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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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3 1 **Contribution of connective tissue components, muscle fibres and marbling to beef**  
4 2 **tenderness variability in *Longissimus thoracis*, *Rectus abdominis*, *Semimembranosus* and**  
5 3 ***Semitendinosus* muscles**

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23 18 # The two authors contributed equally to this work.

24 19  
25 20 **Abstract:**

26 21 **Background:** This study aimed to identify relationships between components of  
27 22 intramuscular connective tissue, the proportions of the different fiber types, intramuscular fat  
28 23 and sensory tenderness of beef cooked at 55°C. For that, four muscles differing in their  
29 24 metabolic and contractile properties as well as in their collagen content and butcher value, from  
30 25 dairy and beef cattle of several ages and sexes were used in order to create variability.

31 26 **Results:** Correlation analyses and/or stepwise regressions were applied on Z-scores to  
32 27 identify the existing and robust associations. Tenderness scores were further categorized into  
33 28 tender, medium and tough classes using unsupervised learning methods. The findings revealed  
34 29 a muscle-dependant role on tenderness of total and insoluble collagen, cross-links and of type  
35 30 IIB+X and IIA muscle fibers. The *Longissimus thoracis* and *Semitendinosus* muscles that, in  
36 31 this study, were extreme in their tenderness potential were very different from each other and  
37 32 of the *Rectus abdominis* (RA) and *Semimembranosus* (SM). RA and SM muscles were very  
38 33 similar for their relationship for muscle components and tenderness. A relationship was present  
39 34 between marbling and tenderness only when the results were analysed irrespective of all the  
40 35 factors of variations of experimental model that are muscle and animal type.

41 36 **Conclusion:** The statistical approaches applied in this trial performed using the z-scores  
42 37 allowed to identify the robust associations between muscle components and sensory beef  
43 38 tenderness and also to found discriminatory variables of beef tenderness classes.

44 39 **Keywords:** Collagen; Cross-links; Proteoglycans; Lipids; Sensory beef quality; Meat.

## 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<sup>1</sup>. 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<sup>1, 2</sup>. Different chemical cross-links (CLs) stabilize the molecule of collagen and consequently are involved in collagen solubilization and its mechanical properties after meat cooking<sup>3</sup>. 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<sup>4</sup>. However, the role of CLs in beef tenderness is still controversial and not fully elucidated<sup>5, 6</sup>. The other main components of IMCT (after collagen) are the proteoglycans (PGs)<sup>1</sup>. 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<sup>7</sup>. From these properties, few authors have suggested that PGs could contribute to meat texture<sup>8-11</sup> but only two studies have investigated their relation with beef tenderness<sup>6, 12</sup>.

Marbling, still 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<sup>5</sup>. However, in less marbled beef, the role of IMF on beef tenderness is unclear and the relationships were mostly curvilinear<sup>13</sup> and weak<sup>2</sup>.

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<sup>12</sup>, strong and consistent in their direction, weak or insignificant and furthermore do not depend on a single component only but would rather be multifactorial<sup>3, 14, 15</sup>.

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

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<sup>th</sup> and 10<sup>th</sup> rib for LT and from the middle part of the muscle for ST, SM and RA <sup>16</sup>. 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<sup>17</sup>. For CLs, 1 mL of the 6N acid hydrolysate was centrifuged

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3 102 at 16,000 x g for 5 min at + 4°C. For ICol, muscle powder (250 mg) was weighted in duplicate  
4 103 and rehydrated for one hour with 1X solubilization buffer containing 0.23 M NaCl, 25 mM  
5 104 Tris–HCl, pH 7.4 and heated in a water bath at 75°C for one hour. The soluble fraction was  
6 105 separated from the insoluble fraction by filtration (pleated filters in cotton cellulose, VWR  
7 106 512–0206) and discarded. Insoluble fraction was hydrolysed according to the same method as  
8 107 for TCol. For TCol and ICol data were expressed in mg of hydroxyproline per g of dry matter  
9 108 (mg OH-pro g<sup>-1</sup> dry matter (DM)). The soluble collagen was determined as following: Soluble  
10 109 Collagen (SCol) = (TCol-ICol)/TCol\* 100.

11 110 For CLs, 600 µL 6 N NaOH and 600 µL 1 M Tris were added to 600 µL acid supernatant.  
12 111 Final pH was adjusted between 7 and 8 by adding some drops of 6 N HCl or NaOH.  
13 112 Pyridinoline cross-links (pyridinoline + deoxypyridinoline) were determined by the enzyme-  
14 113 linked immunoassay Metra Pyd EIA kit (Quidel Corporation, USA) according to the  
15 114 manufacturer and adapted by our group to the muscular tissue<sup>17</sup>. The results were then  
16 115 expressed in nM of pyridinoline per g of dry matter (nM pyr g<sup>-1</sup> DM).

### 17 116 **2.2.2. Total proteoglycan (PGs) content**

18 117 For total PGs, the procedure used was that previously described and updated<sup>17</sup>. Briefly,  
19 118 muscle powder was incubated 24 h at +4°C in the extraction buffer containing 6 M Urea, 1 M  
20 119 NaCl, 2% CHAPS and protease inhibitor cocktail (Complete, Roche Diagnostics GmbH, ref.  
21 120 11 836 145 001). The solid to liquid ratio was 100 mg of muscle powder to 1 mL of extraction  
22 121 buffer. The following day, the samples were centrifuged 40 min at +4°C, 15 000 xg. The  
23 122 supernatant (muscle extract) was recovered and used to determine PGs content. This assay was  
24 123 based on the ability of sulphated glycosaminoglycans (GAGs) to bind the cationic dye 1,9-  
25 124 dimethylmethylene blue (DMMB). Thus, 1 mL of DMMB solution was added in excess to 100  
26 125 µL of muscle extract and shaken 30 min at room temperature. After centrifugation for 15 min  
27 126 at 12 000 x g, the supernatant (DMMB excess) was removed. One mL of 50 mM sodium  
28 127 acetate buffer solution was then added to the residue and shaken for 30 min. Subsequently, the  
29 128 absorbance was measured at 656 nm using a micro-plate reader (TECAN Infinite® M200). The  
30 129 concentrations were determined by comparison to a determined standard curve by  
31 130 chondroitine-4-sulfate, ranging from 0 to 2.5 µg of C4S. Each sample was measured twice and  
32 131 data were expressed in µg of GAGs per g of dry matter (µg GAGs g<sup>-1</sup> DM).

### 33 132 **2.2.3. Myosin heavy chains isoforms quantification by electrophoresis**

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3 133 Myosin heavy chain isoforms were separated with an adapted SDS-PAGE electrophoresis<sup>18</sup>.  
4 134 Briefly, 100 mg of frozen muscle was ground using a Polytron in 5 mL of extraction buffer  
5 135 solution (0.5 M NaCl, 20 mM Na Pyrophosphate, 50 mM Tris, 1 mM EDTA and 1 mM  
6 136 Dithiothreitol). The samples were kept 10 min at 4 °C on ice, and then centrifuged for 5 min at  
7 137 5,000xg. Following centrifugation, the supernatant was diluted 1:1 (v/w) with glycerol at 87%  
8 138 and stored at -20 °C until used. The samples were then mixed with an equal volume of loading  
9 139 buffer (4% SDS (w/v), 125 mM Tris, pH 6.8, 20% glycerol (v/v), 10% β mercaptoethanol  
10 140 (v/v), 0.02% pyronin Y (w/v)) incubated at room temperature 10 min and then heated (70 °C)  
11 141 10 min. The proteins were separated using 9.2% polyacrylamide gels (the lower running buffer  
12 142 consisted : 50 mM Tris (base), 75 mM glycine and 0.05% w/v SDS; the upper running buffer  
13 143 2× the concentration of the lower running buffer added of β-mercaptoethanol (0.07% v/v)) <sup>18</sup>.  
14 144 Ten micrograms of protein extracts were loaded per well onto 0.75-mm-thick gels mounted on  
15 145 a Mini-Protean II Dual Slab Cell electrophoretic system (Bio-Rad). The migration was carried  
16 146 out at 4 °C at a constant voltage of 70 V for 30 h. After migration, the gels were fixed in 30%  
17 147 (v/v) ethanol and 5% acetic acid (v/v) and then stained with colloidal Coomassie Blue R250 for  
18 148 24 h. Gels were destained in a 30% ethanol (v/v) and 5% acetic acid (v/v) solution until the  
19 149 background was sufficiently cleared. After staining, the gels were scanned and the proportions  
20 150 of the different MyHC bands were quantified by densitometry with ImageQuant Software5500  
21 151 (Amersham Biosciences/GE Healthcare). MyHC-IIB isoform was found in only 2 animals,  
22 152 thus, MyHC-IIB percentage was totalised with those of MyHC-IIX creating a new variable  
23 153 “MyHC-IIX+B”.

#### 2.2.4. Intramuscular fat content

24 154  
25 155 Total lipid content of LT, RA, SM and ST was estimated by near infrared spectroscopy  
26 156 (NIRS) according to the procedure previously described by **Guy et al.<sup>19</sup>**. Briefly, muscle  
27 157 samples (about 5 g of muscle lyophilized powder) were scanned in a circular cup (diameter 50  
28 158 mm, depth 10 mm) (Part number IH – 0307, NIRSystems, Infrasoft International, South  
29 159 Atherton St. State College, PA 16801, USA), compressed and sealed with a disposable paper-  
30 160 backed wrap. Samples were scanned in reflectance mode (400 – 2500 nm) in a NIRS 6500  
31 161 scanning monochromator (NIRSystems, Silver Spring, MD, USA) using ISI software, version  
32 162 3.01 (Infrasoft International, South Atherton St. State College, PA 16801, USA) equipped with  
33 163 a spinning module. Reflectance data were recorded at 2 nm intervals and stored as log  
34 164 (1/reflectance). Then the reflectance data were exported into WinISI II version 1.60 (Infrasoft  
35 165 International, South Atherton St. State College, PA 16801, USA) which was used to estimate

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3 166 IMF values. A NIRS model was used to analyse the data based on spectra and biochemical  
4 167 analyses performed on 48 samples used in the current study. For the biochemical analyses,  
5 168 muscle dry matter was assayed gravimetrically after drying at 80°C for 48 h. Then, total lipids  
6 169 were extracted by mixing 6 g of muscle powder with chloroform-methanol<sup>20</sup>. Each sample was  
7 170 measured in triplicate and data were expressed in g per 100 g of dry matter (g/100 g DM). The  
8 171 statistical parameters (coefficient of determination and standard error of prediction) of the  
9 172 prediction model were 0.92 and 1.19 g/100 g of fresh matter respectively.

### 16 173 **2.3. Sensory analysis**

17 174 After thawing at 2 to 5 °C in vacuum packs for at least 24 h before cooking, muscles were  
18 175 cut into pieces of 3 cm cross-section and cooked in an oven at 250°C. They were removed at an  
19 176 internal temperature of 55°C, a medium rare cooking usually used in France<sup>21</sup>. The samples  
20 177 were presented in sequential monadic sessions involving 12 panellists. The expert panellists  
21 178 were trained in accordance with the ISO standards ISO/TC 34 as described by Gagaoua *et al.*<sup>22</sup>.  
22 179 Thus, the panellists rated global tenderness of the grilled meat on a 10 cm unstructured line  
23 180 scale (from 0 to 10), where 0 refers to extremely tough and 10 to extremely tender meat. The  
24 181 sessions were carried out in a sensory analysis room equipped with individual booths under  
25 182 artificial red light to reduce the influence of the appearance of the samples. Each tasting booth  
26 183 was equipped with computer terminals linked to a fileserver running a sensory software (Fizz v  
27 184 2.20 h, Biosystemes, Couternon, France) that facilitated the direct entry of assessor ratings.

### 38 185 **2.4. Statistical analysis**

39 186 The statistical analyses were performed using XLSTAT 2017.19.4 software (AddinSoft,  
40 187 Paris, France). First, normal distribution and homogeneity of the dataset was tested by Shapiro-  
41 188 Wilk test ( $P > 0.05$ ). It is worthwhile to note that there is scarcity in the publications studying  
42 189 the muscle components and sensory quality traits of dairy animals. So, although experimental  
43 190 design was not developed to compare dairy and beef, the differences in sensory tenderness  
44 191 scores among muscles (M) and animal types (AT) (dairy and beef) were investigated using a  
45 192 GLM model at a significance level of 5%. The differences in biochemical composition between  
46 193 the M and AT were presented elsewhere<sup>16</sup>. For any comparisons including interactions,  
47 194 Student–Newman–Keuls (SNK) was used to separate the least-squares means (LSmeans).  
48 195 Differences were considered to be statistically significant if  $P < 0.05$ . The distribution of the  
49 196 variables of interest to investigate the trend of differences among M and AT was illustrated by  
50 197 a principal component analysis (PCA), performed on raw data, by considering all the factors

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3 198 together including tenderness as a supplementary variable. Two factors with eigenvalues >1.0  
4 199 were considered in this PCA on the basis of the scree plot and evaluation of the factor loading  
5 200 matrix after orthogonal rotation following the procedure described by Gagaoua *et al.*<sup>23</sup>. An  
6 201 eigenvalue represents the amount of variance that is captured by a given component.  
7  
8 202 Eigenvalue criterion is the main criteria used for solving the number of components problem,  
9  
10 203 also known as the Kaiser-Guttman criterion (Kaiser, 1974). Accordingly, to check the  
11 204 suitability of the factorial model, the Kaiser-Meyer-Olkin (KMO) measure, known also as  
12 205 Kaiser's Measure of Sampling Adequacy (MSA) was applied to test sampling adequacy was  
13 206 used. The overall KMO value of the PCA was 0.72, which is acceptable<sup>24</sup>.

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19 207 Z-scores were computed in a stepwise manner using multivariate regression analyses to  
20 208 consider the fixed effects of M and to avoid the effects due to the other factors of variations  
21 209 present in the database by taking into account the differences in the number of animals for LT  
22 210 and RA muscle compared to SM and ST. Z-scores represent the deviation of each trait  
23 211 observation relative to the mean of the corresponding animal irrespective of the breed,  
24 212 experiment and other confounded effects and were calculated using PROC STANDARD of  
25 213 SAS, which standardizes data to a mean of 0 and standard deviation of 1. Subsequently and  
26 214 using the Z-scores, Pearson correlations were performed to investigate simple relationships  
27 215 between muscle characteristics and sensory tenderness. Also, multiple regression analyses were  
28 216 applied to propose regression equations explaining the overall tenderness among the  
29 217 investigated factors. The relationships were studied i) irrespective of the M and of other factors  
30 218 of variation; ii) for each M, irrespective of the other factors of variation. For regression  
31 219 analysis, the "optimal model" explaining maximal variability option was used<sup>25</sup>. The  
32 220 percentage of variability in meat tenderness explained by the explanatory variables was based  
33 221 on the  $r^2$  value of the regression analysis x 100.

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45 222 To identify the variables most discriminating potential tenderness of the steaks, a clustering  
46 223 analysis was performed on the whole data<sup>14</sup>. For that and according to the work by Gagaoua *et*  
47 224 *al.*<sup>14</sup>, several unsupervised learning methods: *k*-means, partitioning around medoids (PAM)  
48 225 and hierarchical clustering analysis (HCA) were tested. *K*-means was the method retained,  
49 226 because it gave the best results based on the homogeneity of the classes determined by the  
50 227 average silhouette width ( $S_i$ ) criterion<sup>26, 27</sup>. Silhouette width refers to a succinct graphical  
51 228 representation method for interpretation and validation of consistency within clusters of data. It  
52 229 is a measure of how similar an object is to its own cluster (cohesion) compared to others<sup>26</sup>. The  
53 230 value of silhouette width ranges from -1 to +1, and can be interpreted as follow: i)

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

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

### 240 3. Results

#### 241 3.1. Description of structural, biochemical and quality attributes

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

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

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

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

### 275 **3.2. Associations between sensory tenderness scores and muscle characteristics**

#### 276 **3.2.1. Correlation analyses**

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

#### 292 **3.2.2. Regression analyses**

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  $6.61 \pm 0.12$ . The medium tenderness class (2) grouped 40.47% of the steaks with an average score of  $5.05 \pm 0.09$ . The tough class (3) grouped the lowest number of the steaks (19.64%) and had an average score of  $3.67 \pm 0.09$ .

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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3 324 since on a scale of 0 to 10, the samples used had a tenderness ranging from 1.91 to 8.45. The  
4 325 muscle characteristics measured presented also a large range of variability.

#### 6 326 ***4.1. Differences of tenderness among the muscles***

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9 327 In this trial, LT and RA muscle were mostly classified as tender and intermediate meat  
10 328 while SM and ST were equivalent in tenderness and likely classified as tough muscles. For the  
11 329 LT and RA muscles, these differences were in agreement with earlier studies<sup>28, 29</sup>. For the SM  
12 330 and ST, some studies described that ST was more tender than SM <sup>28, 30, 31</sup> and others, described  
13 331 the opposite<sup>29, 32</sup>. The findings of this study confirmed that meat of dairy was on average  
14 332 tougher than meat of beef cattle. In contrast to our results, an earlier study on cull cows of  
15 333 dairy and beef, the overall meat tenderness was found not different when the animals were  
16 334 slaughtered at the same age, fattening duration and fatness score<sup>32</sup>. On the contrary, other  
17 335 studies have shown that shear force of raw meat from Holstein, a dairy breed, was higher than  
18 336 that of young Charolais or Limousin (two beef breeds) bulls<sup>33</sup>. It is worthy to note that after 10  
19 337 days ageing and cooking at an end-point temperature of 75°C internal temperature, the shear  
20 338 force of meat of these breeds was similar. However, there are very few data in the literature  
21 339 that addressed this point. The findings of the present trial further showed that meat of dairy  
22 340 types presented a greater variability of tenderness than beef types for a period of ageing of 7  
23 341 days and a cooking temperature of 55°C. This is in line to the literature describing a muscle-  
24 342 dependency<sup>34</sup>. Accordingly, the cluster analysis of tenderness revealed that 31% of meat cuts  
25 343 of dairy animals were considered tough by the sensory panel against only 11% for beef  
26 344 category. Within muscles, the meat of dairy animals could be, as least in this study, as tender as  
27 345 meat of beef animals. These results need further validation on a larger number of animals and  
28 346 on a model that have less variability than this trial.

#### 29 347 ***4.2. Relationships between muscle components and meat tenderness***

30 348 The findings of this trial highlighted the impact of different components of IMCT (TCol,  
31 349 ICol, SCol, CLs and PGs), of muscle fibre types (I, IIA, IIX+B) and IMF on beef tenderness.  
32 350 Overall, TCol, ICol and their CLs were negatively correlated with tenderness, this result  
33 351 remaining unaffected in at least three of the four studied muscles. The two statistical  
34 352 approaches that are correlation and regression analyses used to investigate the relationships  
35 353 between the muscle components and tenderness focused on a leading role of TCol, ICol and  
36 354 CLs, in RA and SM muscles. For the LT muscle, only ICol was correlated with tenderness and  
37 355 retained in the regression equations. For ST muscle, a more glycolytic muscle, the PGs were

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3 356 involved in tenderness including TCol, CLs and SCol. In addition to be involved in tenderness  
4 357 of RA, SM and ST muscles, CLs were the only components of IMCT to be significantly  
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6 358 different between the three tenderness classes. This result was coherent, since we previously  
7  
8 359 found in the experimental same design, that ICol and CLs were highly correlated irrespective  
9  
10 360 of muscle<sup>16</sup>. CLs are known to be involved in collagen solubility determinism<sup>35</sup>. The only  
11  
12 361 muscle for which the CLs were not correlated with tenderness was LT muscle, result that we  
13  
14 362 expect related to the fact that this muscle had the lowest CLs content. On the contrary, an  
15  
16 363 earlier study revealed in LT muscle a negative correlation between CLs and tenderness<sup>17</sup>.

17  
18 364 Although many groups investigated the relationship between the composition of connective  
19  
20 365 tissue in TCol, ICol, CLs and meat tenderness, the conclusions have not been clearly  
21  
22 366 established<sup>4, 36, 37</sup>. This can be due to several reasons including the large variability of  
23  
24 367 tenderness within muscle, time of ageing, cooking method, as well as to rearing practices of the  
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26 368 animals<sup>13, 38</sup>. Overall, the authors did not always specify the location of sampling within the  
27  
28 369 muscle, that is very important<sup>39</sup> nor the exact conditions of ageing and method or conditions of  
29  
30 370 cooking. When they do, the cooking and ageing conditions vary so much from one study to  
31  
32 371 another that it is difficult to compare the results. It is worthwhile to cite that tenderness  
33  
34 372 evaluation by sensory panels depend also on the consumer habits and preferences<sup>21, 22</sup>.  
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36 373 Moreover, it is rare that the authors work on standardized data as we did in this experiment.  
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38 374 This approach allowed to test the robustness and accuracy of the associations by investigating  
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40 375 if they exist independently of the fixed effects of factors of variation of the model<sup>40</sup>.

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42 376 Unlike TCol, ICol and CLs, few authors investigated the relationships between TPGs and  
43  
44 377 tenderness. To our knowledge, only two studies evaluated TPGs in the context of meat quality<sup>6,</sup>  
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46 378 <sup>12</sup>. In the present study, the correlation between TPGs and tenderness was muscle-dependent.  
47  
48 379 However, in agreement to earlier studies, PGs were retained in the proposed regression  
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50 380 equations of beef tenderness of young bulls<sup>12</sup>. The non-involvement of TPGs is not surprising  
51  
52 381 considering their low proportion in muscle and their low elastic modulus<sup>3</sup>. On contrary, in LT  
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54 382 muscle of Qinchuan steers<sup>6</sup>, the authors reported a positive correlation with shear force. It is  
55  
56 383 worthwhile to note that the authors expressed their data in mg of GAG per g of total collagen  
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58 384 that would partly explain their findings. We think that if they expressed their data in mg of  
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60 385 GAG per mg of dry matter, they probably would have found any relationship or inverse  
386 relationship as we did in ST muscle.

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3 387 In cattle, the relationships between muscle fiber characteristics and tenderness have been  
4 388 extensively studied<sup>1, 2</sup>. They are complex and vary according to muscle, sex, age, breed and  
5 389 cooking temperature and method<sup>1, 21, 41</sup>. IIA and IIX+B muscle fibers were involved in  
6 390 tenderness of three of four muscles.  
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11 391 As for IMCT components, the same relationships were present between muscle fiber types  
12 392 and tenderness for RA and SM muscles, IIA being negatively correlated and IIX+B, positively.  
13 393 ST and LT muscles were very different from the point of view of type of fibers involved in  
14 394 their tenderness. For LT muscles, tenderness was rather related to IIX+B, while for ST  
15 395 muscles, the three types seemed involved (shown both with correlations and regression  
16 396 analyses). The facts that our results show marked differences between the LT and ST muscles  
17 397 were in agreement with the previous studies<sup>42</sup>. These authors identified that LT and ST muscles  
18 398 were different in the relationship between their metabolic properties and tenderness. For ST  
19 399 muscle, higher degrees of fast glycolytic properties were associated with tenderness whatever  
20 400 the cooking temperature and country origin of the panellists<sup>21</sup>. For LT muscle, higher degrees  
21 401 of fast glycolytic properties were associated with lower tenderness. In the present study, the  
22 402 muscle fiber types were less frequently involved in correlation and regression analyses than  
23 403 IMCT components and not able to separate the three tenderness classes. Then, in this model,  
24 404 muscle fibers were less involved in tenderness variability than IMCT.  
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36 405 Adipocytes of IMF develop inside IMCT and cause remodelling of extra cellular matrix  
37 406 and reduce the mechanical strength of IMCT, contributing to the tenderization of beef<sup>5</sup>. In our  
38 407 model, IMF was generally positively correlated with tenderness, and was only retained in the  
39 408 regression equation for LT muscle, the marbled muscle. As in this study, most of the other  
40 409 studies on continental beef breeds have shown that IMF plays a positive (but weak) role in  
41 410 meat tenderness<sup>5</sup>. This result was interpreted as a decrease of the perception of chewing  
42 411 residues and an increase of the sensation of juiciness<sup>5</sup>. According to Nishimura and co-  
43 412 workers, the adipocytes, during their development, cause a disorganization of the perimysial  
44 413 connective tissue leading to its weakening and dilution of the perimysial fibers<sup>43</sup>. This would  
45 414 have, consequently, a decrease of the compressive strength. Also according to the same  
46 415 authors, this phenomenon is only noticeable when meat has at least 8% of IMF<sup>43</sup>. In the  
47 416 present study, LT muscles contained on average 6% of IMF, a value near the threshold given  
48 417 by Nishimura and co-workers<sup>43</sup> and greater to that of beef tenderness acceptability<sup>13</sup>. This  
49 418 average high IMF content was due to the high levels of IMF in LT muscle of dairy animals.  
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## 419 5. Conclusion

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

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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  $\pm$  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  $\pm$  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*.

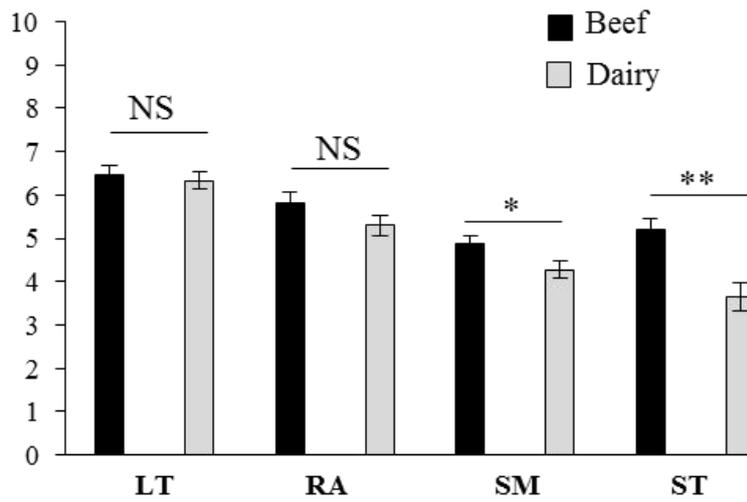
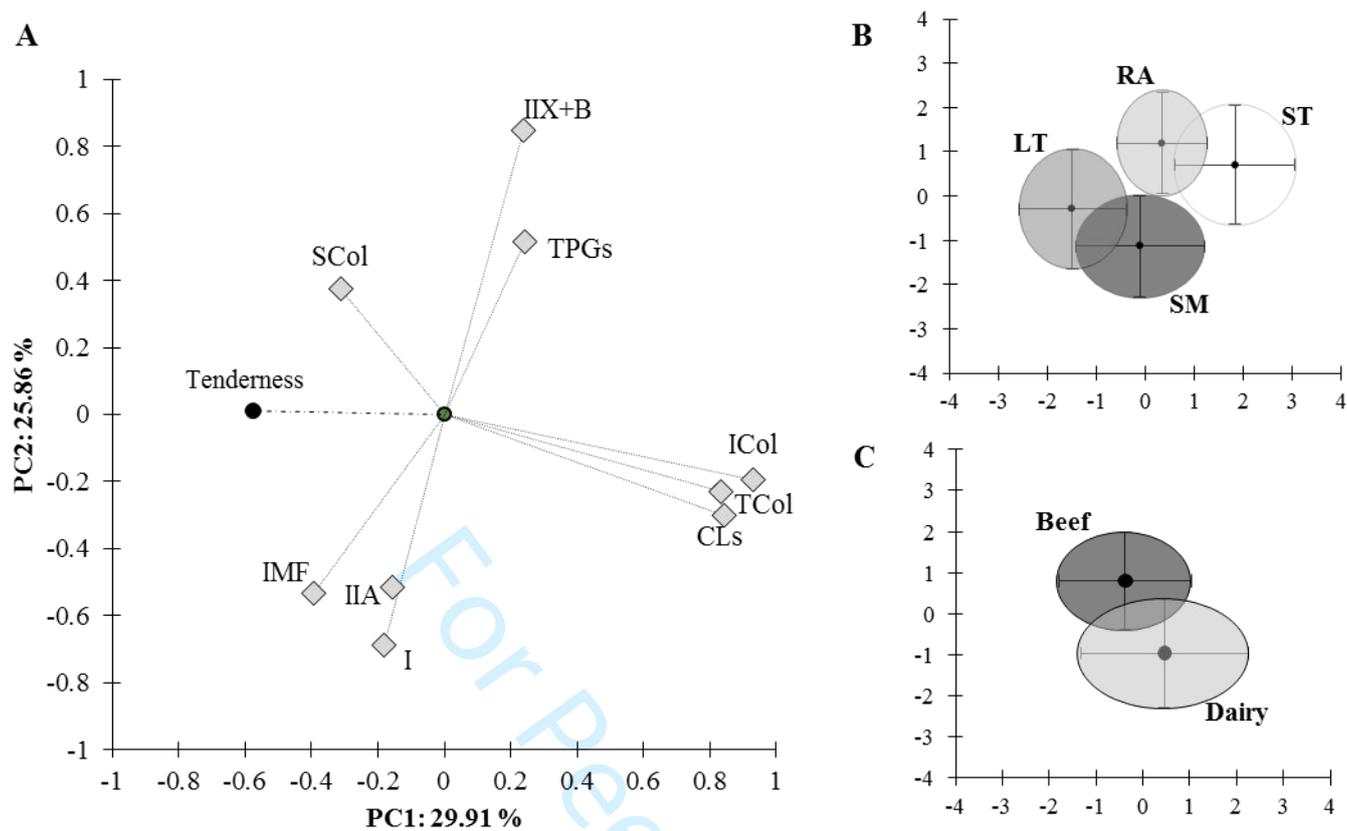


Figure 1.

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**Figure 2.**

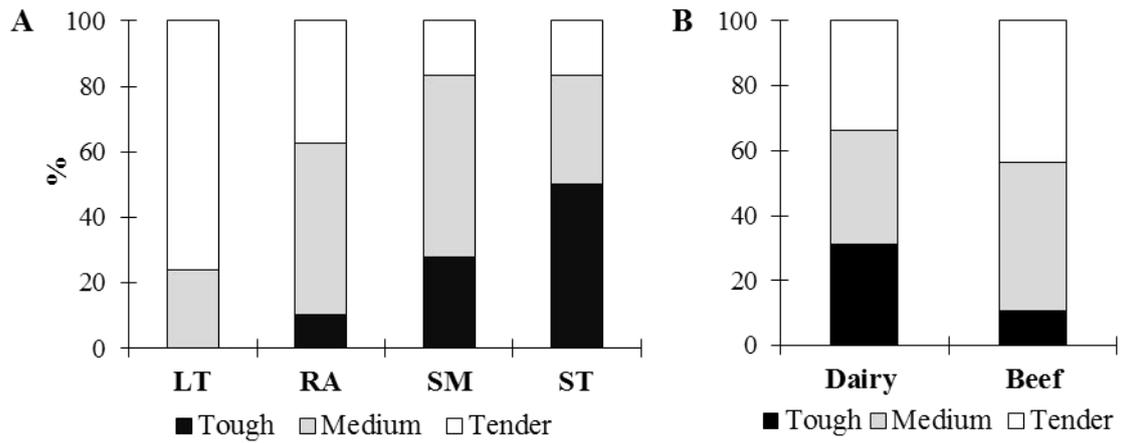


Figure 3.

For Peer Review

**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) |
|---|--------|-------|--------|-------------------|
| <b>IntraMuscular Connective tissue (IMCT)</b> |        |       |        |                   |
| 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    |
| <b>Muscle fiber types (%)</b>                 |        |       |        |                   |
| 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       |
| <b>IMF</b>                                    | 4.50   | 2.02  | 45.4   | 1.83 – 13.88      |
| <b>Sensory tenderness (0 – 10 scale)</b>      | 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 ( $\mu$ g chondroitin 4-sulfate/g dry matter); IMF: IntraMuscular Fat content (mg/100g fresh matter).

**Table 2.** Least square means  $\pm$  SEM of tenderness<sup>1</sup> 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 $\pm$ 1.0 <sup>a</sup> | 5.6 $\pm$ 1.1 <sup>b</sup> | 4.6 $\pm$ 0.8 <sup>c</sup> | 4.5 $\pm$ 1.4 <sup>c</sup> | 4.9 $\pm$ 1.4 <sup>b</sup> | 5.6 $\pm$ 0.2 <sup>a</sup> | 0.10 | 0.001   | 0.001 | 0.005 |

<sup>1</sup> 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.

For Peer Review

**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              |    |
| <b>IMCT</b>                   |  |   |                |                |                 |    |
| TCol                          | <b>-0.37***</b>  | -0.12                                     | <b>-0.29**</b> | <b>-0.45**</b> | <b>-0.28*</b>   | 4  |
| ICol                          | <b>-0.51***</b>  | <b>-0.35*</b>                             | <b>-0.41**</b> | <b>-0.45**</b> | +0.17           | 4  |
| SCol                          | <b>+0.32***</b>  | +0.19                                     | +0.13          | +0.05          | <b>+0.66***</b> | 2  |
| CLs                           | <b>-0.54***</b>  | -0.18                                     | <b>-0.38**</b> | <b>-0.30*</b>  | <b>-0.52***</b> | 4  |
| TPGs                          | -0.12  | -0.17                                     | -0.11          | +0.08          | <b>+0.38*</b>   | 1  |
| <b>Muscle fiber types (%)</b> |  |   |                |                |                 |    |
| I                             | <b>+0.20*</b>  | -0.27                                     | -0.07          | 0.01           | 0.03            | 1  |
| IIA                           | -0.02  | -0.19                                     | <b>-0.31**</b> | <b>-0.46*</b>  | <b>-0.39*</b>   | 3  |
| IIX+B                         | -0.12  | <b>+0.35*</b>                             | <b>+0.37*</b>  | <b>+0.42**</b> | +0.25           | 3  |
| <b>IMF</b>                    | <b>+0.28***</b>  | 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 ( $\mu\text{g}$  chondroitin 4-sulfate/g dry matter); IMF: Intramuscular fat content (mg/100g fresh matter).

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

**Table 4.** Regression equations of best models, performed on Z-values, fitted to predict variation in tenderness using parameters of intramuscular 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 |             |             |             | Fr |
|----------------------|--|---|-------------|-------------|-------------|----|
|                      |  | LT  | RA          | SM          | ST          |    |
| R <sup>2</sup>       | <b>0.39</b>  | <b>0.18</b>                               | <b>0.17</b> | <b>0.31</b> | <b>0.57</b> |    |
| P-value              | 0.001  | 0.03                                      | 0.04        | 0.002       | 0.001       |    |
| <b>IMCT</b>          |  |   |             |             |             |    |
| TCol                 | -  | -   | -           | -           | -           | 0  |
| ICol                 | -0.19*   | -0.65**                                   | -0.41**     | -0.33*      | -           | 4  |
| SCol                 | +0.17*   | -   | -           | -           | +0.68***    | 2  |
| CLs                  | -0.31**  | -   | -           | -           | -           | 1  |
| TPGs                 | -  | -   | -           | -           | -           | 0  |
| <b>Muscle fibers</b> |  |   |             |             |             |    |
| I                    | +0.15*   | -   | -           | -           | +0.51**     | 2  |
| IIA                  | -  | -   | -           | -0.34*      | -           | 1  |
| IIX+B                | -  | -   | -           | -           | +0.38*      | 1  |
| <b>IMF</b>           | +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 ( $\mu$ g 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: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$

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

| Variables                     | Class 1: Tender<br>(39.88%) | Class 2: Medium<br>(40.47%) | Class 3: Tough<br>(19.64%) | SEM  | <i>P</i> -value |
|-------------------------------|-----------------------------|-----------------------------|----------------------------|------|-----------------|
| Mean tenderness score         | 6.6 ± 0.12 <sup>a</sup>     | 5.1 ± 0.09 <sup>b</sup>     | 3.7 ± 0.09 <sup>c</sup>    | 0.10 | ***             |
| <b>IMCT</b>                   |                             |                             |                            |      |                 |
| TCol                          | 3.71 <sup>b</sup>           | 4.52 <sup>a</sup>           | 4.84 <sup>a</sup>          | 0.10 | ***             |
| ICol                          | 1.98 <sup>a</sup>           | 2.62 <sup>b</sup>           | 3.14 <sup>a</sup>          | 0.06 | ***             |
| SCol                          | 36.54 <sup>a</sup>          | 34.44 <sup>a</sup>          | 28.16 <sup>b</sup>         | 0.70 | ***             |
| CLs                           | 23.84 <sup>c</sup>          | 30.00 <sup>b</sup>          | 37.05 <sup>a</sup>         | 0.64 | ***             |
| TPGs                          | 189.39 <sup>b</sup>         | 213.54 <sup>a</sup>         | 204.24 <sup>a</sup>        | 4.75 | *               |
| <b>Muscle fiber types (%)</b> |                             |                             |                            |      |                 |
| I                             | 25.83 <sup>a</sup>          | 26.00 <sup>a</sup>          | 20.99 <sup>a</sup>         | 0.96 | ns              |
| IIA                           | 41.32 <sup>a</sup>          | 39.45 <sup>a</sup>          | 41.12 <sup>a</sup>         | 0.93 | ns              |
| IIX+B                         | 32.85 <sup>a</sup>          | 34.55 <sup>a</sup>          | 37.88 <sup>a</sup>         | 1.34 | ns              |
| <b>IMF</b>                    | 5.39 <sup>a</sup>           | 4.41 <sup>b</sup>           | 3.94 <sup>b</sup>          | 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 ( $\mu$ g chondroitin 4-sulfate/g dry matter); IMF: Intramuscular fat content (mg/100g fresh matter).