MUSCLE TRANSCRIPTOME PROFILES HIGHLIGHT BIOMARKERS OF PIG PRODUCTION SYSTEM AND HIGH MEAT QUALITY

B. Lebret[1, 2], K. Denieul[1, 2] and M. Damon[1, 2]

[1] INRA, UMR 1348 PEGASE, F-35590 Saint-Gilles, France

[2] Agrocampus Ouest, UMR 1348 PEGASE, F-35000 Rennes, France

Abstract – Muscle biological characteristics strongly influence the economic value and the eating quality of pork. However, the muscle properties underlying high sensory and technological meat quality (MQ) remain unclear. The local Basque breed exhibiting high sensory quality of pork could thus be a relevant model to study MQ development. Therefore, the Longissimus muscle (LM) transcriptome and MQ of Basque pigs reared in various production systems themselves influencing MQ (conventional, C; alternative, A; extensive, E; n=10/system) were determined. E pigs had higher ultimate pH and darker meat, less drip loss but higher shear force than A or C pigs. However, meat tenderness and juiciness did not differ according to production system. LM transcriptome differences between systems highlighted up regulation of genes involved in control of muscle structure and thermal response in the E pigs compared with A and C pigs. Besides, associations between microarray expression and pork traits revealed numerous potential biomarkers of MQ. Using quantitative RT-PCR, 90 transcript-trait correlations were confirmed ($P<0.05$, $|r| \leq 0.74$), among which 27 were validated ($P<0.05$, $|r| \leq 0.73$) on complementary experimental data (n=30).

Key Words – Basque pork quality, Microarray, Muscle biology

I. INTRODUCTION

Sensory and technological meat quality (MQ) results from complex muscle properties. Even though some influencing biological traits have been highlighted, the mechanisms underlying high MQ remain unclear. Local pig breeds giving high quality pork [1] are thus of high interest to study MQ development. This study aimed at comparing the muscle transcriptome and MQ of Basque pigs reared in diverse production systems affecting MQ [1, 2]. Besides, associations between transcriptome profile and pork traits aimed at identifying biomarkers of MQ, that were confirmed on the same animals then validated on complementary data using RT-PCR for quantification of gene expression.

II. MATERIALS AND METHODS

Animal design. The experiment involved 2 replicates (R1 and R2) each including 30 Basque castrated male pigs reared in conventional (C; slatted floor, 1.0 m²/pig), alternative (A; bedding and outdoor access, 2.4 m²/pig) or extensive (E, free range, 2.5 ha/group) production system (n=10/system/replicate), from 35 to 145 kg.

Meat quality traits. Longissimus muscle (LM) was sampled 30 min p.m. for pH1 and glycolytic potential (GP) measurements and RNA extraction. After 24 h, LM ultimate pH (pHu) and color (lightness, L*; redness, a*; yellowness, b*; saturation, C*; and hue angle, h°) were measured, and LM sampled for drip loss (3 d p.m.) and intramuscular fat content (IMF) measurements [2]. LM roasts aged 4 d were vacuum-packed and stored (-20°C) before Warner-Bratzler shear force (WBSF) of cooked meat (70°C, 50 min), and sensory analyses. Data were submitted to a variance analysis including the effects of production system, replicate and their interaction.

RNA extraction and transcriptome analysis. After extraction (Trizol), LM total RNA from the R1 pigs and the reference (sample pool) were labeled with Cy3 and Cy5, respectively, and hybridized on a specific 15 K pig skeletal muscle microarray [3]. Image analysis, spot intensity measurement and data normalization [3] led to 11428 spots retained for statistical analysis. Expression data were adjusted for slide effect (n=6) when significant ($P<0.05$) by variance analysis. A differential expression analysis was performed on residuals by variance analysis with production system (C, A, E) as fixed effect and Benjamini-Hochberg (BH) [4] multiple test correction (adjusted P-value<0.05).

Functional analysis. Enrichment analysis of the two lists of genes differentially ($P<0.10$) expressed

between systems (A or C vs E) was undertaken on GO terms biological process, cellular components and molecular function (DAVID bioinformatics resources, http://david.abcc.ncifcfr.gov/).

Identification of biomarkers of MQ. Pearson's correlation coefficients between microarray expression of the 11428 spots and MQ traits were calculated (R software 2.8.1, R Development Core Team, 2008) with BH adjusted P-value P<0.05 to identify potential biomarkers of MQ.

Confirmation and validation of biomarkers by RT-PCR. Expression of 46 annotated genes with high coefficient correlation to one or many MQ traits was determined by RT-PCR [3] on both R1 and R2 LM samples. Gene expression was measured (triplicate) using Fast SYBR® Green reagent and both forward and reverse specific primers (Primer Express Software). Normalized expression level was calculated using a calibrator and 3 stable reference genes. Correlations were calculated between RT-PCR expression and MQ traits within both R1 and R2 data sets. A transcript-trait correlation was confirmed when found significant (P<0.05) and of same sign (+ or -) on both microarray and RT-PCR data on R1 samples, and validated when significant (P<0.05) and of same sign on RT-PCR data of both R1 and R2 samples.

III. RESULTS AND DISCUSSION

Pork quality highly depended on production system, with E pigs exhibiting slightly higher pH1, higher pHu despite similar GP, and lower L*, b* and h° values indicating less light and darker red meat than A or C pigs. Although standardized pre-slaughter handling between systems, E pigs had higher plasma creatine kinase activity at slaughter (2.0 vs 1.2 and 1.0 U/ml for E, C and A pigs respectively, P<0.001) indicating their higher physical activity that may have influenced pHu and color. E pigs also showed less drip and a trend (P=0.07) for less IMF than A, the C pigs being intermediate. Despite higher meat WBSF of E than A or C pigs, sensory traits did not differ between production systems. The strong effects of the E system on MQ traits without impact on eating quality may result from the adverse consequences of the higher pH1, pHu and lower drip on the one hand, and higher WBSF and trend for lower IMF on the other hand, on tenderness and juiciness [5].

Table 1. Pork quality in conventional (C), alternative (A) or extensive (E) production systems (n=60)

Quality trait	C	A	E	RSD[1]	Sign[2].
pH1	6.48a	6.52a	6.53b	0.17	*
pHu	5.58a	5.57a	5.71b	0.15	**
L*	51.2b	51.6b	48.1a	2.6	***
a*	9.57	9.67	9.30	1.6	ns
b*	6.53b	6.85b	4.89a	0.9	***
C*	11.6	11.9	10.5	1.8	ns
h°	34.5b	35.3b	27.8a	3.0	***
Drip, %	0.86ab	1.11b	0.55a	0.53	**
GP, µg/g	136	138	136	17	ns
IMF,%	3.79	4.07	3.28	1.1	ns
WBSF, N/cm²	24.6a	22.7a	30.3b	5.3	***
Tenderness, 0-10	5.1	4.9	5.0	0.6	ns
Juiciness, 0-10	3.3	3.3	3.4	0.8	ns
Flavour, 0-10	4.6	4.4	4.3	0.5	ns

[1]Residual standard deviation; [2] ***: P<0.001; **: P<0.01; *: P<0.05; ns: P>0.05. In a row values with different letters differ (P<0.05)

Differences in MQ traits between A and C pigs were not significant whereas increased drip, b*, IMF and juiciness were found for A compared with C pigs in a previous study [2]. Similar results (P>0.05) were obtained in both replicates except lower (P<0.01) b*, C* and h° in R2 than in R1.

Analysis of microarray data highlighted 400 differentially expressed probes according to production system, with 10 probes differentially expressed between C and A pigs, and 390 probes differentially expressed between E and C or A pigs. These 390 probes corresponded to 267 unique annotated genes, among which 117 were highly expressed in the E pigs, and 150 highly expressed in the A or C pigs. Expression level of 10 highly differentially expressed genes quantified by RT-PCR validated microarray data (not shown).
Functional annotation clustering of biological processes associated to transcriptome differences between production systems emphasized 8 main clusters (Table 2). Six clusters were associated to E, and 2 clusters to A or C systems. Especially, genes related to the control of muscle structure including FLNC, NEB, ANKRD1, ANKRD2, CSRP3, ABRA and involved in thermal response :

CRYAB, HSPB1, HSPB7, HSPB8, HSPB22 were highly expressed in E compared with A or C pigs.

Table 2. Enrichment analysis of differential expression between production systems (E: extensive, C: conventional, A: alternative)

Cluster	System	n genes	Associated ontology	*P*
1	E	11	GO:0030016~myofibril	$7.8E^{-7}$
2	E	23	GO:0005856~cytoskeleton	$4.3E^{-4}$
3	E	9	GO:0009266~response to temperature	$3.9E^{-3}$
4	E	11	GO:0006986~response to unfolded protein	$3.7E^{-2}$
5	E	7	GO:0016791~phosphatase activity	$2.4E^{-2}$
6	E	8	GO:0016564~transcription repressor activity	$2E^{-2}$
7	C or A	8	GO:0006325~chromatin organization	$7.5E^{-2}$
8	C or A	8	GO:0051301~cell division	$2.7E^{-2}$

FLNC (filamin gamma) crosslinks actin filaments into orthogonal network. Nebulin (NEB), a giant protein of the cytoskeletal matrix, coexists with the thick and thin sarcomere filaments. The p.m. degradation of filamin and nebulin would alter myofibrils and sarcormeres integrity, and initiate further physico-chemical and structural changes leading to muscle tenderization [6]. ANKRD1 (ankyrin repeat domain 1) is involved in the myofibrillar stretch-sensor system and in calcium handling in skeletal muscle. It has been suggested as candidate gene for pHu [7] in agreement with the higher pHu and ANKRD1 expression of the E pigs. ANKRD2 is induced upon stretch and has been associated with pork firmness [8]. CSRP3 (cysteine and glycine-rich protein 3) regulates development and cellular differentiation processes and is up regulated during post-exercise recovery in pig muscle [9]. ABRA (actin-binding Rho activating protein, or STARS) expression is also up regulated in skeletal muscle after stimulating resistance training [10]. Thus, the higher CSRP3 and ABRA expression in the LM of E pigs could result from their higher pre-slaughter activity. Up regulation of genes related to muscle structure in these pigs could also be associated to their higher WBSF, even though without impaired tenderness.

Genes up-regulated in E pigs involved in thermal response include many small heat-shock proteins (sHSP). CRYAB (αB crystallin) and HSPB8 act as molecular chaperones. HSPB1 and HSPB7 (HSP27 family), induced by environmental stress, play a role in stress resistance and actin organization. Interestingly, higher protein levels of these sHSP have been reported in dark (low L*) vs light pork [11] in agreement with their higher gene expression in the E pigs exhibiting low L*. HSP up regulation could obviously also result from the extensive conditions with high physical activity and low environmental temperature of the E pigs.

Regarding identification of biomarkers of MQ, numerous significant correlations (BH adjusted P $0<0.05$) were calculated between microarray expression and MQ traits: 7 for C* up to 2920 for tenderness, whereas no significant correlations were found with juiciness or tenderness. Highest individual determination coefficients (R^2) between gene expressions and traits ranged from 0.34 (C*) up to 0.65 (tenderness). Using RT-PCR expression of 46 genes on the R1 pigs, 90 transcript-trait correlations including 32 genes were found with one or several MQ traits ($P<0.05$, $|r|\leq 0.74$). Since 276 significant correlations were found between microarray expression of these 46 genes and MQ traits, the confirmation level is of 33%.

RT-PCR expression of these 46 genes was quantified on the R2 pigs and associated to their MQ data, leading to 27 transcript-trait correlations ($P<0.05$, $|r|\leq 0.73$) between 14 genes and 9 traits: pH1 (1), pHu (5), L* (5), a* (2), b* (5), C* (1), h° (6), IMF (1), GP (1), giving a validation rate of 30%. Table 3 presents thecorrelations found for pHu and color values at the identification, confirmation and validation steps. Many biomarkers were validated for both pHu and color with opposite correlations with pH and L*, b* or h°, in agreement with the negative relationships between these traits [6]. In accordance with present data, ANKRD1, OTUD1, FOS, MB and ZNF503 were recently highlighted as biomarkers of pHu, L*, a* and h° on a data set including both Basque and conventional Large White pigs [12].

IV. CONCLUSION

Muscle transcriptome differences according to pig production system and pork quality have been highlighted in this study. Our results also led to the identification and further validation of 27

transcript-trait correlations including pH, color and IMF ($P<0.05$, $|r| \leq 0.73$) thus highlighting early p.m. biomarkers of pork quality.

Table 3. Correlations ($P<0.05$) between gene expression quantified by transcriptomics (T, n=30) or RT-PCR (R1, n=30; R2, n=30), pHu and colour

Gene[1]	Expression	pHu	L*	a*	b*	h°
	T	0.30			-0.41	
ANKRD1	R1	0.68			-0.71	
	R2	0.73			-0.53	
	T	0.58	-0.34			
ARL5B	R1	0.53	-0.48			
	R2	0.66	-0.45			
	T					0.51
CA3	R1					0.43
	R2					0.52
	T				-0.44	-0.45
FOS	R1				-0.55	-0.40
	R2				-0.45	-0.58
	T		0.39			0.53
HPS1	R1		0.58			0.57
	R2		0.50			0.53
	T			0.60		-0.33
MB	R1			0.48		-0.40
	R2			0.44		-0.43
	T			0.30		
NHEDC2	R1			0.47		
	R2			0.41		
	T	0.49	-0.41		-0.48	-0.43
OTUD1	R1	0.52	-0.38		-0.67	-0.40
	R2	0.39	-0.46		-0.47	-0.51
	T	0.37	-0.37		-0.36	-0.44
PPP1R3C	R1	0.64	-0.64		-0.64	-0.68
	R2	0.38	-0.41		-0.46	-0.58
	T				-0.32	
UBE2Q2	R1				-0.37	
	R2				-0.38	
	T	-0.65	0.37			
ZNF503	R1	-0.42	0.39			
	R2	-0.43	0.40			

[1]ANKRD1, ankyrin repeat domain 1; ARL5B, ADP-ribosylation factor-like 5B; CA3, carbonic anhydrase 3; FOS, FBJ murine osteosarcoma viral oncogene homolog; HPS1, Hermansky-Pudlak syndrome 1 protein; MB, myoglobin; NHEDC2, Mitochondrial sodium/hydrogen exchanger; OTUD1, OTU domain containing 1; PPP1R3C, protein phosphatase 1, regulatory (inhibitor) subunit 3C; UBE2Q2, Ubiquitin-conjugating enzyme E2; ZNF503, zinc finger protein 703-like.

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