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# Are there consistent relationships between major connective tissue components, intramuscular fat content and muscle fibre types in cattle muscle?

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*Intramuscular connective tissue (**IMCT**) is mainly composed of several fibrils (known as total collagen (**TCol**)) linked between each other by different chemical cross-links (**CLs**), the whole being embedded in a matrix of proteoglycans (**PGs**). In the field of beef quality, there is limited information on the role of CLs and PGs. Accordingly, several authors suggest that, to investigate the role of IMCT, it is important to investigate them just like TCol and insoluble collagen (**ICol**). In muscle, there are two other components, the muscle fibres and intramuscular fat (**IMF**) content. There are limited data on the relationships between these three components of muscle and then on possibility to independently manipulate these characteristics in order to control the final quality of meat. The present study aimed to investigate whether consistent relationships exist between these different components of muscle. Therefore, the present study compared four muscles of two cattle types (dairy and beef) to determine associations between TCol, ICol, CLs and PGs. Data were analysed across and within muscle (**M**) and animal type (**AT**) based on residuals. There was a strong M and AT effect for all muscle characteristics and an interaction M × AT for type I muscle fibres and IMF. Correlations between TCol, ICol and their CLs were M- and AT-independent. Total proteoglycans were positively correlated with TCol and ICol in a muscle-dependent manner irrespective of AT, but no correlation was found with CLs. On the contrary, CLs were negatively correlated with the ratio TPGs : TCol in an M-dependent manner, irrespective of AT. TCol, ICol and CLs were positively and negatively correlated with type IIA and IIB+X muscle fibres only in* longissimus thoracis *(**LT**) muscle, regardless the AT. Insoluble collagen was the only parameter of IMCT to be correlated with type I muscle fibres but only in LT muscle, irrespective of AT. There was no correlation between PGs and muscle fibre types, but PGs were the only IMCT component to be related with IMF in an M-dependent manner, irrespective of AT. Finally, there was no correlation between muscle fibre types and IMF content within M and AT. This study revealed that there is a strong relationship between IMCT components irrespective of M, an M-dependent relationship between the IMCT components and muscle fibre types and few (only with PGs) or no relationship between IMF and IMCT and muscle fibres.*

**Keywords:** beef, dairy, collagen, cross-links, proteoglycans

## Implications

Beef producers have been increasingly requested by consumers to guarantee tender meat. Tenderness depends partly on the muscle biochemical components and on the relationships that they have between them. This study identified that the main components of intramuscular connective tissue were all positively related, irrespective of muscles and animal types. Correlations between total and insoluble collagen, its cross-links and the muscle fibre types as well as the proteoglycans and marbling are muscle-dependent. As a result, any management factor that influences one of the above components may also have an effect on tenderness.

## Introduction

Extracellular matrix (**ECM**) of intramuscular connective tissue (**IMCT**) is mainly composed of fibrillar collagens known also as total collagen (**TCol**) (Dubost *et al.*, 2013). There are

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different types of cross-links (**CLs**) between fibrils and fibres of collagen (Hayes *et al.*, 2013). They confer on the collagen fibres' high tensile strength and heating resistance (Lepetit, 2007). Collagens are embedded in a matrix of proteoglycans (**PGs**) that play an important role in the stabilization of the fibrillar collagen network (Nishiumi *et al.*, 1997). Proteoglycans are known to exhibit changes in amount and composition during development, growth and under pathological conditions (Listrat *et al.*, 2019). They are further degraded *postmortem*, the consequence being an increase in the sensitivity of collagen to proteolysis and therefore an increase in meat tenderness (Nishimura *et al.*, 1996). *In vitro*, Etherington (1977) showed that the presence of CLs would increase the resistance of collagen to enzymatic digestion, and that absence of PGs would increase its susceptibility. These results suggest that, in order to study the role of IMCT on beef quality, it is important to study collagen, CLs and PGs simultaneously and the balance between them in different muscles of various cattle types. With regard to beef meat, while collagen to a lesser extent, CLs, has been extensively studied and compared between muscles (Torrescano *et al.*, 2003; Rhee *et al.*, 2004), PGs have not been extensively considered. Accordingly and to the best of our knowledge, only one study has approached this question (Dubost *et al.*, 2013). Then, one of the objectives of this study was to study PGs in several muscles differing in their contractile properties and IMCT composition.

Intramuscular connective tissue is a three-dimensional network that surrounds each muscle fibre but also the muscle fibre bundles and finally the muscle as a whole. In bovine muscles, there are three main fibre types, I, IIA and IIX. A fourth type (IIB) has been observed in few animals (Picard and Cassar-Malek, 2009; Gagaoua *et al.*, 2015). The intramuscular adipose tissue is the third tissue of muscle, and it is expanded inside IMCT. It is well known that the quantity of collagen, the proportion of the different muscle fibre types and the quantity of intramuscular fat (**IMF**) content vary from one type (species, breed, gender, age) of animal to another and from one muscle to another according to muscle position and function, while having a complex link with meat tenderness (Purslow, 2005; Listrat *et al.*, 2016). Whether the existing relationships between the different types of fibres are well known (Lefaucheur, 2010), it is not the case of their relationships with the connective tissue components, nor with IMF. Moreover, there are limited data to clarify if there is any possibility to independently manipulate these characteristics by genetic, nutritional and environmental factors in order to control the final quality of meat and thereby respond to the queries of both industrials and consumers.

Thus, the main objective of the present study was to verify in a holistic approach whether consistent relationships exist among IMCT composition (collagen, PGs, CLs), muscle fibre typology and IMF content independently and within of muscle (**M**) and of animal type (**AT**).

## Material and methods

### *Beef production and muscle sampling*

The experiment was performed on four muscles sampled from the same animals ($n = 48$ for *longissimus thoracis* (**LT**) and *rectus abdominis* (**RA**) and $n = 36$ for *semimembranosus* (**SM**) and *semitendinosus* (**ST**) from two ATs (dairy, Holstein breed, $n = 76$; beef, Charolais breed, $n = 92$). Among the two types, the animals were of four genders: young bulls (18%), heifers (21%), steers (12%) and cows (49%). Young bulls were slaughtered at about 19 (for beef animals) and 21 (for dairy animals) months old, steers at about 35 months old, heifers at about 32 months old and cows between 6 and 8 years old. The meat of animals covered a wide range of collagen, IMF content and muscle fibres composition (Table 1). This is the main reason for which

**Table 1** *Mean, SD, CV and range (min–max) of the bovine muscle characteristics of all samples across the muscles and animal types*

| | Mean | SD | CV (%) | Range (min–max) |
|---|---|---|---|---|
| EUROP conformation score (1 to 15)[a] | 6.46 | 3.35 | 51.8 | 1–11 |
| Carcass weight | 382.15 | 73.10 | 10.9 | 267.50–529.20 |
| Muscle characteristics | | | | |
| IMCT | | | | |
| TCol (mg OH-prol/g DM) | 4.26 | 1.24 | 29.2 | 1.82–7.92 |
| ICol (mg OH-prol/g DM) | 2.47 | 0.83 | 33.7 | 1.10–5.79 |
| CLs (nM Pyd/g DM) | 28.97 | 8.25 | 28.5 | 15.71–57.63 |
| TPGs (μg C4S/g DM) | 202.42 | 60.76 | 30.0 | 49.88–403.28 |
| TPGs (mg C4S/g TCol) | 6.44 | 2.69 | 41.8 | 1.70–15.10 |
| Muscle fibre types (%) | | | | |
| I | 24.93 | 12.24 | 49.1 | 1.78–63.09 |
| IIA | 40.73 | 12.09 | 29.7 | 15.31–74.52 |
| IIX+B | 34.34 | 17.27 | 50.3 | 0–75.48 |
| IMF (mg/100 g fresh matter) | 4.69 | 2.13 | 45.4 | 1.84–13.88 |

IMCT = intramuscular connective tissue; TCol = total collagen (μg OH-prol (OH-proline)/mg DM); TCLs = total cross-links (nM Pyd (pyridinoline)/g DM); ICol = insoluble collagen (μg OH-prol/mg DM); TPGs = total proteoglycans (μg C4S (chondroitin-4-sulphate)/g DM or mg C4S/g TCol (OH-prol × 7.14)); IMF = intramuscular fat.

[a]EUROP classification grid for carcass conformation scores from P− = 1 to E+ = 15.

this trial was designed. Moreover, the whole animals were from 15 different farms but were all slaughtered in the same slaughterhouse (Le Lion d'Angers, France, license number 49176001) under the same conditions when they achieved a fat cover score of 3. The carcasses were chilled 2 days in a cold room (+2°C) then muscles were dissected and matured under vacuum for 7 days. Muscle samples (about 150 g) were cut into pieces of 1 cm cross-section, sealed under vacuum in plastic bags and stored at −20°C until preparation for biochemical analysis. Anatomical location of samples was standardized within muscles.

*Biochemical characteristics of intramuscular connective tissue*

For TCol and insoluble collagen (**ICol**) contents, CLs and total PGs (**TPGs**) measurements, frozen muscle was homogenized in a household grinder (Robot coupe R2, Augère Poumarat, Clermont-Ferrand, France), freeze-dried for 48 h, pulverized in a horizontal blade mill and stored at +4°C in stopper plastic flasks until analyses.

*Total, insoluble collagens and cross-links measurements.* For TCol and CLs, about 250 mg of muscle powder was weighed and acid hydrolysed with 10 ml of 6 N HCl overnight at 110°C in a screw-capped glass tube. For TCol, the acid hydrolysate was diluted five times in 6 N HCl and the subsequent procedure used was that previously described and updated by Dubost *et al.* (2013). For CLs, 1 ml of the 6 N acid hydrolysate was centrifuged at 16 000×**g** for 5 min at + 4°C. For ICol, muscle powder was solubilized and hydrolysed according to the same method as for TCol. For the three parameters, each sample was weighed and measured in duplicate and data were expressed in mg of hydroxyproline per g of DM (mg OH-Pro/g DM).

For CLs, 600 µl of 6 N NaOH and 600 µl of 1 M Tris were added to 600 µl of acid supernatant. Final pH was adjusted between 7 and 8 with 6 N HCl or NaOH. Pyridinoline CLs (pyridinoline + deoxypyridinoline) were determined by the enzyme-linked immunoassay Metra Pyd EIA kit (Quidel Corporation, San diego, CA, USA) according to the manufacturer guidelines. Results were expressed in nM of pyridinoline per g of DM (nM pyr/g DM).

*Total proteoglycan content.* Total proteoglycans were determined following the procedure described and updated for meat samples by Dubost *et al.* (2013). Muscle powder was incubated 24 h at +4°C with extraction buffer containing 6 M Urea, 1 M NaCl, 2% CHAPS and protease inhibitor cocktail (Complete, Roche Diagnostics GmbH, Mannheim, Germany). The solid to liquid ratio was 100 mg of muscle powder to 1 ml of extraction buffer. The next day, samples were centrifuged 40 min at +4°C, 15 000×**g**. Supernatant (muscle extract) was recovered and used to determine PG content. This assay was based on the ability of sulphated glycosaminoglycans (**GAGs**) to bind the cationic dye 1,9-dimethylmethylene blue (**DMMB**). Briefly, 1 ml of DMMB solution was added in excess to 100 µl of muscle extract and shaken 30 min at room temperature. After centrifugation 15 min, 12 000×**g**, the supernatant (DMMB excess) was removed. One millilitre of 50 mM sodium acetate buffer was added to the residue and shaken 30 min. Absorbance was measured at 656 nm, with a micro-plate reader (TECAN Infinite® M200). Concentrations were determined by comparison with a standard curve of chondroitine-4-sulphate, ranging from 0 to 2.5 µg of C4S. Each sample was measured twice and data were expressed by two methods, first in µg of GAGs per g of DM (µg GAGs/g DM) and second, in mg of GAGs per g of TCol. Data expressed in OH-Pro were transformed to be expressed in collagen content, assuming that collagen content corresponded to 7.14 × OH-Pro content and that collagen has a molecular weight of 300 kDa (Dubost *et al.*, 2013).

*Electrophoresis of the myosin heavy chain isoforms.* The proportions of the different myosin heavy chain (**MyHC**) isoforms were determined using an adequate and high-resolution mini-gel electrophoresis according to the method published by Picard *et al.* (2011). 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 5000×**g**. 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% sodium dodecyl sulfate 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) during 10 min. The proteins were separated using 9.2% polyacrylamide gels (the lower running buffer consists of 50 mM Tris (base), 75 mM glycine and 0.05% w/v SDS and the upper running buffer 2× the concentration of the lower running buffer with 0.07% v/v of β-mercaptoethanol). Protein extracts (10 µg) were loaded per well onto 0.75-mm-thick gels mounted on a Mini-Protean II Dual Slab Cell electrophoretic system (Bio-Rad), Marnes-la-Coquette. 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. The 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, Velizy-Villacoublay, France). The quantification of the bands revealed the existence of MyHC-IIb isoform in only two animals. Consequently, MyHC-IIb percentages were totaled with those of MyHC-IIx creating a new variable 'MyHC-IIx+b' (fast glycolytic fibres) as described in Gagaoua *et al.* (2017). Thus, the proportions of three MyHC isoforms (I, IIa and IIx+b) were considered in this report.

*Intramuscular fat content.* Total lipid content of LT, RA, SM and ST was estimated by NIRS according to the procedure previously described by our group (Guy *et al.*, 2011). 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, USA), compressed and sealed with a disposable paper-backed wrap. Samples were scanned in reflectance mode (400 to 2500 nm) in an NIRS 6500 scanning monochromator (NIRSystems, Silver Spring, MD, USA) using ISI software, version 3.01, from 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) which was used to estimate IMF values.

The NIRS model used was proposed by Andueza *et al.* (2019). This model included spectra and biochemical analyses performed on 48 samples used in the current study. For the biochemical analyses, muscle DM 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 according to the method of Folch *et al.* (1957). Each sample was measured in triplicate and data were expressed in 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.

*Statistical analyses*

The statistical analyses were conducted using XLSTAT 2017.19.4 (AddinSoft, Paris, France). The investigation of the variations among the muscle component variables were analysed according to a randomized factorial design, each animal being a replicate, and the model included the effects of M and AT, and that of their interaction. The significance of the effects was tested at the level of 5%. The significance of the difference between factor means was tested using the Student–Newman–Keuls' (equal cell sizes) or the Bonferroni's (unequal cell sizes) multiple comparison procedures. The overall model was as follows (1):

$$y_{ijk} = \mu + a_i + b_j + (ab)_{ij} + e_{ijk} \qquad (1)$$

where $y_{ijk}$ is the dependent variable of IMCT components (TCol, ICol, CLs and TPGs), muscle fibre types (type I, IIA and IIX+B) and IMF content; $\mu$ is the overall population average; $a_i$ is the muscle type (M); $b_j$ is the mu AT; $(ab)_{ij}$ is the effect of interaction between M and AT; and $e_{ijk}$ is the random error.

In order to get an overview of the relationships between the variables (TCol, ICol, CLs, TPGs, type I, IIA and IIX+B muscle fibres and IMF) a principal component analysis (**PCA**) was performed on raw data. On this PCA, the four muscles (LT, RA, SM and ST) and the two AT (beef and dairy) were included as supplementary data to highlight their relationships with the active variables. Sample distribution plots using the two-dimensional coordinate system defined by the first two principal components (**PCs**) with eigenvalues of 2.96 and 2.50, respectively, were used to highlight the variability among the muscles and/or ATs. An eigenvalue represents the amount of variance that is captured by a given component. Eigenvalue criterion is one of the most commonly used criteria for solving the number of components problem, also known as the Kaiser–Guttman criterion. To check the suitability of the factorial model, the Kaiser–Meyer–Olkin test for sampling adequacy was used.

For correlation analyses, *Z*-scores were computed to consider the main factors in this trial and to use the standardized data only. *Z*-scores represent the deviation of each parameter observation (mean of 0 and SD of 1) relative to the mean of the corresponding animal irrespective of the muscle, experiment and other confounded effects related to age or AT. They were computed for each muscle and corresponding AT separately and then used for the analyses. Before the correlation analyses, homoscedasticity requirements were met, that is, experimental errors were independently and normally distributed and possess a constant variance. Pearson correlation coefficients were computed between all the measured parameters using the computed residuals (*Z*-scores) and considered significant at $P < 0.05$. Thus, the correlations were computed (i) irrespective of M and AT, (ii) within M and irrespective of AT and (iii) within AT and irrespective of M. The correlations were not compared in this study but their redundancy within the three situations above allowed to identify the robust and coherent relationships that are worthy for discussion and to draw conclusions.

## Results

Table 1 summarized the means, SDs, CV and range of the variables used in this report.

*Effect of muscle and animal type on intramuscular connective tissue*

Variance analysis showed an M effect (across AT) ($P < 0.001$) and AT effect (across M) ($P < 0.05$) for the whole IMCT characteristics (TCol, ICol, CLs and TPGs) without any significant interaction (Table 2). TCol content differed significantly ($P < 0.05$) between the four muscles. *Semitendinosus* muscle had the highest amount of TCol followed by RA, SM and LT, respectively. On average, ST muscle contained +14% collagen than RA, 26% more than SM and 35% more than LT. *Semitendinosus* muscle also had the greatest amount in ICol and CLs, the RA and SM muscles were intermediate and the LT had the lowest amount giving rise to this following trend: ST > RA = SM > LT. On average, ST muscle contained 27% and 14% more ICol and CLs, respectively, than RA and SM muscles and 45% and 39% more than LT muscle. The content in TPGs was equivalent in ST, RA and LT muscles and higher for SM muscle (ST = RA = LT < SM). The ratio TPGs : TCol was not different between LT and SM muscles and was on average greater (+35%) than those of ST and RA muscles that were equivalent (LT = SM > ST = RA).

**Table 2** *Variance analyses showing the effects of muscle and animal type on the bovine muscle characteristics*

| | M | | | | AT | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Variables | LT | RA | SM | ST | Dairy | Beef | SEM[1] | M | AT | M × AT |
| IMCT | | | | | | | | | | |
| TCol (mg OH-prol/g DM) | 3.46$^{d}$ | 4.56$^{b}$ | 3.99$^{c}$ | 5.36$^{a}$ | 4.70$^{a}$ | 3.99$^{b}$ | 0.10 | 0.001 | 0.001 | 0.77 |
| CLs (nM Pyd/g DM) | 22.03$^{c}$ | 30.70$^{b}$ | 30.99$^{b}$ | 36.01$^{a}$ | 34.37$^{a}$ | 25.49$^{b}$ | 0.64 | 0.001 | 0.001 | 0.08 |
| ICol (mg OH-prol/g DM) | 1.86$^{c}$ | 2.54$^{b}$ | 2.41$^{b}$ | 3.40$^{a}$ | 2.82$^{a}$ | 2.28$^{b}$ | 0.06 | 0.001 | 0.001 | 0.87 |
| TPGs (μg C4S/g DM) | 190.6$^{b}$ | 178.6$^{b}$ | 243.1$^{a}$ | 203.0$^{b}$ | 189.41$^{b}$ | 218.30$^{a}$ | 4.70 | 0.001 | 0.009 | 0.09 |
| TPGs (μg C4S/g TCol) | 7.40$^{a}$ | 5.15$^{b}$ | 8.04$^{a}$ | 4.82$^{b}$ | 5.31$^{b}$ | 7.40$^{a}$ | 0.20 | 0.001 | 0.030 | 0.44 |
| Muscle fibre types (%) | | | | | | | | | | |
| I | 26.24$^{b}$ | 37.21$^{a}$ | 14.77$^{c}$ | 17.54$^{c}$ | 25.16$^{a}$ | 22.72$^{b}$ | 0.95 | 0.001 | 0.001 | 0.001 |
| IIA | 47.10$^{a}$ | 40.95$^{b}$ | 40.24$^{b}$ | 33.83$^{c}$ | 44.80$^{a}$ | 36.54$^{b}$ | 0.94 | 0.001 | 0.001 | 0.08 |
| IIX+B | 26.62$^{b}$ | 21.84$^{b}$ | 44.38$^{a}$ | 48.62$^{a}$ | 30.04$^{b}$ | 40.71$^{a}$ | 1.33 | 0.001 | 0.001 | 0.13 |
| IMF (mg/100g fresh matter) | 5.80$^{a}$ | 5.41$^{a}$ | 3.46$^{b}$ | 3.67$^{b}$ | 5.24$^{a}$ | 3.94$^{b}$ | 0.17 | 0.001 | 0.001 | 0.01 |

M = muscle; AT = animal type; IMCT = intramuscular connective tissue parameters; TCol = total collagen (μg OH-prol (OH-proline)/mg DM); TCLs = total cross-links (nM Pyd (pyridinoline)/g DM); ICol = insoluble collagen (μg OH-prol/mg DM); TPGs = total proteoglycans (μg C4S (chondroitin-4-sulphate)/g DM or mg C4S/g TCol (OH-prol × 7.14)); IMF = intramuscular fat; LT = *longissimus thoracis*; RA = *Rectus abdominis*; SM = *semimembranosus*; ST = *semitendinosus*.
Least square means in the same row for muscle or animal type not followed by a common letter differ significantly, $P < 0.05$. Otherwise the least square means are not significant (NS).
[1]Overall SEM of the population for each variable.

TCol, ICol and CLs amounts were significantly higher in the muscles of dairy than beef animals (Table 2). For PGs (in μg/g DM or in μg/g TCol), beef animals had the greatest amounts.

*Effect of muscle and animal type on the muscle fibre types*

There was a high M and AT ($P < 0.001$) effect on the three muscle fibre types: RA muscle had the greatest percentages of type I fibres, followed by LT then by SM and ST that were equivalent (RA > LT > SM = ST). *Longissimus thoracis* muscle had a higher proportion of type IIA fibres, on average +13% compared to RA and SM that were equivalent and +30% compared to ST that had the lowest percentages (LT > RA = SM > ST). Finally, SM and ST muscles had, as expected, the greatest values of type IIX+B fibres compared to RA and LT that were equivalent (SM = ST > LT = RA). Dairy animals had the highest percentages of type I and type IIA fibres and the lowest IIX+B fibres compared to beef animals. An interaction M × AT was observed for type I muscle fibres ($P < 0.001$) (Table 2). This interaction was due to the difference of proportion of type I muscle fibres between LT and RA muscles (Figure 1).

The RA muscle of dairy animals had a higher proportion of type I muscle fibres than the LT muscle of beef animals (+50%), while the LT and RA muscles of beef animals had an equivalent proportion of type I muscle fibres. SM and ST muscles had an equivalent proportion of type I muscle fibres that did not differ from one AT to another. The following trend was observed for dairy and beef animals: RA > LT = SM = ST and LT = RA > SM = ST.

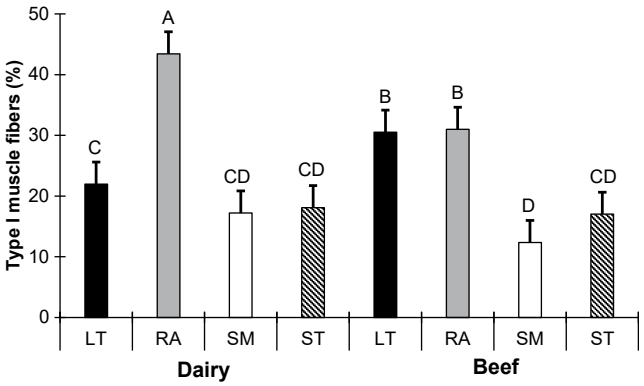


**Figure 1** Percentages of type I muscle fibre according to the four bovine muscles (*longissimus thoracis* (LT), *rectus abdominis* (RA), *semimembranosus* (SM) and *semitendinosus* (ST) muscles) and animal types. The variables followed by the same letter are not different at the significant level of 5%.

*Effect of muscle and animal type on intramuscular fat content*

There was a high M and AT ($P < 0.001$) effect on IMF content (Table 2). Intramuscular fat content was similar between LT and RA muscles and was on average +35% greater compared to SM and ST muscles allowing this following trend: LT = RA > SM = ST (Table 2). Dairy animals had the highest content of IMF (+25%) than beef animals. A significant M × AT interaction ($P < 0.01$) was found for IMF content (Table 2). This interaction was due to the difference of IMF content between the LT and RA muscles of the two ATs (Figure 2). The LT muscle of dairy animals had more IMF than RA muscle (+15%), while the LT and RA muscles of beef muscles had an equivalent IMF content. SM and ST muscles had an equivalent IMF content that did not differ from one AT to another. The following trend was observed for dairy and beef animals: LT > RA > SM = ST and LT = RA = SM = ST.

*Overall relationships between the measured parameters across muscles and animal types by the means of principal component analysis*

The PCA performed on the whole raw data was given in Figure 3a. The first PC, accounting for 32.8% of variability,

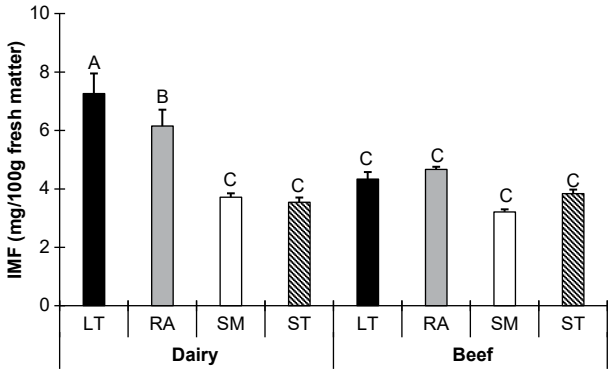


**Figure 2** Intramuscular fat (IMF) content among the four bovine muscles (*longissimus thoracis* (LT), *rectus abdominis* (RA), *semimembranosus* (SM) and *semitendinosus* (ST) muscles) and animal types. The variables followed by the same letter are not different at the significant level of 5%.

was mainly characterized by the opposition between TCol, ICol and CLs (positive side) with TPGs (in mg C4S/g TCol) in the negative side. The second PC, accounting for 27.7% of variability, was mainly characterized by the opposition between type IIX+B muscle fibres, TPGs (μg/g MS) (positive side) and IMF and type I and IIA muscle fibres (negative side).

The bi-plot (Figure 3b) highlighted the separation in the first two PC of the four muscles. ST samples were mainly grouped in the right upper part of the bi-plot which means that they were mainly characterized by high contents of TCol, ICol and CLs. *Rectus abdominis* samples were mainly grouped in right lower part meaning that they are mainly characterized by a high slow oxidative type. *Longissimus thoracis* samples were mainly grouped in the left lower part of the bi-plot, which means that they are mainly characterized by a high IMF content. Finally, SM samples were grouped in left upper part, as they were mainly characterized by high TPG amounts and IIX+B fibre percentages.

For ATs, the samples of dairy animals were mainly located in the right part of the bi-plot (Figure 3c) which means that they had a high collagen content (TCol, ICol and their CLs) and a high IMF content with as expected greater slow oxidative properties of the muscles. Samples from beef animals were mainly located in the left part of the bi-plot, characterized by a high TPGs content, a low IMF content with more fast glycolytic properties.

### *Pearson correlations among variables within muscle and animal type*

The correlations that will be described in this part were computed (i) irrespective of M and AT, (ii) within M and irrespective of AT and (iii) within AT and irrespective of M. These results were presented in Table 3.

### *Between the intramuscular connective tissue parameters*

Total collagen, ICol and their CLs were significantly and positively correlated ($P < 0.05$). This result remained true regardless of the analysis.

Total proteoglycans were positively correlated with TCol (+0.27, $P < 0.05$) and ICol (+0.28, $P < 0.05$) irrespective of M and AT. More specifically, their correlation with TCol remained unaffected for LT (+0.47, $P < 0.001$), ST (+0.49, $P < 0.001$) and beef animals (+0.41, $P < 0.001$) but absent for RA, SM muscles and dairy animals; with ICol, remained unaffected for LT (+0.38, $P < 0.01$), RA (+0.32, $P < 0.05$), ST muscles (+0.32, $P < 0.05$) and beef animals (+0.39,

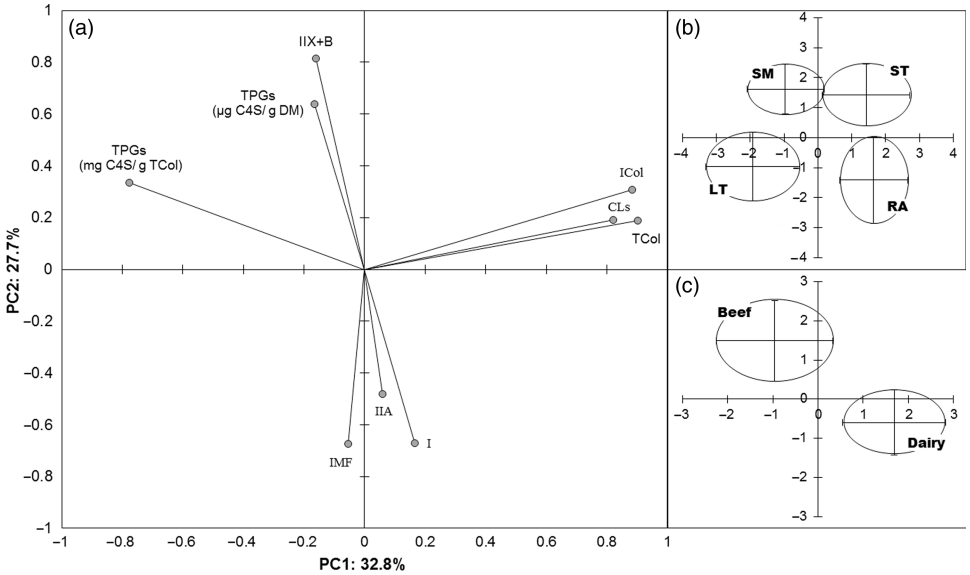


**Figure 3** (a) Principal component analyses depicting the relationships among the four bovine muscle characteristics using the whole raw data as active variables and muscles and animals types as supplementary data. (b) Bi-plot of the projection of the observations of *longissimus thoracis* (LT), *rectus abdominis* (RA), *semimembranosus* (SM) and *semitendinosus* (ST) muscles that are encircled in ellipses (*x*, *y*-means ± *x*, *y*- SD). Furthermore, the barycentres for each muscle are given. (c) Bi-plot of the projection of the observations of animal types following the same procedure of highlighting the distribution of dairy and beef animals. The active variables were TCol = total collagen (μg OH-prol (OH-proline)/mg DM); CLs = cross-links (nM Pyd (pyridinoline)/g DM); ICol = insoluble collagen (μg OH-prol/mg DM); TPGs = total proteoglycans (μg C4S (chondroitin-4-sulphate)/g DM or mg C4S/g TCol (OH-prol × 7.14)); IMF = intramuscular fat content (g/100 g fresh matter); muscle fibre types = I, IIA, IIX+B.

**Table 3** *Pearson correlations between the bovine muscle characteristics (columns A and B), irrespective of muscle (M) and animal type (AT), within muscle and irrespective of animal type and within animal type and irrespective of muscle*

| Column A | Column B | | Within M and irrespective of AT | | | | Within AT and irrespective of M | |
|---|---|---|---|---|---|---|---|---|
| Variables | Variables | Irrespective of M and AT | LT | RA | SM | ST | Dairy | Beef |
| TCol | TCLs | +0.45*** | +0.55*** | +0.57*** | +0.29* | +0.35* | +0.49*** | +0.43*** |
| | ICol | +0.74*** | +0.83*** | +0.78*** | +0.71*** | +0.61*** | +0.69*** | +0.79*** |
| | TPGs | +0.27* | +0.47*** | | | +0.49*** | | +0.41*** |
| | T PGs/TCol | −0.48*** | −0.48*** | −0.54*** | −0.61*** | | −0.43*** | −0.52*** |
| | IIA | +0.16* | +0.29* | | | | | +0.27** |
| | IIX+B | −0.17* | −0.36** | | | | | −0.28** |
| CLs | ICol | +0.58*** | +0.63*** | +0.72*** | +0.45** | +0.45*** | +0.68*** | +0.50*** |
| | TPGs | | | | | | | |
| | T PGs/TCol | −0.27* | | −0.34* | | −0.33* | | −0.38** |
| | I | | | | | | | |
| | IIA | +0.15* | +0.33* | | | | | |
| | IIX+B | −0.18* | −0.30* | | | | −0.34*** | |
| **ICol** | T PGs | +0.28* | +0.38** | +0.32* | | +0.32* | | +0.39*** |
| | T PGs/TCol | −0.29** | −0.32* | −0.28* | −0.36** | | −0.23* | −0.33** |
| | I | +0.16* | +0.33** | | | | +0.30** | |
| | IIA | +0.17* | +0.27* | | | | | +0.26** |
| | IIX+B | −0.30*** | −0.42*** | | −0.34* | | −0.27* | −0.32*** |
| **TPGs** | TPGs/TCol | +0.64*** | +0.43*** | +0.68*** | +0.82*** | +0.70*** | +0.82*** | +0.51*** |
| | IMF | +0.22* | +0.29* | +0.40** | | | +0.22* | +0.21* |

Significances of the correlations: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.
[1]intramuscular connective tissue parameters: TCol = total collagen (μg OH-prol (OH-proline)/mg DM); TCLs = total cross-links (nM Pyd (pyridinoline)/g DM); ICol = insoluble collagen (μg OH-prol/mg DM); TPGs = total proteoglycans (μg C4S (chondroitin-4-sulphate)/g DM or mg C4S/g TCol (OH-prol × 7.14)); I, IIA, IIX+B = muscle fibre types; IMF = intramuscular fat content; LT = *longissimus thoracis*; RA = *Rectus abdominis*; SM = *semimembranosus*; ST = *semitendinosus*.

$P < 0.001$) and absent for SM muscle and dairy animals (+0.39, $P < 0.001$). Whatever the analysis, TPGs were not correlated with CLs.

Total collagen and ICol were globally negatively correlated with TPGs/TCol within M and AT ($P < 0.001$). This relation remained unaffected for LT (−0.48, $P < 0.001$, −0.32, $P < 0.05$), RA (−0.54, $P < 0.001$, −0.28, $P < 0.05$) and SM (−0.61, $P < 0.001$, −0.36, $P < 0.01$) muscles, dairy (−0.43, $P < 0.001$, −0.23, $P < 0.05$) and beef (−0.52, $P < 0.001$, −0.33, $P < 0.05$) animals and absent within ST samples. Cross-links were globally negatively correlated with TPGs/TCol within M and AT (−0.27, $P < 0.01$), this correlation remained unaffected for RA (−0.34, $P < 0.05$) and ST (−0.33, $P < 0.05$) muscles and for beef animals (−0.38, $P < 0.01$) and absent for LT, SM muscles and within dairy animals.

*Between the intramuscular connective tissue parameters and muscle fibre types*

There was no correlation between TCol or CLs and type I fibres. On the contrary, ICol was positively correlated with type I fibres, irrespective of M and AT (+0.16, $P < 0.05$). This relation remained unaffected for LT muscle (+0.33, $P < 0.01$) and within dairy animals (+0.30, $P < 0.01$). Total collagen, ICol and CLs were, respectively, slightly ($P < 0.05$) positively and negatively correlated with type IIA and IIX+B fibres within M and AT. This relation remained unaffected only for LT muscle. TPGs or TPGs/TCol were correlated with none of the three fibre types.

*Between intramuscular fat and the other parameters*

There was no correlation between IMF and TCol, ICol and CLs but IMF was positively correlated with TPGs (within M and AT: +0.22, $P < 0.05$). This relation remained unaffected for LT (+0.29, $P < 0.05$) and RA (+0.40, $P < 0.01$) muscles, for dairy (+0.22, $P < 0.05$) and beef animals (+0.21, $P < 0.05$) but was absent for SM and ST muscles.

Intramuscular fat was not correlated with muscle fibre types.

## Discussion

*Relationships among the intramuscular connective tissue components*

The main CLs in adult bovine muscle are the pyridinolines (Kuypers and Kurth, 1995). Few authors who were interested in variations of CL content between muscles have shown, as in the present study, a large variability (Ngapo *et al.*, 2002). This study is the first to highlight that whatever the muscle and AT, CLs were positively correlated with TCol. This probably reflects the functional differences of the muscles. According to the functional requirements of muscle (contraction and elongation), the quantity and composition of IMCT vary in order to coordinate force transmission in muscle

(Purslow, 2010). The muscles involved in the movement, for example, ST muscle, are characterized by more CLs and collagen than postural muscles, for example, LT muscle. In this study, CLs were also highly correlated with ICol whatever the muscle and AT. Although this result is observed for the first time, it is not surprising since Bailey and Light (1989) reported that the nature of CLs, more specifically heat-stable CLs such as pyridinoline, determined collagen solubility. PGs could also be involved in the determinism of collagen solubility. As a matter of fact, in this study, we identified a relation between PGs, CLs and ICol, this result being muscle-dependent. These results confirm our previous preliminary studies (Dubost *et al.*, 2013). The PGs are either intracellular or cell-surface, pericellular and extracellular (Iozzo and Schaefer, 2015). In muscle, the major PG (decorin) is an extracellular PG that belongs to the family of small leucine-rich proteoglycans (**SLRPs**) (Gillies and Lieber, 2011). The SLRPs could regulate the intermolecular cross-linking of collagen by controlling lysyl oxidase activity and the collagen fibril diameter (Kalamajski and Oldberg, 2010).

*Relationships between the intramuscular connective tissue components and the muscle fibre types*

There is scarcity in the data dealing with the relationship between CT and muscle fibre types. Among existing studies focused on total and ICol and whatever the species, no clear relationship has been reported (Lefaucheur, 2010). In the present study, there was a relationship between CT components and muscle fibre types only in LT muscle that is a rather oxydo-glycolytic muscle according to its high proportion of type IIA fibres. However, it is worthwhile to note that on the same LT muscle differences exist among breeds (Gagaoua *et al.*, 2017). In this muscle, TCol, ICol and CLs were respectively positively and negatively correlated with oxydo-glycolytic (IIA) and glycolytic (IIX+B) fibres. Except ICol, there was no correlation with type I muscle fibre. Overall, our results (about TCol and ICol) are coherent with those reported in robust correlation network by Gagaoua *et al.* (2016) and partially in agreement with those of Kovanen *et al.* (1984). These latter showed that, in rat, slow twitch muscle fibres are more collagenous than the glycolytic ones. About PGs, the results of the present study failed to demonstrate any relation between them and muscle fibre types. There are no, to our knowledge, data on this subject in the literature.

*Relationships between intramuscular fat, the intramuscular connective tissue components and the muscle fibre types*

Few studies investigated the relationships between IMCT and IMF and among the very few examples that exist; only TCol and ICol were mostly considered. Our results did not support any relationship among TCol, ICol and IMF. These are in line with those of Seideman (1986) and Gagaoua *et al.* (2016) in LT muscle of animals of different sex and breeds and in disagreement with those of Christensen *et al.* (2011). In this last study, the authors showed a positive correlation between TCol, ICol and IMF using raw data from 436 young bulls of 15 European breeds.

The only significant correlation between IMCT and IMF was with TPGs when data were analysed within M and AT, this correlation being unaffected for the two more fatty muscles, LT and RA. To differentiate, the adipocytes need a specific force that would be imposed by ECM (Cristancho and Lazar, 2011). Yet, it is known that the composition of ECM influences its stiffness, the molecules supposed to be regulators of the rigidity of the ECM being the CLs (Depalle *et al.*, 2015) and some SLRPs such as the decorin and biglycan (Reese *et al.*, 2013; Saneyasu *et al.*, 2016; Listrat *et al.*, 2019). These results, obtained *in vitro*, would partly explain the correlation found between PGs and IMF and between CLs, and the ratio TPGs : TCol, but further studies are needed to clarify this statement.

The correlations between type I and IIX+B muscle fibre types and IMF content were in agreement with the results of literature when calculated across M and AT. As in the present study, several authors reported that IMF content was typically positively correlated with the percentage of oxidative fibres and negatively with the glycolytic fibres (Lefaucheur, 2010). When the results were computed within M and AT, there were no more relationships between IMF and muscle fibre types. This explains that, in literature, there are so many contradictory results on the subject (Listrat *et al.*, 2016)

## Conclusion

This study is the first to describe the relationships among the components of intramuscular connective tissue, muscle fibre types and IMF in bovine muscle. The associations between TCol, ICol and their CLs were M- and AT-independent. The other correlations were all M-dependent, irrespective of AT. Total proteoglycans were correlated with TCol and ICol and CLs and ICol were correlated with the ratio TPGs: TCol or with TPGs. This result suggested that CLs and PGs could be involved in collagen solubility. The results of this report suggest further that TCol, ICol and their CLs have an M-dependent relationship (specific of LT muscle), irrespective of AT, with muscle fibre typology. PGs were the only CT component to be correlated with IMF. Muscle fibre types had no relationship with IMF content within M and AT.

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### Declaration of interest

The authors declare no conflict of interest.

### Ethics statement

In this trial, there was no need to get any further approval because the animals were not from experimental farms. In addition, the experimental samples were dissected at a commercial slaughterhouse.

### Software and data repository resources

None of the data were deposited in an official repository.

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