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1 **The associations between proteomic biomarkers and beef tenderness depend on the end-**
2 **point cooking temperature, the country origin of the panelists and breed**

3

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18 **Abstract:**

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

31

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

34 **1. Introduction**

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

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

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

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

68 **2. Materials and Methods**

69 ***2.1. Animals handling and slaughtering***

70 This study is part of the European FP6 ProSafeBeef project (FOODCT-2006-36241) under
71 the INRA reference AQ284 (Gagaoua *et al.* 2015a). Seventy-four young bulls of three pure
72 breeds (24 Aberdeen Angus (AA), 25 Limousin (LIM), and 25 Blond d'Aquitaine (BA)) were
73 used. The animals were fattened in the same experimental station (INRA-UE 1414 Herbipôle)
74 during a 105 – 107 days finishing period until slaughter at around 16.0 ± 0.99 months
75 (average of 17.2 ± 0.92 months for AA, 16.4 ± 1.10 months for BA, 16.9 ± 0.79 months for
76 LIM). The fattening diet given *ad libitum* was based on straw (25%) and concentrate (75%)
77 (Gagaoua *et al.* 2016b). The animals were housed in groups of 4 animals of the same breed in
78 6×6 m pens with straw bedding.

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

89 ***2.2. Muscle sampling***

90 Muscle samples from *Longissimus thoracis* (LT, mixed fast oxido-glycolytic) were excised
91 45 min *post-mortem* from the right side of carcasses (6th rib) of each animal. Approximately
92 5g of the samples was frozen in liquid nitrogen and kept at -80°C until fiber characterization
93 by myosin heavy chains isoforms (MyHC) quantification and protein extractions for the
94 measure of tenderness biomarkers by Dot-Blot. At 24 h *post-mortem* another part was cut into
95 5 cm thick steaks and placed in sealed plastic bags in a Multivac A300/42 vacuum packager
96 (Multivac UK, Swindon, UK) to -980 mbar and kept between 2 and 4°C for 14 days for
97 ageing. Each steak was then frozen and stored at -20°C until tenderness assessments.

98 **2.3. Protein content determination**

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

103 **2.4. Dot-Blot analysis**

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

112 **2.5. Electrophoresis separation of Myosin Heavy Chain isoforms**

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

122 **2.6. Tenderness evaluation by sensory panels**

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

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

131 For the French sensory protocol, the steaks were thawed without stacking or overlapping,
132 at 2 to 5°C in vacuum packs for at least 24h or 48 h before cooking. One hour before sensory
133 assessment, the meat samples were cut into four approximately 1.50 cm thick steaks, 2 steaks
134 were assigned for cooking to 55°C and 2 steaks for cooking to 74°C. After exposure to air for
135 1 h at 18°C, the steaks were grilled on a double grooved plate griddle (SOFRACA, Morangis,
136 France) heated to 310°C for 30 min before cooking. Steaks were heated for 2 min between
137 two aluminum foil sheets, until the end-points temperature of 55°C or 74°C in the geometric
138 center of the steak was reached (measured using a temperature probe (Type K, HANNA HI
139 98704, Newark, USA)). After grilling, each grilled sample was cut into 20 mm cubes that
140 were immediately served to 12 panelists. Thus, sensory panels rated global tenderness of the
141 grilled meat on a 10 cm unstructured line scale (from 0 to 10), where 0 refers to extremely
142 tough and 10 to extremely tender meat (Gagaoua *et al.* 2016a). The sessions were carried out
143 in a sensory analysis room equipped with individual booths under artificial red light to reduce
144 the influence of the appearance of the samples. Each tasting booth was equipped with
145 computer terminals linked to a fileserver running a sensory software (Fizz v 2.20h,
146 Biosystemes, Couternon, France) that facilitated the direct entry of assessor ratings.

147 For the UK panel, the samples were defrosted overnight at 4°C and then cut into 2.0 cm
148 thick steaks. The steaks were then grilled under the overhead heat from grill elements of a
149 Tricity double oven domestic cooker producing approximately 120°C at the meat surface;
150 turning every two minutes until reaching the internal temperatures of 55 or 74°C in the
151 geometric center of the steak (measured by a thermocouple probe). After grilling, all fat and
152 connective tissue was trimmed and each steak was cut into 3×2×2 cm blocks. The blocks were
153 wrapped in pre-labelled foil, placed in a heated incubator for no more than 15 min before
154 testing by 10 panelists. The sensory evaluation was conducted in individual booths
155 illuminated with red light and equipped with the same sensory software than the French panel.
156 The assessors used 8-point category scales to evaluate tenderness from 0 – extremely tough to
157 8 – extremely tender.

158 **2.7. Statistical analyses**

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

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

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

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

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

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

200 **3. Results and Discussion**

201 ***3.1. Cooking temperature and breed effects on tenderness of muscle cuts assessed by*** 202 ***panelists from France (FR) and the United Kingdom (UK)***

203 Tenderness scores were irrespective of the panelists significantly different ($P < 0.05$) among
204 the three breeds at 74°C only (**Table 2**). At 55°C, the steaks of Aberdeen Angus were scored
205 higher but without any significant difference with the two other breeds. For all the samples
206 taken together and regardless the panelists, tenderness scores were greater (+12 (FR) and
207 +23% (UK), $P < 0.001$) at 55°C than 74°C. However, no interaction of breed \times cooking
208 temperature was observed ($P > 0.05$). The increase in internal endpoint temperature may be
209 related to higher water loss during cooking, with a direct influence on the tenderness scores.
210 This is in agreement with previous studies reporting greater tenderness when meat was
211 cooked at $< 60^\circ\text{C}$ (Gomes *et al.* 2014). More precisely, meat tenderness decreases in two
212 distinct phases, the first from 40 to 50°C and the second from 60 to 80°C with a significant
213 increase between 50 and 60°C (Christensen *et al.* 2000). The more tender meat of Aberdeen
214 Angus at 74°C only would be explained by the highest intramuscular fat (IMF) content
215 (3.49% *vs.* 1.52% and 1.18% for Limousin and Blond d'Aquitaine, respectively (Gagaoua *et*
216 *al.* 2016b)). Particularly, IMF levels of 3–4% or more enhances beef tenderness at higher
217 cooking temperatures due to fat breakdown giving meat a succulent mouthfeel (Savell &
218 Cross 1988), which was the case for the Aberdeen Angus bulls in this study (Gagaoua *et al.*
219 2016b).

220 ***3.2. Protein biomarkers to explain beef tenderness using principal component regression***

221 Principal component regression (PCR) models were used to describe the relationships
222 between the 21 biomarkers and tenderness scores (**Fig. 1** and **Table 3**) according to breed
223 (Aberdeen Angus, Blond d'Aquitaine, Limousin), end-point cooking temperature (55 and
224 74°C) and country origin of the panelists (France and the UK). These models are based on z-

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

227 The 12 equations explained 68 to 95% of the variability in tenderness (**Table 3**). The
228 number of the entered proteins in the PCR models as well as their biological families varied
229 according to breed, end-point cooking temperature and panelists (**Table 3** and **Fig. 2A-C**).
230 For Aberdeen Angus, 7 to 9 proteins were retained and explained 84%, 95% (at 55°C and
231 74°C) and 73%, 88% of tenderness for the French and the UK panelists, respectively. For
232 Limousin, 9 to 11 proteins were retained and explained 84%, 86% (at 55°C and 74°C) and
233 88%, 86% of tenderness for the French and the UK panelists, respectively. For Blond
234 d'Aquitaine, 10 to 14 proteins were retained and explained 90%, 87% (at 55°C and 74°C) and
235 68%, 93% of tenderness for the French and the UK panelists, respectively. The Venn
236 diagrams by breed (**Fig. 2A-C**) present the biomarkers according to their biological family for
237 the two end-point cooking temperatures (55 and 74°C) and country origin of the panelists,
238 France (FR) and the United Kingdom (UK).

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

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

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

257 the cooking temperature or processing/environmental conditions (Kemp *et al.* 2009).
258 Specifically, the heat capacities of sarcoplasmic, myofibrillar, and stromal proteins differ and
259 the cooking temperature determines the overall spectrum of thermal denaturation of different
260 meat proteins inducing strong differences on final meat texture (Tornberg 2005; Kemp *et al.*
261 2009). This explains the major role of myofibrillar or sarcoplasmic proteins and intramuscular
262 connective tissue in cooked meat texture (Brunton *et al.* 2006). Results of our study agree
263 with this knowledge. In the following subsections, we discuss the relationships between
264 tenderness and the proteins of the different biological families in their order of entrance in the
265 regression equations, taking into account the treatment factors.

266 3.2.1. Heat shock proteins

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

276 sHSPs were retained at least 6 times in the regression equations (**Fig. 1** and **Table 3**).
277 These consistent associations are of particular interest. Thus, CRYAB and HSP20 were
278 strongly negatively and HSP40 (*DNAJA1*) positively correlated with tenderness in the 4
279 equations for Blond d'Aquitaine bulls (**Table 3**). HSP20 and CRYAB are important
280 modulators of intermediate filament assemblies and are able to bind most unfolded proteins in
281 a non-specific manner as well as important anti-apoptotic properties role in *post-mortem*
282 muscle (Ouali *et al.* 2013; Dubińska-Magiera *et al.* 2014). HSP40 is a protein that is located
283 in the cytosol and cochaperones the intermediate filament with HSP70 to retard apoptosis.
284 This protein was also positively correlated with tenderness for the Limousin (UK55 and
285 UK74°C) but negatively for Aberdeen Angus breed (FR55 and UK74). In cattle, only one
286 work has shown a negative relationship between *DNAJA1* expression (HSP40) in LT muscle
287 of young Charolais bulls and tenderness score assessed by a sensory panel (Bernard *et al.*
288 2007).

289 The stronger involvement of sHSPs in tenderness of Blond d'Aquitaine compared to the
290 other breeds may be explained by some of their other characteristics. Particularly, compared
291 to Limousin and Aberdeen Angus, Blond d'Aquitaine has greater muscularity with low
292 connective tissue and intramuscular fat contents. In addition, isolated skeletal muscle myosin
293 retains much of its native structure as well as its enzymatic activity when incubated at high
294 temperature in the presence of sHSPs (Melkani *et al.* 2006). Hence, sHSPs may have
295 contributed to the development of meat texture, irrespective of cooking temperature and
296 consequently have influenced tenderness. In support of this, tenderness of the Blond
297 d'Aquitaine breed was quite strongly positively related to certain structural proteins, such as
298 CapZ- β and MyHC-IIx, whatever the end-point cooking temperature or panelist origin (**Fig.**
299 **1**).

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

316 3.2.2. Structural proteins

317 Many studies indicate that meat tenderization is strongly related to the disruption of muscle
318 structure by the breakdown of myofibrillar proteins (Bjarnadottir *et al.* 2010; Ouali *et al.*
319 2013; Carvalho *et al.* 2014; Marino *et al.* 2015) and our results are coherent with this.
320 Particularly for the Limousin and Blond d'Aquitaine breeds, the 7 structural proteins were
321 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 (Egelanddal *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 = $27.36 \pm 7.69\%$ vs.
342 LIM = $23.65 \pm 2.67\%$); MyHC-IIa (AA = $54.47 \pm 6.78\%$ vs. LIM = $30.75 \pm 5.51\%$) and
343 MyHC-IIx+b (AA = $18.17 \pm 10.53\%$ vs. LIM = $45.62 \pm 6.66\%$). 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 & Bamberg 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 H₂O₂ 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 Guillemain *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 al.*
439 *et 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 O₂⁻ to O₂ and H₂O₂.
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 (Guillemain *et al.* 2011a) and Rouge des Près

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

455 3.2.5. Proteolytic enzymes

456 Proteolytic enzymes play a central role in the fragmentation of structural proteins, and
457 consequently, in the tenderization process (Ouali & Talmant 1990; Ouali *et al.* 2013). This
458 study investigated only one proteolytic enzyme, CAPN1 or μ -calpain, which was as expected
459 positively related to tenderness and entered 7 times in the regression equations, including the
460 4 equations of the Blond d'Aquitaine breed (**Table 3** and **Fig. 1**). The latter is coherent with
461 the presence of structural proteins, CapZ- β and MyHC-IIx in the equations of this breed:
462 increased proteolytic activity increases the fragmentation, and therefore the extractability of
463 those structural proteins. Calpain-activity depends on many other biological pathways, related
464 to Ca^{2+} homeostasis, structure, energy metabolism, heat stress, mitochondrial activity, and
465 apoptosis (Ouali *et al.* 2013; Gagaoua *et al.* 2015a; Picard & Gagaoua 2017; de Oliveira *et al.*
466 2019). For example, calpain-activity needs the presence of Ca^{2+} , and therefore depends on
467 Ca^{2+} -regulating proteins, including the ryanodine receptor (Brulé *et al.* 2010). In addition,
468 calpain autolysis during the proteolytic process and levels decrease therefore concomitantly.
469 If there are great amounts of the oxidized form of HSP70-1A/B, one of the substrates for
470 calpain, less calpain will be available to degrade structural proteins (Gagaoua *et al.* 2015b).
471 This illustrates the strong interconnectedness of the processes that underlie *post-mortem*
472 proteolysis and consequently, final meat tenderness.

473 4. Conclusion

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

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

490 **Conflict of interest**

491 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 (<i>gene</i>)	UniProtKB ID	Antibody dilutions
<i>Heat Shock Proteins</i>		
α B-Crystallin (<i>CRYAB</i>)	P02511	1/500
Hsp20 (<i>HSPB6</i>)	O14558	1/200
Hsp27 (<i>HSPB1</i>)	P04792	1/3000
Hsp40 (<i>DNAJA1</i>)	P31689	1/250
Hsp70-1A/B (<i>HSPA1B</i>)	P08107	1/2000
Hsp70-8 (<i>HSPA8</i>)	P11142	1/250
Hsp70-Grp75 (<i>HSPA9</i>)	P38646	1/250
<i>Metabolism</i>		
Enolase 3 (<i>ENO3</i>)	P13929	1/45000
LDH-B (<i>LDHB</i>)	P07195	1/50000
MDH1 (<i>MDH1</i>)	P40925	1/1000
<i>Structure</i>		
CapZ- β (<i>CAPZB</i>)	P47756	1/250
α -actin (<i>ACTA1</i>)	P68133	1/1000
MyLC-1F (<i>MYL1</i>)	P05976	1/1000
MyBP-H (<i>MYBPH</i>)	Q13203	1/4000
<i>Oxidative resistance</i>		
DJ-1 (<i>PARK7</i>)	Q99497	1/250
Prdx6 (<i>PRDX6</i>)	P30041	1/500
SOD1 (<i>SOD1</i>)	P00441	1/1000
<i>Proteolysis</i>		
μ -calpain (<i>CAPNI</i>)	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)		<i>P</i> -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, <u>MyHC-IIx</u> , 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, <u>MyHC-I</u> , CAPN1	0.73	0.68	0.0016
	UK74	8	CRYAB, HSP27, HSP40 , HSP70-1A/B, ENO3, <u>MyHC-I</u> , DJ-1, PRDX6	0.88	0.48	0.0012
Limousin	FR55	9	HSP20, HSP70-8 , ENO3, LDH-B , MDH1, MyBP-H, <u>MyHC-I</u> , <u>MyHC-IIa</u> , <u>MyHC-IIx</u>	0.84	0.52	0.0001
	FR74	10	HSP20, HSP27 , HSP70-1A/B, ENO3, LDH-B , α-Actin, MLC-1F, <u>MyHC-IIa</u> , 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, <u>MyHC-I</u> , <u>MyHC-IIa</u> , CAPN1	0.88	0.47	0.0002
	UK74	10	HSP27, HSP40, HSP70-8 , ENO3, CapZ-β , MLC-1F, <u>MyHC-IIa</u> , <u>MyHC-IIx</u> , DJ-1, SOD1	0.86	0.51	0.0002
Blond d'Aquitaine	FR55	13	CRYAB, HSP20, HSP27, HSP40 , ENO3, CapZ-β , α-Actin, <u>MyHC-I</u> , <u>MyHC-IIx</u> , 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, <u>MyHC-I</u> , <u>MyHC-IIa</u> , <u>MyHC-IIx</u> , SOD1, CAPN1	0.87	0.55	0.0003
	UK55	10	CRYAB, HSP20, HSP40, LDH-B, CapZ-β , <u>MyHC-IIx</u> , 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, <u>MyHC-IIx</u> , 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.

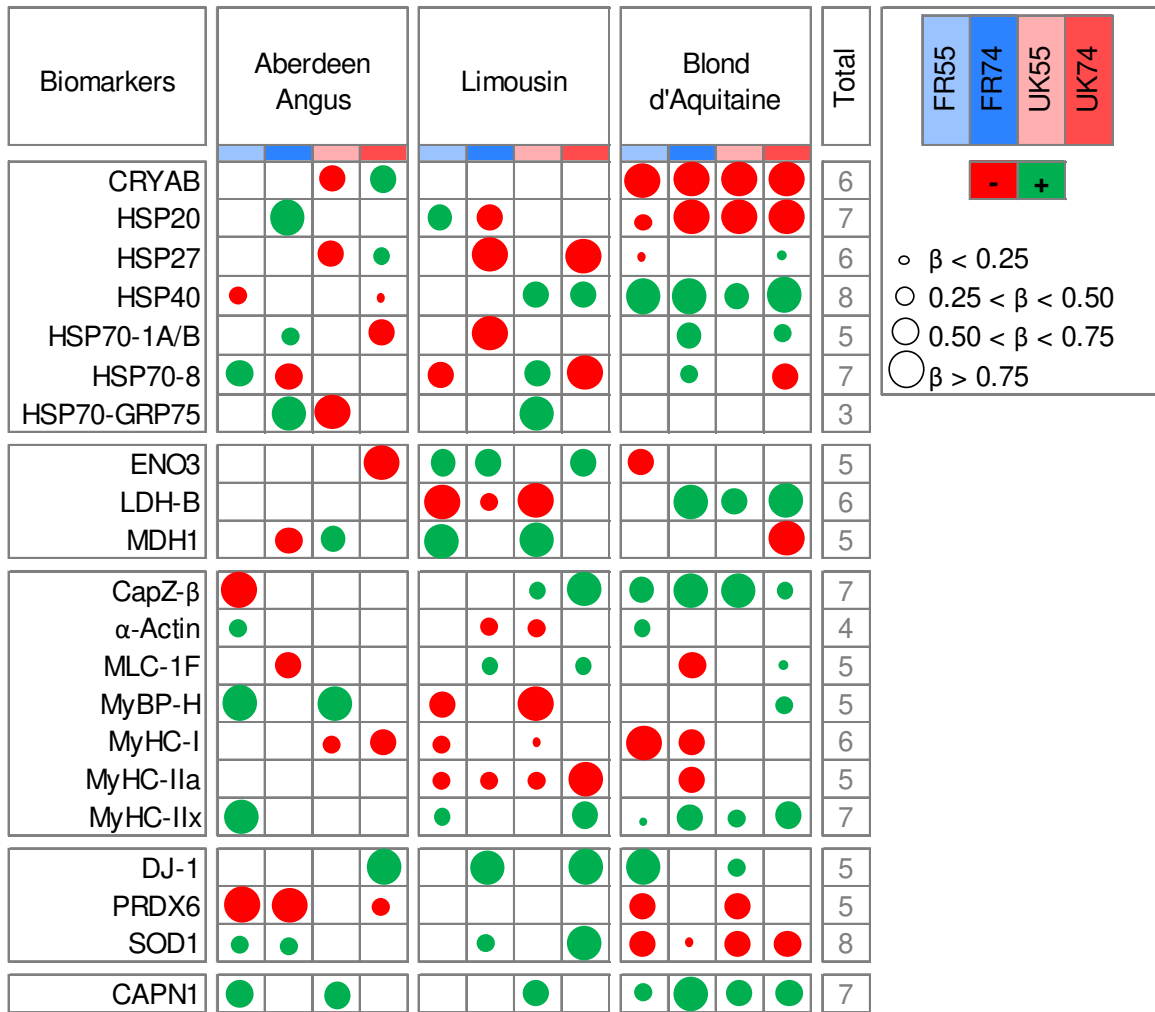


Fig. 1.

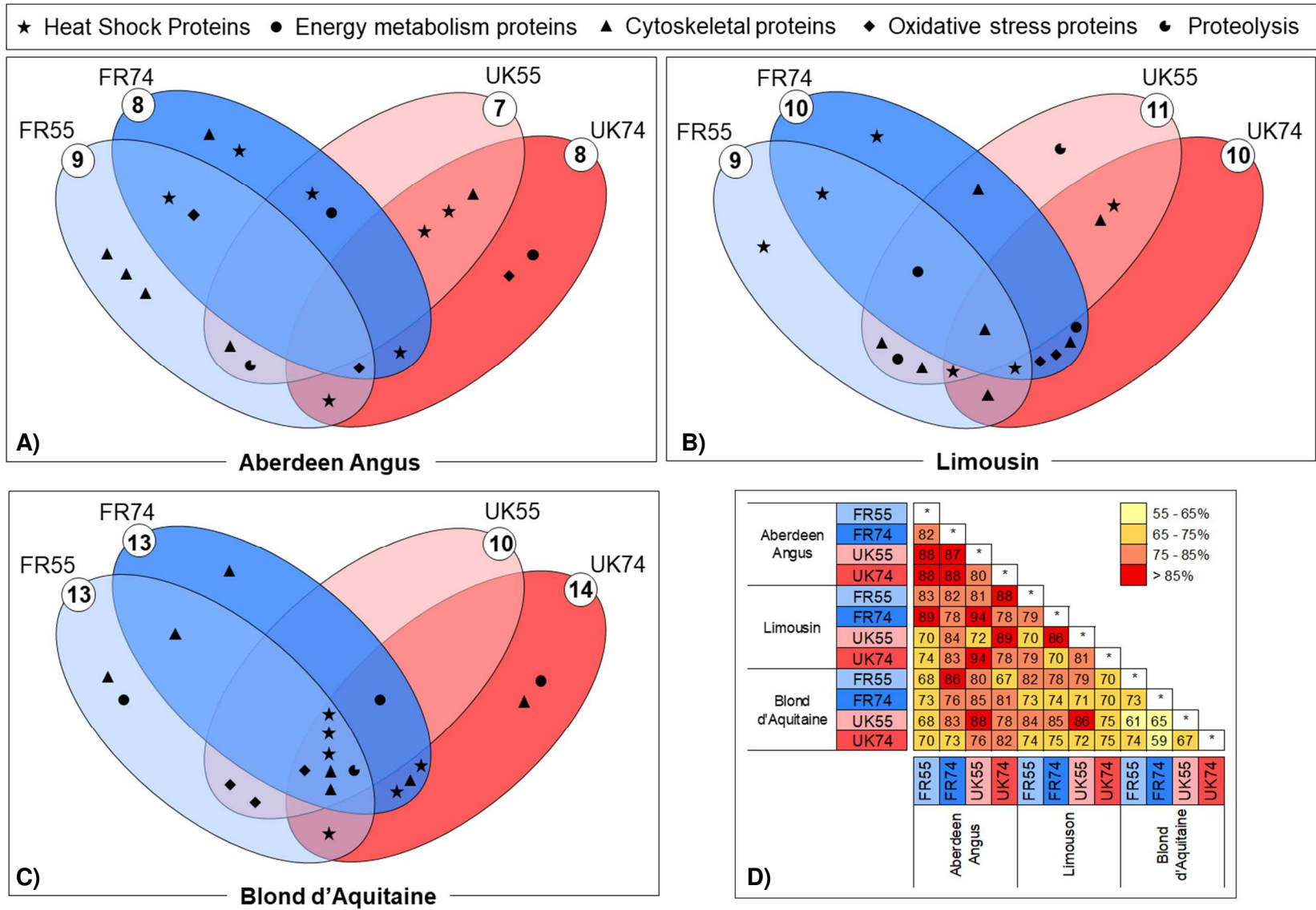
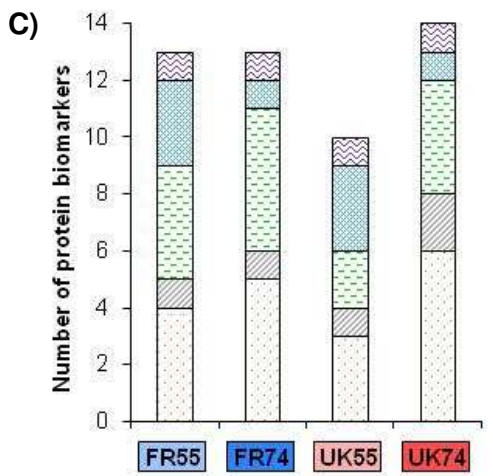
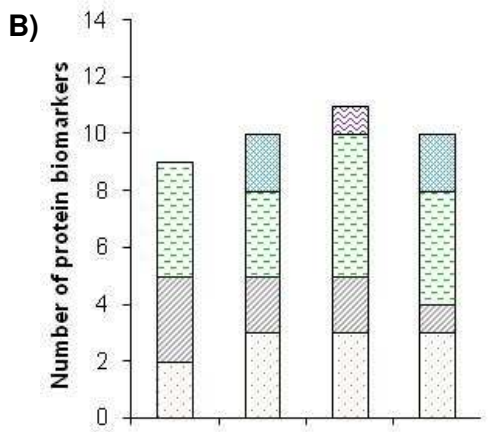
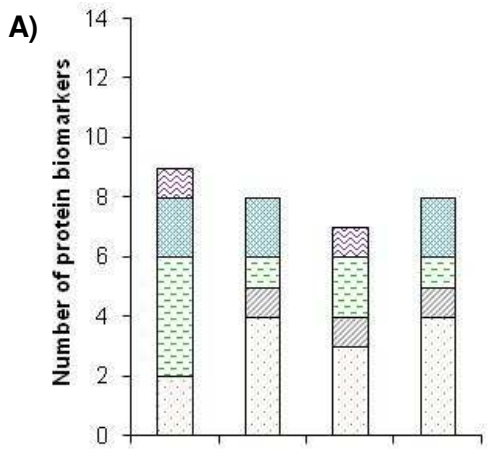


Fig. 2.



Cooking temperature/panel origin

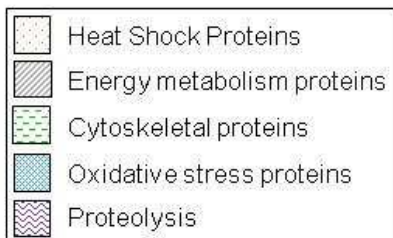


Fig. 3.