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1 **Identification of biomarkers associated with the rearing practices, carcass**
2 **characteristics and beef quality: an integrative approach**

3 Mohammed Gagaoua¹, Valérie Monteils¹, Sébastien Couvreur², Brigitte Picard^{1*}

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5 ¹ UMR1213 Herbivores, INRA, VetAgro Sup, Clermont université, Université de Lyon,
6 63122 Saint-Genès-Champanelle, France

7 ² URSE, Université Bretagne Loire, Ecole Supérieure d'Agriculture (ESA), 55 rue Rabelais,
8 BP 30748, 49007 Angers Cedex, France

9

10 *Corresponding author: Dr. Brigitte Picard

11 Email: brigitte.picard@inra.fr ; phone: +33 4 73 62 40 56. Fax: +33 4 73 62 46 39

12

13 **Abstract**

14 Data from birth to slaughter of cull cows allowed using PCA-based approach coupled with
15 the iterative *K*-means algorithm the identification of three rearing practices classes. The
16 classes were different in their carcass characteristics. Old cows raised mainly on pasture have
17 better carcass characteristics, while having an equivalent tenderness, juiciness, flavor,
18 intramuscular fat content and pHu to those fattened with hay or haylage. The *Longissimus*
19 *thoracis* muscle of the cows raised on pasture (with high physical activity) showed greater
20 proportions of IIA fibers at the expense of the fast IIX ones. Accordingly, the meat of these
21 animals have better color characteristics. Superoxide dismutase (SOD1) and α B-crystallin
22 quantified by Dot-Blot were the only other biomarkers to be more abundant in “Grass” class
23 compared to “Hay” and “Haylage” classes. The relationships between the biomarkers and the
24 six carcass and eleven meat quality traits were investigated using multiple regression analyses
25 per rearing practices. The associations were rearing practice class and phenotype trait-
26 dependent. ICDH and TP53, were common for the three classes, but the direction of their
27 entrance was different. In addition, rearing practices and carcass traits were not related with
28 Hsp70-Grp75 and μ -calpain abundances. The other relationships were specific for two or one
29 rearing practices class. The rearing practices dependency of the relationships was also found
30 with meat quality traits. Certain proteins were for the first time related with some beef quality
31 traits. MyHC-IIX, PGM1, Hsp40, ICDH and Hsp70-Grp75 were common for the three rearing
32 practices classes and retained to explain at list one beef quality trait. A positive relationship
33 was found between PGM1 and hue angle irrespective of rearing practices class. This study
34 confirms once again that production-related traits in livestock are the result of sophisticated
35 biological processes finely orchestrated during the life of the animal and soon after slaughter.

36 **Key words:** Cull cows, Rearing practices, Carcass characteristics, Meat quality, Biomarkers.

37 Introduction

38 In beef production systems, meat comes from various types of animals, of different breeds
39 and ages, which have been also managed and finished using different rearing practices.¹⁻³
40 Beef quality properties are well known to be impacted by these factors and also others related
41 to slaughtering and carcass handling practices.⁴⁻⁶

42 Today, consumers are (re)requesting stable sensory beef quality traits, namely meat color,
43 tenderness, juiciness and flavor attributes. Thus, stakeholders of meat chain must be able to
44 deliver and guarantee high-quality products to reduce perceived risk and gain consumer
45 confidence and, at the same time, producers must provide a viable farm holding, by increasing
46 animal and carcass performances or the added-value. This is a challenging task due to the
47 nature of beef itself. Beef is biochemically dynamic, hence it is naturally susceptible to
48 variation in acceptability which prompted the use of innovative and high-throughput
49 processing technologies for its characterization.⁷ Numbering among the innovative methods
50 that have been introduced to identify biomarkers of muscle food quality attributes are ‘-
51 omics’ based research. Proteomics approach was extensively implemented for better
52 understanding of several aspects of muscle to meat conversion in relation to different but
53 individual rearing practices factors. They have provided a broader and deeper understanding
54 of how these factors could contribute to meat quality variations,^{8,9} but less has been done in
55 relation with carcass characteristics. Consequently, the use of high-throughput processing
56 technologies for joint management of carcasses and meat quality will be very useful for the
57 beef sector, since numerous countries are seeking powerful tools that can help the
58 stakeholders to allow both a high **i**) remuneration for the farmers which is based on the
59 properties of the carcasses and **ii**) satisfaction of the consumer expectations in terms of beef
60 quality traits.

61 Proteomic investigations have further confirmed and elucidated previous concepts and
62 provided dozens of protein biomarkers belonging to heat shock stress, energy metabolism,
63 structure, oxidative stress, apoptosis, signaling and proteolysis pathways to be involved.⁹
64 These protein biomarkers may help beef stakeholders seeking better marketing options
65 through efficient beef production systems. These would also help to determine which rearing
66 practices and animal traits, as well as live-animal performances and carcass characteristics,
67 need to be improved to reach the targeted market specifications. Thus, there is a need to go
68 beyond the stage of identifying protein markers between proteomics data and the animals’
69 traits or phenotypes of interest. In this study, we report the use of Dot-Blot¹⁰ as a quantitative

70 approach for the quantification of protein biomarkers related to meat quality of Protected
71 Designation of Origin (PDO) Maine-Anjou. This PDO exclusively included animals from the
72 Rouge des prés breed which originated in the northwestern part of France. This area is
73 excellent for beef production as it has both grassland and tillable land. The Rouge des Prés
74 breed was the second among the 4 breeds allowed to be used in France for PDO meat
75 production¹⁰. The PDO Maine-Anjou is composed of around 80% of cows, younger than 10
76 years of age, having calved at least once and a minimal carcass weight of 380 kg. Steers over
77 30 months of age with carcass weight of 400 kg minimum can also be found (20%). PDOs are
78 of special economic importance for the valorization of local breeds as it is a dual-purpose
79 breed, used for both beef and milk. The specifications of animal products under PDO label are
80 also paid increasing attention in Europe (European Union Regulation (EEU) 2081/92). These
81 labels are meant to support diversity in agricultural production, satisfy consumers, and protect
82 product names against fraud and imitation. Thus, there is a great interest and economical
83 value as an initiative of the breeders association, for the implementation of high-throughput
84 techniques, likely omics techniques, for meat quality management of PDO meat to guarantee
85 high quality for the consumers^{10,11}. In this study, the biomarkers were associated in addition to
86 other muscle characteristics measured using conventional methods, with carcass properties
87 and meat quality according to the rearing practices of PDO Maine-Anjou cows. Furthermore,
88 the main objective was also to address an innovative approach by identifying the cows
89 according to their rearing practices, which is valued not only the muscle, carcass, sensory and
90 technological quality of meat but also the whole life of the animal affecting the beef quality
91 obtained under different production systems. For this purpose, multiple regression analyses
92 were performed to relate each carcass and meat trait with the muscle characteristics according
93 to the rearing practices identified. A focus on the use of biomarkers to understand the
94 relationships with carcass and meat quality potential was further explored.

95 **Materials and methods**

96 *Animals, handling, slaughtering and sampling*

97 A total of 110 French PDO Maine-Anjou cows originated in the northwestern part of
98 France were collected from a cooperative of livestock farmers located in the department of
99 Maine-et-Loire. The rearing practices of each animal were surveyed by a questionnaire.¹¹
100 Briefly, the questionnaire included information about **i**) the finishing period [part of hay,
101 haylage and/or grass in the finishing diet (% w/w); daily and global amount of concentrate
102 (kg); fattening duration (days); physical activity (% days out)] and **ii**) the animal

103 characteristics [Animals with meat or dairy-ability; birth month/season ; birth weight (kg);
104 age at weaning (month); weaning duration (week); age of first calving; number of calving;
105 suckling value (0-10) and age at slaughter]. Before slaughter, all animals were food deprived
106 for 24 h and had free access to water. All the animals were slaughtered in the same
107 commercial abattoir (Elivia, Lion d'Angers, 49220, France), stunned using captive-bolt pistol
108 prior to exsanguination and dressed according to standard commercial practices. Slaughtering
109 was performed in compliance with the French welfare regulations and respecting EU
110 regulations (Council Regulation (EC) No. 1099/2009). After slaughter, the carcasses were
111 characterized and graded according to the European beef grading system (CE 1249/2008).
112 Thus, information for each carcass were measured, namely hot carcass weight (HCW, kg),
113 EUROP conformation score (EUROP grid), weight of the whole ribeye, 12th rib
114 characterization by *Longissimus* muscle weight, muscle carcass weight, carcass fat weight and
115 fat to muscle ratio (% w/w). The carcasses were not electrically stimulated and they were
116 chilled at 4°C until 24 h *p-m*.

117 Muscle samples of *Longissimus thoracis* (LT, mixed fast oxido-glycolytic muscle) were
118 excised from the right-hand side of the carcass of each animal 24 h *p-m* and four parts were
119 distinguished. The first part of the samples was subsequently frozen in liquid nitrogen and
120 kept at -80°C until analyzed for fiber characterization (fiber area, myosin heavy chains
121 isoforms (MyHC) and metabolic enzymes activities) and for protein extractions for the
122 quantification of the proteins by Dot-Blot. The second part of the sample was cut into pieces
123 (1–2 cm cross-section), vacuum packed and stored at -20 °C until analyzed for intramuscular
124 fat content. Another part was used for meat color measurement. The last part was cut into
125 steaks (20 mm thick) and placed in sealed plastic bags under vacuum and kept at 4°C for
126 ageing (14 days). Each loin sample was then frozen and stored at -20°C waiting sensory
127 evaluation and shear force measurements.

128 ***Muscle characteristics determination***

129 The metabolic muscle type was determined by measuring glycolytic and oxidative enzyme
130 activities of respectively lactate dehydrogenase (LDH; EC 1.1.1.27) and isocitrate
131 dehydrogenase (ICDH; EC 1.1.1.42).¹² These enzymes representative of different steps of the
132 glycolytic and oxidative pathways are currently used for the characterization of the metabolic
133 types of beef muscles.⁵

134 The contractile properties of the muscle were determined by the quantification of the
135 myosin heavy chains (MyHC) isoforms according to the method of Picard *et al.*¹³ using an
136 adequate electrophoresis protocol. Thus, three isoforms of myosin Heavy Chain (MyHC)
137 isoforms were quantified using this technique. Briefly, the myofibrillar proteins were
138 extracted using a buffer containing 0.5 M NaCl, 20 mM Na Pyrophosphate, 50 mM Tris, 1
139 mM EDTA and 1 mM Dithiothreitol. After homogenization of approximately 100 mg of
140 frozen muscle in 5 mL of the extraction buffer using a Polytron (x22 000) and an incubation
141 time of 5 min at 4°C on ice, the sample was subjected to a centrifugation at 5000 x g for 10
142 min at 4°C. Following centrifugation, the supernatant was diluted 1:1 (v/w) with glycerol at
143 87% and stored at -20°C until use. The samples were then mixed with an equal volume of
144 loading buffer (basic 2 x Laemmli) containing 4% SDS (w/v), 125 mM Tris (pH 6.8), 20%
145 glycerol (v/v), 10% β-mercaptoethanol (v/v) and 0.02% pyronin Y (w/v), incubated at room
146 temperature for 10 min and then heated (70°C) in a water bath for 10 min. The protein
147 extracts (10 μg) were loaded per well onto 0.75-mm-thick gels and were separated using 9.2%
148 polyacrylamide gels. The lower running buffer consisted of 50mM Tris (base), 75mM glycine
149 and 0.05% w/v SDS. The upper running buffer was at 2x the concentration of the lower
150 running buffer and β-mercaptoethanol (0.07% v/v) was added. Ten micrograms of protein
151 extracts were loaded per well onto 0.75-mm-thick gels mounted on a Mini-Protean II Dual
152 Slab Cell electrophoretic system (Bio-Rad). The migration was carried out at a constant
153 voltage of 70 V for 30 hours at 4°C. Controls of bovine muscle containing three (MyHC-I, IIA
154 and IIX) or four (MyHC-I, IIA, IIX and IIB) muscle fibers were added at the extremities of each
155 gel.¹⁰ After migration, the gels were fixed in 30% (v/v) ethanol and 5% acetic acid (v/v) and
156 then stained with colloidal Coomassie Blue R250 for 24 h. Gels were destained in a 30%
157 ethanol (v/v) and 5 % acetic acid (v/v) solution until the background was sufficiently cleared.
158 After staining in a Coomassie Blue dye solution, the gels were scanned and the proportions of
159 the different MyHCs bands were quantified by densitometry with ImageQuant Software 5500
160 (Amersham Biosciences/GE Healthcare) and expressed in percentage. The quantification of
161 the bands revealed no existence of MyHC-IIb isoform in PDO Maine-Anjou breed, thus only
162 MyHC-I, IIA and IIX isoforms are reported.

163 The muscle mean cross sectional fiber area for all the animals was determined on 10-μm
164 thick sections cut perpendicular to the muscle fibers with a cryotome.¹⁴ Between 100 and 200
165 fibers in each of two different locations in the muscle were used to determine the mean fiber
166 area (in μm²) by computerized image-analysis.

167 The protein concentrations of the protein extracts used in this study were determined
168 according to the dye binding method of Bradford¹⁵ using the Bio-Rad Protein assay (Bio-Rad
169 Laboratories Inc.). Bovine serum albumin (BSA) at a concentration of 1 mg/mL was used as
170 standard, and the protein content was calculated from the standard curve.

171 The relative abundance of 21 protein biomarkers was evaluated using the immunological
172 Dot-Blot technique.^{16,17} The biomarkers belong to 6 biological pathways:

173 1) *heat shock proteins* [α B-crystallin, Hsp20, Hsp27, Hsp40, Hsp70-1A, Hsp70-1B,
174 Hsp70-8 and Hsp70-Grp75];

175 2) *metabolism* [Enolase 3 (ENO3) and Phosphoglucomutase 1 (PGM1)];

176 3) *structure* [α -actin, Myosin binding protein H (MyBP-H), Myosin light chain 1F (MyLC-
177 1F) and Myosin heavy chain IIx (MyHC-IIx)];

178 4) *oxidative stress* [Superoxide dismutase [Cu-Zn] (SOD1), Peroxiredoxin 6 (PRDX6) and
179 Protein deglycase (DJ1)];

180 5) *proteolysis* [μ -calpain and m-calpain] and

181 6) *apoptosis* and *signaling* [Tumor protein p53 (TP53) and H2A Histone (H2AFX)].

182 The conditions retained and suppliers for all primary antibodies dilutions and details of the
183 protocol were according to our previous studies.^{10,17} An antibody was considered specific
184 against the studied protein when only one band at the expected molecular weight was detected
185 by western blot. Briefly, the protein extracts (15 μ g) of each muscle sample were spotted in
186 quadruplicate on a nitrocellulose membrane with the Minifold I Dot-Blot apparatus from
187 Schleicher & Schuell Biosciences (Germany) in a random order on the 96-spot membrane. In
188 addition, a mixed standard sample (15 μ g) was deposited for data normalization. The Dot-Blot
189 membranes were air-dried for 5 min, blocked in 10% PBS milk buffer at 37°C for 20 min, and
190 then incubated to be hybridized with the specific primary antibody of each protein.
191 Subsequently, the membranes were incubated at 37°C for 30 min with the anti-mouse
192 fluorochrome-conjugated LICOR-antibody IRDye 800CW (1 mg/mL). Infrared fluorescence
193 detection was used for quantification of the relative protein abundances. Subsequently, the
194 membranes were scanned using the Odyssey NIR imager (LI-COR Biosciences), with an 800
195 nm laser, a 169 μ m spatial resolution and a fixed gain of 5. Finally, the Dot-Blot images were
196 quantified with GenePix PRO v6.0 (Axon). Each dot volume was calculated as the total dot
197 intensity from which the median local background value multiplied by the dot area was
198 subtracted. Because Dot-Blot offers the possibility of replicates, a data-prefiltering approach
199 was implemented to eliminate outlier values mainly due to dust. The exclusion technique of

200 outliers was based on the Medium Absolute Difference (MAD) and applied before repeated
201 values were averaged. Finally, to make the data comparable between assays, the data were
202 normalized using a regression-approach based on the used mix standard specific for each
203 muscle. Thus, the relative protein abundances were based on the normalized volume and were
204 all expressed in arbitrary units (A.U).

205 **Meat quality evaluation**

206 For muscle ultimate pH evaluated at 24h *post-mortem*, the measurements were conducted
207 by inserting a glass electrode into each muscle sample using a Hanna pH meter (HI9025,
208 Hanna Instruments Inc., Woonsocket, RI, USA) suitable for meat penetration.¹⁰ The
209 measurements were done between the 6th and 7th rib. For that, five measurements were made
210 and the average value was used. The pH meter was calibrated at chilling temperature (average
211 temperature 3.5°C) using pH 4 and pH 7 buffers.

212 For surface meat color determination, a portable colorimeter (Minolta CR400, Konica
213 Minolta, Japan) was used to measure the L^* , a^* and b^* color coordinates of the muscle cuts
214 according to Gagaoua *et al.*¹⁰ The parameters Chroma (C^*), related to the intensity of color,
215 and hue angle (h^*), related to the change of color from red to yellow, were further calculated
216 using the following equations:

$$217 \quad C^* = [(a^{*2} + b^{*2})^{1/2}] \quad (1)$$

$$218 \quad h^* = [\text{ATAN}^{-1}(b^*/a^*)] \quad (2)$$

219 The measurements were done in three replicate series, at nine different locations on each
220 steak to obtain three measures for each muscle cut, and an average value was used for
221 analysis.

222 For intramuscular fat (IMF) content, a Dionex ASE 200 Accelerated Solvent Extractor
223 (Dionex Corporation, Sunnyvale, CA, USA) was used. From 1 ± 0.001 g of meat powder
224 were placed in a 22 mL extraction cell previously prepared with a cellulose filter and silicon
225 balls. The extraction was performed with petroleum ether at a temperature of 125 °C and a
226 pressure of 103 bar. The extract, containing fat content and petroleum ether, was collected and
227 transferred in an evaporation vial previously weighted (± 0.001 g). After 15 min of
228 evaporation, the vial was placed 17h in a drying oven at 105°C and then weighted (± 0.001 g)
229 to determine the IMF content in the meat sample.

230 For sensory beef assessment the protocol recently described by Gagaoua *et al.*⁶ was used.
231 Briefly, steaks all aged for 14 days were thawed, without stacking or overlapping, at 5°C in
232 vacuum packs for 48 h before cooking and sensory assessment at 55 °C. One hour before
233 sensory assessment, the meat samples were cut into approximately 1.50 cm thick steaks and
234 grilled on a double grooved plate griddle (SOFRACA, Morangis, France) heated to 300°C for
235 30 min before cooking. Steaks were heated for 2 min until the end-points temperature of 55°C
236 in the geometric center of the steak was reached (measured using a temperature probe (Type
237 K, HANNA HI 98704, Newark, USA)). After grilling, each steak was cut into 20 mm cubes
238 that were immediately served to 12 panelists chosen according to the criteria described by
239 Gagaoua *et al.*⁶ Briefly, the expert panelists, grouping 6 males and 6 females, were trained in
240 accordance with the ISO standards ISO/TC 34 (ISO_8586, 2012) and were selected based on
241 their sensory sensitivity, appropriate training and experience in sensory testing. Formal
242 evaluation allowed selection of those panelists capable of making consistent and repeatable
243 sensory assessments of meat products that is, making comparative judgements both within a
244 session and from one session to another. The continuous accuracy and precision of these
245 panelists have been already assured by regular training sessions and subsequent evaluation of
246 performance, according to the ISO 8588-1 standards. For the present study, we have used
247 panelists with several years experience of sensory evaluation of meat and meat products.

248 The panelists rated the steaks on a 10-cm unstructured line scale (from 0 to 10) measured
249 in mm for the following attributes: global tenderness (0 – extremely tough, 10 – extremely
250 tender), juiciness (0 – extremely dry, 10 – extremely juicy) and beef flavor intensity (0 –
251 extremely weak, 10 – extremely strong). The sessions were carried out in a sensory analysis
252 room equipped with individual booths under artificial red light to reduce the influence of the
253 appearance of the samples. At each session, a monadic presentation of 6 samples was done,
254 each sample being selected in random order. Each tasting booth was equipped with computer
255 terminals linked to a fileserver running a sensory software program (Fizz v 2.20 h,
256 Biosystemes, Couternon, France) that facilitated the direct entry of assessor ratings, which
257 were later formatted in Excel. Within each sensory session, scores were averaged across
258 panelists for each steak, and the means were used in the statistical analyses.

259 For objective tenderness, the Warner-Bratzler shear force (WBSF) was measured
260 according to Lepetit and Culioli,¹⁸ Briefly, from the sample cuts cooked as previously
261 described, two to five 1 x 1 x 4 cm cores per steak sample were removed parallel to the
262 longitudinal orientation of the muscle fiber of LT muscle. WBSF was assessed using an

263 Instron 5944 two or three times per core in order to obtain around 10 repetitions per sample.
264 Force at rupture during shear compression testing was expressed in N/cm².

265 *Statistical analysis*

266 The collected data concern the animal life and fattening period of 110 PDO Maine-Anjou
267 cows data (p = 16), carcass characteristics (p = 7), muscle properties (p = 27) and ribeye steak
268 (*Longissimus thoracis*) quality traits (p = 11).

269 Rearing practices classes were created using an innovative statistical approach based on
270 principal component analysis (PCA). We first projected the 16 variables related both to the
271 *fattening period* [part of hay, haylage and/or grass in the finishing diet (% w/w); amount of
272 concentrate (daily and overall in kg); fattening duration (days) and physical activity (% days
273 out)] and *animal characteristics* [Animals with meat or milk-ability; birth season ; birth
274 weight (kg); age of weaning (month); weaning duration (week); age of first calving; number
275 of calving; suckling value (0-10) and age at slaughter]. Afterwards, a *K*-means cluster analysis
276 ($k = 3$) via the variability explained by PCA allowed the clustering of the animals into distinct
277 clusters. *K*-means clustering is a widely used classification method that generates a specific
278 number of classes (nonhierarchical). This method assumes a certain number of clusters, k ,
279 fixed *a priori* and produces a separation of the objects into nonoverlapping groups coming
280 from Euclidean distances minimized at each step of an iterative procedure¹⁰. We proceeded by
281 using the variability explained by the axes of the PCA with eigenvalues >1.0 after Z-scores
282 calculation.¹⁰ Five factors with eigenvalues >1.0 were extracted on the basis of the scree plot
283 and evaluation of the factor loading matrix after orthogonal rotation. To check the suitability
284 of the factorial model, the Kaiser-Meyer-Olkin (KMO) test for sampling adequacy was used.
285 Three clusters representing three different rearing practices classes were identified and named
286 to simplify the discussion as follow: Class 1= “**Hay class**”; Class 2 = “**Grass class**” and Class
287 3 = “**Haylage class**”, respectively. The use of *K*-means provides an important justification for
288 PCA-based data reduction and allows good separation by grouping the animals according to
289 their similarities in terms of their properties.

290 To determine the significance of differences between the least squares means of the animal
291 life and fattening period, carcass, muscle and meat quality traits between the three rearing
292 practices classes, PROC GLM procedure of SAS was performed. The significant differences
293 were performed using Tukey's test at a significance level of $P < 0.05$.

294 For better understandings of the relationships and hence of mechanisms, simple and
295 multiple regression analyses were performed using the PROC REG of SAS to create best
296 regression equations for meat quality traits and carcass characteristics (as dependent variables,
297 x) using the protein muscle characteristics and protein biomarkers (as independent variables,
298 y) per rearing practice class. For that, the option “optimal model” was chosen to produce the
299 model with the highest r^2 value. We maximized at 4 the number of the explanatory variables
300 to be retained in the models to meet to the principle of parsimony [one variable each ten
301 observations (animals)]. Regression coefficients and significance of each retained variable are
302 calculated for the models. The absence of collinearity was systematically verified for each
303 regression model, by producing condition indices and variance proportions using the COLLIN
304 procedure of SAS. Variables were identified as collinear if they possessed both a high
305 condition index >10 and a proportion of variation >0.5 for two or more traits.⁵

306 ***Results and Discussion***

307 The present study aimed to **i)** separate into classes using an innovative multivariate
308 analysis a total of 110 PDO Maine-Anjou cows according to their rearing practices applied
309 throughout their live; and **ii)** compare these classes for their carcass, meat quality and muscle
310 characteristics. In addition, this study aims also **iii)** to relate for the first time a list of
311 biomarkers quantified by Dot-Blot and other muscle characteristics, with carcass and meat
312 quality traits according to the rearing practices of PDO Maine-Anjou and explore the
313 consistencies and differences.

314 ***Characteristics of the three identified rearing practices classes***

315 The data concerning the succession of main management events from birth to slaughter of
316 cows allowed, using PCA-based approach coupled with the iterative *K*-means algorithm, the
317 identification of three rearing practices (**Figure 1**). These multivariate analyses shows that the
318 cows with similar characteristics were clustered together as can be seen on the ellipses of the
319 insert at the upper left corner of **Figure 1**. The first class (hereafter “**Hay class**”) grouped 44
320 young and light cows with the lowest age of weaning and finished mainly with hay,
321 moderately active, with low calving and milking ability (**Table 1**). The second class (hereafter
322 “**Grass class**”) was very different from the two other classes, grouped 30 old cows with the
323 highest age at weaning, the highest number of calving and suckling score and finished under
324 grass diet, with greater physical activity **Table 1**. The third class (hereafter “**Haylage class**”)
325 grouped 36 old cows, with higher birth weight, finished with haylage, and characterized by

326 low activity **Table 1**. PCA has been numerously used to characterize muscle foods as this
327 method point out quickly the relationships among the variables themselves. This method was
328 recommended to reduce a whole set of related parameters of animal and meat where coherent
329 patterns can be detected more clearly.¹⁹ Hence, plotting of the five axis with eigenvalues > 1.0
330 on a Cartesian axis revealed that, on the whole, the cows might be discriminated.

331 *Differences and similarities in carcass, meat quality and muscle characteristics*

332 The carcass characteristics of the cows for the three rearing practices classes are
333 summarized in **Table 2**. Cows within the Grass class had different carcass characteristics in
334 comparison to those of Hay and Haylage classes. The only parameter that is not different is
335 the fat to muscle ratio. It is recognized that management practices, such as rearing method,
336 can alter carcass characteristics through differences in finishing practices and diet.²⁰
337 Nevertheless, it is known that the specific effects on carcass are not easy to evaluate.²¹ In this
338 study, the old animals reared under Grass had the highest carcass conformation, as well as hot
339 carcass, muscle and carcass fat weights. Muscle protein turnover and better vascularization
340 may be played a great role in the high muscularity of the animals as it is known to be highly
341 dependent on energy intake and protein supply.²² Further, in spite of their high age, the cows
342 of Grass class gave carcass properties that are economically advantageous for breeders whom,
343 according to the EUROP classification, will be paid more. Earlier investigations observed that
344 cattle finished on pasture can grade as well as feedlot cattle.²³ We may then speculate that
345 fattening the animals with the same amount of concentrate ($P > 0.05$) but managing them
346 differently, induced significant differences in favor of the cows of Grass class in comparison
347 with the Haylage class. Thereby, one can suppose that the farmers by their expertise were able
348 to identify the importance to keep the cows in the herd because they fit with the objectives of
349 PDO Maine-Anjou: reproductive performance, suckling ability and good skeletal and
350 muscular development, as confirmed in this study (**Table 1** and **2**). Moreover, this study
351 showed clearly that the Grass class is more suitable for carcass properties of PDO Maine-
352 Anjou and there is a need to encourage farmers to maintain this practice to produce added-
353 value beef. These might also satisfy consumers requesting pasture meat, perceived as
354 environmentally friendly. Still, little information is available on the effect of the feeding or
355 rearing system on the proportion of various tissues, thus carcass properties, in the studied
356 breed, and this work is the first to provide these comparisons in addition to a recent
357 preliminary work by our group on these animals.²⁴

358 The comparison of the meat eating quality properties between the three rearing practices
359 classes are given in **Table 3**. The levels of ultimate pH did not differ. The values were within
360 the normal range for cattle^{10,25} where the mean values in each individual were all below the
361 threshold of 6.0.

362 Tenderness evaluated using instrumental (WBSF) or trained sensory panels was not
363 different between the rearing practices (**Table 3**). Similarly, juiciness, a texture attribute
364 positively related irrespective of cooking temperature and country panel with tenderness⁶ was
365 not different. Texture is one of the most important component of meat quality.⁸ Our results
366 emphasize that the cows of the three rearing practices classes, characterized by differences in
367 slaughter age, carcass weights and individual (zoo-technical) characters, led to the same meat
368 texture properties. These findings may be partly explained by the ageing period of meat cuts
369 (14 days), which is common for high-quality beef.²⁶ Moreover, French *et al.*²⁷ found in line to
370 our findings, no difference in meat quality with respect to shear force between cattle finished
371 on different regimen practices, including those of pasture. Thus, these results speculate that
372 irrespective of the differences in rearing practices and carcass properties, the same texture
373 properties of PDO Maine-Anjou ribeye steaks are achieved.

374 Intramuscular fat (IMF) content and beef flavor scores were not different between the
375 classes. These variables are closely related and known to affect the palatability of meat cuts.⁵
376 In contrast to our findings, finishing practices were reported to influence these two traits,
377 namely grass feeding.²⁸ The similar IMF contents in this work may be explained by the high
378 fat deposition in the studied breed which is not influenced by the rearing practice in this
379 experiment. On another hand and in agreement to earlier investigations,^{5,29} none to weak
380 effects of IMF on sensory traits were found in this study (data not shown). A meta-analysis by
381 our group showed that IMF accounted for a small percentage (4 to 5%) of the variation
382 observed in palatability traits in *Longissimus thoracis* muscle of cows and young bulls.²⁹

383 Beef color is the first attribute evaluated by consumers and is the major factor that
384 determines and influences the decisions of meat purchase at the point of sale.³⁰ In the present
385 study, there is no difference in lightness (L^*) between the meat of the 3 classes. Whereas, the
386 meat of the animals raised on pasture (Grass class) was redder in color than of Hay and
387 Haylage classes (**Table 4**). Causes of this effect are difficult to evaluate because several
388 factors, not a specific one, are responsible for these differences. In agreement to earlier
389 reports, Dunne *et al.*³¹ reviewed that, due to higher physical activity as it is the case in this
390 study (**Table 1**), animals reared on pasture have higher concentrations of myoglobin in

391 response to the high oxidative fibers.¹² It was also reported that beef color is influenced by
392 factors such as carcass fatness, animal age and carcass weight, all were the highest as
393 described above in Grass class than the two other rearing practices.²¹

394 Concerning muscle properties of the three classes, among the biomarkers analyzed, only 4
395 muscle proteins were different ($P < 0.05$, **Table 4**). MyHC-IIa, Superoxide dismutase (SOD1)
396 and α B-crystallin were more abundant in Grass class. MyHC-IIx level was the lowest in this
397 class, as confirmed by both electrophoresis and Dot-Blot analyses (**Table 4**). The results
398 highlight a switch to more oxidative fibers (IIA fibers) at the expense of IIX fibers in grass
399 class (**Figure 2b**), a finding in agreement to previous studies.¹² The higher levels of SOD1
400 and α B-crystallin in Grass class are coherent with earlier studies reporting higher abundances
401 of SOD1 and α B-crystallin in oxidative fibers.^{32,33} This may be partly due to the grass diet and
402 high physical activity (~98% vs. ~49% for Hay and ~27% for Haylage classes) of the cows,
403 allowing hence the development of intermediate rather than fast contractile properties.

404 ***Relationships between protein biomarkers with carcass characteristics per rearing*** 405 ***practices class***

406 The relationships between the biomarkers and carcass traits were investigated using
407 multiple regression analyses per rearing practice, as the explanatory power of the global
408 regression equations were very low (**Table S1-supplementary data**). Thus, the regression
409 coefficients and significance of the proteins retained in each explaining equation by rearing
410 practice are given in **Tables 5 – 7** for Hay, Grass and Haylage classes, respectively. A
411 summary of these associations, which aimed to better understand the relationships, and
412 underpinning mechanisms are given in **Figure 2a**. To our knowledge, proteomic studies
413 reporting links between protein biomarkers and carcass properties are still scarce and this
414 study is the first to show the abundances of biomarkers that may be affected according to
415 different rearing practices. Meanwhile, the findings confirm the previous investigations³⁴
416 reporting the dependency of the relationships between biomarkers and phenotypes, which are
417 in this study, rearing practices and carcass trait-dependent.

418 For each rearing practices class, 14 biomarkers (common or different) entered in the
419 regression equations of the 6 carcass characteristics studied (**Tables 5 – 7 and Figure 2a**).
420 The explained variability ranged from 24 to 62% according to the carcass properties and the
421 rearing practices classes. Two proteins only, ICDH and TP53, were common for the three
422 classes and entered in at least one carcass trait for each rearing practices class. ICDH,

423 representative of oxidative metabolism, had negative and positive coefficient for
424 conformation score for Hay and Haylage classes, respectively. TP53, a protein representative
425 of apoptosis, entered negatively and positively in HCW equations of Hay and Grass classes,
426 respectively. These inverted relationships, highlight the complexity of the effect of rearing
427 practices on the development of muscle and carcass properties, and thereby those of protein
428 abundances, as earlier postulated in several studies by our group for muscles, meat quality
429 traits and breeding practices.^{5,26,33,34} Further, it must be recognized that the lack of consistency
430 would be a consequence of the impact of animal performance effects and the existence of
431 individual animal performances x rearing practice interactions.

432 Irrespective of rearing practice, Hsp70-Grp75 and μ -calpain were not associated with any
433 of the carcass traits. These two proteins have a common denominator, Ca^{2+} , as they are both
434 involved on its trafficking from the endoplasmic reticulum (ER) to the sarcoplasm and
435 mitochondria.¹⁷ It seems that rearing practices and carcass traits are not related with these two
436 biomarkers as for meat quality traits.

437 Other relationships were specific for two or one rearing practices class (**Figure 2a**). SOD1,
438 Hsp70-1A and MyLC-1F involved in carcass traits of Hay and Haylage classes. Hsp70-1B,
439 MyBP-H, H2AFX, Hsp20 and Prdx6 entered in the regression equations of Grass and Hay
440 classes. MyHC-IIx, Hsp40, α -actin, PGM1 and ENO3 entered in the regression equations of
441 Grass and Haylage classes. The other proteins were related with carcass traits in one rearing
442 practices class only, likely α B-crystallin, m-calpain, LDH and MyHC-IIa for Hay class;
443 Hsp70-8 and fiber area for Grass class; and finally DJ-1, MyHC-I and Hsp27 for Haylage
444 class. The analysis of these relationships by rearing practices class with a focus on the
445 relevant proteins only, *i.e.*, proteins that are retained in the equations more than twice for
446 carcass traits, was highlighted in the following.

447 **For Hay class**, MyBP-H and α B-crystallin retained 4 and 3 times to explain the carcass
448 traits, respectively (**Figure 3a** and **Table 5**). MyBP-H was negative in the regression
449 equations of conformation, ribeye, *m. longissimus* and hot carcass weights. MyBP-H has
450 significant effects on length, thickness, and lateral alignment of myosin filaments.³⁵ Located
451 in the A-band of the myofibrils, it is closely related with the thick filaments, however its
452 primary association is with the fibers of fast twitch muscles.³⁵ The relationship of α B-
453 crystallin with ribeye, muscle and fat carcass weights agree with the importance of Hsp in the
454 regulation of intermediate filament network, likely desmin, involved in the structural integrity
455 and function of muscle cells and hence, those of carcass traits.³⁶ In pig, Hou *et al.*³⁷ reported

456 $\alpha\beta$ -crystallin implication in the regulation of skeletal muscle growth and body size. Small Hsp
457 proteins were found in several studies to be also related with fat accumulation in bovine
458 skeletal muscle. For example, Kim *et al.*³⁸ pointed out that Hsp27 has a negative correlation
459 with fat deposition in agreement with our findings. For instance, it is difficult to discuss more
460 there relationships. Nevertheless, they offer some important perspectives.

461 **For Grass class**, MyHC-IIx, H2AFX, MyBP-H and Hsp40 were the proteins mostly
462 related with the carcass traits (**Figure 2a** and **Table 6**). The first three proteins in addition to
463 ICDH were retained in the model of *Longissimus* muscle weight (LMW) and explained 62%
464 of variance. The negative relationship with MyHC-IIx agree with the findings discussed
465 above, and suggest that more the animals are grazed, more are the oxidative properties of the
466 muscle, herein by ICDH showed with a strong regression coefficient ($\beta = 0.53$; $P < 0.001$). As
467 expected, the impact of MyHC-IIx is more pronounced within ribeye and muscle carcass
468 weights (**Table 6**). Similarly, an earlier study revealed that the protein content in skeletal
469 muscle is affected by the regulation of *MyHC* expression when steers are grazed.³⁹ MyBP-H,
470 common to Hay class, was further retained in the regression equations of ribeye, *Longissimus*
471 muscle and fat carcass weights of Grass class. These findings support its involvement in
472 carcass traits and may partly explain the inverse relationships found within Grass and Hay
473 rearing practices class, described above to differ in their contractile properties. The precise
474 role of MyBP-H in carcass composition has not yet been described and we suggest this
475 biomarker to play a pivotal role in muscularity development under a marked rearing practice
476 effect. The relation with Hsp40, agrees well with the results found for Hay class, involving
477 cellular protein trafficking and stress defense. Accordingly, our group was the first to report
478 Hsp40 expression during cattle muscle development.⁴⁰ In the present study, we show for the
479 first time links between Hsp40 and carcass traits in a rearing practices dependency manner.
480 Hsp40 is a co-chaperone that would assist the regulation of complex formation between
481 Hsp70 and client proteins using rapid and transient interaction by activating ATP hydrolysis.⁴¹
482 Changes in Hsp40 expression might further reflect a shift in muscle metabolism.⁴⁰ Our
483 findings agree to this proposal. Thus, the rearing practices effect and specificity on Hsp40
484 abundance is of great significance, as the cows of Grass class showed a clear orientation of
485 muscle metabolism towards the oxidative type.¹² The involvement of H2AFX in
486 conformation, *Longissimus* muscle and fat carcass weights highlights the high level of
487 regulation that occurs during animal growth and carcass development of cows reared under
488 Grass class. In Hay class, H2AFX was negatively related with ribeye and fat carcass weights.

489 This study is the first to show these relationships. We may suggest H2AFX to play a pivotal
490 role in the balance between different biological processes. This histone was described to bind
491 to DNA and to various enzymes and protein entities, thereby modulating many cellular
492 pathways⁴². H2AFX was further reported to be involved in the underlying mechanisms of
493 muscle to meat conversion, namely of tenderness.^{16,42, 43}

494 *For Haylage class*, MyHC-I, PGM1 and Hsp27 were the main proteins involved in
495 different carcass traits (**Figure 2a** and **Table 7**). The first two proteins reflect both contractile
496 and metabolic properties of the muscle. Their involvement together with that of Hsp27 agrees
497 with the results described above for Hay and Grass classes. It seems that the retained proteins
498 and their direction differ between classes but they share common biological pathways that
499 would underpin muscle growth and carcass development. These proteins reveal a great role of
500 energy metabolism, heat stress and signaling pathways. Moreover, the findings highlight
501 specific sophisticated mechanisms behind adaptation of muscle and carcass to each rearing
502 practices management. This agrees with the previously published literature indicating that
503 physiology of growth in meat animals is an orchestrated process characterized by fine
504 coordination of cell proliferation and increase in cell size in organs, skeletal muscle, bone, and
505 adipose tissue from birth to market weight (for review see ²²). The involvement of Hsp27
506 supports the relationships found with carcass traits at both protein^{36,38} and gene levels.⁴⁴ Hsps
507 protect proteins of the cytoskeleton and have the ability to collaborate to control the redox
508 status.⁴¹ In this work, the explaining regression equations confirmed mechanisms reported in
509 certain studies exploring relationships with few proteins or genes. For example, the equation
510 of fat carcass weight retained MyHC-I and Hsp70-1A (positive) and DJ-1 and SOD1
511 (negative). The positive involvement of both MyHC-I and Hsp70-1A agrees with the specific
512 expression of large Hsp70 in slow-twitch fibers.⁴¹ More precisely, these authors reported that
513 the inducible form of Hsp70 is constitutively expressed in rat muscles comprised of type I
514 muscle fibres, but not in those comprised of type IIb fibres.⁴¹ The oxidative properties of cells
515 are continuously affected along these mechanisms, which may explain the implication of the
516 two anti-oxidant proteins (DJ-1 and SOD1). These results need further investigations since
517 little is known about these proteins in bovine muscle in relation to carcass, according to the
518 rearing practices of meat animals.

519 *Associations between muscle biomarkers with meat quality traits per rearing practice*
520 *class*

521 The relationships between the biomarkers and meat quality traits were performed using the
522 same approach discussed above. Similarly, the regression coefficients and significance of the
523 proteins retained in each equation by rearing practices class are given in **Tables 5 – 7** for Hay,
524 Grass and Haylage classes, respectively. A summary of these associations is given in **Figures**
525 **2b,c** for each rearing practices class. A total of 17 biomarkers for Hay class, and 18 for both
526 Grass and Haylage classes, were entered in the regression equations of the 11 meat quality
527 traits (**Tables 5 – 7** and **Figure 2b**). Furthermore, 3 to 5 carcass traits were entered in the
528 equations by class. These later did not explain a great variability compared to biomarkers,
529 unless for fat carcass weight which as expected, strongly and positively affected intramuscular
530 fat irrespective of rearing practices class. The explained variance of the regression equations
531 ranged from 20 to 80% (**Tables 5 – 7**). The findings support the numerous studies
532 highlighting the relationships between protein biomarkers identified using proteomic studies
533 and meat quality traits as recently reviewed in two comprehensive reviews^{9,42} The
534 implementation of high-throughput analytical tools over the last two decades was an
535 important step toward a better understanding of the complex biological systems that define
536 muscle to meat conversion.^{8,16,17} The results reveal the rearing practice dependency of the
537 relationships. Certain proteins were for the first time related with some beef quality traits,
538 namely juiciness, beef flavor and color parameters.

539 Among the biomarkers, five were common for the three rearing practices class and retained
540 to explain at list one beef quality trait. These are MyHC-IIx, PGM1, Hsp40, ICDH and
541 Hsp70-Grp75. From these, PGM1 was positively related with h^* irrespective of rearing
542 practice class. This robust relationship was earlier reported in cattle and identified PGM1 to
543 be positively related with a^* and h^* .¹⁰ This is in line with the idea that in the early *post-*
544 *mortem* period, glycolysis will continue, thereby affecting meat color and other sensory
545 qualities. PGM1 catalyzes the production of 3-phosphoglycerate and ATP from 1.3-
546 bisphosphoglycerate during aging and the phosphorylation of PGM1 would play an important
547 role in pH drop and hence of meat quality traits development (for review see ⁸).

548 The other metabolic enzyme, ICDH, entered positively with WBSF of Grass and Hay
549 classes, was further positively related with pHu of Hay and Haylage classes. ICDH involved
550 in the tricarboxylic acid (TCA) cycle was already identified to play a great role in *post-*
551 *mortem* beef metabolism. This agrees with the above results comparing classes and describing
552 relationships with carcass traits, supported mainly by the entrance of MyHC-IIx fibers in the
553 regression equations numerous times.

554 The results about heat shock proteins are in line with the great role that these chaperones
555 would play in muscle to meat conversion.^{16,17,45} Hsp40 was negative in the regression
556 equations of meat color coordinates of Hay (with C^* and h^*), Grass (with b^*) and Haylage
557 (with L^*) classes. It was further positively related with juiciness (for Hay class) and
558 tenderness (for Haylage class). The primary studies have shown negative relationships
559 between *DNAJ1* (the gene coding for Hsp40 protein) expression in *Longissimus thoracis*
560 muscle and tenderness.⁴⁶ Later on, this protein was reported by our group to be related with
561 beef color.^{10,17} Moreover, the central role of the large Hsp70-Grp75 in color and sensory meat
562 qualities was recently proposed.¹⁷ In the present study, Hsp70-Grp75 was positive in C^* of
563 Hay class, negative and positive respectively with juiciness and pHu for Grass class, and
564 positive with L^* for Haylage class. After animal bleeding, apoptosis is induced and in
565 response, Hsp proteins are recruited to impede the onset of apoptosis and to chaperone the
566 unfolding of muscle proteins.⁸ We assume that HSPs have essential roles in *post-mortem*
567 skeletal muscle since they contribute to proper conformation of proteins and preservation of
568 their biological functions. They act as molecular chaperones during protein assembly, protein
569 folding and unfolding, and in the refolding of damaged proteins, thus maintaining cell
570 homeostasis.⁴⁵ Hsp70-Grp75, a 75 kDa glucose regulated protein retained in the three classes,
571 is the only mitochondrial Hsp present in nearly all cellular compartments and may affect
572 muscle to meat conversion in different ways.^{17,47} Further Hsp proteins were also involved but
573 in just two or one rearing practice. For example, α B-crystallin was as described above for
574 carcass traits positively related with IMF for Haylage class and negatively with juiciness, L^*
575 and b^* for Hay class. Hsp70-8 entered positively in beef flavor and negatively as expected in
576 sensory tenderness and C^* equation of Hay class and positively with juiciness and a^* for
577 Grass class. Hsp27 was negative with pHu in Hay class as described by Sevane *et al.*⁴⁴ and
578 positive with WBSF and negative with a^* and b^* for Grass class. Hsp20, another small Hsp,
579 was negative with C^* for Grass class and positive with L^* for Hay class. Finally, Hsp70-1A
580 was specific for Haylage class only and related negatively with pHu. Comparisons concerning
581 most of these relationships were extensively reported for different beef quality traits, namely
582 tenderness. The reader would refer to recent reviews in the field.^{9,42}

583 The other similarities found in this study are the positive relation of H2AFX with h^* for
584 both Grass and Hay classes. This study is the first to found this relation. It was further
585 involved negatively in beef flavor and ultimate pH and positively with b^* for grass class.
586 H2AFX is an histone involved in the cellular response to DNA damage, notably during

587 oxidative stress, which may explain its relationships with these traits where oxidation would
588 play an important role. This protein was proposed by our group using *in silico* to be related
589 with beef tenderness.¹⁶

590 From the other antioxidant proteins reported, Prdx6 was positive with C^* for both Grass
591 and Haylage classes. It was also negatively and positively related with h^* and a^* in the same
592 classes, respectively. These findings are in line with our recent investigations on beef
593 color^{10,17} and those of other laboratories.⁴⁸ Prdx6 is expressed in nearly all tissues including
594 skeletal muscle, and protects cells against oxidative stress. In addition, it was positively
595 related and in agreement to Guillemain *et al.*¹⁶ with both tenderness and juiciness in Haylage
596 class. SOD1, a member of antioxidant enzymes, entered in both Hay and Grass classes. It was
597 negative in the regression equations of juiciness and beef flavor for Hay class, and positive
598 with WBSF and a^* for Grass class. SOD1 is an antioxidant, which allows a fast dismutation
599 of O^{2-} to O_2 and H_2O_2 . Accordingly, Grabež *et al.*⁴⁹ reported great abundance of SOD1 in
600 tough meat. The authors suggest that stronger oxidative stress may cause ROS accumulation
601 in tough meat and reduce cell protection.

602 Structural proteins were also retained in several regression equations, but were specific for
603 each quality trait and the direction of their entrance is not always the same. The first example
604 concern α -actin negatively and positively related with WBSF of Haylage and Grass classes,
605 respectively. Meat tenderness is achieved primarily through ageing, during which structural
606 changes occur in the muscle owing to complex mechanisms involving pH and ionic strength,
607 combined with the action of cellular proteolytic systems.⁸ In this study, both μ -calpain and m-
608 calpain, two proteolytic systems, were related with meat quality traits, but rearing practice and
609 trait-dependent. For example, m-calpain was retained negatively and positively in L^*
610 equations for Haylage and Hay classes, respectively. Furthermore, m-calpain was negatively
611 related with WBSF and positively with sensory tenderness for Hay class. For further
612 interpretation of the involvement of both proteolytic and structure proteins and their influence
613 on meat quality, the reader would refer to the comprehensive reviews published recently.^{8,50}

614 Overall, taking all of the results together, this study allowed us to explore the protein
615 biomarkers relationships with Grass, Hay and Haylage rearing practices of PDO Maine-Anjou
616 beef. This study confirms that many production traits or production-related traits in livestock
617 are the result of sophisticated biological processes finely orchestrated during the life of the
618 animal and soon after slaughter. These results may serve as a first guideline of the
619 relationships between muscle proteome with carcass and beef quality traits according to the

620 rearing practices. Furthermore, these protein biomarkers related to the carcass traits offer
621 scientific community a starting point from which to study the complex protein-networks
622 managing muscle to meat conversion that underlie economically important phenotype traits.
623 In this study, direct relationships using a consistent number of animals and biomarkers in
624 comparison to proteomic studies investigating generally little animals allowed confirming the
625 earlier statements and reveals for the first time direct relationships with carcass traits.
626 Furthermore, the most original in this integrative approach is that we have associated for the
627 first time myriad proteins belonging to several biological pathways in relation to carcass
628 qualities that were not often considered in the same study. The relationships were also with
629 eleven meat quality traits grouping texture, flavor, pH and color, according to the rearing
630 practices of the animals.

Notes

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Supporting Information

Table S1. Described the multiple regression analyses of carcass characteristics and meat quality traits for the 110 m cows as one dataset using protein biomarkers. The Supporting Information is available free of charge on the ACS Publications website.

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Figure captions

Figure 1. Principal component analysis showing both the loading and score plots of the variables used to discriminate between the 110 cows. The rearing practice classes 1 – 3 (ellipses of the insert at the upper left corner of PCA) were highlighted in blue, green and red respectively. The variables in bold character are significantly different ($P < 0.05$) between classes and those in italic are not different as depicted in **Table 1**.

Figure 2. A summary of the entrance frequency of the protein biomarkers in the regression equations of **A)** carcass characteristics and **B)** meat quality traits within rearing practice classes from **Tables 5 – 7**. The regression equations of meat quality traits (**B**) were built by including **C)** carcass characteristics as explanatory variables (FCW: Fat Carcass weight; LMW: *Longissimus* muscle weight; MCW: muscle carcass weight; HCW: hot carcass weight; EUROP CS: EUROP carcass score and RW: ribeye weight). The relevance of the proteins in the traits of each rearing practice are presented by a red gradient color from none (0) with white to the high (4) in intense red.

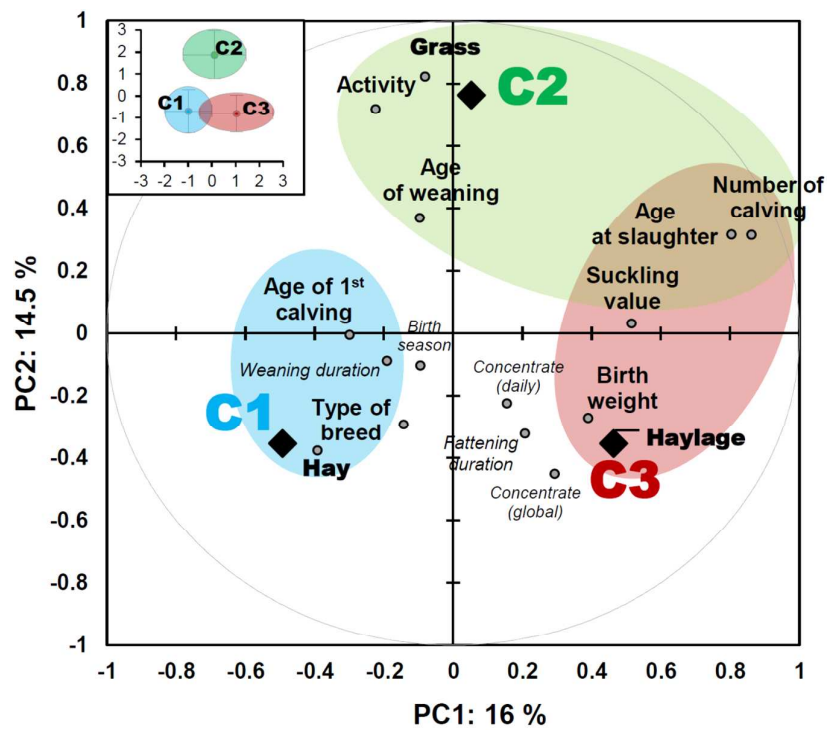


Figure 1.

**Carcass quality traits regressed
with protein biomarkers only**

A)	« Hay »	« Grass »	« Haylage »	Total
ICDH	1	1	1	3
TP53	1	1	1	3
SOD1	1	0	1	2
Hsp70-1A	2	0	2	4
MyLC-1F	2	0	2	4
αB-crystallin	3	0	0	3
m-calpain	2	0	0	2
LDH	2	0	0	2
MyHC-IIa (%)	1	0	0	1
Hsp70-1B	1	1	0	1
MyBP-H	4	3	0	7
H2AFX	2	3	0	5
Hsp20	1	1	0	2
Prdx6	1	1	0	2
MyHC-IIx (AU)	0	4	1	5
Hsp40	0	3	2	5
α-actin	0	1	1	2
PGM1	0	1	3	4
ENO3	0	2	1	3
Hsp70-8	0	1	0	1
Fiber area	0	1	0	1
DJ1	0	0	1	1
MyHC-I (%)	0	0	4	4
Hsp27	0	0	3	3
MyHC-IIx (%)	0	0	1	1



Variable entrance

**Meat quality traits regressed
with protein biomarkers (B) and
carcass characteristics (C)**

B)	« Hay »	« Grass »	« Haylage »	Total
MyHC-IIx (AU)	1	3	4	8
PGM1	3	2	2	7
Hsp40	3	1	2	6
ICDH	2	2	2	6
Hsp70-Grp75	1	2	1	4
m-calpain	4	0	2	6
αB-crystallin	3	0	1	4
Fiber area	3	0	1	4
TP53	2	0	2	4
MyBP-H	1	0	2	3
ENO3	1	0	1	2
H2AFX	1	4	0	5
SOD1	2	2	0	4
MyIIC-IIx (%)	3	2	0	5
Hsp70-8	3	2	0	5
Hsp27	1	3	0	4
Hsp20	1	1	0	2
Prdx6	0	2	4	6
MyLC-1F	0	2	3	5
MyHC-IIa (%)	0	1	3	4
α-actin	0	2	2	4
μ-calpain	0	3	1	4
HSP70-1A	0	0	1	1
DJ1	0	0	1	1
MyHC-I (%)	0	2	0	2
LDH	0	1	0	1

C)	« Hay »	« Grass »	« Haylage »	Total
FCW	3	1	4	8
LMW	2	1	2	5
MCW	2	0	3	3
HCW	2	1	0	1
EUROP CS	0	1	0	3
RW	0	3	0	5

Figure 2.

Table 1. Comparison of the three rearing practices identified according to variables related to animal life and fattening period.

Variables	Class 1 ^a “Hay” (n = 44)	Class 2 “Grass” (n = 30)	Class 3 “Haylage” (n = 36)	SEM ^b	p-value ^c
Birth weight (kg)	49.3 ^b	48.3 ^b	52.2 ^a	0.49	**
Age of weaning (month)	6.9 ^b	7.6 ^a	7.2 ^{a,b}	0.10	*
Weaning duration (week)	7.7	9.8	9.1	0.90	ns
Age at first calving (month)	33.5 ^a	32.1 ^{a,b}	31.3 ^b	0.39	*
Number of calving	2.1 ^b	3.7 ^a	3.4 ^a	0.20	***
Suckling score (/10)	5.6 ^b	6.4 ^a	5.9 ^{a,b}	0.13	*
Fattening duration (day)	98.9	91.8	104	2.87	ns
Haylage diet (%)	3.6 ^b	6.1 ^b	75.4 ^a	3.54	***
Hay diet (%)	84.6 ^a	28.8 ^b	19.7 ^b	3.58	***
Grass diet (%)	11.8 ^b	65.0 ^a	4.9 ^b	3.08	***
Daily concentrate diet (kg)	7.8	7.6	7.7	0.20	ns
Global concentrate diet (kg)	760	683	757	23.37	ns
Activity (%)	49.1 ^b	93.9 ^a	26.7 ^c	4.43	***
Age at slaughter (month)	57.9 ^b	76.1 ^a	72.2 ^a	2.37	**

^a Least square means in the same row for class effect not followed by a common letter (a-c) differ significantly: $P < 0.05$.

^b Standard error of mean

^c Significances : ns: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Table 2. Comparison of the three rearing practices identified according to variables related to carcass characteristics.

Variables	Class 1 ^a “Hay” (n = 44)	Class 2 “Grass” (n = 30)	Class 3 “Haylage” (n = 36)	SEM ^b	p-value ^c
Hot carcass weight (HCW, kg)	428 ^b	459 ^a	435 ^b	3.46	***
EUROP conformation score (EUROP CS)	4.8 ^{a,b}	5.1 ^a	4.5 ^b	0.08	*
Ribeye weight (g) (RW)	2956 ^b	3333 ^a	2903 ^b	61.10	**
<i>Longissimus</i> muscle weight (LMW)	370 ^b	407 ^a	345 ^c	7.57	**
Muscle carcass weight (MCW)	1811 ^b	2026 ^a	1773 ^b	38.60	*
Fat carcass weight (FCW)	551 ^b	650 ^a	553 ^b	18.23	*
Fat to muscle ratio (% w/w)	30.9	32.8	31.2	0.97	ns

^a Least square means in the same row for class effect not followed by a common letter (a-c) differ significantly: $P < 0.05$.

^b Standard error of mean

^c Significances : ns: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Table 3. Comparison of the three rearing practices identified according to variables related to meat quality traits.

Variables	Class 1 ^a “Hay” (n = 44)	Class 2 “Grass” (n = 30)	Class 3 “Haylage” (n = 36)	SEM ^b	p-value ^c
Ultimate pH	5.59	5.60	5.60	0.01	ns
Lightness (<i>L</i> *)	39.5	39.9	39.6	0.22	ns
Redness (<i>a</i> *)	8.65 ^b	9.27 ^a	8.67 ^b	0.12	*
Yellowness (<i>b</i> *)	7.00 ^b	8.31 ^a	7.24 ^b	0.14	***
Chroma (<i>C</i> *)	11.2 ^b	12.5 ^a	11.3 ^b	0.15	**
Hue angle (<i>h</i> *)	38.9 ^b	41.6 ^a	39.8 ^b	0.47	*
Tenderness (0 – 10 scale)	4.5	4.6	4.4	0.05	ns
Juiciness (0 – 10 scale)	3.3	3.2	3.3	0.04	ns
Beef flavor (0 – 10 scale)	4.7	4.7	4.7	0.03	ns
Warner-Bratzler shear force (N/cm ²)	44.6	42.8	45.8	1.07	ns
Intramuscular fat content (% w/w)	15.7	17.6	16.4	0.59	ns

^a Least square means in the same row for class effect not followed by a common letter (a-c) differ significantly: $P < 0.05$.

^b Standard error of mean

^c Significances : ns: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Table 4. Comparison of the three rearing practices identified according to variables related to muscle characteristics.

Variables	Class 1 ^a “Hay” (n = 44)	Class 2 “Grass” (n = 30)	Class 3 “Haylage” (n = 36)	SEM ^b	p-value ^c
<i>Contractile properties by myosin fiber characterization</i>					
Fiber area, μm^2	2885	2940	2894	61.8	ns
MyHC-I (%)	30.5	31.9	31.4	0.71	ns
MyHC-IIa (%)	55.0 ^b	61.2 ^a	54.8 ^b	1.22	*
MyHC-IIx (%)	14.5 ^a	6.9 ^b	13.8 ^a	1.34	*
<i>Metabolic properties by metabolic enzyme activities</i>					
LDH ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$)	713	693	708	10.4	ns
ICDH ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$)	1.02	1.07	1.03	0.03	ns
<i>Protein biomarkers quantified Dot-Blot (in arbitrary units)</i>					
<i>Heat shock proteins</i>					
αB -crystallin	207.5 ^b	252.5 ^a	217.3 ^b	8.04	*
Hsp20	159.3	159.8	176.6	4.35	ns
Hsp27	75.7	81.8	79.6	1.90	ns
Hsp40	129.9	126.5	135.1	2.01	ns
Hsp70-1A	120.1	115.4	124.7	2.51	ns
Hsp70-1B	192.4	174.4	187.9	4.73	ns
Hsp70-8	112.7	113.7	109.7	2.38	ns
Hsp70-Grp75	143.6	145.1	146.2	2.92	ns
<i>Metabolism</i>					
Enolase 3 (ENO3)	141.8	145.8	146.7	3.47	ns
Phosphoglucosmutase 1 (PGM1)	106.5	94.3	102.5	2.61	ns
<i>Structure</i>					
α -actin	129.4	120.1	119.6	3.87	ns
Myosin binding protein H (MyBP-H)	125.9	122.9	127.7	1.78	ns
Myosin light chain 1F (MyLC-1F)	60.7	61.3	62.2	1.24	ns
Myosin heavy chain IIx (MyHC-IIx)	99.1 ^a	78.2 ^b	92.3 ^a	2.63	*
<i>Oxidative stress</i>					
Superoxide dismutase [Cu-Zn] (SOD1)	94.1 ^b	118.5 ^a	96.4 ^b	3.63	*
Peroxiredoxin 6 (PRDX6)	105.8	102.4	111.2	1.67	ns
Protein deglycase (DJI)	93.4	87.7	91.5	1.33	ns
<i>Proteolysis</i>					
μ -calpain	145.4	155.9	157.5	3.66	ns
m-calpain	96.3	94.0	98.7	1.21	ns
<i>Apoptosis and signaling</i>					
Tumor protein p53 (TP53)	114.8	119.5	122.1	1.82	ns
H2A Histone Family Member X (H2AFX)	99.8	97.1	99.2	2.14	ns

^a Least square means in the same row for class effect not followed by a common letter (a-c) differ significantly: $P < 0.05$.

^b Standard error of mean

^c Significances : ns: not significant; *: $P < 0.05$

Table 5. Multiple regression analyses of carcass characteristics and meat quality traits for Hay class using protein biomarkers and carcass characteristics.

#	Variables	Carcass quality parameters						Meat quality parameters										
		HCW	EUROP CS	RW	LMW	MCW	FCW	WBSF	GT	JUIC	BF	IMF	pHu	L*	a*	b*	C*	h*
	R-squared	0.31**	0.41***	0.29**	0.33**	0.24*	0.40***	0.50***	0.46***	0.40***	0.22*	0.42***	0.52***	0.29*	0.26*	0.30*	0.20*	0.40***
6	α B-crystallin			-0.48**		-0.50*	-0.51***			-0.43*				-0.41*		-0.29*		
6	m-calpain	0.52**			0.61***			-0.47**	0.35*					0.50**			0.35*	
5	MyBP-H		-0.53***	-0.41*	-0.59***	-0.43*		0.37*										
3	SOD1						0.20*			-0.43**	-0.25*							
3	ICDH		-0.40**					0.34**					0.47***					
3	H2AFX			-0.31*			-0.46**											0.61**
3	TP53	-0.48*							0.57***			0.34*						
3	PGM1									-0.35*					-0.33*			0.35*
3	Fiber area							0.42**			-0.25*				-0.33*			
3	MyHC-IIx (%)											-0.24*		0.41*				-0.34*
3	Hsp40									0.75***								-0.37*
3	Hsp70-8								-0.32*		0.48*							-0.51**
2	Hsp20					-0.35*								0.35*				-0.45*
2	Hsp70-1A		-0.29*				0.25*											
2	LDH	0.38*			0.40*													
2	MyLC-1F			0.30*		0.34*												
1	Hsp27												-0.36**					
1	MyHC-IIx (AU)										-0.29*							
1	ENO3															0.54**		
1	Hsp70-Grp75																	0.59*
1	MyHC-IIa (%)	0.39*																
1	Hsp70-1B		0.69***															
1	Prdx6				-0.30*													
3	FCW								0.36**		0.18*	0.36**						
2	LMW												-0.28*					-0.57*
2	MCW													-0.22*				0.50*
2	HCW									0.30*					0.33*			

#: frequency of the entrance of the variables in the regression equations.

Abbreviations: **WBSF:** Warner-Bratzler Shear Force; **GT:** global tenderness; **JUIC:** juiciness; **BF:** beef flavor; **IMF:** intramuscular fat; **L*:** lightness; **a*:** redness; **b*:** yellowness; **C*:** Chroma; **h*:** hue angle; **HCW:** hot carcass weight; **RW:** ribeye weight; **LMW:** *Longissimus* muscle weight; **MCW:** muscle carcass weight; **FCW:** fat carcass weight.

Significances: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 6. Multiple regression analyses of carcass characteristics and meat quality traits for Grass class using protein biomarkers and carcass characteristics.

#	Variables	<i>Carcass quality parameters</i>						<i>Meat quality parameters</i>										
		HCW	EUROP CS	RW	LMW	MCW	FCW	WBSF	GT	JUIC	BF	IMF	pHu	L*	a*	b*	C*	h*
	R-squared	0.49**	0.48**	0.52***	0.62***	0.59***	0.58***	0.50**	0.53***	0.56***	0.57***	0.80***	0.44**	0.42**	0.55***	0.43**	0.45**	0.52***
7	H2AFX		0.73***		0.40**		-0.43*						-0.49*			0.55**		0.67***
7	MyHC-IIx (AU)			-0.68***	-0.38*	-0.72***	-0.36*			-0.71**						0.64*	0.95**	
4	Hsp40			0.28*		0.31*	0.45**									-0.43*		
3	α -actin	0.83**						0.40*	0.27*									
3	Prdx6	-0.46*															0.89**	-0.37*
3	Hsp70-8					0.46*				0.76**					0.46**			
3	ICDH				0.53***			0.50**			-0.41*							
3	PGM1	-0.56**													-0.47**			0.49**
3	MyBP-H			0.47**	0.47**		0.63**											
3	μ -calpain												-0.60*	0.66**			0.28*	
3	Hsp27							0.45*							-0.36*	-0.71**		-0.35*
2	Hsp20		-0.30*															
2	MyLC-1F										-0.61***	-0.16*						
2	Hsp70-Grp75												0.98***					
2	SOD1							0.47**		-0.58**						-0.31*		
2	MyHC-IIx (%)													-0.49*				-0.36*
2	MyHC-I (%)								0.51**				0.39*					
2	ENO3			0.35*		0.33*												
1	Fiber area		0.24*															
1	HSP70-1B		0.57**															
1	TP53	0.87***																
1	LDH																	
1	MyHC-IIa (%)									-0.87***				-0.28*				
3	RW								0.36*			-1.23***		-0.49**				
1	EUROP CS								-0.44**									
1	HCW										0.48**							
1	LMW											0.45**						
1	FCW											1.34***						

#: frequency of the entrance of the variables in the regression equations.

Abbreviations: **WBSF:** Warner-Bratzler Shear Force; **GT:** global tenderness; **JUIC:** juiciness; **BF:** beef flavor; **IMF:** intramuscular fat; **L*:** lightness; **a*:** redness; **b*:** yellowness; **C*:** Chroma; **h*:** hue angle; **HCW:** hot carcass weight; **RW:** ribeye weight; **LMW:** *Longissimus* muscle weight; **MCW:** muscle carcass weight; **FCW:** fat carcass weight.

Significances: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 7. Multiple regression analyses of carcass characteristics and meat quality traits for Haylage class using protein biomarkers and carcass characteristics.

#	Variables	Carcass quality parameters						Meat quality parameters										
		HCW	EUROP CS	RW	LMW	MCW	FCW	WBSF	GT	JUIC	BF	IMF	pHu	L*	a*	b*	C*	h*
	R-squared	0.30*	0.34**	0.38**	0.43**	0.37**	0.44**	0.33*	0.43**	0.44**	0.56***	0.69***	0.35**	0.46***	0.40**	0.53***	0.61***	0.35**
5	MyHC-IIx (AU)		0.36*								-0.23*			0.97***	0.81***	0.72***		
5	MyLC-1F	0.31*			-0.35*			-0.36*							0.26*	0.36**		
5	PGM1	0.36*		0.35*		0.40*					-0.58**							0.47*
4	Hsp40			-0.40*		-0.41*			0.44*					-0.46**				
4	PRDX6								0.44*	0.43**				0.34*		0.28*		
4	MyHC-I (%)	0.53*		0.37*	0.31*		0.50**											
3	α -actin	-0.54**						-0.34*		-0.53**								
3	TP53				-0.62***					-0.63***					0.32*			
3	HSP70-1A		-0.35*				0.45**						-0.30*					
3	ICDH		0.53**										0.47**	-0.36*				
3	Hsp27		-0.38*	0.55**		0.42**												
3	MyHC-IIa (%)													-0.49*	-0.78**	-0.80***		
2	ENO3				0.50**				-0.59**									
2	DJ1						-0.36*		-0.77***									
2	MyBP-H										0.31*							-0.44*
2	m-calpain										-0.33*			-0.31*				
1	SOD1						-0.29*											
1	MyHC-IIx (%)					-0.34*												
1	Hsp70-Grp75												0.47**					
1	μ -calpain																	
1	α B-crystallin																	
1	Fiber area																	-0.20*
4	FCW											0.45***	-0.25*	0.40**				0.45**
3	MCW							0.48*		0.43**			0.37*					
2	LMW							-0.45*										

#: frequency of the entrance of the variables in the regression equations.

Abbreviations: **WBSF:** Warner-Bratzler Shear Force; **GT:** global tenderness; **JUIC:** juiciness; **BF:** beef flavor; **IMF:** intramuscular fat; **L*:** lightness; **a*:** redness; **b*:** yellowness; **C*:** Chroma; **h*:** hue angle; **HCW:** hot carcass weight; **RW:** ribeye weight; **LMW:** *Longissimus* muscle weight; **MCW:** muscle carcass weight; **FCW:** fat carcass weight. Significances: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

TOC

