MOLECULAR BIOMARKERS TO DISCRIMINATE PORK QUALITY CLASSES BASED ON SENSORY AND TECHNOLOGICAL ATTRIBUTES

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Abstract – Meat quality (MQ) is a complex phenotype assed by different indicators measured by using costly and/or invasive analyses. Early post-mortem (p.m) biomarkers of MQ refer to single indicators, but not to the overall quality of pork samples. This study aimed at determining pork quality classes combining both sensory and technological dimensions. Then, combinations of biomarkers discriminating between quality classes were identified to further predict quality level of pork loins. Sensory, technological and gene expression data were collected on 100 pig *Longissimus* [b1] (8th dorsal to 2nd lumbar vertebrae level) samples exhibiting a wide and gradual variability in MQ. Scientific and statistical approaches were combined to select indicators and their thresholds specifying quality classes differing in sensory and technological attributes: low (=defective; L), acceptable (A) and extra (E) quality. Gene expressions were used as predictive variables in a generalized linear model to discriminate quality classes. The best model (selected with the Akaike information criterion) included expression levels of 12 genes (18% error rate on known data, 24% after cross validation). Besides, a classification tree to predict quality categories was developed, including six branches with only five genes but a higher error rate than the linear model. External validation of predictive models is currently undertaken using 250 commercial pig samples.

Key Words – Commercial pork, gene expression levels, muscle

I. INTRODUCTION

Sensory and technological attributes of pork are determined by complex interactions between pig genotype, animal rearing conditions, slaughtering conditions and meat processing techniques. Meat quality (MQ) is thus a complex phenotype that can be determined only few days after slaughter considering physical, biochemical, or sensory indicators assessed by (usually) costly and invasive analyses. MQ is therefore difficult to predict, and presents a high variability even when controlling variation factors.

Functional genomics has been recently used in various research programs to identify early post-mortem (p.m.) biomarkers of MQ in various species [1, 2]. In a recent study, 100 pigs from two different breeds and various production systems were used, giving rise to pork loin samples (*Longissimus* muscle [b2], LM, 8th dorsal to 2nd lumbar vertebrae level) exhibiting a wide and gradual variability in technological and sensory quality [3]. Combining MQ data with transcriptomic profiles of a subset of 50 LM samples taken at 30 min p.m., biomarkers of MQ traits were identified and further validated by RT-PCR on the remaining 50 LM samples. Sixty associations between gene expression and MQ levels were thus validated, with expression of one gene explaining up to 46% of the phenotypic variation of a single MQ trait [3]. These results are promising but highlight that predictive capacity of biomarkers should be improved to foresee the development of control tools for pork industry. Therefore, we recently considered another approach based on the identification and validation of biomarkers of sensory and technological MQ classes, i.e. low, acceptable or extra pork quality levels [4]. The final objective is to propose molecular tools to classify carcasses or primary cuts early after slaughter, according to their predicted sensory or technological quality level. The aim of the present study was to determine sensory and technological pork quality classes, and then to determine combinations of early p.m. biomarkers discriminating between quality classes, in order to predict quality level of pork loins in meat industries.

II. MATERIALS AND METHODS

Pork quality classes. To establish pork quality categories based on sensory and technological attributes, data of MQ traits recorded on 100 loin samples (LM, fresh raw and cooked meat) including pH 30 min p.m., ultimate pH (pHu), colour: lightness (L*), saturation (C*) and hue angle (h°), drip loss, cooking loss, intramuscular fat (IMF) content, shear force, and tenderness, juiciness and flavor scores determined by a trained panel, were considered. Scientific expertise and literature data [5, 6, 7, 8, 9, 10, 11] as well as statistics (principal component analysis (PCA), multiple correspondence analyses and ascending hierarchical classification (FactoMineR package, R software 3.1.1, R Foundation for Statistical Computing, 2014)) approaches were combined to select MQ indicators and their threshold values leading to define different quality classes [12]. Differences in MQ traits between categories were analyzed by Anova (proc GLM, SAS software version 9.4, 2013, SAS Inst., Cary, NC).

Gene expression levels of LM samples. Available dataset of expression level (RT-PCR) of 40 genes previously identified and validated as biomarkers of single MQ traits [3] and obtained on n=98 of the LM samples was used. Firstly, the dataset of gene expression level was checked for outliers data by PCA analysis (FactorMineR package, R software). Because 52 LM samples exhibited missing values for at least one gene expression level, data were imputed using multidimensional analysis. Lack of any variation in data distribution after imputation was verified (*missMDA* package, R software) [13].
Five samples exhibiting at least 8 missing values for gene expression data were then discarded from the data set. Finally, gene expression data of 93 LM samples was considered for further analyses.

Molecular biomarkers discriminating pork quality classes. To predict the quality level of any given pork sample based on its expression levels of few genes, a multinomial generalized linear model was adjusted using a stepwise selection with the Akaike information criterion (*multinom* and *step* R functions). The chosen probability cut-point to predict the quality class "A" on the multinomial model was 0.3. Afterwards a cross validation was undertaken using the "leave-one out" method to estimate the error rate of the selected model.
Another strategy to predict the belonging of a given sample to any quality category, based on decision tree, was also applied. Classification trees were established (*tree* R function) and submitted to cross validation, which resulted in a selected tree with six branches.

III. RESULTS AND DISCUSSION

Pork quality classes. Scientific expertise and literature allowed to highlight important MQ traits to be considered to discriminate pork quality levels, i.e., pH 30, pHu, L*, drip loss, IMF, and tenderness score. Descriptive statistical methods highlighted relationships between MQ traits thereby allowing select the most discriminant ones. Finally, 4 MQ traits: pH 30, pHu, drip loss and IMF and their threshold values were considered, to define 3 quality classes: *low* (impaired) quality (L; pH30 < 6.10 or pHu 24h < 5.50, i.e. PSE and PSE-tendency or acid and acid-tendency meat, respectively) and among non-defective pork, *acceptable* (A; drip $\geq$ 1% or IMF < 2.5%) and *extra* (E; drip < 1% and IMF $\geq$ 2.5%). The classes were first defined using 98 pork samples under study. Then the 5 LM samples with at least 8 missing data for gene expression were removed, leading to a total of 93 pork samples with both MQ and gene expression data. The characteristics of pork quality classes are presented in Table 1.

Table 1. Characteristics of pork quality classes

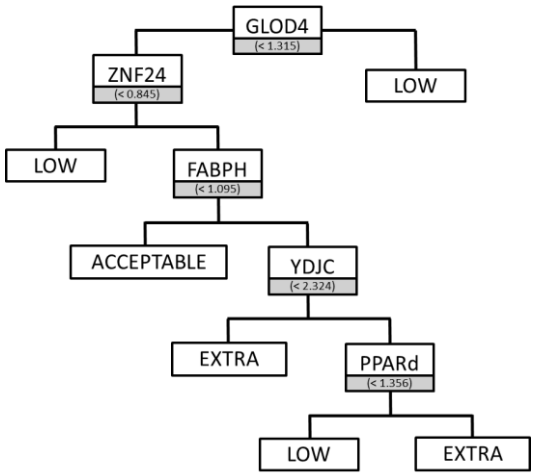
Quality trait	Low	Acceptable	Extra	Sign[1].
n	34	25	34	
pH 30 [2]	6.39 a	6.48 b	6.59 c	***
pHu [2]	5.43 a	5.57 b	5.66 c	***
Drip loss, % [2]	2.52 c	1.84 b	0.65 a	***
IMF, % [2]	2.90 a	2.71 a	3.67 b	**
Lightness	54.3 b	51.3 b	49.5 a	***
Hue angle	37.6 b	35.8 b	31.5 a	***
Shear force, N	28.8	29.2	26.2	P=0.11
Tenderness [3]	4.07 a	4.40 ab	4.92 b	***
Juiciness [3]	2.81 a	3.19 ab	3.36 b	*
Flavour [3]	4.24	4.40	4.43	ns

[1] ***: P<0.001; **: P<0.01; *: P<0.05; ns: P>0.05. In a row values with different letters differ (P<0.05)
[2] Traits used to establish pork quality classes.
[3] Scored on a 0 (low) to 10 (high intensity) scale.

As expected, the L class exhibited the lowest pH30 and pHu values and the highest drip, and the E class the highest pH values and lowest drip, the A class being intermediate. IMF content was higher in E, but did not differ between L and A classes.
Regarding the other MQ traits not used for class determination, the E class showed lower L* and h° values, indicating redder meat, than L and A classes. Differences in shear force between classes did not reach significance, but the E class showed the highest tenderness and juiciness scores and the L class the lowest, the A being intermediate. Altogether this indicates that the 3 classes thus defined actually correspond to varying levels of both sensory and technological qualities of fresh pork reflecting consumer and industry demands.

Biomarkers discriminating pork quality classes.
The multinomial generalized linear model to predict pork quality classes selected the expression levels of 12 genes (GLOD4, PPARd, GUP1, HSPD1, YDJC, CCDC91, NAP1, FOS, LIPE, SPARC, IGF1, MCAT) as best predictive variables. The error rate estimated on the known data was 18%. After cross validation using the "leave-one-out" method, the error rate was 24%.
These genes were associated to various biological functions known to play important roles in the determination of technical and sensory quality of fresh pork [3], including energy metabolism at mitochondrial level (GLOD4), lipid metabolism (PPARd, LIPE, MCAT), carbohydrates metabolism (YDJC), control of gene expression (GUP1), cell regulation and apoptotic processes (HSPD1), protein transport (CCDC91), calcium transport (FOS, SPARC), muscle structure and contraction (SPARC), muscle hypertrophy (IGF1).
External validation of this discriminant model (in progress) is undertaken using 250 LM samples issued from various pig crossbreeds produced in French commercial pork chains and thus totally different from the 93 samples used for modelling. Gene expression level of these LM samples will be quantified and using the above equations, predicted quality class of each sample will be compared with its quality level determined on the basis of its actual pH30, pHu, drip and IMF values. Besides, the decision tree method led to different trees varying in number of branches and genes involved. However, cross validation step highlighted that the minimum misclassification was obtained with trees including between 4 and 6 branches. Therefore, a tree including 6 branches and 5 genes (GLOD4, ZNF24, FABPH, YDJC and PPARd) has been retained (error rate of 41%) (Figure 1).

Figure 1. Decision tree for determination of pork quality classes according to LM expression level of 5 genes



Genes involved in decision tree also belong to important muscle features associated to determination of pork quality, such as lipid metabolism (FABPH, PPARd), mitochondrial activity (GLOD4), carbohydrates metabolism (YDJC) and transcription (ZNF24).
External validation of this model will be also undertaken using the aforementioned 250 LM samples issued from commercial pork chains, even though present results suggest a better prediction accuracy of linear model than decision tree to classify pork samples in quality classes.

IV. CONCLUSION

In conclusion, this study shows a classification in 3 classes of MQ including the sensory and technological attributes of fresh pork. The capacity of both the multinomial generalized model and the tree method to predict the belonging of a given pork sample to a quality class by gene expression is very accurate. These models are currently evaluated for external validation using 250 commercial pork samples before being used as predictive tools in meat industry.

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