deviation (SD)) represent the variation in the distribution of the individuals in the first two axis.

**Figure 3. A)** Distribution of muscles within their tenderness classes across animal types (Tough, Medium, Tender). **B)** Distribution of meats of dairy and beef animals within their tenderness class across muscles. Muscles = LT: *Longissimus thoracis*; RA: *Rectus Abdominis*; SM: *Semimembranosus* and ST: *Semitendinosus*.

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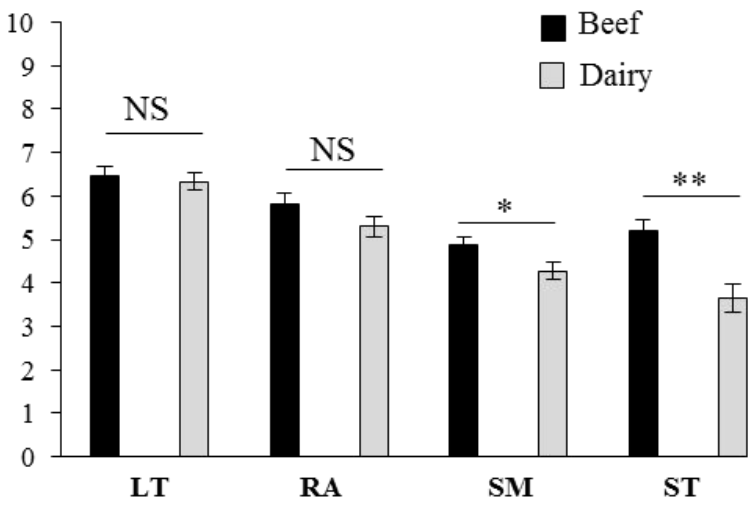


**Figure 1.**

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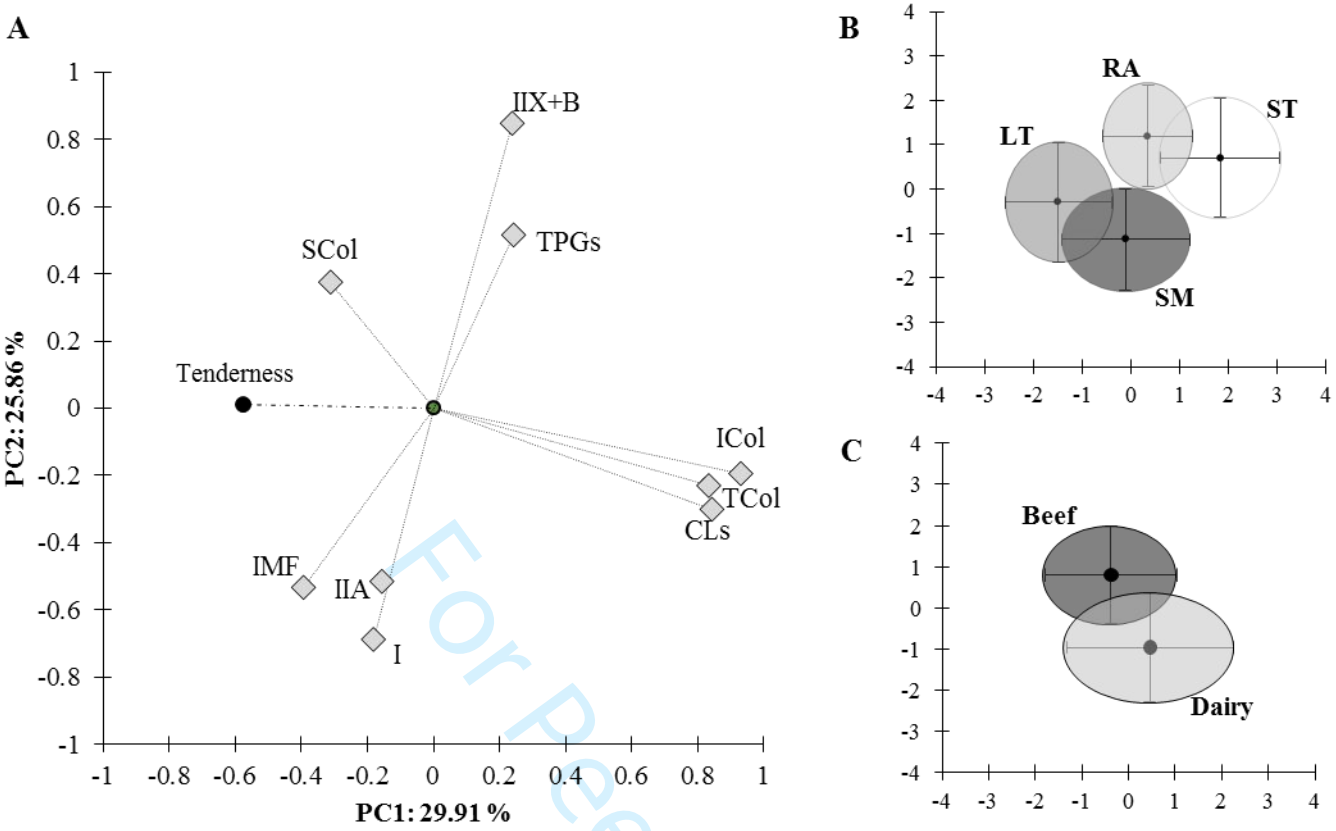


**Figure 2.**

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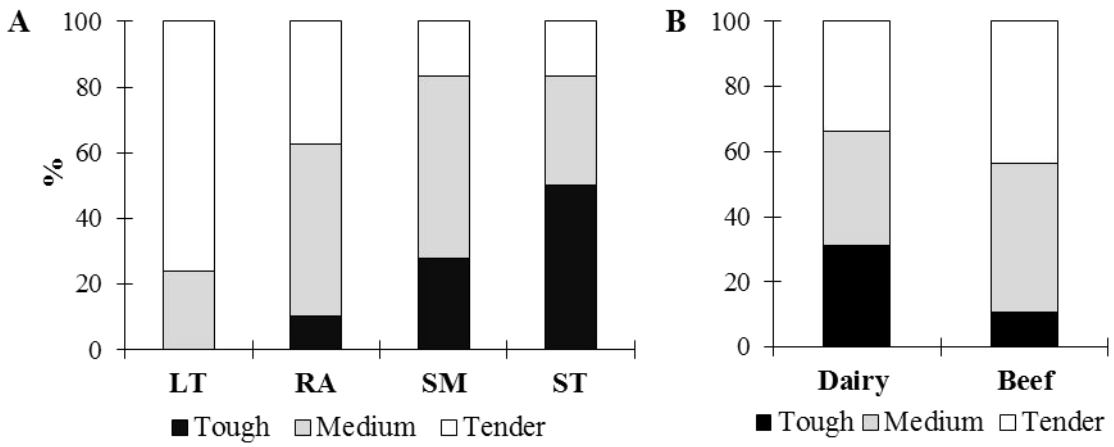


**Figure 3.**

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**Table 1.** Mean, standard deviation (SD), coefficient of variation (CV) and range of muscle characteristics and sensory tenderness of all samples across the muscles and animal types.

| Variables | Mean | SD | CV (%) | Range (Min – Max) |
|---|---|---|---|---|
| **IntraMuscular Connective tissue (IMCT)** | | | | |
| TCol | 4.42 | 1.25 | 39.2 | 1.82 – 7.92 |
| ICol | 2.62 | 0.89 | 33.7 | 1.10 – 5.80 |
| SCol | 33.55 | 9.40 | 27.5 | 10.61 – 59.65 |
| CLs | 29.92 | 8.45 | 28.5 | 15.71 – 57.62 |
| TPGs | 202.92 | 59.73 | 30.0 | 49.88 – 403.28 |
| **Muscle fiber types (%)** | | | | |
| I | 23.71 | 12.06 | 49.1 | 23.71 – 63.09 |
| IIA | 39.26 | 12.20 | 29.7 | 15.31 – 74.52 |
| IIX+B | 37.02 | 18.02 | 50.3 | 4.2 – 75.48 |
| **IMF** | 4.50 | 2.02 | 45.4 | 1.83 – 13.88 |
| **Sensory tenderness (0 – 10 scale)** | 5.4 | 1.34 | 25.8 | 1.91 – 8.45 |

TCol: Total Collagen (mg OH-proline/g dry matter); ICol: Insoluble Collagen (mg OH-proline/g dry matter); SCol: Soluble Collagen (%); CLs: Cross-Links (nM pyridinolline/g dry matter); TPGs: Total Proteoglycans (µg chondroitin 4-sulfate/g dry matter); IMF: IntraMuscular Fat content (mg/100g fresh matter).

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**Table 2.** Least square means ± SEM of tenderness[1] per muscle and animal type.

| Variable | Muscle (M) | | | | Animal type (AT) | | SEM | Effects | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LT | RA | SM | ST | Dairy | Beef | | M | AT | M*AT |
| Tenderness | 6.4 ± 1.0[a] | 5.6 ± 1.1[b] | 4.6 ± 0.8[c] | 4.5 ± 1.4[c] | 4.9 ± 1.4[b] | 5.6 ± 0.2[a] | 0.10 | 0.001 | 0.001 | 0.005 |

[1] Least square means in the same row for muscle (M) or animal type (AT) effect not followed by a common letter (a-c) differ significantly ($P<0.05$).

Abbreviations: *Longissimus thoracis* (LT), *Rectus abdominis* (RA), *Semimembranosus* (SM) and *Semitendinosus* (ST) muscles. SEM: Standard Error of the Mean.

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**Table 3.** Pearson correlations between muscle characteristics and sensory tenderness scores performed on Z-values.

| Variables | Irrespective of muscle and other factors of variation (all data) | Irrespective of factors other than muscle | | | | Fr |
|---|---|---|---|---|---|---|
| | | LT | RA | SM | ST | |
| **IMCT** | | | | | | |
| TCol | **-0.37*** ** | -0.12 | **-0.29** ** | **-0.45** ** | **-0.28*** | 4 |
| ICol | **-0.51*** ** | **-0.35*** | **-0.41** ** | **-0.45** ** | +0.17 | 4 |
| SCol | **+0.32*** ** | +0.19 | +0.13 | +0.05 | **+0.66*** ** | 2 |
| CLs | **-0.54*** ** | -0.18 | **-0.38** ** | **-0.30*** | **-0.52*** ** | 4 |
| TPGs | -0.12 | -0.17 | -0.11 | +0.08 | **+0.38*** | 1 |
| **Muscle fiber types (%)** | | | | | | |
| I | **+0.20*** | -0.27 | -0.07 | 0.01 | 0.03 | 1 |
| IIA | -0.02 | -0.19 | **-0.31** ** | **-0.46*** | **-0.39*** | 3 |
| IIX+B | -0.12 | **+0.35*** | **+0.37*** | **+0.42** ** | +0.25 | 3 |
| **IMF** | **+0.28*** ** | 0.07 | 0.10 | -0.12 | -0.20 | 1 |

Abbreviations: *Longissimus thoracis* (LT), *Rectus abdominis* (RA), *Semimembranosus* (SM) and *Semitendinosus* (ST) muscles (M); Fr: Frequency of presence of significant correlation; IMCT: Intra Muscular Connective tissue; TCol: Total Collagen (mg OH-proline/g dry matter); ICol: Insoluble Collagen (mg OH-proline/g dry matter); SCol: Soluble Collagen (%); CLs: Cross-Links (nM pyridinolline/g dry matter ); TPGs: Total Proteoglycans (µg chondroitin 4-sulfate/g dry matter); IMF: Intramuscular fat content (mg/100g fresh matter).

Significance of the correlations in bold character: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$

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**Table 4.** Regression equations of best models, performed on Z-values, fitted to predict variation in tenderness using parameters of intramucular connective tissue (IMCT), muscle fibre types and intramuscular fat content (IMF).

| **Variables** | Irrespective of muscle and other factors of variation (all data) | Irrespective of factors other than muscle | | | | |
|---|---|---|---|---|---|---|
| | | **LT** | **RA** | **SM** | **ST** | Fr |
| $R^2$ | **0.39** | **0.18** | **0.17** | **0.31** | **0.57** | |
| *P*-value | 0.001 | 0.03 | 0.04 | 0.002 | 0.001 | |
| **IMCT** | | | | | | |
| TCol | - | - | - | - | - | 0 |
| ICol | -0.19* | -0.65** | -0.41** | -0.33* | - | 4 |
| SCol | +0.17* | - | - | - | +0.68*** | 2 |
| CLs | -0.31** | - | - | - | - | 1 |
| TPGs | - | - | - | - | - | 0 |
| **Muscle fibers** | | | | | | |
| I | +0.15* | - | - | - | +0.51** | 2 |
| IIA | - | - | - | -0.34* | - | 1 |
| IIX+B | - | - | - | - | +0.38* | 1 |
| **IMF** | +0.18** | +0.19 | - | - | - | 2 |

Abbreviations: *Longissimus thoracis* (LT), *Rectus abdominis* (RA), *Semimembranosus* (SM) and *Semitendinosus* (ST) muscles (M); IMCT: Intra Muscular Connective tissue; TCol: Total Collagen (mg OH-proline/g dry matter); ICol: Insoluble Collagen (mg OH-proline/g dry matter); SCol: Soluble Collagen (%); CLs: Cross-Links (nM pyridinolline/g dry matter); TPGs: Total Proteoglycans (µg chondroitin 4-sulfate/g dry matter); IMF: Intramuscular fat content (mg/100g fresh matter); Fr: Frequency entrance of each variable in the regression equations.

Significance of regression coefficient of each retained variable in the models: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$

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**Table 5.** Variance analysis comparing the 3 tenderness classes identified by the *k*-means method as Tender, Medium and Tough.

| Variables | Class 1: Tender (39.88%) | Class 2: Medium (40.47%) | Class 3: Tough (19.64%) | SEM | *P*-value |
|---|---|---|---|---|---|
| Mean tenderness score | 6.6 ± 0.12[a] | 5.1 ± 0.09[b] | 3.7 ± 0.09[c] | 0.10 | *** |
| **IMCT** | | | | | |
| TCol | 3.71[b] | 4.52[a] | 4.84[a] | 0.10 | *** |
| ICol | 1.98[a] | 2.62[b] | 3.14[a] | 0.06 | *** |
| SCol | 36.54[a] | 34.44[a] | 28.16[b] | 0.70 | *** |
| CLs | 23.84[c] | 30.00[b] | 37.05[a] | 0.64 | *** |
| TPGs | 189.39[b] | 213.54[a] | 204.24[a] | 4.75 | * |
| **Muscle fiber types (%)** | | | | | |
| I | 25.83[a] | 26.00[a] | 20.99[a] | 0.96 | ns |
| IIA | 41.32[a] | 39.45[a] | 41.12[a] | 0.93 | ns |
| IIX+B | 32.85[a] | 34.55[a] | 37.88[a] | 1.34 | ns |
| **IMF** | 5.39[a] | 4.41[b] | 3.94[b] | 0.17 | ** |

Least square means in the same row for class effect not followed by a common letter (a-c) differ significantly ($P<0.05$).

*P*-value significance: ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Abbreviations: IMCT: Intramuscular Connective tissue; TCol: Total Collagen (mg OH-proline/g dry matter); ICol: Insoluble Collagen (mg OH-proline/g dry matter); SCol: Soluble Collagen (%); CLs: Cross-Links (nM pyridinolline/g dry matter); TPGs: Total Proteoglycans (µg chondroitin 4-sulfate/g dry matter); IMF: Intramuscular fat content (mg/100g fresh matter).

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