

Research Article

Biomarker Abundance in Two Beef Muscles Depending on Animal Breeding Practices and Carcass Characteristics

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Abstract

This work sets out a methodological approach to assess the relationships that exist between four sets of data: breeding practices, carcass characteristics, m. *Longissimus thoracis* biomarkers and m. *Semitendinosus* biomarkers. Seventy-one young bulls of 3 breeds were characterized by 78 variables.

The variables in each set were arranged into 4, 3, 5 and 3 homogeneous clusters respectively using the clust of Var approach. A second clustering of variables established from these 15 clusters was then used to build 5 pools of clusters that were precisely described to characterize the interaction between the 4 sets of variables. Some practices have a universal impact on biomarker patterns eg. increasing straw proportion in the diet leads to an increase in MyLC-1F and Hsp40 biomarkers whatever the breed and the muscle. Nevertheless, most breeding practices appear to have varying impacts on biomarkers depending on animal type and muscle. For example, ST biomarkers are less related to breeding practices and/or carcass characteristics than in LT. Moreover, some biomarker expressions appear to be breed-dependant, especially for the Angus breed which is very specific in comparison to continental breeds. The breed where muscle biomarkers are the most closely linked to breeding practices and carcass characteristics is the Blonde d' Aquitaine. In this breed, MDH1 and DJ-1 biomarkers are positively linked to muscle yield and carcass development in both muscles and some other LT biomarkers might also be considered as good indicators of carcass fatness (small Hsp, ENO3, Capz- β , MyBP-H).

Several of the tested biomarkers have been identified as being relevant for carcass quality prediction suggesting that biomarker abundance could represent an interesting possibility for the early management of carcass and meat quality.

ABBREVIATIONS

LT: Longissimus Thoracis; ST: Semitendinosus

INTRODUCTION

Farm management systems, especially during the finishing period, are known to impact final carcass and meat quality traits. In order to predict the most important meat quality traits as early as possible, it is therefore useful to reveal the gene networks involved in the development of the desired quality phenotype. The modern beef industry currently lacks fast, objective and noninvasive tools to estimate and/or predict the beef quality of live animals or online in abattoirs, paying specific attention on sensory traits. The identification of relevant genetic and genomic

markers that could permit the detection of live animals' potential desired quality traits would be helpful for beef producers to help them select the most appropriate production systems, animal types, and markets.

Gene expression controls the biological characteristics of muscles and thanks to advances in genomics, researchers have identified a number of genes that are associated with different meat qualities [1-4]. Over the last few decades, the implementation of genomic research programs has been very important in revealing the underlying biological mechanisms behind several meat quality traits [5]. These programs concern DNA level, RNA level and also protein expression. As an emerging method, proteomics has been applied to characterize the

physiology behind animal growth and development, reproduction, welfare and beef products and to identify potential meat quality markers [6,7]. These markers, which may be over- or under-expressed depending on meat quality grade, are involved in many different biological mechanisms, such as energy metabolism, muscle structure, stress and oxidative stress reactions, cell cycle, proteolysis and apoptosis [7, 8].

The aim of the present report is to study the link between breeding practices and/or beef carcass characteristics with the abundances of a series of muscle biomarkers. In an attempt to establish, if possible, universal links and / or predictive equations, two muscles (differing in their metabolic and contractile characteristics) were studied in young bulls from three continental breeds differing in terms of their earliness and physiological characteristics.

MATERIALS AND METHODS

Animals, Management, Slaughtering and Sampling

The method was developed using data from the EU FP6 Integrated Project Pro Safe Beef (FOODCT-2006-36241) under the INRA reference AQ284. This study used 71 young bulls of three pure breeds: Aberdeen Angus ($n_A=22$), Blond d'Aquitaine ($n_{BA}=24$) and Limousin ($n_{Lim}=25$).

The French Blond d'Aquitaine breed is highly muscled with low intramuscular fat content ; the Aberdeen Angus breed is known to be precocious and thus fat with a marbled meat; the Limousin meat is intermediate between the other two breeds [9].

Animals (12 months-old at the beginning of the experiment) were assigned during the finishing period (105 days before slaughter) to a feeding regime given *ad libitum* based on straw (25%) and concentrate (75%) supplemented with antioxidant or not. They were housed in groups of four animals of the same breed in 6 × 6 m pens with straw bedding. Individual daily feed intake and body weight were recorded every 2 weeks. They were slaughtered at about 17 months of age at a live weight of around 665 kg.

The study was carried out in compliance with current French welfare recommendations for the use of experimental animals.

All animals were slaughtered under standard conditions in the experimental abattoir of the INRA Research Centre, after 24 hours of food deprivation. Slaughter was carried out in compliance with French welfare regulations. The carcasses were not electrically stimulated and they were chilled and stored at 4°C up to 24 h *post-mortem* (*p-m*). Final pH was recorded between the 6th and 7th rib using a pH meter equipped with a glass electrode at 24h *p-m*.

Animal measurements and carcass characteristics

Age, live weight and empty body weight were recorded before slaughter. Average daily gain (ADG), feed conversion efficiency (ADG / dry matter) and feed conversion efficiency based on net energy intake (ADG / net energy intake) were calculated using initial live weight and feed consumption (expressed as dry matter or energy intake) during the finishing period. Carcass weight was determined at slaughter. The 6th rib joint was

removed and dissected in order to estimate the composition of the carcass (content and proportions) based on the regression equations previously established [10]. Carcass and meat yield were estimated using the determined weights. The carcass was dissected in order to weigh the various organs and tissues (leather weight; head weight; digestive and urinary tract content; shinbone weights). Measurements were also recorded (total carcass length; thigh thickness; length shank-symphysis; sirloin thickness, kidney length) yielding measurement ratios (sirloin thickness / kidney length; thigh thickness / total length; thigh thickness / length shank-symphysis). In this way, weights, measures and ratios yielded 36 variables characterizing animal performances (Table 1).

Meat quality evaluation

Muscle samples from *m. longissimus thoracis* (LT, mixed fast oxido-glycolytic muscle: 23% of type I fibers, 36% IIA and 39% IIX) and *m. semitendinosus* (ST, mixed fast glycolytic muscle: 8% of type I fibers, 24% of IIA and 64% of IIX) [9, 10] were excised from the carcass of each animal within 15 min *p-m*.

The 21 protein biomarkers analyzed for LT and ST muscle are given in Table (1). The conditions for use and specificity of primary antibodies against these 21 proteins in bovine muscle were previously determined by [11] using Western blotting.

Total protein extractions were performed according to the method of (Bouley, Chambon and Picard 2004) in a denaturation extraction buffer containing 8.3 M urea, 2 M thiourea, 1% DTT and 2% CHAPS. The protein concentration was determined using the Bradford protein assay [12]. The protein extractions were stored at -20 °C until use.

Relative abundances of the 21 protein biomarkers were evaluated following the Dot-Blot technique as described by Gagaua *et al.* [13]. Briefly, protein samples were spotted in quadruplicate on a nitrocellulose membrane with the Minifold I dot blot from Schleicher & Schuell Biosciences (Germany) and hybridized with the specific antibody of each protein in the conditions recently reported by [9]. Secondary fluorescent conjugated IR Dye 800CW antibodies (anti-mouse) were supplied by LI-COR Biosciences (Lincoln, NE, USA) and used at 1/20000. Subsequently, membranes were scanned by an Odyssey (LI-COR Biosciences) scanner at 800 nm. The relative abundance of protein for each sample, given in arbitrary units, was standardized by comparison to a reference sample constituted by mixing all samples from the three young bulls in equal proportions.

Statistical Analysis

For each breed, the statistical analysis was organised in 3 steps as shown following the method recently reported by our group [14] and highlighted in Figure (1).

For each set of variables (breeding practices / animal measurements and carcass performances / LT biomarkers /ST biomarkers), a hierarchical clustering of variables was applied to assess the links between the different variables making up the set. To do this, we used a combination of two dimension reduction approaches (implemented in R software): i) the *Clust of Var* approach, developed to arrange variables into homogeneous

Table 1: Variables of each of the four sets of variables investigated in this study.

Breeding practices (BP)	Carcass characteristics (CC)	<i>M. Longissimus thoracis</i> biomarkers (LTB)	<i>M. Semitendinosus</i> biomarkers (STB)
Breed Age (day) Straw intake (kg DM) Concentrate intake (kg DM) Dry matter Intake (kg) % straw % concentrate Energy level of the ration (expressed in UF) Protein level of the ration (expressed in g of PDIN and g of PDIE) Initial live weight (kg) Slaughter live weight (kg) Average daily gain (g / day) Feed efficiency (expressed per kg of DMI) Feed efficiency (expressed per UF)	Carcass yield (% empty body weight) Muscle yield (% empty body weight) Trim fat weight (kg) Shin bone weigh (SBW; kg) Leather weight (kg) Liver weight (kg) Head weight (HW; kg) Empty body weight (EBW; kg) Cold carcass weight (CCW; kg) 5th quarter fat (5QF; kg) Carcass fat tissues (CFT; kg) Carcass fat tissues (CFT ; % of carcass fat) Muscles (kg) Muscles (% of carcass weight) Bone (kg) Bone (% of carcass weight) Total fat tissues (TFT; kg) Total fat tissues (TFT ; % empty body weight)	Heat shock protein αB-crystallin Hsp20 HSp27 Hsp40 Hsp70-1B Hsp70-8 Hsp70-Grp75 Metabolism ENO3 (enolase 3) LDH-B (lactate dehydrogenase chain B) MDH1 (malate dehydrogenase 1) Structure CapZ-β (F-actin-capping protein subunit β) α-actin MyLC-1F (myosin light chain 1F) MyBP-H (myosin binding protein H) MyHC-I (myosin heavy chain-I) MyHC-II (myosin heavy chain-II) MyHC-IIx (myosin heavy chain-IIx) Oxidative resistance DJ-1 (Parkinson disease protein 7) PRDX6 (cis-peroxiredoxin) SOD1 (superoxide dismutase Cu/ Zn) Proteolysis μ-calpain	Heat shock protein αB-crystallin Hsp20 HSp27 Hsp40 Hsp70-1B Hsp70-8 Hsp70-Grp75 Metabolism ENO3 (enolase 3) LDH-B (lactate dehydrogenase chain B) MDH1 (malate dehydrogenase 1) Structure CapZ-β (F-actin-capping protein subunit β) α-actin MyLC-1F (myosin light chain 1F) MyBP-H (myosin binding protein H) MyHC-I (myosin heavy chain-I) MyHC-II (myosin heavy chain-II) MyHC-IIx (myosin heavy chain-IIx) Oxidative resistance DJ-1 (Parkinson disease protein 7) PRDX6 (cis-peroxiredoxin) SOD1 (superoxide dismutase Cu/ Zn) Proteolysis μ-calpain

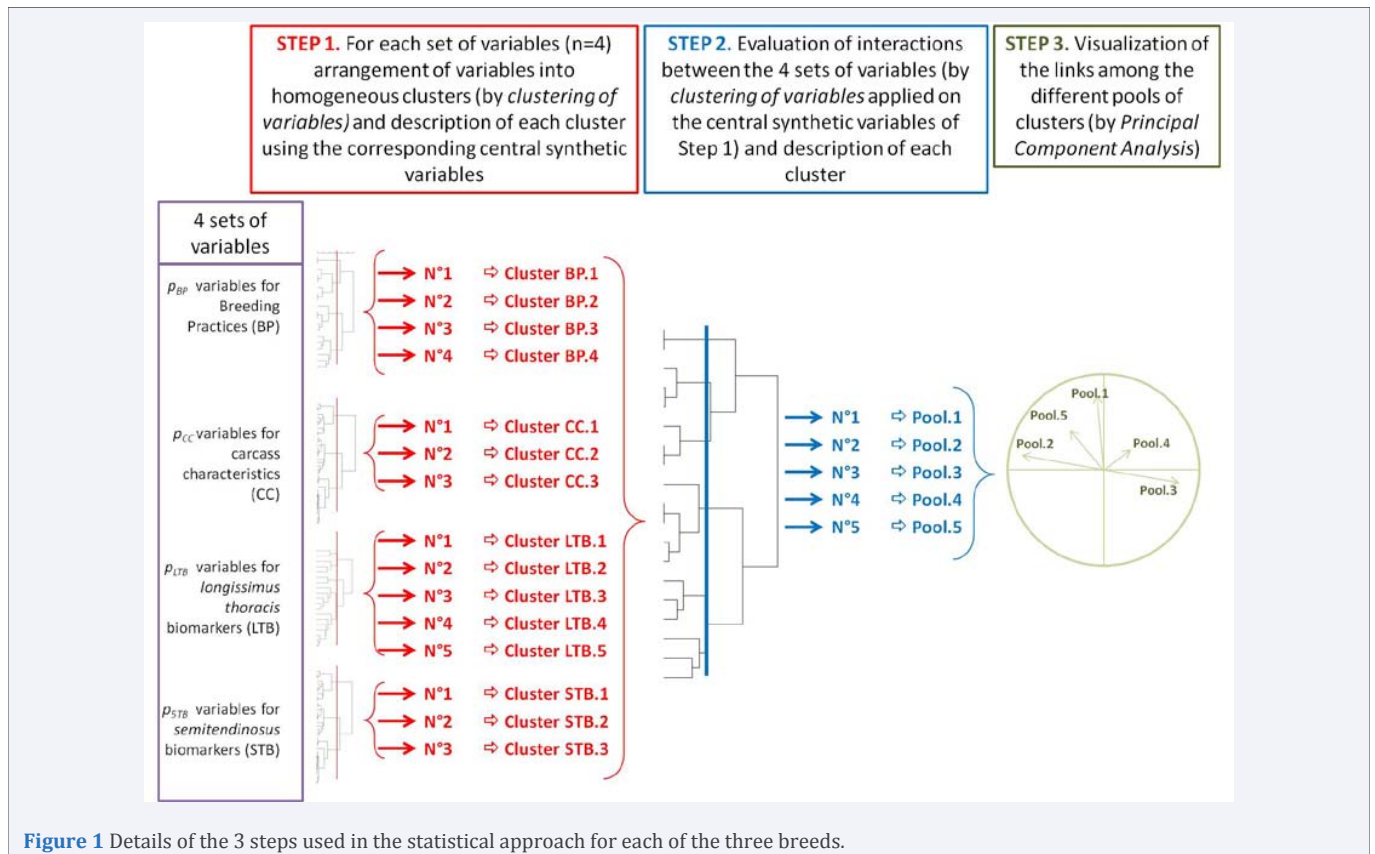


Figure 1 Details of the 3 steps used in the statistical approach for each of the three breeds.

clusters and to allow dimension reduction and variable selection, and ii) principal component analysis (PCA). Neither pre-treatment, nor normalisation techniques was applied before PCA. However, the methodology of the PCA implies to center and reduce the variables that are characterised by different units and dispersion profiles. A bootstrap approach allowed to evaluate the stability of the partitions of variables and to determine the appropriate number of clusters. Moreover, this method is useful to identify the links between the variables and to highlight redundancy in a data set. Thus, for each set of data, we obtained different clusters.

Step 1: For each cluster, a central quantitative synthetic variable, defined as the first principal component of PCA applied to all the variables within the cluster, was calculated. To characterize each cluster, only the variables having a square correlation with the central quantitative synthetic variables greater than 0.50 were taken into consideration. Nevertheless, all variables were taken into account in the following steps of the analysis. To visualize and evaluate the correlation between the different clusters established for each set of variables, a PCA and different correlation tests were carried out (data not shown).

Step 2: To establish the links between breeding practices / animal measurements and carcass performances / LT biomarkers / ST biomarkers, a *Clust of Var* PCA was carried out on the different clusters taken together (all of the clusters obtained in Step 1, corresponding to the four data sets). Different pools of clusters were created according to the first component of the PCA applied to the clusters and characterized, using the variables having a square correlation with the central quantitative synthetic variables greater than 0.50.

Step 3: To visualize and evaluate the correlation between the four pools of clusters, a PCA was performed.

RESULTS

A preliminary analysis was carried out on only 2 sets of variables (LT biomarkers and ST biomarkers) in order to evaluate the relationships between the list of protein biomarkers from both muscles (see supplementary material). This allowed us to establish that different biological mechanisms may underlie protein expression depending on the muscle. Moreover, although some generic results can be obtained by taking all breeds together, it appears more pertinent to establish the links between breeding practices, carcass performances and muscles biomarkers breed by breed as biomarker abundances appear to be breed-specific. These results confirm those reported by our group [9,15,16], indicating a muscle-dependent and a breed-dependent evolution of meat quality biomarkers hence the decision to study the relations between the 4 set of variables breed per breed.

Step 1: arrangement of variables in each set of variables into homogeneous clusters and description of each cluster

As indicated above, each cluster of variables is characterized by variables highly correlated to a central quantitative synthetic variable. Using variable clustering thus aims to maximize the homogeneity within a cluster. For each breed ($n = 3$) and each set of variables (number of set of variable = 4; breeding practices BP, carcass characteristics CC, LT biomarkers (LTB), ST biomarkers (STB)), variable clusters were constituted (Table 2).

As regards breeding practices, the clusters were quite similar and more or less coherent from breed to breed i.e. a higher concentrate intake in the feeding ration was positively linked with higher energy (UF) and protein (PDIN – PDIE) intake (A-BP-3, L-BP-2, BA-BP-2); the proportions of concentrates and straw in the feeding ration was negatively correlated (A-BP-1, L-BP-1, BA-BP-1).

As regards carcass characteristics, variable associations were also found to be relatively stable from one breed to another and also consistent with the expectations: adipose tissues (TFT, CFT, 5QF, Trim Fat) expressed in amount and in proportion being opposed to muscle and bone development (A-CC-1, L-CC-1, BA-CC-1). The increase in muscle and bone weight are in line with the increase in global animal development (EBW, CCW, SBW, HW; A-CC-2, L-CC-2, BA-CC-2). In continental breeds, muscle and bone development is also associated with carcass (Limousin) or muscle yields (Blond d'Aquitaine). On the other hand, in the Angus breed, these yields appear to be independent.

In the Longissimus thoracis muscle, some biomarkers are similarly associated in both continental breeds: Hsp70-1, Hsp70-8, μ -calpain, DJ-1 and MDH1 for example. The other clusters were not stable among breeds, confirming the conclusions established in the first phase and justifying a study breed per breed.

In the Semitendinosus muscle, only one pair of biomarkers is associated in the same manner in the two continental breeds: Hsp20 and Hsp70-Grp75. However, great similarity is noted when the Angus and Blond d' Aquitaine breeds are considered together, with two identical associations: on the one hand Hsp70-Grp75 and μ -calpain and on the other hand MyHC-II, MyBP-H and MDH1.

Step 2: evaluation breed per breed of the interaction between the 4 sets of clusters, and constitution of 4 to 5 pools of clusters per breed

i) Links between LT and ST biomarkers

Whatever the breed, the clusters constituted on ST biomarkers were located in very close proximity, with 3 pools (Pool-A5, -L4, -B4) and an association of respectively 11, 6 and 11 ST biomarkers in Angus, Limousin and Blond d'Aquitaine breeds (Figure 1). In the two continental breeds, Hsp70-1B, MyHC-I, Hsp70-8 and μ -calpain biomarkers of both ST and LT muscles were shared by the same pools (Pool-L1 and -B1). Even though the retained biomarkers are not similar from one breed to another. For example, in the Angus breed, ST and LT biomarkers are not included in the same pool (Table 3).

Links between biomarkers and breeding practices

None of the ST biomarker clusters established in Angus and Limousin were associated with breeding practices or carcass characteristics clusters. This may indicate that ST biomarkers are not linearly correlated to the breeding practices implemented in this work.

Whatever the breed, increasing straw proportion in the diet at the expense of concentrates is linked to a modification in biomarker expression in the ST muscle (for Blond d'Aquitaine) or LT muscle (for the other two breeds). The proportion of straw in

Table 2: Variables associated in each cluster and direction of the evolution, depending on the variable domain and breed.		
Breed	Variable domain	Characterization of each cluster (<i>only the well represented variables in each cluster ($R^2 > 0.5$) are given</i>)
Angus (A)	BP	A-BP-1 : ↗ Straw intake ; ↗ % Straw ; ↘ % Concentrates A-BP-2 : ↗ ADG ; ↗ Feed Efficiency per DMI ; ↗ Feed Efficiency per UF A-BP-3 : ↗ Concentrate intake ; ↗ DMI ; ↗ UF ; ↗ PDIN ; ↗ PDIE
	CC	A-CC-1 : ↘ % Muscle ; ↘ % Bone ; ↗ % CFT ; ↗ % TFT ; ↗ CFT ; ↗ TFT ; ↗ 5QF ; ↗ Leather weight A-CC-2 : ↗ HW ; ↗ SBW ; ↗ CCW ; ↗ EBW ; ↗ Muscles ; ↗ Bone A-CC-3 : ↗ Carcass yield ; ↗ Muscle yield
	LTB	A-LTB-1 : ↗ MyHC-I A-LTB-2 : ↗ μ -calpain ; ↗ Hsp70-Grp75 ; ↘ SOD1 A-LTB-3 : ↗ MyHC-II ; ↗ MyLC-1F ; ↗ LDH-B
	STB	A-STB-1 : ↗ Hsp70-8 A-STB-2 : ↗ B-crystallin ; ↗ MyHC-I ; ↗ CapZ- β ; ↗ DJ-1 A-STB-3 : ↗ MyHC-II ; ↗ MyBP-H ; ↗ μ -calpain ; ↗ LDH-B ; ↗ MDH1 ; ↗ Hsp70-Grp75
Limousin (L)	BP	L-BP-1 : ↗ Straw intake ; ↗ % Straw ; ↘ % Concentrates L-BP-2 : ↗ Concentrate intake ; ↗ DMI ; ↗ UF ; ↗ PDIN ; ↗ PDIE L-BP-3 : ↗ SLW ; ↗ ADG ; ↗ Feed Efficiency per DMI ; ↗ Feed Efficiency per UF
	CC	L-CC-1 : ↘ % Muscle ; ↘ % Bone ; ↗ % CFT ; ↗ % TFT ; ↗ CFT ; ↗ TFT ; ↗ 5QF ; ↗ Leather weight L-CC-2 : ↗ HW ; ↗ SBW ; ↗ CCW ; ↗ EBW ; ↗ Muscles ; ↗ Bone ; ↗ Carcass yield
	LTB	L-LTB-1 : ↗ Hsp20 ; ↗ DJ-1 ; ↗ MyHC-II ; ↗ MDH1 L-LTB-2 : ↗ B-crystallin ; ↗ Hsp70-1B ; ↗ MyHC-I ; ↗ Hsp70-8 ; ↗ μ -calpain L-LTB-3 : ↗ SOD1 ; ↗ Hsp40 ; ↗ MyLC-1F
	STB	L-STB-1 : ↗ SOD1 ; ↗ Eno 3 L-STB-2 : ↗ Hsp20 ; ↗ DJ-1 ; ↗ Hsp70-Grp75 L-STB-3 : ↗ MyHC-I ; ↗ MyLC-1F ; ↗ PRDX6
Blond d'Aquitaine (BA)	BP	BA-BP-1 : ↗ Straw intake ; ↗ % Straw ; ↘ % Concentrates BA-BP-2 : ↗ Concentrate intake ; ↗ DMI ; ↗ UF ; ↗ PDIN ; ↗ PDIE ; ↗ ADG
	CC	BA-CC-1 : ↘ % Muscle ; ↗ % CFT ; ↗ % TFT ; ↗ CFT ; ↗ TFT ; ↗ 5QF ; ↗ Leather weight BA-CC-2 : ↗ HW ; ↗ SBW ; ↗ CCW ; ↗ EBW ; ↗ Muscles ; ↗ Bone ; ↗ Muscle yield
	LTB	BA-LTB-1 : ↗ Hsp27 ; ↗ Hsp20 ; ↗ CapZ- β ; ↗ Eno3 ; ↗ MyBP-H BA-LTB-2 : ↗ MyHC-I ; ↗ DJ-1 ; ↗ MDH1 BA-LTB-3 : ↗ Hsp70-1B ; ↗ Hsp70-8 ; ↗ MyLC-1F ; ↗ μ -calpain ; ↗ PRDX6
	STB	BA-STB-1 : ↗ α B-crystallin ; ↗ Hsp70-1B BA-STB-2 : ↗ Hsp27 ; ↗ SOD1 ; ↗ MyHC-IIx BA-STB-3 : ↗ Hsp20 ; ↗ CapZ- β ; ↗ Hsp70-8 ; ↗ μ -calpain ; ↗ PRDX6 ; ↗ Hsp70-Grp75 BA-STB-4 : ↗ MyHC-I ; ↗ Hsp40 ; ↗ MyLC-1F BA-STB-5 : ↗ DJ-1 ; ↗ MyHC-II ; ↗ MyBP-H ; ↗ MDH1
Abbreviations: BP: Breeding Practices ; CC: Carcass Characteristics ; LTB: LT Biomarkers ; STB: ST Biomarkers Identification of each cluster: Breed – Set of variable - Number of the Cluster in the set of variable. Example: A-BP-1 for the first cluster of breeding practices in the Angus Breed.		

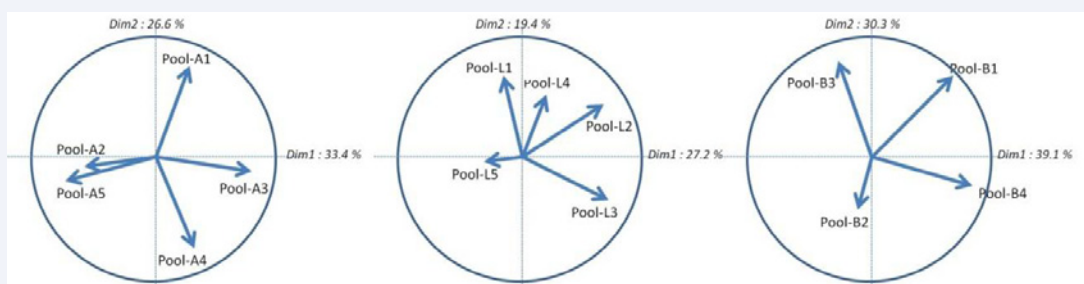


Figure 2 Evaluation breed per breed of the links among the different pools of clusters for Angus (A), Limousin (L) and Blond d'Aquitaine (B). Details of clusters and pools are given in Tables 2 and 3 respectively

the diet is positively linked to MyLC-1F and Hsp40 biomarkers either in the LT muscle (for Limousin) or in the ST muscle (for Blond d'Aquitaine). These findings may indicate a universal relation between straw proportion and these biomarkers, whatever the breed and the muscle considered.

While it is positively linked to SOD1 expression in the LT from Limousin animals, straw proportion is negatively linked to this biomarker in the ST muscle from Angus animals. Also, straw proportion is positively linked to the LT MyHC-I biomarker in the Blond d'Aquitaine breed, whereas this factor is positively

Table 3: Constitution of pool of clusters after a clustering of variable on the clusters.

Breed	Name of the Pool	Characterization of each pool
Angus (A)	Pool-A1	A-CC-2 ; A-LTB-1
	Pool-A2	A-BP-1 ; A-LTB-2 ; A-LTB-3
	Pool-A3	A-BP-3 ; A-CC-1
	Pool-A4	A-BP-2 ; A-CC-3
	Pool-A5	A-STB-1 ; A-STB-2 ; A-STB-3
Limousin (L)	Pool-L1	L-LTB-2 ; L-STB-1
	Pool-L2	L-BP-1 ; L-LTB-3
	Pool-L3	L-CC-1 ; L-CC-2 ; L-BP-3 ; L-BP-2*
	Pool-L4	L-STB-2 ; L-STB-3
	Pool-L5	L-LTB-1
Blond d'Aquitaine (BA)	Pool-B1	BA-CC-2 ; BA-LTB-2 ; BA-LTB-3 ; BA-STB-5
	Pool-B2	BA-BP-1 ; BA-STB-4
	Pool-B3	BA-LTB-1 ; BA-CC-1 ; BA-BP-2
	Pool-B4	BA-STB-1 ; BA-STB-2 ; BA-STB-3

(only the well represented clusters in each pool ($R^2 > 0.5$) are given except for that indicated with an asterisk ($R^2 < 0.5$); all the clusters of a single pool are positively correlated ; composition of clusters is specified in Table 2)
Identification of each pool: Breed –Number of the Pool; Example: Pool-A1 for the first pool in the Angus Breed.

linked to the LT MyHC-II biomarker in the Angus breed. Thus, breeding practices appear to have various impacts on biomarkers depending on the animal type and the muscle studied. The results revealed that depending on breeding practices, the Angus muscles appear to have opposite reactions to those from continental breeds.

Protein and energy content of the diet has no significant impact on muscle biomarkers except for Blond d'Aquitaine animals. For this breed, the increase of the diet value is linked to an increase in enolase 3, small heat shock proteins (Hsp27, Hsp20) and two structural proteins (CapZ- β and MyBP-H):

Links between biomarkers and carcass characteristics

Whatever the breed, increasing the diet in concentrate, UF and PDI content infers an increase in adipose tissue development in the whole animal, in the carcass and in the 5th quarter of the carcass (Table 3). In the Blond d'Aquitaine breed which is known to be rather lean, the fatter carcasses (higher CFT, TFT, 5QF, leather weight, %TFT, % CFT) are characterized by a higher expression of previously indicated biomarkers (Hsp27, Hsp20, enolase 3, CapZ- β and MyBP-H), whereas no linear relation appears between these parameters in the other two breeds.

In the Limousin breed, variation in studied carcass characteristics does not appear to be linked to muscle biomarker modification. In the other two breeds, muscle and bone development (including carcass, total bone, head and shin bone weights) appears to be positively linked to MyHC-I biomarker abundance in LT muscle.

When considering the three breeds as one data set, it appears that the most significant links between breeding practices and biomarkers, as well as between carcass characteristics and biomarkers, are found in the Blond d'Aquitaine breed.

In this breed, muscle (weight and yield) and bone development is linked to an increase in MDH1 and DJ-1 biomarkers in both muscles. In LT muscle these carcass characteristics are linked to MyHC-I biomarker abundance, whereas in ST muscle they are

positively correlated with the abundance of MyHC-II, confirming as indicated before that the response of biomarkers to variation factors is muscle dependant. Finally, in LT muscle, bone and muscle development is also correlated with large Hsp (Hsp70-1B and Hsp70-8) and oxidative resistance (DJ-1, PRDX6) proteins.

Step 3: evaluation breed per breed of the links among the different pools of clusters

A principal component analysis was performed breed per breed in order to evaluate the proximities between the different pools of clusters (Figure 2). It allowed us to establish in the Angus breed that:

- pools A2, A3 and A5 are positively linked together on the first axis (33.4 % inertia, Figure 2). The positive values are associated with richer diets (UF, PDI, DMI, concentrate and straw ingested) and the fatter carcasses (% CFT, % TFT, CFT, TFT, 5QF, Leather weight). The diets richer in straw (amount and proportion) and the carcasses with a higher development (% Muscle and % Bone) are located on the opposite side of the axis. Some biomarkers are negatively correlated with fatter carcasses on both muscles, namely μ -calpain, Hsp70-Grp75, MyHC-II and LDH-B. Fatter carcasses are also negatively linked to Hsp70-8, CapZ- β and MyBP-H in ST muscle. Only the SOD1 biomarker of the LT muscle is positively linked with the carcass characteristics.

- pools A1 and A4 are positively linked on the second axis (26.6 % inertia), comparing heavier carcasses (EBW, CCW, SBW, HW, Muscle and Bone weights; positive values) versus yields (carcass and muscle) and animal efficiency (ADG, Feed efficiency ; negative values) (Figure 2). The MyHC-I biomarker of the LT muscle is well represented on this second axis (positive values).

In the Limousin breed, the first axis is linked to pools L2 and L3 (27.2 % inertia). As previously observed for the Angus breed, the richer diets and fatter carcasses were positively linked to SOD1, Hsp40 and MyLC-1F of LT muscle. On the second axis (19.4% inertia), only pool L1 appears to be well represented.

Finally, in the Blond d'Aquitaine breed, the first axis associated

pools B1 and B4 (39.1 % inertia), whereas the second axis is only associated with pool B3 (30.3 % inertia). These associations confirmed the similarities reported previously between some protein abundances in both muscles (Hsp70-8, μ -calpain, Hsp70-1B, DJ-1, PRDX6 and MDH1). These proteins are higher in heavier (EBW, CCW, HW, SBW) and higher meat yield carcasses.

Some other LT muscle biomarkers (Hsp20, Hsp27, CapZ- β , enolase 3 and MyBP-H) were positively associated with carcass fat development (%CFT, %TFT, CFT, TFT, 5QF, leather weight), in accordance with an increase in diet intake (UF, PDI, DMI, concentrates) and a higher daily gain (ADG).

DISCUSSION AND CONCLUSION

The aim of the present work was to study the evolution of the abundances of a list of 21 biomarkers in two divergent beef muscles depending on animal breeding practices and carcass characteristics using an innovative clustering approach recently reported by our group [14-16]. The findings revealed large differences between the muscles studied and confirmed the well documented literature data that the two analyzed muscles have different characteristics in terms of muscle fibre composition [17]. In comparison to LT, the ST muscle was reported to have lower oxidative and higher glycolytic activities [18]. It also has a lower proportion of slow fibres, a lower oxidative metabolism and a higher fibre cross-sectional area than LT muscle [19].

This study showed that the impacts of breeding practices on muscle biomarkers are diverse depending on muscle and/or breed. Indeed, it seems that it is not easy to quantify the impact of these breeding practices but they appear to have a significant impact on ST biomarkers in the Blond d'Aquitaine, whereas in the two other breeds, the impact is more pronounced on the LT muscle.

The findings suggest that whatever the breed, ST biomarkers are less related to breeding practices and/or not linked to carcass characteristics than in LT. The rather limited impact of breeding practices on ST biomarkers was unexpected. It has been reported in the past that breeding practices affect muscles differently and certain young bull muscles, namely the rather glycolytic ST has been considered more responsive than the more oxidative ones (such as the m. *Supraspinatus*) [20]. Accordingly, it could be hypothesized that the feeding diets were not contrasted enough in the present work to underline the various biomarker expressions in the ST muscle.

Otherwise, some practices appear to have an unchanged impact on biomarker patterns whatever the breed and the muscle. For example, increasing the proportion of straw in the diet leads to an increase in MyLC-1F and Hsp40 biomarkers both in the LT of Limousin and Angus and in the ST of Blond d'Aquitaine. This may be in part related to protein turnover [21]. The proteomic investigations on the effect of feeding and feed intake in beef muscles are very scarce and the few reported studies concentrate on other species. For example, Almeida *et al.* [22] showed that three structural and contractile apparatus proteins (among them a Myosin Light Chain spot) in sheep are affected when subjected to restricted feed. Moreover, MyLC has been associated with higher muscle deposition ability in cattle [23] and its expression increases in animals fed on pasture vs. concentrate [24, 25].

However, most of the biomarkers evolve differently in the two muscles according to breeding practices. The muscle-type effect detected in this study confirms those found in the literature for the same muscles, indicating a muscle effect on 14 proteins among the 24 proteins tested [26]. These differences according to muscle type are in accordance with previous conclusions established on the links between biomarkers and meat quality traits. These two different muscles being related for example to tenderness through two distinct molecular networks [26].

Even though this "universal impact" is true for some biomarkers, it does not always hold for others, especially as biomarker expression seems to be breed-dependant. Breed differences were also investigated using comparative proteomic profiling on two muscles from five different pure pig breeds and the findings showed that there are potential biomarkers for breed classification [27]. Indeed, the patterns of some Angus biomarkers when breeding practices are modified are the opposite of those of continental ones. For example, increasing straw proportion in the diet leads to an increase/a decrease in SOD1 biomarker, or an increase in oxidative/glycolytic biomarkers depending on the breed under consideration (Limousin vs. Angus and Blond d'Aquitaine vs. Angus, respectively). The reverse impact of straw proportion increase on the SOD1 biomarker depending on the muscle could be explained by a differing expression of oxidative resistance proteins, irrespective of contractile and metabolic muscle type. This hypothesis was formulated previously by [26], who indicated that the glycolytic ST has a lower oxidative protection system and that in case of stress, a strong and active pathway could be engaged to protect cells against reactive oxygen species (ROS) by Cu/Zn superoxide dismutase (SOD1). Moreover, the fibre type, especially the slow twitch ones may be the source of these differences. As reported above, there are more type I fibres in LT (23%) compared to ST muscle (8%) and these fibres are known to contain high levels of mitochondria and to be involved in the scavenging mechanisms of ROS [27,28]. Furthermore, it has been reported that oxidative and glycolytic fibres show marked differences even in ROS metabolism and enzymatic activities of SOD (lower in fast glycolytic muscles) [29,30]. Finally, it is worth mentioning that cattle with lower levels of IMF were reported to have a lower muscle oxidative capacity and more glycolytic capacity [31] and the breeds investigated in this study have different IMF content (Angus > Limousin $\approx$ Blond d'Aquitaine [32]. It was proposed that individual cattle with a lower level of MyLC expression during growth have more IMF deposition because the lower MyLC level promoted the proliferation of myoblasts, which can be transdifferentiated into IMF when nutritionally rich diets are given during the fattening period and thus increase deposition of IMF [33,34].

In the Blond d'Aquitaine breed, biomarkers are somewhat more closely linked with breeding practices and carcass characteristics than in the two other breeds. Thus, it might be supposed that it would be easier to predict breeding practices and carcass characteristics using biomarker abundance in that breed than in the other two, which might make it possible to direct the carcass towards the most suitable market earlier. Among the interesting biomarkers retained, we found MDH1 and DJ-1 that are both positively linked to muscle yield and carcass development (weights of the different parts) in both LT and ST

muscles. These findings confirm those obtained by Guillemain *et al.* [35] revealing no significant muscle-effect on the MDH1 oxidative enzyme biomarker. It is well-known that MDH1 plays a pivotal role in the malate-aspartate shuttle operating between cytosol and mitochondria and to be involved in the contractile function [36,37]. So, the involvement of MDH1 with an increase in expression is required for the high demands of energy metabolism in developing tissues, especially in skeletal muscle as previously reported [38]. Once again, the associations of these two potential biomarkers may be related to the production of ROS associated with increased metabolic activity during muscle (carcass) development. During oxidative stress, DJ-1 (a highly conserved protein involved in the regulation of oxidative stress by directly quenching ROS upon oxidative modification of a conserved cysteine residue) is re-localized to the mitochondria, where it has a key role in scavenging mitochondrial H_2O_2 and limiting mitochondrial fragmentation. MDH1 is involved in the final steps of glycolysis before malate enters the mitochondrion; hence we suggest that the relationship between DJ-1 and MDH-1 with carcass development and other animal characteristics may be relatively direct.

In the LT muscle, these carcass properties are also positively linked to the large Hsp proteins (Hsp70-1 and Hsp70-8) and once again with the oxidative resistance biomarkers (DJ-1 and PRDX-6). These biomarkers could be particularly interesting as they are known as good biomarkers for meat tenderness and other meat quality traits [39]. Concerning large Hsp proteins, they are reported to be important for *p-m* muscle changes and to correlate in numerous studies with tenderness [9,26,40]. Large Hsp proteins are involved in folding of newly or denatured proteins and promote the recovery of cell membranes, thus maintaining cell homeostasis [41].

Moreover, it appears that Hsp70-1 could be a relatively general biomarker of tenderness whatever the breed and the muscle considered. Thus, it is interesting to establish that in the LT muscle large HSP proteins could be considered as a good indicator of carcass quality. Hsp70-1 being linked both to carcass characteristics and to meat tenderness suggests that evaluating Hsp70-1 abundance could offer a way to predict and to control these two parameters. Thus, we confirm a number of profiling proteomic studies that have reported the involvement of large Hsp proteins in the determination of tenderness and other quality traits [7].

Finally, muscle yield is positively linked to MyHC-I in the LT and MyHC-II in ST muscle. The contradiction between the two muscles has already been observed and discussed above. Indeed, in the LT muscle of young bull and cows muscles, tenderness appears to be positively correlated with the slow oxidative state while in the ST muscle, tenderness is positively correlated with glycolytic properties [14]. Thus, it could be supposed that muscle yield is positively linked to tenderness whatever the muscle being considered.

In the Blond d'Aquitaine breed, some LT muscle biomarkers might be considered as good indicators of carcass fatness: namely small Hsp, ENO3, CapZ- β and MyBP-H. When considering previous results established with the same experiment, it appears that some of these biomarkers could be of great value

for meat tenderness prediction since small Hsp by Hsp20, Hsp27 or α B-crystallin, the glycolytic ENO3 enzyme (involved in the development and regeneration of muscle) and the structural protein CapZ- β , are all reported to be positively related to meat tenderness [10] and meat color [15].

In the Angus breed, considered as precocious and relatively fat in comparison to the late and lean Blond d'Aquitaine breed, the relationship between fat development and muscle biomarkers may involve only the ST muscle. The links between fat development and biomarkers are inverted in this muscle, as fatter carcasses were negatively linked to ST Hsp70-8, CapZ- β and MyBP-H proteins. This may confirm our previous findings showing that relationships between breeding practices and/or carcass characteristics and biomarkers are muscle and breed dependent. Also, it may be due to the particularity of Angus breed which manifested specific patterns in comparison to continental breeds, probably linked to its precociousness and its higher and earlier fat development. In agreement with our findings, various recent proteomic studies have previously reported that numerous proteins are involved in fat development. These papers revealed some of the biological pathways that determine the bovine intramuscular fat (IMF) deposition [34, 42,43].

Finally, it should be noted that for a given breed (eg. Blond d'Aquitaine), some biomarkers are impacted by breeding practices in the same way. For example, DJ-1 or MDH1 are positively correlated from one muscle to the other. On the contrary, other biomarkers appear to be negatively correlated. For example, the increase in MyHC-I biomarker in the mixed fast oxido-glycolytic LT muscle are to be associated with the increase of MyHC-II biomarker in the mixed fast glycolytic ST muscle. These data suggest that several animal factors impact muscle protein expression, some of which are common between muscles, whereas other differ. The findings of this study are quite coherent with previous conclusions reported in the significant literature by most of the studies conducted by our group [7, 9].

The present paper's originality resides in the fact that the topic is still a new one. Indeed, the relationships between breeding practices and biomarker abundance have not, to our knowledge, really been explored in the literature, the relations between carcass performances and biomarker abundance being even less fully developed in previous studies. Thus, the combination of the topic and the statistical method used in this study appears to be creative and unlocks new perspectives for early prediction of carcass and meat quality.

Overall, several of the tested biomarkers have been identified as being relevant for the traits of interest, and their relationships with these desired traits are discussed in terms of the possible underlying biological mechanisms. Before these biomarkers can be used as an industrial tool, further developments are needed, above all the investigation of the breed and muscle impacts, protein per protein [44-48]. To better understand the similarities but also the differences that may exist among breeds/muscles, a similar study is in progress using a large database of more than 40 experiments bringing together about 20 breeds and a large number of muscles. Finally these explorations might allow early (at the beginning of the finishing period, by a muscular biopsy or a blood sampling) determination of potential carcass performances

and meat quality traits. This could facilitate the management of carcass and meat quality by breeding practices, using prediction equations involving the tested muscle biomarkers.

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