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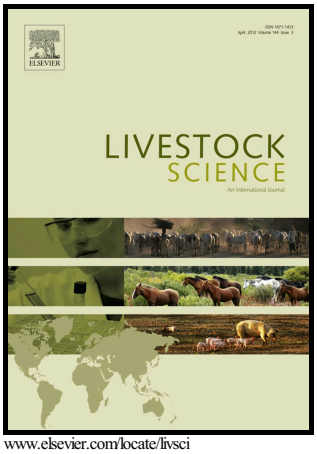

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COLLAGEN XII AND XIV, TWO COLLAGEN TYPES BOTH ASSOCIATED WITH BOVINE MUSCLE AND INTRAMUSCULAR LIPID METABOLISM

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Abstract:

In continental Europe, intramuscular fat (IMF) content in beef is low but plays a key role in determining flavour. IMF expands inside the extracellular matrix (ECM) of intramuscular connective tissue. This ECM contains among others, collagen XII and XIV, two minor collagens probably involved in muscle metabolism. So, our purpose was to determine if there was an association between the contents in collagen XII and XIV and muscle metabolism. *Rectus abdominis* (RA, oxidative) and *Semitendinosus* (ST, oxido-glycolytic) muscles were sampled from steers of two genotypes with high (Angus [Ang], n=10) or low (Limousine [Lim], n=10) intramuscular fat level. Collagen XII and XIV relative abundance was evaluated by Western blot analysis. The characteristic parameters of metabolism were previously measured. Ang muscles contained more collagen XIV than Lim muscles ($p < 0.001$). RA muscle contained more collagen XII than ST muscle ($p < 0.01$). Across the two breeds and muscles, collagen XII was positively correlated with A- and H-fatty acid binding protein (FABP) contents (+0.43 and +0.53, respectively, $p < 0.05$) and activities of three oxidative enzymes (cytochrome-*c* oxidase: +0.41, isocitrate dehydrogenase [ICDH]: +0.40, citrate synthase [CS]: +0.32) ($p < 0.05$) and collagen XIV was positively correlated with

triacylglycerol content (+0.35, $p < 0.35$), A- and H-FABP protein contents (+0.35 and +0.38, respectively, $p < 0.05$) and ICDH (+0.34, $p < 0.05$) and negatively with activities of two enzymes of glycolytic metabolism (phosphofructokinase: -0.31 and lactate dehydrogenase: -0.42, $p < 0.05$). After removing the genotype and muscle effects, only collagen XII remained correlated with A- and H-FABP and CS activity (+0.30, +0.34 and +0.46, respectively, $p < 0.0505$). H-FABP is expressed in various tissues but predominantly in cardiac and oxidative skeletal muscles, whereas A-FABP is exclusively expressed within adipocytes. From these results, we can conclude that collagen XII and XIV are both associated with bovine muscle fiber and intramuscular lipid metabolism, but probably by different mechanisms. As a matter of fact, collagen XII is associated with intramuscular fat differentiation (from its correlation with A-FABP content) and oxidative metabolism (from its correlation with H-FABP content and CS activities) regardless of breed and muscle, whereas type XIV collagen is associated with the same parameters plus enzymes of glycolytic metabolism that discriminate breeds.

Key Words: bovine, collagen, muscle, intramuscular lipid, metabolism

Introduction

Intramuscular fat (IMF) content referred as marbling is one of the factors determining the quality grade of beef meat in North America, Asia and Australia. In continental Europe, IMF content in beef is low (5% on average), however it plays a key role in determining flavour (Gandemer, 2002). The number and the diameter of intramuscular adipocytes are major factors determining IMF content and hence marbling (Cianzio et al., 1985). IMF expands inside the extracellular matrix (ECM) or ground substance of intramuscular connective tissue (IMCT) (Nishimura et al., 1999). The ECM is a dynamic network of molecules mainly composed of fibrillar collagens often referred as total collagen and other minor components. Among ECM molecules, there are 2 minor (by opposition to major ones that are fibrillary

collagens) collagens, namely type XII and XIV. Collagen XII and XIV belong to the family of molecules called Fibril Associated Collagen with Interrupted Triple Helix (FACIT) collagens. They are present in perimysium of skeletal muscle, where they are co-localized (Listrat et al., 2000).

Collagen XII and XIV were previously thought to play a role in differentiation of adipocytes, in which IMF accumulates (Tahara et al., 2004; Ruehl et al., 2005), and hence in marbling development. Collagen XII could also play a role in oxidative metabolism of muscle fibres (Zou et al., 2014). From all these fragmented results, obtained in cell culture and in collagen XII knock-out mice, respectively, we can suppose that collagen XII and XIV would be involved in muscle metabolism as a whole (both IMF and muscle fibres).

To verify the hypothesis of any association of collagen content and more particularly of collagen XII and XIV with muscle metabolism, we have studied the relationships between total collagen, collagen XII and XIV contents and some enzymes of muscle energy metabolism and markers of metabolism of intramuscular lipids in two muscles with a great difference in their metabolic properties (Jurie et al., 2007). To increase the variability in fatness of muscles, our study was performed in two breeds (Angus [Ang] *vs* Limousin [Lim]) with different ability to develop marbling as previously described (Hocquette et al., 2003). We have used this experimental design to test the hypothesis that breed type and muscle type will affect the contents of type XII and XIV collagens within skeletal muscle.

Material and methods

The study was carried out in compliance with the French recommendations and those of the Animal Care and Use Committee (IACUC) of the National Institute for Agricultural Research (INRA) of Clermont-Ferrand/Theix, France for the use of experimental animals including animal welfare.

Animals and muscle sampling

Two groups of 10 steers from two different breeds (Lim and Ang) were used as previously described. Although reared in two different countries, in the experimental unit of INRA Theix Research Center in France for Lim and in the experimental farm of Murdoch University in Australia for Ang, both breeds have received the same diets during a 6-month finishing period (Table 1) (Bonnet et al., 2007). All animals were slaughtered at 23 months of age.

Carcasses were chilled in a cold room (+2 °C) and muscle samples were taken at 24 h *post-mortem*. Two muscles were taken for each animal: *Rectus abdominis* [RA] (oxidative), and *Semitendinosus* [ST] (glycolytic). For each muscle and each analysis, samples were taken at the same location, the centre of the muscles. For total collagen measurement, muscle samples (about 150 g) were cut into pieces of 1 cm cross-section, sealed under vacuum in plastic bags and frozen. Frozen muscle was homogenized in a household cutter, freeze-dried for 48 h, pulverized in a horizontal blade mill and finally stored at +4 °C in stopper plastic flasks until analyses. For collagen XII and XIV measurements, 60-80 g of muscle was cut into small pieces and frozen in liquid nitrogen. They were ground in liquid nitrogen in a mill to produce a fine homogeneous powder and stored at−80 °C.

Collagen content

Muscle collagen content was determined from the hydroxyproline (OH-prol) concentration and expressed in OH-prol / mg of muscle dry weight (Dubost et al., 2013).

Western blot analyses of type XII and XIV collagens

Type XII and XIV collagens were extracted with RIPA (Radio Immunoprecipitation Assay) lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, adjusted at pH =

7.4 and completed with 100 mM sodium fluoride, 4 mM sodium pyrophosphate and 2 mM orthovanadate, 1% Triton 100X, 0.5% Igepal CA-630 and protease inhibitor cocktail [Complete, Roche Diagnostics GmbH, ref. 11 836 145 001]). After extraction, protein concentration was determined by spectrophotometry (UVIKON 860) with the Bradford assay (Bradford, 1976).

Proteins were separated in denaturing conditions (10% sodium dodecyl sulphate-polyacrylamide and β-mercaptoethanol). Samples were loaded on the gel (stacking gels of 4% and separation gels of 6%) at the rate of 50 µg of protein for collagen XII and XIV. Gels were run at 80 V for 20 min and then 120 V for 1 h, at +4°C.

Bands were transferred to a PVDF membrane (ref. IPVH00010, Millipore) at 120 mA for 5h at +4°C. Unspecific binding of antibodies to the membranes were blocked with 10% milk T-TBS 1X blocking buffer at 37°C for 20 min. Membranes were washed 3 x 5 min in T-TBS 1X and then incubated overnight at +4°C with the primary antibodies. The three primary antibodies were monoclonal mouse anti-bovine antibodies. Anti-type XII (clone 20H2) and anti-type XIV collagen (clone 15B8) were diluted to 1/50. Anti-type XII and XIV were previously characterized (Aubert-Foucher et al., 1992; Berthod et al., 1997). Membranes were washed 2 x 10 min in T-TBS 1X and hybridized with the second antibody associated with horseradish peroxidase for chemiluminescence detection (IgG sheep anti-mouse, NA931 Amersham) diluted to 1/5000.

Each collagen type presented two or three specific bands which were considered and quantified as single band under Image Quant software. A mix of all samples was deposited on all the gels for normalization. The value obtained for all bands of each samples was normalized by the value obtained for the bands of the mix of samples loaded on each gel. Each sample was measured in triplicate and results were expressed in arbitrary units or per mg of OH-proline.

Muscle and lipid metabolism

Total lipids were extracted from muscle samples using chloroform-methanol (2:1, vol/vol) according to the method of Folch et al. (1957). Muscle triacylglycerol content (TAG) was determined from total lipid extracts, as described by Leplaix-Charlat et al. (1996) and was expressed in g/100 g of fresh muscle. As the adipocytes (cells of IMF) contain almost exclusively TAGs (Gandemer, 2002) and that total IMF content is highly correlated with TAG content (Hocquette et al., 2012), we have chosen to present only TAG content. The fatty acid binding protein (FABP) contents were determined by ELISA, as described by Piot et al. (2000), on cytosolic protein preparations from muscles, using a polyclonal antibody raised against rat heart and skeletal muscle (H-FABP) or rat adipocyte (A-FABP) isoforms, respectively. Contents of two isoforms of FABP were expressed in µg/mg of cytosolic protein. Maximal activity levels of enzymes reflecting the glycolytic metabolic pathway (phosphofructokinase [PFK] and lactate dehydrogenase [LDH]), the mitochondrial density (isocitrate dehydrogenase [ICDH], citrate synthase [CS]), the oxidative phosphorylation (cytochrome-*c* oxydase [COX]), and the potential for fatty acid β-oxidation (hydroxyacyl-CoA dehydrogenase [HAD] were measured spectrophotometrically as previously described by Jurie et al. (2007) and Hocquette et al. (2012). The results were expressed in nanomoles.minute^{-1}.milligram of protein^{-1} or micromoles.minute^{-1}.milligram of protein^{-1}

Statistical analysis

Differences between genotypes and muscles were analysed by analysis of variance using XL-Stat, (2013). Differences were considered to be statistically significant if $P < 0.05$ and to show a tendency for a statistical significance if $P < 0.10$. Fixed effects included genotype, animal nested within genotype, muscle type and interaction between muscle and genotype. All results were presented as least square means ± SEM.

In order to get an overview of the relationships between variables of energy metabolism (ICDH, COX, PFK, LDH), lipid metabolism (TAG, H-FABP and A-FABP and LPL) and collagen characteristics (contents of total collagen and of collagens XII and XIV), a principal component analysis (PCA) was performed (Destefanis et al., 2000) with muscle and genotype effects. PCA allows calculating new variables, called principal components (PC), which account for the variability in the data across breeds and muscles. This enables a description the information with fewer variables than originally present. Pearson correlation coefficients between collagen characteristics (total collagen content, collagen XII and XIV contents, ratios of collagen XII or XIV to total collagen content) and the other variables (total TAG content, A-FABP and H-FABP protein contents as well as enzymatic activities) were calculated using XL-Stat, (2013) firstly with pooled data of the two muscles and of the two breeds. Then, as some parameters presented a genotype effect and others a muscle effect, Pearson's correlation coefficients were also calculated on all the residual observations after removing the muscle and the genotype effects.

Results

Least square mean values of parameters of muscle metabolism according to muscles and breeds are presented in Table 2. The muscle effect (except for CS) and the breed effect (except for HAD) were significant for all measured parameters. The effect of the muscle x breed interaction was also significant in some cases. These results were not detailed because they were previously published by Jurie et al. (2007).

Difference in total collagen and collagen XII and XIV contents between muscles and genotypes

Total collagen content significantly differed between breeds (Table 3). Its content was on average higher in Ang than in Lim muscles across muscles (+62%). Across genotype, there

was a tendency for a muscle effect; on average, ST muscle had more collagen content than RA muscle (+15%).

In RA and ST muscles of Ang and Lim steers, we observed the presence of both collagen XII and XIV. They were present as two bands of 220 and 290 kD (Fig.1). For collagen XIV, there were no muscle effect but a strong breed effect, while, for collagen XII, there was a muscle effect, and no breed effect. There was no muscle x breed interaction for both total collagen content and collagen XII and XIV abundances (Table 3). Collagen XIV relative abundance was on average higher in Ang than in Lim breed across muscles (+71%) (Table 1) and collagen XII relative abundance was higher in RA than in ST (+145%). For the ratios of collagen XIV or XII to total collagen, there was a significant muscle effect with higher values in RA than in ST muscle across breeds (+64% for collagen XIV / total collagen and +149% for collagen XII / total collagen, $p < 0.05$ and 0.01, respectively). For these parameters, the breed and the muscle x breed interaction were not significant (Table 3).

Relationships between all measured parameters

The principal component analysis performed on the pooled data (40 values: two breeds x 10 animals and two muscles) is illustrated in Fig. 2. The first principal component explained 49.79% of variance and the second principal component explained 11.19% of variance. The first principal component was mainly characterized by the opposition between activities of enzymes of glycolytic and oxidative metabolism (PFK and LDH *vs* COX and ICDH, $-0.46 < r < -0.60$ $p < 0.05$).

The correlation analysis (Table 4) showed that total collagen content was positively correlated with TAG content and the activity of the enzyme CS which is associated with oxidative metabolism, (+0.47 and +0.48 respectively, $p < 0.05$). On the contrary, total collagen was negatively correlated with activity of two enzymes of glycolytic metabolism, namely PFK and

LDH (-0.34 and -0.36, respectively, $p < 0.05$). Collagen XII content was positively correlated with A- and H-FABP protein contents (+0.43 and +0.53 respectively, $p < 0.05$) and with activities of three oxidative enzymes, COX, ICDH and CS (+0.41, +0.40 and +0.32, respectively, $p < 0.05$). Collagen XIV was positively correlated with TAG content (+0.35, $p < 0.05$), A- and H-FABP protein contents (+0.35 and +0.38 respectively, $p < 0.05$), with activity of an enzyme of oxidative metabolism, namely ICDH (+0.34, $p < 0.05$) and negatively with activities of two enzymes of glycolytic metabolism, namely PFK and LDH (-0.31 and -0.42 respectively, $p < 0.05$). The ratio of collagen XII / total collagen was positively correlated with A- and H-FABP contents (+0.32 and +0.37 respectively, $p < 0.05$) and with activity of ICDH (+0.31, $p < 0.05$). The ratio of collagen XIV / total collagen was positively correlated with H-FABP content +0.47, $p < 0.05$) and with activity of ICDH (+0.43, $p < 0.05$) and negatively with activity of LDH (-0.41, $p < 0.05$). Collagen XII and XIV contents were not correlated to total collagen content (r=0.19 and 0.16 respectively) and not correlated to each other (0.20, not significant) (Table 4).

The score plot (Fig. 3) was drawn with all samples in the multivariate space of the two first principal components. RA samples from Ang tended to be located in the right part of the score plot which means that they were mainly characterized by high contents of TAG and collagen. Other samples (RA and ST from Lim and ST from Ang) were in the left part of the score plot which indicates that they contained less fat. In addition, the score plot shows that, regardless the breed, ST contains less collagen XII than RA muscles since these muscles are clearly discriminated according to collagen XII. Similarly, the two breeds are discriminated according to collagen XIV.

Relationships between all measured parameters after removing the breed and muscle effects

Because the variance analysis underscored a breed or a muscle effect for all studied parameters (Table 3), we performed correlation analyses on the residual observations after removing the breed and muscle effects. It was characterized by positive remaining correlations between collagen XII content and some markers of lipid metabolism or oxidative metabolism. Content of collagen XII was positively correlated with A- and H-FABP protein contents (+0.30 and +0.34, respectively, $p < 0.05$) and with CS activity (+0.46, $p < 0.05$). These results are detailed in Table 2.

Discussion

It is known that late-maturing cattle breeds (such as Lim) deposit more muscle and less fat, compared to early-maturing cattle breeds (such as Ang). In addition, the muscles of breeds such as Ang contain more collagen (Christensen et al., 2011; Dubost et al., 2013) and have a more oxidative and less glycolytic metabolism than those of breeds such as Lim (Jury et al., 2007). This combination of factors is the reason why we have chosen to use Ang and Lim animals from a previous experimental design (Jurie et al., 2007) to study the association between collagen content and muscle metabolism.

Total collagen content

In the present study, we confirmed that the Ang muscles contain more collagen than the Lim muscles. The two selected muscles showed no significant difference in their content of total collagen when all animals were analyzed simultaneously. When the difference in content of total collagen between muscles was analyzed within a breed, the RA muscle of Lim showed significantly less total collagen than the ST and the same tendency was observed for the Ang.

To our knowledge, these are the first data in the literature concerning the comparison of total collagen content between ST and RA muscles.

When all the samples were taken in account, there was a significant positive correlation between total collagen content, TAG content and activity of CS, an enzyme of oxidative metabolism. These results confirmed statements of Bacou and Vigneron in a review paper (Bacou and Vigneron, 1988) who indicated that the more oxidative muscles had more intramuscular fat and more collagen. These results also confirmed results of Christensen et al. (2011) who observed that total collagen and total lipid content were partially correlated. However, our results clearly demonstrated that this correlation depends on the breed of animals. When this correlation was calculated, as in this study, for two extreme breeds (Lim and Ang) for their muscle content in total collagen and lipids, it was higher (0.47) compared to the situation when 15 European breeds were compared (r=0.20, Christensen et al. (2011). Furthermore, when the correlation between TAG and collagen contents was calculated after removing the breed effect, it became non-significant. So we conclude that the total collagen content of muscle is more likely associated with genetic (breed) effects and less likely associated with oxidative and lipid metabolism. We could also speculate that, the Lim cattle having more muscle mass, there is muscle cross sectional dilution of collagen so giving a lower final collagen value (Jurie et al., 2007).

Collagen XII and XIV

Collagen XII and XIV, two disulphide bonded and structurally homologue polypeptides, (Agarwal et al., 2012) were present in all studied samples. Because of the reducing migration conditions (Aubert-Foucher et al., 1992), both presented two main bands of respectively 220 and 290 kD. This band is due to incomplete denaturation in electrophoresis sample buffer in absence of urea. These two collagens may present differences in their expression pattern but

also be co-localized. For example, during chicken embryonic development (Wälchli et al., 1994) and in skin, they have distinct localizations (Berthod et al., 1997), but in fetal and adult skeletal musclen, they are expressed at the same time in the perimysium (Listrat et al., 2000). They have functional homologies. They interact with other ECM molecules such as decorin (Font et al., 1998) or tenascin-X (Lethias et al., 2006), they promote collagen gel contraction *in vitro* (Nishiyama et al., 1994), but their respective roles are still unclear. Collagen XII could form flexible bridges between collagen fibrils by interacting with them both directly and indirectly by its binding partners. This could allow the tissues to absorb shear stresses upon loading (Chiquet et al., 2014). Collagen XIV could regulate collagen fibrillogenesis. This was suggested by the fact that tendons of collagen XIV deficient mouse showed larger collagen fibrils than wild type mouse (Ansorge et al., 2009).

In the present study, collagen XII and XIV were present in the two muscles of the two breeds, but for collagen XII there was a muscle effect, and for collagen XIV, a breed effect. For collagen XIV, the results about the lack of muscle effect were comparable to data of Dubost et al. (results in press), even though these authors analysed different muscles. The same authors did not find any breed effect, although there was a tendency for higher collagen XIV content in Angus than in Blonde d'Aquitaine muscles (results in press). This difference may be explained by effects of age and hormones since these authors worked with 18-month-old bulls while we worked with 23-month-old steers. However, to our knowledge, there are no data in the literature to support this hypothesis. These results associated to those cited above suggested that collagen XII and XIV could have distinct roles in skeletal muscle. This idea is reinforced by the fact that during fetal life in the muscle, when the expression of collagen XII decreases, collagen XIV expression increases (unpublished data).

Collagen XII

Collagen XII content was positively correlated with A- and H-FABP contents and with activities of some oxidative enzymes (namely with COX, ICDH, CS) with no correction for the breed and muscle effects. These correlations, except those with COX and ICDH activities, remained valid independently of breed and muscle type, i.e. after removing the breed and muscle effects. A- and H-FABP are small cytosolic proteins that specifically bind and transport non esterified long chain fatty acids intracellularly. A-FABP is correlated with TAG, independently of muscle and of genotype, and therefore would be an indicator of the intramuscular fat content (Jurie et al., 2007). It is a marker for the differentiation of intramuscular adipocytes (He et al., 2012). *In vitro* studies on bovine intramuscular pre-adipocytes lines (Tahara et al., 2004) have suggested that collagen type XII could be related to adipogenesis. Thus, collagen XII (from its correlation with A-FABP) would be associated with intramuscular lipid metabolism. As hypothesized by Tahara et al. (2004), collagen XII could be involved in the process of adipocytes differentiation. Collagen XII (from its correlations with some enzymes of oxidative metabolism) would be also associated with the oxidative muscle metabolism. This assumption is corroborated by the results of Zou et al. (2014) which showed that the lack of collagen XII (in Col12a1-/- mice) is associated with a decrease in oxidative metabolism in two types of muscle. In *m. soleus*, there was a decrease in type I fiber number and an increase in type IIA fiber number. While in the *m. tibialis anterior*, the lack of collagen XII was associated with a decrease in type IIX fiber number and an increase in type IIB fiber number, the fibers I being more oxidative than IIA, the IIX oxido/glycolytic and the IIB, glycolytic fibers.

Collagen XIV

Collagen XIV content was positively correlated with TAG content and with A-and H-FABP, protein contents as well as with the activity of one oxidative enzyme (ICDH). Furthermore,

collagen XIV content was negatively correlated with activities of two glycolytic enzymes (PFK, LDH). As for collagen XII, collagen XIV appeared to be associated with intramuscular lipid metabolism and with the muscle metabolic characteristics (oxidative and glycolytic). But these results were mainly dependent on the breed since all positive correlations between collagen XIV content and muscle metabolism were not significant after removing the breed effect. The higher proportion of type XIV collagen in the Angus muscles could favour the development of IMF as suggested by Ruehl et al. (2005). As adipocytes have functions in energy storage, organizing energy supply and also as a shock absorber, their size has to change continuously. Consequently, they would need a more flexible ECM. Collagen XII and XIV could participate in ECM deformability, by making it more or less flexible according to the constraints on the tissue (Nishiyama et al., 1994) and then allow to adipocytes to grow in perimysium.

Conclusions

We can thus conclude that type XII collagen would be associated with intramuscular fat differentiation (from its correlation with A-FABP content) and oxidative metabolism (from its correlation with H-FABP content, COX, ICDH and CS activities) regardless of muscle and breed. Type XIV collagen is also associated with muscle metabolism (including IMF content) but across breeds. Then, type XII and XIV collagens are both related to muscle metabolism and IMF accumulation, but probably by different biological mechanisms. Fat plays a role in the taste of meat, probably retaining fluids during cooking leading to an increase in juiciness. The nature of the collagen tissue around the fat cells could be important because the tensions generated during cooking could lead to significant losses of fat and thus affecting juiciness. Our results are a step in the understanding of the relationships between the components of connective tissue and intramuscular fat.

Acknowledgements

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Figure captions

Figure 1: Western blots of Angus and Limousin *Rectus abdominis* (RA) and *Semitendinosus* (ST) muscle extracts. The monoclonal antibodies against collagen XII and XIV, two disulphide bonded polypeptides, recognized two bands on Western blots of bovine muscle extracts; one band migrated at 220 kDa and the other migrated at around 290 kDa. The presence of these two bands is due to the fact that migration conditions are reducing.

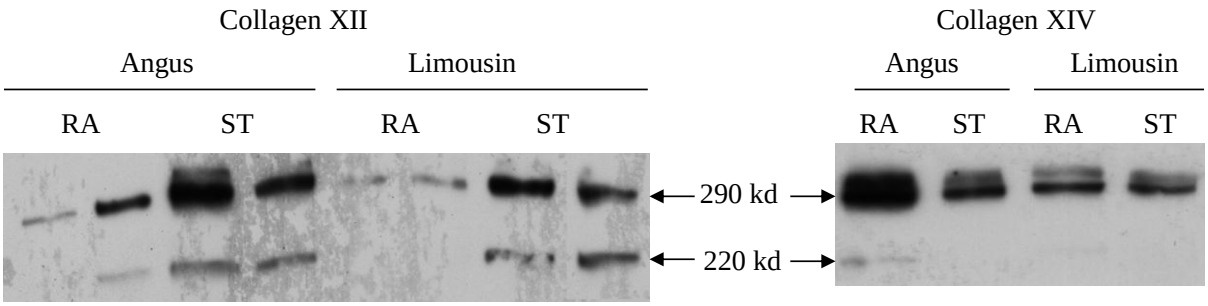


Figure 2: Principal component analyses showing relationships between muscle characteristics performed on the pooled data (40 values: two breeds x 10 animals and two muscles). The variables were triacylglycerol (TAG) content (g/100 g of fresh muscle), activities of phosphofructokinase (PFK) and lactate dehydrogenase (LDH) (µmol.min^{-1}.mg^{-1} of protein), hydroxyacyl-CoA dehydrogenase (HAD), isocitrate dehydrogenase (ICDH), citrate synthase (CS), cytochrome-*c* oxidase (COX) (nmol.min^{-1}.mg^{-1} of protein), total collagen content (tot col) (µg OH-prol.mg^{-1} of dry matter) and contents of collagens XII and XIV (Col XII and XIV) (arbitrary units).

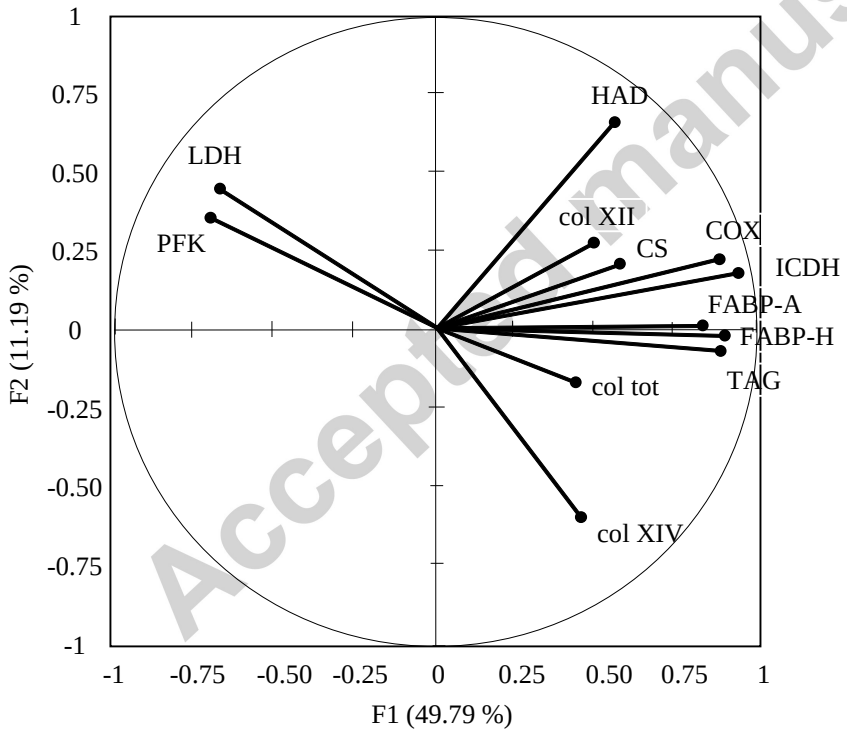
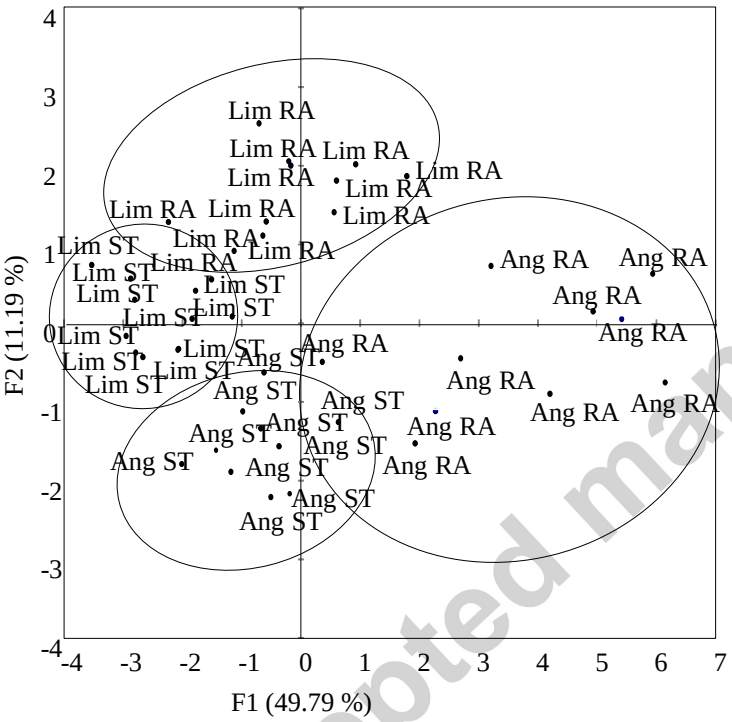


Figure 3: Principal component analyses showing projection of muscle samples from each breed in the same 'xy' plane as for Figure 1. Each muscle from each breed is indicated by the name of the breed (Ang: Angus or Lim: Limousin followed by the name of the muscle (RA: *Rectus abdominis*; ST: *Semitendinosus*).



References

Agarwal, P., Zwolanek, D., Keene, D.R., Schulz, J.-N., Blumbach, K., Heinegard, D., Zaucke, F., Paulsson, M., Krieg, T., Koch, M., Eckes, B., 2012. Collagen XII and XIV - new partners of cartilage oligomeric matrix protein in the skin extracellular matrix suprastructure. J. Biol. Chem.

Ansorge, H.L., Meng, X.M., Zhang, G.Y., Veit, G., Sun, M., Klement, J.F., Beason, D.P., Soslowsky, L.J., Koch, M., Birk, D.E., 2009. Type XIV collagen regulates fibrillogenesis: premature collagen fibril growth and tissue dysfunction in null mice. J. Biol. Chem. 284, 8427-8438.

Aubert-Foucher, E., Font, B., Eichenberger, D., Goldschmidt, D., Lethias, C., van der Rest, M., 1992. Purification and characterization of native type XIV collagen. J. Biol. Chem. 267, 15759-15764.
Bacou, F., Vigneron, P., 1988. Propriétés des fibres musculaires squelettiques. 1. Influence de l'innervation motrice. Reprod. Nutr. Dev. 28, 1387-1453.
Berthod, F., Germain, L., Guignard, R., Lethias, C., Garrone, R., Damour, O., Van der Rest, M., Auger, F.A., 1997. Differential expression of collagens XII and XIV in human skin and in reconstructed skin. J. Invest. Dermatol. 108, 737-742.
Bonnet, M., Faulconnier, Y., Leroux, C., Jurie, C., Cassar-Malek, I., Bauchart, D., Boulesteix, P., Pethick, D., Hocquette, J.F., Chilliard, Y., 2007. Glucose-6-phosphate dehydrogenase and leptin are related to marbling differences among Limousin and Angus or Japanese Black × Angus steers. J. Anim. Sci. 85, 2882-2894.
Bradford, M.M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. 72, 248-254.
Chiquet, M., Birk, D.E., Bönnemann, C.G., Koch, M., 2014. Collagen XII: Protecting bone and muscle integrity by organizing collagen fibrils. The International Journal of Biochemistry & Cell Biology 53, 51-54.
Christensen, M., Ertbjerg, P., Failla, S., Sanudo, C., Richardson, R.I., Nute, G.R., Olleta, J.L., Panea, B., Alberti, P., Juarez, M., Hocquette, J.F., Williams, J.L., 2011. Relationship between collagen characteristics, lipid content and raw and cooked texture of meat from young bulls of fifteen European breeds. Meat Sci. 87, 61-65.
Cianzio, D.S., Topel, D.G., Whitehurst, G.B., Beitz, D.C., Self, H.L., 1985. Adipose Tissue Growth and Cellularity: Changes in Bovine Adipocyte Size and Number1. J. Anim. Sci. 60, 970-976.
Destefanis, G., Barge, M.T., Brugiapaglia, A., Tassone, S., 2000. The use of principal component analysis (PCA) to characterize beef. Meat Sci. 56, 255-259.
Dubost, A., Micol, D., Meunier, B., Lethias, C., Listrat, A., 2013. Relationships between structural characteristics of bovine intramuscular connective tissue assessed by image analysis and collagen and proteoglycan content. Meat Sci. 93, 378-386.
Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. The Journal of Biological Chemistry 226, 497-509.
Font, B., Eichenberger, D., Goldschmidt, D., Boutillon, M., Hulmes, D., 1998. Structural requirements for fibromodulin binding to collagen and the control of type I collagen fibrillogenesis--critical roles for disulphide bonding and the C-terminal region. Eur. J. Biochem. 254, 580-587.
Gandemer, G., 2002. Lipids in muscles and adipose tissues, changes during processing and sensory properties of meat products. Meat Sci. 62, 309-321.
He, J., Tian, Y., Li, J.J., Shen, J.D., Tao, Z.R., Fu, Y., Niu, D., Lu, L.Z., 2012. Expression pattern of adipocyte fatty acid-binding protein gene in different tissues and its regulation of genes related to adipocyte differentiation in duck. Poult. Sci. 91, 2270-2274.
Hocquette, J.-F., Cassar-Malek, I., Jurie, C., Bauchart, D., Picard, B., Renand, G., 2012. Relationships between muscle growth potential, intramuscular fat content and different indicators of muscle fibre types in young Charolais bulls. Anim. Sci. J. 83, 750-758.
Hocquette, J.F., Jurie, C., Ueda, Y., Boulesteix, P., Bauchart, D., Pethick, D.W., 2003. The relationship between muscle metabolic pathways and marbling of beef. In: Souffrant W.B., C.C., M. (Eds.), EAAP, Rostock-Warnemünde, Germany, pp. 513-516.
Jurie, C., Cassar-Malek, I., Bonnet, M., Leroux, C., Bauchart, D., Boulesteix, P., Pethick, D.W., Hocquette, J.F., 2007. Adipocyte fatty acid-binding protein and mitochondrial enzyme activities in muscles as relevant indicators of marbling in cattle. J. Anim. Sci. 85, 2660-2669.

Leplaix-Charlat, L., Durand, D., Bauchart, D., 1996. Effects of Diets Containing Tallow and Soybean Oil with and Without Cholesterol on Hepatic Metabolism of Lipids and Lipoproteins in the Preruminant Calf. J. Dairy Sci. 79, 1826-1835.
Lethias, C., Carisey, A., Comte, J., Cluzel, C., Exposito, J.Y., 2006. A model of tenascin-X integration within the collagenous network. FEBS Lett. 580, 6281-6285.
Listrat, A., Lethias, C., Hocquette, J.F., Renand, G., Menissier, F., Geay, Y., Picard, B., 2000. Age-related changes and location of types I, III, XII and XIV collagen during development of skeletal muscles from genetically different animals. Histochem. J. 32, 349-356.
Nishimura, T., Hattori, A., Takahashi, K., 1999. Structural changes in intramuscular connective tissue during the fattening of Japanese Black cattle: Effect of marbling on beef tenderization. J. Anim. Sci. 77, 93-104.
Nishiyama, T., McDonough, A.M., Bruns, R.R., Burgeson, R.E., 1994. Type XII and type XIV collagens mediate interactions between banded collagen fibers in vitro and may modulate extracellular matrix deformability. J. Biol. Chem. 269, 28193-28199.
Piot, C., Hocquette, J.F., Herpin, P., Veerkamp, J.H., Bauchart, D., 2000. Dietary coconut oil affects more lipoprotein lipase activity than the mitochondria oxidative capacities in muscles of preruminant calves. J. Nutr. Biochem. 11, 231-238.
Ruehl, M., Erben, U., Schuppan, D., Wagner, C., Zeller, A., Freise, C., Al-Hasani, H., Loesekann, M., Notter, M., Wittig, B.M., Zeitz, M., Dieterich, W., Somasundaram, R., 2005. The elongated first fibronectin type III domain of collagen XIV is an inducer of quiescence and differentiation in fibroblasts and preadipocytes. J. Biol. Chem. 280, 38537-38543.
Tahara, K., Aso, H., Yamasaki, T., Rose, M.T., Takasuga, A., Sugimoto, Y., Yamaguchi, T., Takano, S., 2004. Cloning and expression of type XII collagen isoforms during bovine adipogenesis. Differentiation 72, 113-122.
Wälchli, C., Koch, M., Chiquet, M., Odermatt, B.F., Trueb, B., 1994. Tissue-specific expression of the fibril-associated collagen XII and collagen XIV. J. Cell Sci. 107, 669-681.
Zou, Y.Q., Zwolanek, D., Izu, Y.Y., Gandhy, S., Schreiber, G., Brockmann, K., Devoto, M., Tian, Z.Z., Hu, Y., Veit, G., Meier, M., Stetefeld, J., Hicks, D., Straub, V., Voermans, N.C., Birk, D.E., Barton, E.R., Koch, M., Bonnemann, C.G., 2014. Recessive and dominant mutations in COL12A1 cause a novel EDS/myopathy overlap syndrome in humans and mice. Hum. Mol. Genet. 23, 2339-2352.

Table 1. Composition of the finishing diet of the Limousin and Angus steers (Table from Bonnet et al. (2007))

Item	Limousin[1]	Angus[2]
Ingredient[3], % of DM		
Hay	17.6	14.0
Lupin	8.9	9.0
Soya	1.7	-
Triticale	17.5	18.5

Rolled wheat	47.5	49.5
Lime	-	1.0
Molafos	6.9	8.0
Vitamin mineral premix[4]	1.2	-
Chemical composition		
ME, KJ/kg of DM	12.5	11.9
Crude protein, g/kg of DM	155	151

[1]Limousin steers were reared in the experimental unit of INRA Theix Research Center and finished for 6 months.

[2]Angus steers were reared in the experimental farm of Murdoch University in Australia and were finished for 6 months.

[3]All steers received a similar finishing diet with a cereal-rich (approximately 75%) diet to allow them to express their genetic potential for the development of adipose tissue.

[4]Minerals, %: Ca, 18; P, 12; Mg, 4; Na, 2; trace elements, mg/Kg: Zn, 11,300; Mn, 8,400; Cu, 2,250; I, 120; Co, 30; and Se, 24; vitamins, UI or mg/kg: vitamin A, 2.7 mg; vitamin D3, 135,000 UI; and vitamin E, 500 mg

Table 2: Least square means for metabolic parameters in *Rectus abdominis* (RA) and *Semitendinosus* (ST) muscles of Angus (Ang) and Limousin (Lim) steers

	Breed		Muscle		Effects		
Item	Ang	Lim	RA	ST	Breed (B)	Muscle (M)	BxM
TAG[1]	45.72	10.14	42.26	14.96	0.001	0.001	0.001
A-FABP[1]	31.08	17.75	33.87	14.96	0.010	0.001	0.010

H-FABP[1]	2.91	1.94	3.37	1.49	0.001	0.001	0.001
HAD [1]	9.31	9.10	10.83	7.58	0.770	0.001	0.200
COX [1]	46.95	28.45	51.67	23.73	0.001	0.001	0.452
ICDH[1]	6.87	4.25	7.20	3.93	0.001	0.001	0.172
CS[1]	21.03	15.57	18.39	18.20	0.001	0.878	0.092
PFK[1]	0.13	0.16	0.12	0.17	0.001	0.001	0.010
LDH[1]	4.39	5.53	4.66	5.26	0.001	0.010	0.140

[1]: Triacylglycerol (TAG) content (g/100 g of fresh muscle), A-and H-fatty acid binding protein (A-and H-FABP) contents (µg/mg of protein), activities of phosphofructokinase (PFK), lactate dehydrogenase (LDH) (µmol.min^{-1}.mg^{-1} of protein), hydroxyacyl-CoA dehydrogenase (HAD), isocitrate dehydrogenase (ICDH), citrate synthase (CS), cytochrome-*c* oxidase (COX) (nmol.min^{-1}.mg^{-1} of protein), total collagen content (tot col) (µg OH-prol.mg^{-1} of dry matter) and collagens XII and XIV contents (Col XII and XIV) (arbitrary units).

Table 3: Least square means ± Standard Error of the Mean, for total collagen, collagen XII and XIV contents of *Rectus abdominis* (RA) and *Semitendinosus* (ST) muscles of Angus and Limousin steers

	Breed		Muscle		Effect	
Item	Ang[2]	Lim[2]	RA[2]	ST[2]	Breed	Mus[3]

					(B)	(M)	Bx M
Tot col[1]	2.83±0.180	1.75±0.09	2.13±0.20	2.45±0.15	0.001	0.100	0.320
Col XII[1]	1299±323.70	1063±280.04	1677±377.31	685±100.46	0.250	0.010	0.120
Col XIV[1]	3457±345.84	2020±160.42	2943±381.47	2535±252.43	0.001	0.270	0.180
Col XII[1]/tot col [1]	487±117.20	746±162.75	880±114.10	353±75.23	0.190	0.010	0.810
Col XIV[1]/tot col [1]	1338±164.42	1163±158.00	1552±161.88	948±117.74	0.380	0.010	0.580

[1] Total collagen content (Tot col) (µg OH-prol.mg^{-1} of dry matter), collagen XII and XIV (Col XII and XIV) (arbitrary units)

[2]Ang: Angus; Lim: Limousins; RA: *Rectus Abdominis* muscle; ST: S*emitendinosus* muscle;

[3]Mus: muscle

Table 4: **Pearson's** correlation coefficients between measured connective tissue molecules (total collagen, collagen XII and XIV and theirs ratios to total collagen) and some parameters of metabolism of muscle

	Tot col[1]	Col XII[1]	Col XIV[1]	Col XII[1]/ tot col[1]	Col XIV[1]/ tot col[1]
Lipids					
TAG[1]	**+0.47***	+0.23	**+0.35***	+0.03	+0.22
Residues[2]	+0.25	+0.11	+0.05	-0.41	-0.17

A-FABP[1]	+0.28	**+0.43***	**+0.35***	**+0.32***	+0.30
Residues[2]	+0.06	**+0.30***	+0.16	-0.14	+0.04
H-FABP[1]	+0.22	**+0.53***	**+0.38***	**+0.37***	**+0.47***
residues[2]	-0.06	**+0.34***	+0.10	+0.24	+0.31
Muscle					
Oxidative enzymes					
HAD[1]	+0.12	+0.24	+0.08	+0.21	+0.03
residues[2]	+0.03	+0.30	+0.23	-0.16	-0.10
COX[1]	+0.25	**+0.41***	+0.24	+0.24	+0.21
residues[2]	+0.13	+0.08	+0.29	+0.14	+0.13
ICDH[1]	+0.23	**+0.40***	**+0.34***	**+0.31***	**+0.43***
residues[2]	-0.10	+0.20	+0.25	-0.10	+0.03
CS[1]	**+0.48***	**+0.32***	+0.20	+0.16	+0.20
Residues[2]	+0.01	**+0.46***	+0.20	+0.20	+0.25
Glycolytic enzymes					
PFK[1]	**-0.34***	+0.15	**-0.31***	-0.06	-0.23
residues[2]	-0.08	+0.11	-0.28	+0.36	+0.30

LDH[1]	**-0.36***	-0.15	**-0.42***	-0.08	**-0.42***
residues[2]	-0.42	+0.12	-0.04	-0.38	-0.06
Col XII[1]	0.19	-	0.20		
residues[2]	0.25	-	0.09		
Col XIV[1]	0.16	0.20	-		
residues[2]	-0.06	0.09	-		

[1]: Triacylglycerol (TAG) content (g/100 g of fresh muscle), A-and H-fatty acid binding protein (A-and H-FABP) contents (µg/mg of protein), activities of phosphofructokinase (PFK), lactate dehydrogenase (LDH) (µmol.min^{-1}.mg^{-1} of protein), hydroxyacyl-CoA dehydrogenase (HAD), isocitrate dehydrogenase (ICDH), citrate synthase (CS), cytochrome-*c* oxidase (COX) (nmol.min^{-1}.mg^{-1} of protein), total collagen content (tot col) (µg OH-prol.mg^{-1} of dry matter) and collagens XII and XIV contents (Col XII and XIV) (arbitrary units).

[2] Correlations with residual observations after removing the muscle and the genotype effects

*: P<0.05

Highlights :

- Collagen XII and XIV are both associated with muscle metabolism
- Collagen XII is associated with muscle metabolism regardless of breeds and muscles
- Collagen XIV is associated with muscle metabolism across breeds