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Abstract:

Steaks of 74 animals from 3 young bull breeds (Aberdeen Angus, Limousin and Blond d'Aquitaine) were cooked at two end-point cooking temperatures (55 and 74°C) and evaluated for tenderness by trained panelists from France (FR) and the United Kingdom (UK). Using principal component regressions, the tenderness scores of each breed, country origin of the panelists and cooking temperature were linked with the abundances of 21 protein biomarkers belonging to five biological pathways. Twelve regression equations were built and explained 68 to 95% of tenderness variability. A high dissimilarity in the retained biomarkers was observed among the equations and differences exist among breeds, cooking temperatures and country origin of the panelists. Among the 21 biomarkers, 6 proteins including structural (MyHC-I, MyHC-IIa, MyHC-IIx), oxidative stress (DJ-1, PRDX6) and proteolysis (CAPN1) were retained robustly in positive or negative directions in the tenderization process of *Longissimus thoracis*, regardless the breed, end-point cooking temperature or panelist.

Key words: Beef tenderness; Biomarkers; Dot-Blot; Cooking temperature; Chemometrics; Prediction.

1. Introduction

Beef tenderness is among the palatability traits that has been extensively studied due to its importance for both consumer satisfaction and (re)purchasing decisions (Ouali *et al.* 2013). Various studies indicate that consumers are willing to pay a premium for beef meat guaranteed to be tender (Miller *et al.* 2001). However, beef tenderness is variable due to several intrinsic and extrinsic factors, which are measurable from the farm-to-fork continuum levels (Ferguson *et al.* 2001; Gagaoua *et al.* 2018b), with many of these factors interacting with others.

To understand better the causes of variability in tenderness and the biochemical mechanisms underlying tenderization, functional and comparative proteomics were applied (Gagaoua *et al.* 2015a; Picard & Gagaoua 2017). Protein biomarkers potentially related to tenderness were identified using two-dimensional gel electrophoresis and mass spectrometry. These biomarkers belong to several biological pathways including heat shock, metabolism (glycolytic and oxidative), structure and contraction, oxidative stress, apoptosis, transport, signaling and proteolysis (Picard & Gagaoua 2017). Experiments using high-throughput technologies including Dot-Blot and Reverse Phase Protein Array found that their relationships with tenderness depend on breed (Chaze *et al.* 2013; Picard *et al.* 2014), gender/animal type (Guillemin *et al.* 2011b), rearing factors (Gagaoua *et al.* 2017b), muscle type (Guillemin *et al.* 2011b; Picard *et al.* 2014), texture trait (Gagaoua *et al.* 2018a) and evaluation method of tenderness (Gagaoua *et al.* 2018b).

Cooking temperature is a crucial important factor influencing meat texture (Tornberg 2005) that further differ among countries as it may be to an internal temperature of 55°C (rare cooking) in France or at 74°C (medium to well done) in the United Kingdom (UK) (Gagaoua *et al.* 2016a). Particularly, denaturation and coagulation of muscle proteins by heating have a major influence on the final texture (Obuz *et al.* 2003). Moreover, meat preferences of members of a taste panel depend upon their previous cultural experiences and eating habits. Therefore, in this study we evaluated the effect of end-point cooking temperature and country origin of the panelists on the associations of tenderness with the abundances of a list of protein biomarkers from young bulls of different breeds. Specifically, using chemometrics, it determined the relationships between the relative abundance of 21 protein biomarkers and tenderness of the same meat samples cooked to end-point temperatures of 55 or 74°C and assessed by sensory panels in France and the UK. These data will increase our understanding

about the implication of various biomarkers in tenderness in order to propose in the future, generic biomarker-based tools for early sorting of carcasses to meet consumer expectations.

2. Materials and Methods

2.1. Animals handling and slaughtering

This study is part of the European FP6 ProSafeBeef project (FOODCT-2006-36241) under the INRA reference AQ284 (Gagaoua *et al.* 2015a). Seventy-four young bulls of three pure breeds (24 Aberdeen Angus (AA), 25 Limousin (LIM), and 25 Blond d'Aquitaine (BA)) were used. The animals were fattened in the same experimental station (INRA-UE 1414 Herbipôle) during a 105 – 107 days finishing period until slaughter at around 16.0 ± 0.99 months (average of 17.2 ± 0.92 months for AA, 16.4 ± 1.10 months for BA, 16.9 ± 0.79 months for LIM). The fattening diet given *ad libitum* was based on straw (25%) and concentrate (75%) (Gagaoua *et al.* 2016b). The animals were housed in groups of 4 animals of the same breed in 6×6 m pens with straw bedding.

Animals were slaughtered when they achieved fat class 3 on the EUROP grid of carcass classification, and similar live weights of 665 kg to avoid weight and age effects on muscle characteristics and meat quality (Gagaoua *et al.* 2015a). Before slaughter, all animals were fasted for 24 h and had free access to water. After unloading, they were slaughtered in the experimental slaughterhouse of INRA Auvergne-Rhône-Alpes Research center in compliance with the current French welfare recommendations for the use of experimental animals. The animals were stunned using a penetrative captive bolt, prior animal bleeding. The carcasses were not electrically stimulated as usual in the experimental slaughterhouse of INRA Auvergne-Rhône-Alpes Research center and they were stored between 2 and 4°C up to 24 h *post-mortem* according to standard commercial practices.

2.2. Muscle sampling

Muscle samples from *Longissimus thoracis* (LT, mixed fast oxido-glycolytic) were excised 45 min *post-mortem* from the right side of carcasses (6^{th} rib) of each animal. Approximately 5g of the samples was frozen in liquid nitrogen and kept at −80°C until fiber characterization by myosin heavy chains isoforms (MyHC) quantification and protein extractions for the measure of tenderness biomarkers by Dot-Blot. At 24 h *post-mortem* another part was cut into 5 cm thick steaks and placed in sealed plastic bags in a Multivac A300/42 vacuum packager (Multivac UK, Swindon, UK) to −980 mbar and kept between 2 and 4°C for 14 days for ageing. Each steak was then frozen and stored at −20°C until tenderness assessments.

2.3. Protein content determination

Proteins were extracted according to the protocol set up previously in our laboratory (Bouley *et al.* 2004). The protein concentration was determined according to the dye binding method of Bradford using the Bio-Rad Protein assay (Bio-Rad Laboratories Inc.). Bovine serum albumin (BSA) at a concentration of 1 mg/mL was used as standard.

2.4. Dot-Blot analysis

Using Dot-Blot, we quantified (Gagaoua *et al.* 2015a) a list of 18 protein biomarkers of meat tenderness belonging to 5 biological pathways (**Table 1**): *1)-Heat shock proteins*: αB-crystallin, HSP20, HSP27, HSP40, HSP70-8, HSP70-1A/B, and HSP70-Grp75; *2)-Metabolism:* ENO3, LDH-B and MDH1; *3)-Structure and contraction*: α-actin, MyLC-1F, CapZ-β and MyBP-H; *4)-Oxidative stress*: SOD1, PRDX6 and DJ-1; and *5)-Proteolysis*: μ-calpain (CAPN1). The protocol describing the conditions of quantification, use and specificity of primary antibodies against the 18 proteins in bovine muscle was previously reported (Gagaoua *et al.* 2015a).

2.5. Electrophoresis separation of Myosin Heavy Chain isoforms

The abundance of the 3 other protein biomarkers corresponding to myosin heavy chain (MyHC) isoforms (structural proteins) was determined using an appropriate high-resolution mini-gel electrophoresis technique (Picard *et al.* 2011). Controls of bovine muscle containing three (MyHC-I, IIa and IIx) or four (MyHC-I, IIa, IIx and IIb) muscle fibers were added at the extremities of each gel. The quantification of the bands revealed the existence of MyHC-IIb isoform in only some animals (8 animals of 74). Consequently, MyHC-IIb percentages were totaled with those of MyHC-IIx creating a new variable "MyHC-IIx+b", *i.e.,* fast glycolytic fibers (Gagaoua *et al.* 2015a). Thus, the proportions of three MyHC isoforms (I, IIa and IIx+b) were considered in this report.

2.6. Tenderness evaluation by sensory panels

Tenderness evaluation was conducted in two dedicated laboratories in the UK (University of Bristol) and France (INRA, Le Magneraud). In both laboratories, the expert panelists were trained in accordance with the ISO standards ISO/TC 34 (Gagaoua *et al.* 2016a). Meat samples from France were transported to the UK while maintained at -20°C and were clearly and appropriately labelled. Within each sensory protocol, scores of steaks cooked at end-point temperatures of 55 and 74°C were averaged across panelists for each steak, and the means

were used in the statistical analyses. The statistical analyses among panelists and sessions revealed coefficient of variations < 5%.

For the French sensory protocol, the steaks were thawed without stacking or overlapping, at 2 to 5°C in vacuum packs for at least 24h or 48 h before cooking. One hour before sensory assessment, the meat samples were cut into four approximately 1.50 cm thick steaks, 2 steaks were assigned for cooking to 55°C and 2 steaks for cooking to 74°C. After exposure to air for 1 h at 18°C, the steaks were grilled on a double grooved plate griddle (SOFRACA, Morangis, France) heated to 310°C for 30 min before cooking. Steaks were heated for 2 min between two aluminum foil sheets, until the end-points temperature of 55°C or 74°C in the geometric center of the steak was reached (measured using a temperature probe (Type K, HANNA HI 98704, Newark, USA)). After grilling, each grilled sample was cut into 20 mm cubes that were immediately served to 12 panelists. Thus, sensory panels rated global tenderness of the grilled meat on a 10 cm unstructured line scale (from 0 to 10), where 0 refers to extremely tough and 10 to extremely tender meat (Gagaoua *et al.* 2016a). The sessions were carried out in a sensory analysis room equipped with individual booths under artificial red light to reduce the influence of the appearance of the samples. Each tasting booth was equipped with computer terminals linked to a fileserver running a sensory software (Fizz v 2.20h, Biosystemes, Couternon, France) that facilitated the direct entry of assessor ratings.

For the UK panel, the samples were defrosted overnight at 4°C and then cut into 2.0 cm thick steaks. The steaks were then grilled under the overhead heat from grill elements of a Tricity double oven domestic cooker producing approximately 120°C at the meat surface; turning every two minutes until reaching the internal temperatures of 55 or 74ºC in the geometric center of the steak (measured by a thermocouple probe). After grilling, all fat and connective tissue was trimmed and each steak was cut into 3×2×2 cm blocks. The blocks were wrapped in pre-labelled foil, placed in a heated incubator for no more than 15 min before testing by 10 panelists. The sensory evaluation was conducted in individual booths illuminated with red light and equipped with the same sensory software than the French panel. The assessors used 8-point category scales to evaluate tenderness from 0 – extremely tough to 8 – extremely tender.

2.7. Statistical analyses

Statistical analyses were performed using SAS statistical software (SAS 9.1, SAS Institute INC, Cary, NC, USA) and XLSTAT 2017.19.4 (AddinSoft, Paris, France). Raw data means

were scrutinized for data entry errors and outliers and the normal distribution and homogeneity was tested by Shapiro-Wilk test ($P>0.05$). Variance analyses were carried out using PROC GLM of SAS for cooking temperature (55 *vs.* 74°C) and breed type effects for each country panel separately. Least square means separation was carried out using Tukey test and differences were considered significant at $P<0.05$.

For chemometrics analyses, z-scores were computed to remove breed type and country effects. Z-scores represent the deviation of each observation relative to the mean of the corresponding steak in each condition and were calculated using PROC STANDARD of SAS, which standardizes data to a mean of 0 and standard deviation of 1 (Gagaoua *et al.* 2015a). Similarly, the protein abundances of the biomarkers were standardized for breed effect.

Principal component regression (PCR) analyses on standardized data were conducted per treatment, that is, end-point cooking temperature, breed and sensory panel, with the 21 biomarkers to generate explanatory models of tenderness using the optimal number of components in each case. This tool allows managing multicollinearity correctly and considers all the biomarkers to explain tenderness variability by highlighting also the direction of the retained biomarkers, i.e., positive, negative or none. The principle of PCR is to calculate the principal components (PCs) and then use some of these components in a linear regression model fitted using the typical least squares procedure (Rougoor *et al.* 2000). PCR can be divided into three steps: the first step is to run a PCA on the table of the explanatory variables, *i.e.* the biomarkers of tenderness. The second step is to run an ordinary least squares regression on the selected components. Finally, the parameters of the equation corresponding to the variable to be explained, *i.e.* tenderness, is computed (Gagaoua *et al.* 2018a). To select PCs for regression, the forward selection approach was used. At each stage of selection process, a test was run after a new PC was added to check if some PCs could be deleted without considerably increasing the root mean square error (RMSE). This selection process ended when a minimum RMSE was obtained. Besides the percentage of variance explained, the eigenvalues of the PCs can be of use to decide how many PCs to include in the PCR. The best model was regarded as the final forecast to explain tenderness variability by the list of the biomarkers. The maximal number of explanatory variables in the models was unconstrained (unlimited), because the objective was to compare the biomarkers and pathways retained, between the models.

The degree of similarity between the protein biomarkers to explain tenderness in the twelve models was estimated as the percentage of biomarkers shared based on the *F*

coefficient (López-Pedrouso *et al.* 2014): $F = 2n_{xy}/(n_x + n_y)$. Where n_{xy} is the number of tenderness biomarkers shared by groups x and y; and n_x and n_y are the total numbers of biomarkers retained in groups x and y, respectively. Then, the pairwise biomarker distance (D) expressed as a percentage based on the abundances of the biomarkers under the different factors (treatments) *i.e.,* breed, cooking temperature and country origin of the panelists was estimated as $D = 1 - F$.

3. Results and Discussion

3.1. Cooking temperature and breed effects on tenderness of muscle cuts assessed by panelists from France (FR) and the United Kingdom (UK)

Tenderness scores were irrespective of the panelists significantly different ($P<0.05$) among the three breeds at 74°C only (**Table 2**). At 55°C, the steaks of Aberdeen Angus were scored higher but without any significant difference with the two other breeds. For all the samples taken together and regardless the panelists, tenderness scores were greater (+12 (FR) and +23% (UK), $P<0.001$) at 55°C than 74°C. However, no interaction of breed × cooking temperature was observed ($P>0.05$). The increase in internal endpoint temperature may be related to higher water loss during cooking, with a direct influence on the tenderness scores. This is in agreement with previous studies reporting greater tenderness when meat was cooked at <60°C (Gomes *et al.* 2014). More precisely, meat tenderness decreases in two distinct phases, the first from 40 to 50°C and the second from 60 to 80°C with a significant increase between 50 and 60°C (Christensen *et al.* 2000). The more tender meat of Aberdeen Angus at 74°C only would be explained by the highest intramuscular fat (IMF) content (3.49% *vs.* 1.52% and 1.18% for Limousin and Blond d'Aquitaine, respectively (Gagaoua *et al.* 2016b)). Particularly, IMF levels of 3–4% or more enhances beef tenderness at higher cooking temperatures due to fat breakdown giving meat a succulent mouthfeel (Savell & Cross 1988), which was the case for the Aberdeen Angus bulls in this study (Gagaoua *et al.* 2016b).

3.2. Protein biomarkers to explain beef tenderness using principal component regression

Principal component regression (PCR) models were used to describe the relationships between the 21 biomarkers and tenderness scores (**Fig. 1** and **Table 3**) according to breed (Aberdeen Angus, Blond d'Aquitaine, Limousin), end-point cooking temperature (55 and 74°C) and country origin of the panelists (France and the UK). These models are based on z-

scores to avoid all the differences rising from breeds, sensory protocols between the two countries as well as the two end-point cooking temperatures.

The 12 equations explained 68 to 95% of the variability in tenderness (**Table 3**). The number of the entered proteins in the PCR models as well as their biological families varied according to breed, end-point cooking temperature and panelists (**Table 3** and **Fig. 2A-C**). For Aberdeen Angus, 7 to 9 proteins were retained and explained 84%, 95% (at 55°C and 74°C) and 73%, 88% of tenderness for the French and the UK panelists, respectively. For Limousin, 9 to 11 proteins were retained and explained 84%, 86% (at 55°C and 74°C) and 88%, 86% of tenderness for the French and the UK panelists, respectively. For Blond d'Aquitaine, 10 to 14 proteins were retained and explained 90%, 87% (at 55°C and 74°C) and 68%, 93% of tenderness for the French and the UK panelists, respectively. The Venn diagrams by breed (**Fig. 2A-C**) present the biomarkers according to their biological family for the two end-point cooking temperatures (55 and 74°C) and country origin of the panelists, France (FR) and the United Kingdom (UK).

Considering all models, 11 biomarkers were retained at least 6 times in the equations (**Table 3** and **Fig. 1**) including 5 heat shock proteins (CRYAB, HSP20, HSP27, HSP40, HSP70-8) and 3 structural proteins (CapZ-β, MyHC-I, MyHC-IIx); the 3 remaining proteins being involved in energy metabolism (LDH-B), oxidative resistance (SOD1) and proteolysis (CAPN1). Six proteins were retained robustly in the equations irrespective of the treatment, with the same direction: MyHC-I, MyHC-IIa, PRDX6 (negative), MyHC-IIx, DJ-1 and CAPN1 (positive).

The comparison of the regression equations in terms of the involved proteins revealed a strong dissimilarity based on the pairwise biomarker distance (**Fig. 2D**). The highest (>75%) and lowest (55–75%) dissimilarity scores were found for Aberdeen Angus and Blond d'Aquitaine, respectively. The dissimilarity was particularly strong for Aberdeen Angus, where most of the proteins were specific to the end-point cooking temperature or country origin of the panelists. In contrast, for Blond d'Aquitaine 7 proteins were common irrespective of the treatment factors (**Table 3**). Similar to the present study, earlier studies found different protein expression or levels according to breed or gender type (Guillemin *et al.* 2011b; Chaze *et al.* 2013).

The main biological pathways affected by the number of proteins that entered in each model (**Fig. 3A-C**) agree with the observation that meat proteins are denatured by changing

the cooking temperature or processing/environmental conditions (Kemp *et al.* 2009). Specifically, the heat capacities of sarcoplasmic, myofibrillar, and stromal proteins differ and the cooking temperature determines the overall spectrum of thermal denaturation of different meat proteins inducing strong differences on final meat texture (Tornberg 2005; Kemp *et al.* 2009). This explains the major role of myofibrillar or sarcoplasmic proteins and intramuscular connective tissue in cooked meat texture (Brunton *et al.* 2006). Results of our study agree with this knowledge. In the following subsections, we discuss the relationships between tenderness and the proteins of the different biological families in their order of entrance in the regression equations, taking into account the treatment factors.

3.2.1. Heat shock proteins

The 7 HSPs including small (CRYAB, HSP20 and HSP27), large (HSP70-1A/B, Hsp70-8 and HSP70-GRP75) and HSP40 co-chaperone were all involved in the tenderness regression equations but in a specific manner depending on breed, end-point cooking temperature or country origin of the panelists (**Fig. 1**). sHSPs are intracellular stabilizers that are dynamically expressed in muscle after slaughter to respond in specific regions of muscle cells to prevent the irreversible denaturation and aggregation during heat stimulation of muscle proteins and thus maintain cellular homeostasis (for review: (Lomiwes *et al.* 2014)). They regulate actin polymerization, actin-myosin interactions or actin-binding protein including some roles in the management of the proper assembly of intermediate filaments.

sHSPs were retained at least 6 times in the regression equations (**Fig. 1** and **Table 3**). These consistent associations are of particular interest. Thus, CRYAB and HSP20 were strongly negatively and HSP40 (*DNAJA1)* positively correlated with tenderness in the 4 equations for Blond d'Aquitaine bulls (**Table 3**). HSP20 and CRYAB are important modulators of intermediate filament assemblies and are able to bind most unfolded proteins in a non-specific manner as well as important anti-apoptotic properties role in *post-mortem* muscle (Ouali *et al.* 2013; Dubińska-Magiera *et al.* 2014). HSP40 is a protein that is located in the cytosol and cochaperones the intermediate filament with HSP70 to retard apoptosis. This protein was also positively correlated with tenderness for the Limousin (UK55 and UK74°C) but negatively for Aberdeen Angus breed (FR55 and UK74). In cattle, only one work has shown a negative relationship between *DNAJA1* expression (HSP40) in LT muscle of young Charolais bulls and tenderness score assessed by a sensory panel (Bernard *et al.* 2007).

The stronger involvement of sHSPs in tenderness of Blond d'Aquitaine compared to the other breeds may be explained by some of their other characteristics. Particularly, compared to Limousin and Aberdeen Angus, Blond d'Aquitaine has greater muscularity with low connective tissue and intramuscular fat contents. In addition, isolated skeletal muscle myosin retains much of its native structure as well as its enzymatic activity when incubated at high temperature in the presence of sHSPs (Melkani *et al.* 2006). Hence, sHSPs may have contributed to the development of meat texture, irrespective of cooking temperature and consequently have influenced tenderness. In support of this, tenderness of the Blond d'Aquitaine breed was quite strongly positively related to certain structural proteins, such as CapZ-β and MyHC-IIx, whatever the end-point cooking temperature or panelist origin (**Fig. 1**).

The implication of HSPs in meat quality was earlier proposed; these proteins would slow *post-mortem* muscle apoptosis onset (Ouali *et al.* 2013; Picard & Gagaoua 2017). HSPs are abundant and dynamically expressed in *post-mortem* muscle (Chaze *et al.* 2013; Carvalho *et al.* 2014; Gagaoua *et al.* 2015a; Picard & Gagaoua 2017) including during the heating process of meat (Pulford *et al.* 2008). In line to our findings, other studies found that variations in the expression and level of various HSPs correlated with tenderness in different experiments on cattle and other species, including different breeds, animal types, and muscles (for review: (Picard & Gagaoua 2017)). However, there are some contradictions between studies (Gagaoua *et al.* 2018b) that may be explained by individual reactivity of animals to stress and other factors related to the contractile and metabolic properties of the muscle including oxidative stress regulation that are not yet fully elucidated (Picard *et al.* 2014). Although the exact underlying mechanisms in *post-mortem* muscle and in cooked meat are not yet understood, they may be related to the maintenance of the correct conformation of proteins and preservation of their biological functions, protein assembly, folding and unfolding, and refolding of damaged protein entities including those of contractile and myofibrillar proteins (for review: (Picard & Gagaoua 2017)).

3.2.2. Structural proteins

Many studies indicate that meat tenderization is strongly related to the disruption of muscle structure by the breakdown of myofibrillar proteins (Bjarnadottir *et al.* 2010; Ouali *et al.* 2013; Carvalho *et al.* 2014; Marino *et al.* 2015) and our results are coherent with this. Particularly for the Limousin and Blond d'Aquitaine breeds, the 7 structural proteins were associated with tenderness (**Fig. 1** and **Fig. 2**). Myosin is the most abundant protein in muscle

322 tissue and belongs to a family of ATP-dependent motor proteins (Picard *et al.* 2002). For both
323 breeds, irrespective of end-point cooking temperature and origin of panelists, tenderness was
324 negatively and positively associated with MyHC-I, MyHC-IIa and MyHC-IIx, respectively.
325 CapZ-β, MyHC-I and MyHC-IIx were retained at least 6 times in the models. For Blond
326 d'Aquitaine, CapZ-β and MyHC-IIx were correlated positively and for Limousin, MyHC-IIa
327 negatively with tenderness. MLC-1F was retained in several models, and had a relatively
328 strong negative impact on tenderness for FR74. Regardless of the panelists, MyBP-H, a
329 protein of approximately 55 kDa that was reported in several studies to be correlated with
330 beef tenderness (Picard & Gagaoua 2017), was in this study positively and negatively
331 associated with tenderness at 55°C for Aberdeen Angus and Limousin, respectively, while it
332 was positively associated with tenderness of Blond d'Aquitaine for UK74. As mentioned
333 previously, the denaturation caused by heat varies according to the protein. For example, fast-
334 twitch fibers have lower thermal stability and are more susceptible to denaturation, than their
335 oxidative counterparts (Egelandsdal *et al.* 1994). Accordingly, we think that the interesting
336 finding in the inversion of direction with MyBP-H among breeds at the same temperature of
337 55°C would be related to the slightly different proportions of MyHC isoforms in the LT
338 muscle of Aberdeen Angus and Limousin (Gagaoua *et al.* 2017c). Indeed, in this last cited
339 paper we identified that LT muscle of Aberdeen Angus bulls as relatively oxidative and that
340 of Limousin as more glycolytic oriented. The corresponding distribution of MyHC fibers
341 between these two breeds are as follow: MyHC-I (AA = 27.36 ± 7.69% *vs.*
342 LIM = 23.65 ± 2.67%); MyHC-IIa (AA = 54.47 ± 6.78% *vs.* LIM = 30.75 ± 5.51%) and
343 MyHC-IIx+b (AA = 18.17 ± 10.53% *vs.* LIM = 45.62 ± 6.66%). In addition, other effects of
344 cooking involve protein aggregation due to protein modifications and intermolecular
345 interactions of hydrophobic surfaces which also vary according to temperature. Hence, the
346 different relationships between structural proteins and tenderness according to breed and
347 cooking temperature may be partly explained by different protein profiles between breeds and
348 the way heat acts on them.

349 Differences in the relationships between tenderness and structural proteins may also be
350 related to the differences in the metabolic and contractile properties of the muscle fibres
351 (Picard *et al.* 2014). Compared to oxidative fibers, the glycolytic entities are more susceptible
352 to proteolysis, a process which is central to tenderization (Ouali 1990). Moreover, MyHC-IIx
353 fibers have further greater glycogen contents (Lefaucheur 2010) which together with their
354 specific enzymatic characteristics influence rate and extent of *post-mortem* pH decline, and

355 consequently, sensory eating qualities of beef (Gagaoua *et al.* 2017a). MyHC-IIx was earlier
356 identified as a robust biomarker of tenderness irrespective of the evaluation method, sensory
357 panel or instrumental (Gagaoua *et al.* 2018b). Overall, results indicate that MyHC isoforms
358 are potential biomarkers of tenderness independently of breed, cooking temperature and
359 panel.

360 The association between Myosin light chains (MLC) and tenderness are coherent with
361 earlier reports indicating that their release due to *post-mortem* hydrolysis was associated with
362 beef tenderness (Picard *et al.* 2002; Anderson *et al.* 2012). MLC have substantial hydrophobic
363 areas to which certain HSPs attach, which may slow proteolysis.

364 The relationship between CapZ-β, MyBP-H and α-actin with tenderness differed according
365 to the treatment factors. These structural proteins are degraded during *post-mortem* ageing by
366 the endogenous muscle proteolytic systems. α-Actin (Zapata *et al.* 2009; Polati *et al.* 2012;
367 Gagaoua *et al.* 2017b; Picard & Gagaoua 2017), MyBP-H (Morzel *et al.* 2008; Guillemin *et*
368 *al.* 2011a; Gagaoua *et al.* 2018a) and CapZ-β (Chaze *et al.* 2013; Gagaoua *et al.* 2018b) were
369 earlier identified as biomarkers of beef tenderness. α-Actin is believed to play a pivotal role in
370 the onset of apoptosis during muscle to meat conversion (Ouali *et al.* 2013). CapZ-β also
371 known as F-actin-capping protein subunit β is located in the Z band of the muscle sarcomere
372 and the most abundant barbed-end-capping protein anchoring the barbed ends of sarcomeric
373 actin to the Z-disc, inhibiting polymerization (Maiti & Bamburg 2013). A proteomic study
374 found that CapZ-β was retained in the insoluble fraction of the protein extraction and the
375 levels declined during the 48 hr following slaughter (Bjarnadottir *et al.* 2010), indicative of
376 progressive CapZ-β fragmentation or detachment from the Z band. MyBP-H is located in the
377 A-band of the myofibrils and has significant effects on length, thickness, and lateral alignment
378 of myosin filaments (Gilbert *et al.* 1999), possibly modifying its accessibility to proteolytic
379 processes and effect of heat. Higher levels of α-actin, MyBP-H and CapZ-β in the early *post-*
380 *mortem* period suggest a relatively fast fragmentation of these proteins allowing their easier
381 extraction. However, further studies are needed to better understand these different links with
382 meat tenderness under various heat treatments.

383 *3.2.3. Energy metabolism proteins*

384 MDH1 plays a pivotal role in the malate-aspartate shuttle operating between cytosol from
385 glycolysis towards lipid oxidation in mitochondria (Lo *et al.* 2005). ENO3 is a glycolytic
386 enzyme involved in the conversion of 2-phosphoglycerate to phosphoenolpyruvate. LDH-B

387 catalyzes the interconversion of pyruvate and lactate. These 3 metabolic enzymes were often
388 retained in the equations for the Limousin (8/12), but were also correlated with tenderness of
389 the Blond d'Aquitaine (5/12) and Aberdeen Angus breeds (3/12).

390 We reported earlier that LT muscles with more oxidative properties produce more tender
391 meat (Picard *et al.* 2014) which is coherent with the positive and negative associations of
392 MDH1 and LDH-B with tenderness, respectively, for the Limousin breed, and the negative
393 association between tenderness and ENO3 in the Aberdeen Angus breed (**Fig. 1**). Inverse
394 relationships between tenderness and small HSPs in Aberdeen Angus (with slow oxidative
395 muscle properties) and Limousin/Blond d'Aquitaine (with fast glycolytic muscle properties)
396 was previously described by our group. Level of expression and abundance of ENO3 in the
397 muscle was also linked (positively and negatively) with eating qualities of meat as a function
398 of breeds, muscles or species (Picard & Gagaoua 2017).

399 Certain relationships between these metabolic enzymes and tenderness were however
400 opposite to those described above. For example, in contrast to the Limousin, for the Blond
401 d'Aquitaine breed, LDH-B was positively correlated and for both the Blond d'Aquitaine and
402 Aberdeen Angus breeds, ENO3 and MDH1 were negatively correlated with tenderness
403 (except for MDH1 Aberdeen Angus; UK55). The contrasting results between breeds may be
404 related the individual variability of the breeds and animals (Gagaoua *et al.* 2016b) as the
405 relationships between these enzymes and tenderness probably involve complex interactions.
406 For example, ENO3 participates in multi-enzyme complexes present on the sarcomere (Keller
407 *et al.* 2000) and their role in the tenderization process may further depend on the presence of
408 other proteins such as HSPs (Wulff *et al.* 2012).

409 *3.2.4. Oxidative resistance proteins*

410 The 3 oxidative stress proteins were retained in many of the tenderness equations (**Table 3**
411 and **Fig. 1**). DJ-1 was positively associated with tenderness for Aberdeen Angus (UK74),
412 Limousin (FR74 and UK74), and Blond d'Aquitaine (FR55 and UK55). PRDX6 was
413 negatively associated with tenderness for Aberdeen Angus (FR55, FR74 and UK74) and
414 Blond d'Aquitaine (FR55 and UK55). SOD1 was negatively associated with tenderness in the
415 4 equations of the Blond d'Aquitaine breed and positively for the Aberdeen Angus (FR55 and
416 FR74) and Limousin breeds (FR74 and UK74). Hence, within a breed, there was good
417 coherence within panel and/or temperature for these markers.

418 The oxidative properties of *post-mortem* muscle cells evolve continuously, as well as
419 during cooking, due to cellular damage and the generation of reactive oxygen species (ROS)
420 able to react with lipids and proteins (Brand 2010; Malheiros *et al.* 2019) thereby affecting
421 muscle structure and meat tenderness (Malheiros *et al.* 2019). The modifications of muscle
422 proteins due to oxidation is responsible for protein aggregation, changes in conformation and
423 solubility, and susceptibility to proteolysis (For review: (Estévez 2011)). The 3 oxidative
424 stress proteins may act as protective scavenger agents and therefore play an important role in
425 the tenderization process (for review: (Picard & Gagaoua 2017)). For example, during
426 oxidative stress, the protein deglycase DJ-1 is re-localized in the mitochondria, where it has a
427 pivotal role in scavenging mitochondrial H_2O_2 and limits mitochondrial fragmentation
428 (Thomas *et al.* 2011). DJ-1 abundance increases during ageing in beef *Longissimus* muscle
429 starting early *post-mortem* (Polati *et al.* 2012) until 21 days *post-mortem* (Laville *et al.* 2009).
430 The protective action of this protein against oxidative stress may also influence color of beef
431 (Gagaoua *et al.* 2017c).

432 In contrast to DJ-1, PRDX6, the sixth member of peroxiredoxins superfamily was
433 negatively associated with tenderness. PRDX6 protects cells from oxidative damage
434 particularly by controlling hydrogen peroxide amount in cells (Rhee *et al.* 2005). The negative
435 associations are explained by the anti-apoptotic properties of this protein (for review: (Fisher
436 2017). PRDX6 was earlier described as a marker of beef tenderness (Jia *et al.* 2009;
437 Guillemin *et al.* 2011a; Gagaoua *et al.* 2017b; Gagaoua *et al.* 2018a; Gagaoua *et al.* 2018b)
438 and is further related with other quality traits, such as pH decline and meat color (Gagaoua *et*
439 *al.* 2015b; Gagaoua *et al.* 2017c). Other members of peroxiredoxins are also related with
440 tenderness, including PRDX1 in Charolais breed (Polati *et al.* 2012), PRDX2 in Angus
441 crossbred (Malheiros *et al.* 2019) and PRDX3 in Norwegian Red cattle (Grabez *et al.* 2015).

442 SOD1, or superoxide dismutase (Cu-Zn) is an antioxidant metalloenzyme eliminating
443 excess ROS to limit damage caused by free radicals by the dismutation of O_2^- to O_2 and H_2O_2.
444 In line with the negative association of SOD1 in all the tenderness equations of the Blond
445 d'Aquitaine breed, a recent study reported greater abundance of this protein in tougher meat
446 (Grabez *et al.* 2015). In these observations, stronger oxidative stress may have caused ROS
447 accumulation and reduce cell protection leading to tough meat. However, for the Aberdeen
448 Angus and Limousin breeds the opposite relationship was found and such positive
449 relationships were also reported earlier. SOD1 correlated positively with tenderness in
450 *Semitendinosus* muscle of Charolais young bulls (Guillemin *et al.* 2011a) and Rouge des Près

cows (Gagaoua *et al.* 2017b). According to (Rowe *et al.* 2004), antioxidant enzymes such as superoxide dismutase may protect proteases from oxidative damage, thus leading to tender meat. Hence, as already noted above, the direction of the correlations between certain proteins and tenderness depends on the interactions with other proteins.

3.2.5. Proteolytic enzymes

Proteolytic enzymes play a central role in the fragmentation of structural proteins, and consequently, in the tenderization process (Ouali & Talmant 1990; Ouali *et al.* 2013). This study investigated only one proteolytic enzyme, CAPN1 or μ-calpain, which was as expected positively related to tenderness and entered 7 times in the regression equations, including the 4 equations of the Blond d'Aquitaine breed (**Table 3** and **Fig. 1**). The latter is coherent with the presence of structural proteins, CapZ-β and MyHC-IIx in the equations of this breed: increased proteolytic activity increases the fragmentation, and therefore the extractability of those structural proteins. Calpain-activity depends on many other biological pathways, related to Ca^{2+} homeostasis, structure, energy metabolism, heat stress, mitochondrial activity, and apoptosis (Ouali *et al.* 2013; Gagaoua *et al.* 2015a; Picard & Gagaoua 2017; de Oliveira *et al.* 2019). For example, calpain-activity needs the presence of Ca^{2+}, and therefore depends on Ca^{2+}-regulating proteins, including the ryanodine receptor (Brulé *et al.* 2010). In addition, calpain autolysis during the proteolytic process and levels decrease therefore concomitantly. If there are great amounts of the oxidized form of HSP70-1A/B, one of the substrates for calpain, less calpain will be available to degrade structural proteins (Gagaoua *et al.* 2015b). This illustrates the strong interconnectedness of the processes that underlie *post-mortem* proteolysis and consequently, final meat tenderness.

4. Conclusion

This study shows that the relationships between a list of potential biomarkers and beef tenderness depend on the end-point cooking temperature, country origin of the panelists and breed, although there were also certain consistencies. Particularly, proteins of structure (MyHC-I, MyHC-IIa, MyHC-IIx), oxidative stress (DJ-1, PRDX6) and proteolysis (CAPN1) are robustly involved in the LT tenderization process. These 6 biomarkers were retained in the same directions irrespectively of end-point cooking temperature, country origin of the panelists or breed, and can be considered as predictors of tenderness. Consistencies were found for Blond d'Aquitaine breed with various robust relationships whatever the treatment factors. In this breed, rather CRYAB, HSP20, HSP40, CapZ-β, MyHC IIx, SOD1 and CAPN1

may be robust biomarkers of global tenderness evaluated by sensory analysis, as the relationships are the same whatever the cooking temperature and the country origin of the panelists. The findings of this study allowed understanding a part of the underlying mechanisms in tenderness determinism that are due to cooking temperature and breed and specific link of the proteins at those treatments. Further investigations are needed to compare the muscle proteome of fresh and cooked meat to understand the mechanisms underlying the effects of heating on final tenderness of cooked meat and the validation of the biomarkers.

Conflict of interest

The authors have declared that no competing interests exist.

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686

687 **Figure captions**

688 **Fig. 1.** Principal component regression (PCR) analyses on beef tenderness of the three young
689 bull breeds (Aberdeen Angus, Limousin and Blond d'Aquitaine) obtained at the two cooking
690 temperatures (55 and 74°C) assessed by the trained panelists from France (FR) and the United
691 Kingdom (UK). The size of colored circles indicate the retained biomarkers in the models and
692 the level of their association with tenderness based on the normalized regression coefficients
693 in the equations (β) as shown in the legend. Colors indicate direction of the association of the
694 biomarkers, with green and red reflecting positive and negative relationships, respectively.
695 The non-significant proteins (not retained in the models) were left in blank. The total
696 frequency of entrance of the biomarkers in the regression equations are shown on the right of
697 the graph.
698
699 **Fig. 2.** Venn diagrams (**A-C**) by breed highlighting the number of the retained biomarkers in
700 the PCR models and those shared by biological family according to the two end-point cooking
701 temperatures (55 and 74°C) and country origin of the panelists from France (FR) and the
702 United Kingdom (UK). **D**) Pairwise biomarker distance (*D*) expressed as a percentage based
703 on the abundances of the proteins quantified by Dot-Blot and SDS-PAGE for the different
704 factors *i.e.,* breed, cooking temperature and country origin of the panelists.
705
706 **Fig. 3.** Number of the retained proteins from each of the 5 biological families for **A)**
707 Aberdeen Angus, **B)** Limousin and **C)** Blond d'Aquitaine at the two end-point cooking
708 temperatures (55 and 74°C) as assessed by the trained panelists from France (FR) and the
709 United Kingdom (UK).

Table 1. List of the 18 protein biomarkers quantified using the Dot-Blot technique.[1]

Protein biomarker (*gene*)	UniProtKB ID	Antibody dilutions
Heat Shock Proteins		
αB-Crystallin (*CRYAB*)	P02511	1/500
Hsp20 (*HSPB6*)	O14558	1/200
Hsp27 (*HSPB1*)	P04792	1/3000
Hsp40 (*DNAJA1*)	P31689	1/250
Hsp70-1A/B (*HSPA1B*)	P08107	1/2000
Hsp70-8 (*HSPA8*)	P11142	1/250
Hsp70-Grp75 (*HSPA9*)	P38646	1/250
Metabolism		
Enolase 3 (*ENO3*)	P13929	1/45000
LDH-B (*LDHB*)	P07195	1/50000
MDH1 (*MDH1)*	P40925	1/1000
Structure		
CapZ-β (*CAPZB)*	P47756	1/250
α-actin (*ACTA1*)	P68133	1/1000
MyLC-1F (*MYL1*)	P05976	1/1000
MyBP-H (*MYBPH*)	Q13203	1/4000
Oxidative resistance		
DJ-1 (*PARK7*)	Q99497	1/250
Prdx6 (*PRDX6*)	P30041	1/500
SOD1 (*SOD1)*	P00441	1/1000
Proteolysis		
μ-calpain (*CAPN1*)	P07384	1/1000

[1] The suppliers and conditions for each primary antibody are given as described in (Gagaoua *et al.* 2017c).

Abbreviations: LDH-B: Lactate dehydrogenase chain B ; MDH1: Malate dehydrogenase 1 ; CapZ-β: F-actin-capping protein subunit β; MyLC-1F: Myosin Light chain 1F; MyBP-H: Myosin Binding protein H; DJ-1: Parkinson diseaseprotein 7; Prdx6: Cis-peroxiredoxin; SOD1: Superoxide dismutase Cu/Zn

Table 2. Effect of cooking temperature (55 *vs.* 74°C) and breed on beef tenderness of the steaks assessed by the two panelists from France (FR) and the United Kingdom (UK).

Country panel	Cooking temperature	Breed (B)			SEM	Cooking temperature (CT)		*P*-values [1]		
		Aberdeen Angus	Limousin	Blond d'Aquitaine		55°C	74°C	B	CT	B*CT
France (FR) (0 – 10 scale)	55°C	5.10	4.76	4.85	0.07	4.90[a]	4.29[b]	ns	***	ns
	74°C	4.68[a]	4.17[b]	4.05[b]	0.06			**		
United Kingdom (UK) (0 – 8 scale)	55°C	4.10	3.92	3.78	0.07	3.93[a]	3.04[b]	ns	***	ns
	74°C	3.29[a]	3.03[b]	2.80[b]	0.03			*		

Within a row and breed or end-point cooking temperature, least squares means lacking a common superscript letter differ ($P<0.05$).

[1] Significance level: ns: not significant; * $P< 0.05$; ** $P < 0.01$; *** $P< 0.001$

Table 3. Principal component regression models for beef tenderness of the three young bull breeds (Aberdeen Angus, Limousin and Blond d'Aquitaine) obtained at the two cooking temperatures (55 and 74°C) assessed by the two panelists from France (FR) and the United Kingdom (UK).

Breeds	Temperature and panel origin	n proteins	Protein biomarkers [a]	Model characteristics		
				R-squared	RMSE[b]	*P*-value
Aberdeen Angus	FR55	9	**HSP40, HSP70-8, CapZ-β,** α-Actin, MyBP-H, **MyHC-IIx,** PRDX6, **SOD1, CAPN1**	0.84	0.59	0.0011
	FR74	8	**HSP20,** HSP70-1A/B, **HSP70-8,** HSP70-GRP75, MDH1, MLC-1F, PRDX6, **SOD1**	0.95	0.31	0.0000
	UK55	7	**CRYAB, HSP27,** HSP70-GRP75, MDH1, MyBP-H, **MyHC-I, CAPN1**	0.73	0.68	0.0016
	UK74	8	**CRYAB, HSP27, HSP40,** HSP70-1A/B, ENO3, **MyHC-I,** DJ-1, PRDX6	0.88	0.48	0.0012
Limousin	FR55	9	**HSP20, HSP70-8,** ENO3, **LDH-B,** MDH1, MyBP-H, **MyHC-I,** MyHC-IIa, **MyHC-IIx**	0.84	0.52	0.0001
	FR74	10	**HSP20, HSP27,** HSP70-1A/B, ENO3, **LDH-B,** α-Actin, MLC-1F, MyHC-IIa, DJ-1, **SOD1**	0.86	0.50	0.0002
	UK55	11	**HSP40, HSP70-8,** HSP70-GRP75, **LDH-B,** MDH1, **CapZ-β,** α-Actin, MyBP-H, **MyHC-I,** MyHC-IIa, **CAPN1**	0.88	0.47	0.0002
	UK74	10	**HSP27, HSP40, HSP70-8,** ENO3, **CapZ-β,** MLC-1F, MyHC-IIa, **MyHC-IIx,** DJ-1, **SOD1**	0.86	0.51	0.0002
Blond d'Aquitaine	FR55	13	**CRYAB, HSP20, HSP27, HSP40,** ENO3, **CapZ-β,** α-Actin, **MyHC-I, MyHC-IIx,** DJ-1, PRDX6, **SOD1, CAPN1**	0.90	0.48	0.0001
	FR74	13	**CRYAB, HSP20, HSP40,** HSP70-1A/B, **HSP70-8, LDH-B, CapZ-β,** MLC-1F, **MyHC-I,** MyHC-IIa, **MyHC-IIx,SOD1, CAPN1**	0.87	0.55	0.0003
	UK55	10	**CRYAB, HSP20, HSP40, LDH-B, CapZ-β, MyHC-IIx,** DJ-1, PRDX6, **SOD1, CAPN1**	0.68	0.76	0.0030
	UK74	14	**CRYAB, HSP20, HSP27, HSP40,** HSP70-1A/B, **HSP70-8, LDH-B,** MDH1, **CapZ-β,** MLC-1F, MyBP-H, **MyHC-IIx, SOD1, CAPN1**	0.93	0.41	0.0004

[a] The proteins in bold font are those that were retained more than 6 times (>50% of chance) in the models for the 3 breeds, the two cooking temperatures and the two panels (**Fig. 1**). The direction of the proteins (+ or -) are highlighted in **Fig. 1** and the underlined proteins are those that were already entered robustly in the models with the same direction (+ or -) irrespective of breed, cooking temperature and country origin of the panelists.

[b] RMSE: Root Mean Standard Error of the model.

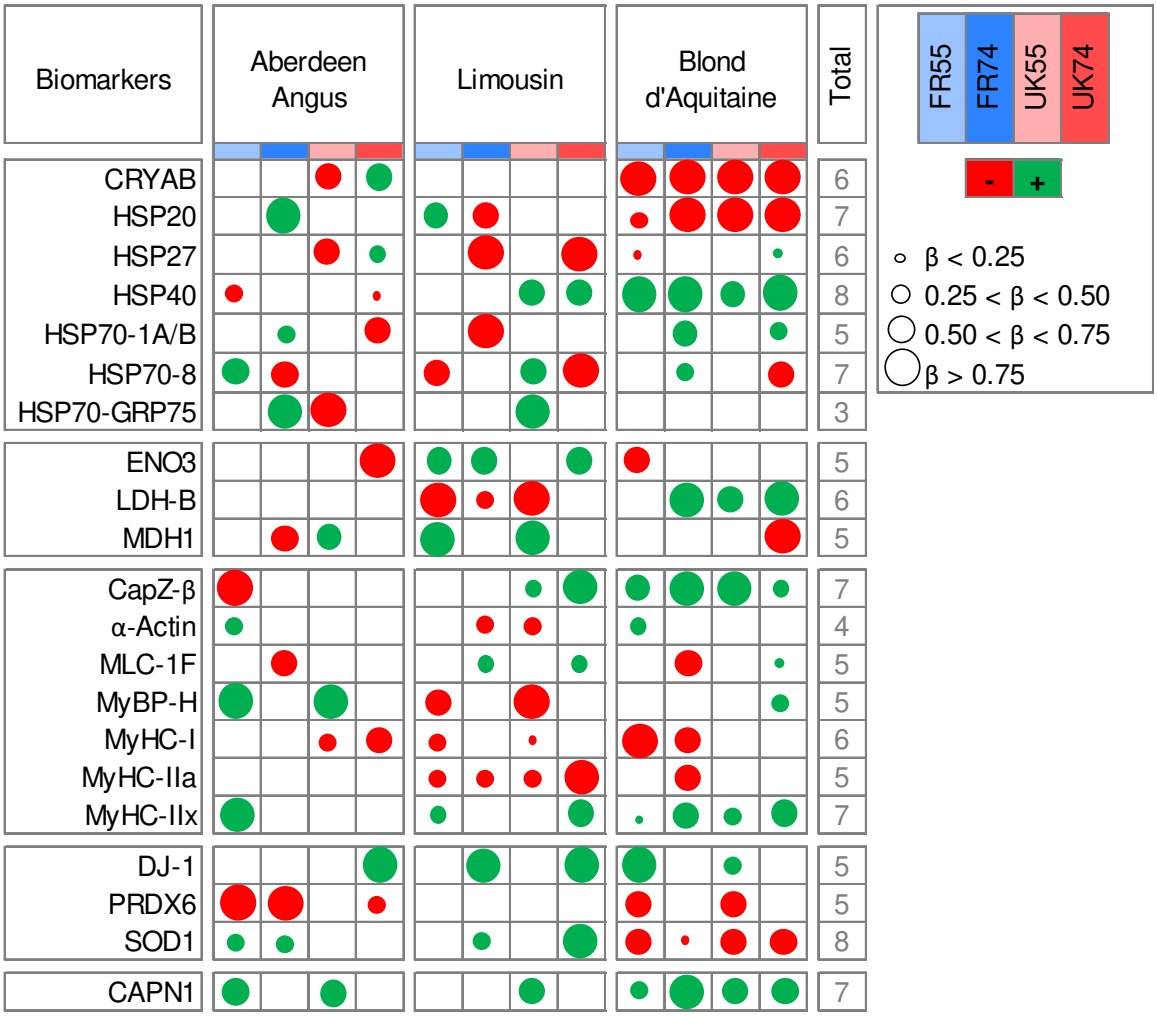
Biomarkers
Aberdeen Angus
Limousin
Blond d'Aquitaine
Total
FR55
FR74
UK55
UK74
-
+
β < 0.25
0.25 < β < 0.50
0.50 < β < 0.75
β > 0.75
CRYAB 6
HSP20 7
HSP27 6
HSP40 8
HSP70-1A/B 5
HSP70-8 7
HSP70-GRP75 3
ENO3 5
LDH-B 6
MDH1 5
CapZ-β 7
α-Actin 4
MLC-1F 5
MyBP-H 5
MyHC-I 6
MyHC-IIa 5
MyHC-IIx 7
DJ-1 5
PRDX6 5
SOD1 8
CAPN1 7

Fig. 1.

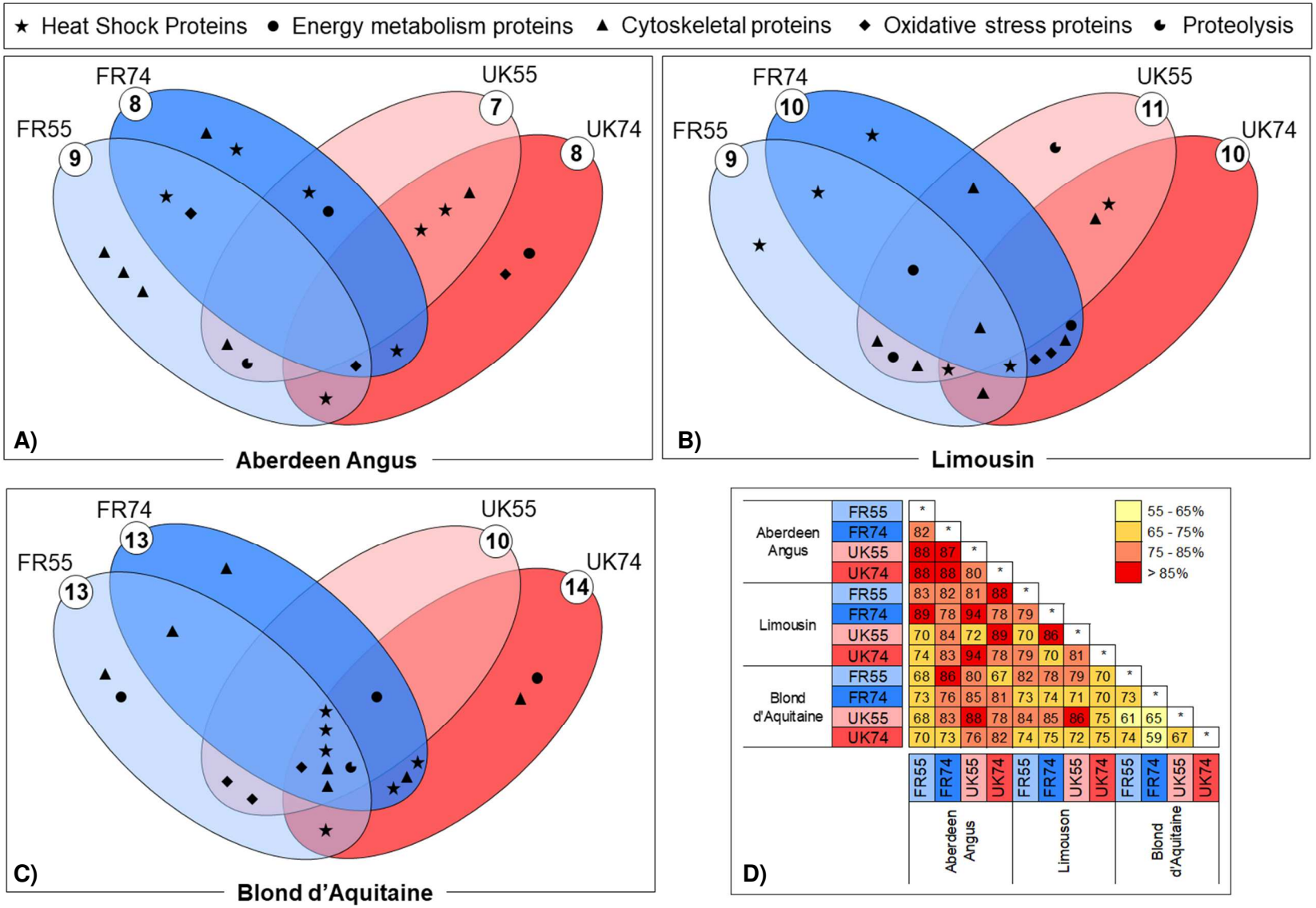
★ Heat Shock Proteins ● Energy metabolism proteins ▲ Cytoskeletal proteins ◆ Oxidative stress proteins ◔ Proteolysis
FR74
UK55
FR55
UK74
8
7
9
8
A)
Aberdeen Angus
10
11
9
10
B)
Limousin
13
10
13
14
C)
Blond d'Aquitaine
Aberdeen Angus
Limouson
Blond d'Aquitaine
55 - 65%
65 - 75%
75 - 85%
> 85%
D)

Fig. 2.

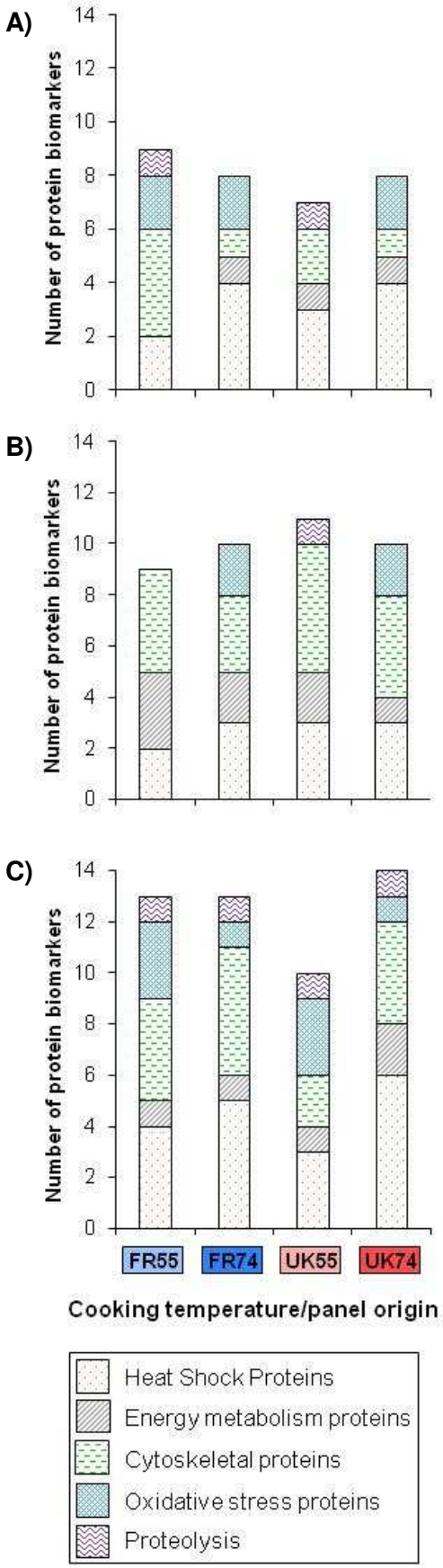
A)
B)
C)
Number of protein biomarkers
0
2
4
6
8
10
12
14
FR55
FR74
UK55
UK74
Cooking temperature/panel origin
Heat Shock Proteins
Energy metabolism proteins
Cytoskeletal proteins
Oxidative stress proteins
Proteolysis

Fig. 3.