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Phenotypic prediction based on metabolomic data for growing pigs from three main European breeds¹

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ABSTRACT: Predicting phenotypes is a statistical and biotechnical challenge, both in medicine (predicting an illness) and animal breeding (predicting the carcass economical value on a young living animal). High-throughput fine phenotyping is possible using metabolomics, which describes the global metabolic status of an individual, and is the closest to the terminal phenotype. The purpose of this work was to quantify the prediction power of metabolomic profiles for commonly used production phenotypes from a single blood sample from growing pigs. Several statistical approaches were investigated and compared on the basis

of cross validation: raw data vs. signal preprocessing (wavelet transformation), with a single-feature selection method. The best results in terms of prediction accuracy were obtained when data were preprocessed using wavelet transformations on the Daubechies basis. The phenotypes related to meat quality were not well predicted because the blood sample was taken some time before slaughter, and slaughter is known to have a strong influence on these traits. By contrast, phenotypes of potential economic interest (e.g., lean meat percentage and ADFI) were well predicted ($R^2 = 0.7$; $P < 0.0001$) using metabolomic data.

Key words: metabolome, phenotypic prediction, pig, variable selection, wavelet transformation

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INTRODUCTION

The accurate and reliable prediction of production phenotypes may open new perspectives for livestock selection. For example, phenotypes of interest could be those that are of considerable economic importance and have top priority in selection objectives but are too expensive to measure routinely or for which measurement is too invasive. Metabolomics is a relatively cheap and easy way to predict (reviewed by Rochfort, 2005) or discover promising biomarkers (Zhang et al., 2011). Recently, this approach was used successfully with pigs

to compare highly phenotypically differentiated breeds (D'Alessandro et al., 2011; He et al., 2012) but not to predict commercially important phenotypes in various breed × gender-determined conditions involving European pig breeds.

The present work was motivated by the hypothesis that the blood metabolome could statistically predict some production phenotypes. The rationale is that blood metabolism reflects the general physiological state of the animal, resulting from the functional metabolic state of the different tissues because blood carries many metabolites and metabolic regulators. The objective of this study was to quantify the power of prediction of several production phenotypes obtained by metabolomic data derived from a single blood sample. The chosen strategy was to evaluate the influence of breed and batch. Concurrent statistical tools evaluated the signal pretreatment step, and the final biological coherence of the results is discussed.

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MATERIALS AND METHODS

All procedures and facilities were approved by French veterinary services (Direction Départementale de la Cohésion Sociale et de la Protection des Populations in Rennes, France; agreement number A35-240-7).

Animal Handling and Zootechnical Data

A total of 506 animals from a Large White dam breed (**LW**), a Landrace dam breed (**LR**), and a Piétrain sire breed (**PI**) were considered in the analysis.

The animals (male castrates of LW and LR and females of PI) were raised at the French central test Station in Le Rheu (France) in 2007 and 2008 in 8 different batches. The sampling design for breeds and batches is given in Table 1. Pigs were grouped in pens of 12 animals from the beginning of the test period (~10 wk of age) until the day before slaughter, considered as the end of the test period (~110 kg BW). They were given ad libitum access to water and a standard pelleted diet formulated to contain 13.2 MJ DE/kg and 164 g CP/kg feed. Pens were equipped with ACEMA 64 electronic feeders (ACEMO, Pontivy, France), allowing the recording of individual feed consumption (Labroue et al., 1993). Animals were individually weighed at the beginning of the test period, at the end of the test period (**LWETP**), and before departure to the slaughterhouse (**LWS**) after at least 16 h of feed deprivation. The duration of the test period, LWETP, and individual feed consumption during the test period were used to calculate ADG, feed conversion ratio (**FCR**), and ADFI during the test period. Slaughters occurred at a given BW on a fixed day in the week in a commercial slaughterhouse (Cooperl-Hunaudaye, Montfort-sur-Meu, France). Carcass weight with the head (**CW**) and carcass weight without the head (**CWwtH**) and the weight of the right half-carcass (**HCW**) were recorded after evisceration on the day of slaughter, and the dressing percentage (**DP**) was calculated as $CW \times 100/LWS$. The day after slaughter, the length of the carcass from the pubis to the atlas (**Length**) as well as the backfat thickness at the shoulder (**BFsh**), backfat thickness at the last rib (**BFlr**), and backfat thickness at the hip joint (**BFhj**) at the sectioned edge of the carcass were recorded. The mean of

these 3 fat measurements was calculated [mean backfat thickness (**mBF**)]. The measurements used for carcass commercial grading (i.e., backfat thickness between the third and fourth lumbar vertebrae and between the third and fourth last ribs as well as loin eye depth between the third and fourth last ribs) were performed using the “fat and lean sensor” CGM probe (Sydel Sa, Lorient, France) and were combined to estimate the commercial lean meat percentage (ComLMP) as described by Daumas et al. (1998). Finally, a standardized cutting procedure of the right half-carcass was performed as described previously (Anonymous, 1990), and ham, loin, backfat, shoulder, and belly were weighed [ham weight (**hamW**), loin weight (**loinW**), backfat weight, shoulder weight (**shW**), and belly weight (**beW**), respectively] and combined to obtain a second estimate of the lean meat percentage (**LMP**) of the carcass (Métayer and Daumas, 1998). On the same day, several meat quality measurements were taken: ultimate pH of the semimembranous muscle (**pH₂₄**), color of the gluteus superficialis muscle through the 3 coordinates L*, a* and b* of the international CIELAB color scale (International Commission on Illumination, Vienna, Austria) using a CR-300 Chromameter (Konica Minolta; Les Ulis, France), and water holding capacity of the gluteus superficialis muscle (**WHC**). The WHC, pH₂₄, and L* were combined to compute a synthetic meat quality index (**MQI**) defined as a predictor of the technological yield of cured-cooked Paris ham processing, as described by the Tribout et al. (1996). In total, 27 traits were recorded.

Metabolomic Data

Blood samples were collected on sodium heparin once for every animal during the test period when animals weighed approximately 60 kg. Samples were immediately centrifuged at $2,500 \times g$ for 15 min at 4°C to separate plasma from red cells and stored at -80°C until analysis.

Fingerprinting was performed by proton nuclear magnetic resonance (**¹H NMR**) spectroscopy after a rapid sample preparation performed as follows: D₂O (500 μL) was added to plasma (200 μL) and mixed, the sample was then centrifuged for 10 min at $3,000 \times g$ at room temperature, and the supernatant (600 μL) was transferred to 5-mm nuclear magnetic resonance (**NMR**) tubes for ¹H NMR analysis.

All ¹H NMR spectra were acquired on a Bruker Avance DRX-600 spectrometer (Bruker SA, Wissembourg, France) operating at 600.13 MHz for ¹H resonance frequency and equipped with a pulsed-field gradients z system, an inverse ¹H-¹³C-¹⁵N cryoprobe attached to a cryoprotectant (the preamplifier cooling unit), and a temperature control unit maintaining the sample temperature at $300 \pm 0.1^\circ\text{K}$.

The ¹H NMR spectra of plasma samples were ac-

Table 1. Number of pigs in each breed × batch combination

	Batch							
	1	2	3	4	5	6	7	8
Large White, dam breed	42	45	54	13	16	20	9	0
Landrace, dam breed	22	39	51	0	21	28	27	0
Pietrain, sire breed	0	37	29	5	0	33	0	17

quired at 300K using the Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence with presaturation with a total spin-echo delay ($2n\pi$) of 320 ms to attenuate broad signals from proteins and lipoproteins, which otherwise display a wide signal and hide the narrower signals of low molecular weight metabolites. The ^1H signal was acquired by accumulating 128 transients over a spectral width of 12 ppm (note: chemical shift units kept ppm), collecting 32,000 data points. The interpulse delay of the CPMG sequence was set at 0.4 ms with $n = 400$ as defined in the following sequence: $[90-(\tau-180-\tau)n$ acquisition]. A 2-s relaxation delay was applied. The Fourier transformation was calculated on 64,000 points. All ^1H NMR spectra were phased and the baseline corrected. The ^1H chemical shifts were calibrated on the resonance of lactate at 1.33 ppm. Then serum spectra were data-reduced before statistical analysis using AMIX software (Analysis of Mixtures version 3.8; Bruker Analytische Messtechnik; Rheinstetten, Germany). The spectral region δ 0.5 to 10.0 ppm was segmented into consecutive nonoverlapping regions of 0.01 ppm (buckets) and normalized according to the total signal intensity in every spectrum. The region around δ 4.8 ppm corresponding to water resonance was excluded from the pattern recognition analysis to eliminate artifacts of residual water. Eight hundred eleven quantitative variables were obtained for every spectrum and were processed by a multidimensional scaling-based procedure to select only informative metabolic variables. More precisely, the multidimensional scaling step, which was repeatedly used ($n = 8$) to select fully informative variables, was performed on the transposed matrix of data. Multidimensional scaling is a multidimensional statistical technique that corresponds here to a principal components analysis of the matrix of distances between variables. Fully informative metabolic variables display a larger variance than baseline variables; therefore, the distances between these 2 types of variables are larger than the distances between the sole baseline variables. Therefore, at each selection step and for every variable, we calculated a distance between the origin and projection coordinates of the variable on the first factorial plan, and variables displaying the larger distances were subsequently selected. After 8 selection steps, only baseline relevant variables were remaining in the unselected dataset and were not included in the informative dataset on which further statistical analyses were achieved. Finally, each metabolomic profile or spectrum was observed on a discrete sampling grid of size $p = 375$ (number of buckets) as plotted in Figure 1.

Technical duplicates were performed on a limited number of animals and showed a reasonable repeatability between them (not shown) as expected. Because it was impossible to standardize feeding conditions on the farm and

exact age, large samples within breeds were performed.

The result of a metabolomic experiment is a spectrum in which some points are known to correspond to 1 or several metabolites but not all. Identification of candidate informative metabolites (after the statistical treatment described below) was performed from known chemical shift references acquired on standard compounds and found in the literature or in a locally developed reference databank. The 2-dimension homonuclear ^1H - ^1H correlation spectroscopy (COSY) and 2D heteronuclear ^1H - ^{13}C heteronuclear single quantum coherence spectroscopy (HSQC) NMR spectra also were registered for selected samples as an aid to spectral assignment. For COSY NMR spectra, a total of 32 transients were acquired into 1,024 data points. A total of 256 increments were measured in F1 using a spectral width of 10 ppm and an acquisition time of 0.28 s was used. The data were weighted using a sine-bell function in the 2 dimensions before Fourier transformation. For HSQC NMR spectra, a relaxation delay of 2.5 s was used between pulses, and a refocusing delay equal to $1/4^1J_{\text{C-H}}$ (1.78 ms) was used. A total of 1,024 data points with 64 scans per increment and 512 experiments were acquired with spectral widths of 10 ppm in F2 and 180 ppm in F1. The data were multiplied by a shifted Qsine-bell function before Fourier transformation.

Wavelet Preprocessing: Online Supplemental Data (Figures S1 to S4)

As proposed by Davis et al. (2007) and Xia et al. (2007), each metabolomics profile was written as the sum of weighted elementary functions, describing hier-

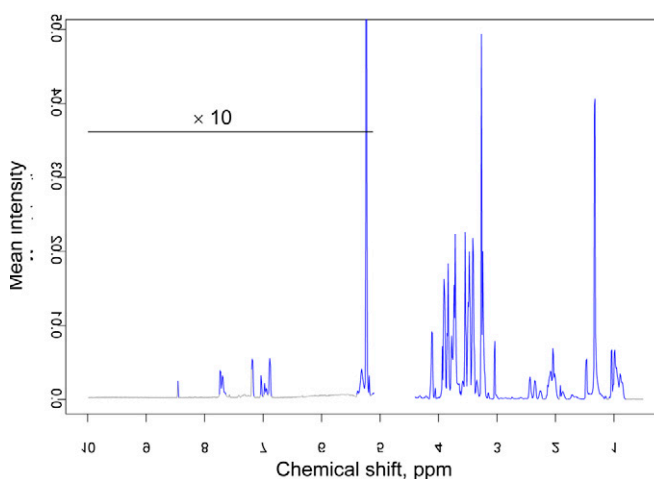


Figure 1. Proton nuclear magnetic resonance (^1H NMR) spectrum acquired on plasma collected on a single growing pig weighing 60 kg. Informative variables preselected by a multidimensional scaling procedure performed on the transposed matrix of metabolomic data transformed into 0.01-part per million (ppm) buckets are colored in blue, when residual information found in baseline is colored in grey. A 10-fold magnification of the spectrum in the aromatic region above 5.15 ppm is applied. See online version for figure in color.

archically the signal from a rough tendency to the finest details, in a finite number of resolution levels. Here, each of the 506 spectra was decomposed onto a Haar basis (elementary step functions). The corresponding wavelet coefficients were thresholded with a soft-thresholding method (see Mallat, 1999, for details) to reduce signal noise by applying low smoothing. We decided to keep the wavelet coefficients of every resolution level, from which the original spectrum can be rebuilt. In the data described in this article, the number q of wavelet coefficients was equal to 367. Another basis, the Daubechies basis made of smooth trimodal elementary functions was used also and gave $q = 388$ wavelet coefficients. A more detailed description of the wavelet decomposition can be found in the Online Supplemental Data (see online version of article at <http://journalofanimalscience.org>).

Selection of Variables for Prediction

Many prediction methods are described in the literature. Among the most well known, the partial least squares (PLS; Wold, 1966) and random forest (Breiman, 2001) methods use all variables whereas the least absolute shrinkage and selection operator (LASSO; Tibshirani, 1996), elastic net (Zou and Hastie, 2005), or sparse PLS (Lê Cao et al., 2008) methods incorporate a feature selection step leading to a reduced number of explanatory variables in the model. Some of these methods (Y. Baraud, C. Giraud, and S. Huet, personal communication; R package available at http://w3.jouy.inra.fr/unites/miaj/public/perso/SylvieHuet_en.html) were performed on our dataset and provided similar results in terms of predictive power (data not shown).

In the case of high dimensionality of the explanatory variables, a feature selection approach is useful for highlighting a limited number of variables of high predictive importance. In general, retaining in the prediction model only a set of useful variables avoids overfitting and ensures a smaller prediction error.

Any variable selection method could have been used here, either on the raw metabolomic data or on the thresholded wavelet coefficients, to select the relevant set of parameters. In both cases, this represents a classical problem for variable selection in a linear model. We decided to present here only the most widely used method: the LASSO technique. Introduced by Tibshirani (1996), the LASSO method is a penalized least squares approach used to solve ill-posed or badly conditioned linear regressions. The main interest of this approach comes from the fact that the solution leads to a restricted number of nonzero coefficients and this number depends on the value of the regularization parameter.

Identifying the points (buckets) of the metabolomic profile that contributes the most to phenotype prediction

can then lead to a biological interpretation step. Indeed, some “peaks” (not all) in the profile have already been identified by biochemists to correspond to specific metabolites (1 or more metabolites per peak). However, in the case of data preprocessing, a single wavelet coefficient can correspond to a large interval in the metabolomic profile, making further interpretation more delicate. Therefore, only lists of biomarkers obtained from raw data are presented in the following sections.

Estimation of Predictive Power

The LASSO technique was applied on 3 versions of the data collected for the 27 phenotypes described in the Data subsection: raw data and thresholded wavelet coefficients obtained with the Haar basis and with the Daubechies basis.

The parameters of each model (see Models below) were estimated first on a subset of the data (learning set with 400 observations) and then performances were calculated on the remaining dataset (test set with 106 observations). The regularization parameter was tuned by cross validation on the learning set.

The global procedure (estimation of the set of relevant parameters on the learning set and estimation of performances on the test set) was repeated 100 times on several random splits of the whole dataset. These random splits took into account the experimental setting of Table 1. This led to a collection of performance values that could be displayed in a boxplot to evaluate the degree of accuracy of each method as well as its variability.

Performances were evaluated using the mean squared errors of prediction (MSEP) standardized by the variance of the observations averaged on the 100 test sets. Note that the MSEP is not upper bounded, so it can go to infinity for very low predictive powers. However, the smaller the MSEP is, the better is the predictive power. A Kolmogorov-Smirnov test of distribution equality was computed for the MSEP on the 100 replicates to test whether 2 methods were comparable. Paired t -tests were used to test the superiority of 1 method against another in terms of MSEP.

To achieve a more detailed comparison between the results of all tested methods, we counted the number of appearances of each selected variable (bucket, Haar coefficient, and Daubechies coefficient) over the 100 replications, for each dataset (raw data and wavelet coefficients obtained either with Haar basis or with Daubechies basis).

Models

We focused on 3 different problems in this article: the prediction of a phenotype based on the metabolomic data alone (Model 1), on breed information and the me-

tabolomic data (Model 2), and on batch and breed information and the metabolomic data (Model 3). We considered a linear relationship between a phenotype and the explanatory variables in all 3 models described above.

Model 1 had the following explanatory variables: intercept (always in the model) and the metabolome variables (subject to variable selection: 375 for raw data and 367 or 388 for wavelet coefficients with Haar or Daubechies, respectively). Model 2 included a breed effect (always in the model), and the following effects that were subject to variable selection: metabolome variables and breed \times metabolome interactions. Finally, Model 3 included breed and batch effects (both always in the model) as well as metabolome variables, breed \times metabolome, and batch \times metabolome interactions (subject to variable selection).

Canonical Analyses

Complementary statistical analyses were performed by regularized canonical analysis using the R package mixOmics (Lê Cao et al., 2009). Two datasets consisting of phenotypic and metabolomic variables were represented to evidence the maximal correlations between variables, both within and between the 2 datasets.

RESULTS

Comparison of Models

For all phenotypes, the models based on a wavelet preprocessing step were in general slightly better or at least equal in terms of prediction error than the one based on the direct use of raw metabolomic data (Supplemental Figures S5, S6, and S7). The efficiency of the preprocessing step was most obvious when only metabolomic information was considered in the model (Model 1). This is well exemplified in the 3 data versions of ADFI, both in terms of MSEP and number of selected coefficients (Figure 2), using only the metabolomic information as explanatory variables (Model 1). Indeed, MSEP values were observed to decrease as was the median number (and strikingly the range) of selected coefficients of the LASSO regression when wavelet preprocessing of data using the Daubechies basis but not the Haar basis was applied. This was corroborated by the comparison of preprocessing methods given by the Kolmogorov-Smirnov test for the MSEP (P -values for raw data vs. Haar = 0.58, raw vs. Daubechies = 1.3×10^{-5} , and Haar vs. Daubechies = 1.6×10^{-2}). Therefore, transformation of the signal with wavelets implied significant differences in the prediction errors for ADFI. Moreover, the results also showed that a phenotype of interest such as ADFI could be well predicted with no need for any additional information on the individuals.

When looking into which preprocessing methods gave the best MSEP on average over all phenotypes, no clear conclusion appeared for Model 1 (Supplemental Figures S11 and S12), but Daubechies was overall to be preferred to Haar for Model 2 (Figure 3 or Supplemental Figures S6, S11, and S12) and Model 3 (Supplemental Figures S7, S11, and S12). Moreover, the wavelet transform with the Haar basis gave numerous extreme results in terms of MSEP. This was more detectable in Model 2 than in Model 1 (Supplemental Figures S5 and S6). Finally, the P -value of the 2-sided Kolmogorov-Smirnov test was equal to 4×10^{-5} for ADFI, meaning that there can be a significant difference due to preprocessing in the prediction results for some phenotypes.

Prediction of Phenotypes Related to Animal Breeding and Carcass Characteristics Using Metabolomic Data

The variation of the prediction levels among all phenotypes was very similar whatever the statistical method used. We present here the results obtained using 1) the best wavelet transform (with Daubechies basis) and 2) the simplest approach, namely the LASSO method applied to the raw dataset (Supplemental Table S2; Figure 3), hence retaining the possibility for a more direct biological interpretation of the results than when a wavelet transform preprocessing step is applied (see below). The mean prediction errors (expressed in phenotypic variance units) varied from 0.3 to >1 . The worst predictions (largest values of MSEP) are obtained for weights measured near slaughter time (i.e., LWETP, CWwtH, HCW, CW, and LWS) and for some phenotypes related to postmortem meat processing (i.e., pH₂₄ and L*). For LMP, which was the best predicted phenotype with a MSEP value of approximately 0.3, the squared correlation (R^2) between observed values and fitted values obtained on the training sample set was equal to 0.82. A R^2 value between observed and predicted values of 0.69 was observed for the

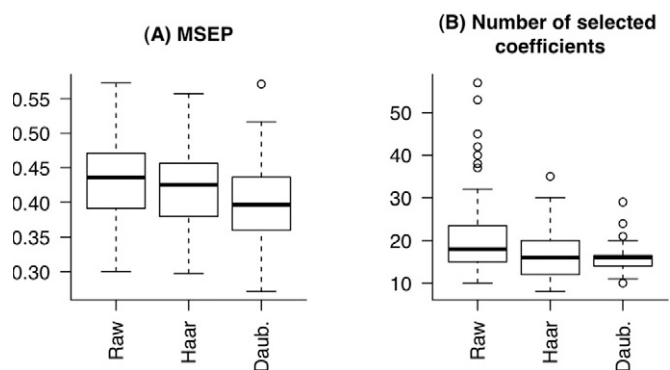


Figure 2. Prediction of ADFI. Boxplot of the preprocessing methods considered over 100 resampling replicates, in the model with metabolomic data only, on raw data (Raw), preprocessed data with Haar wavelet transformation (Haar), and Daubechies wavelet (Daub.). (A) Mean square error of prediction (MSEP) and (B) number of selected coefficients.

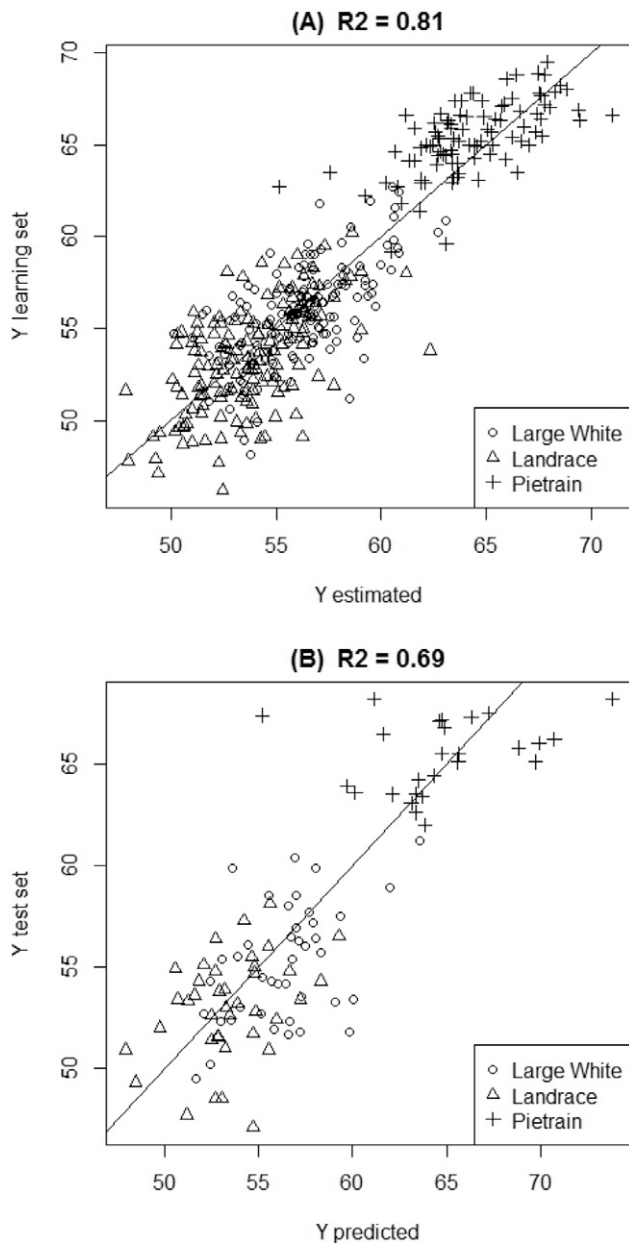


Figure 3. Lean meat percentage phenotype. Estimated values on the learning set (A) and predicted values on the test set (B), both against the true values. Predictive model with metabolomic data only.

test sample set, showing reasonable adequacy between observations and adjustments from the model (Figure 4). Use of more complex models was useful to obtain greater prediction scores for some traits as described hereafter.

Reinforced Phenotypic Prediction Using Both Metabolomic and Breed Information (Model 2). The phenotypes considered here could be sorted into 4 classes depending on their degree of predictability as shown in Figure 3, ranging from the best (class C1 with a MSE/P less than 0.2) to the worst (class C4 with a relative error rate greater than 0.70). All phenotypes belonging to the classes C1 and C2 were better predicted when the breed was considered in the

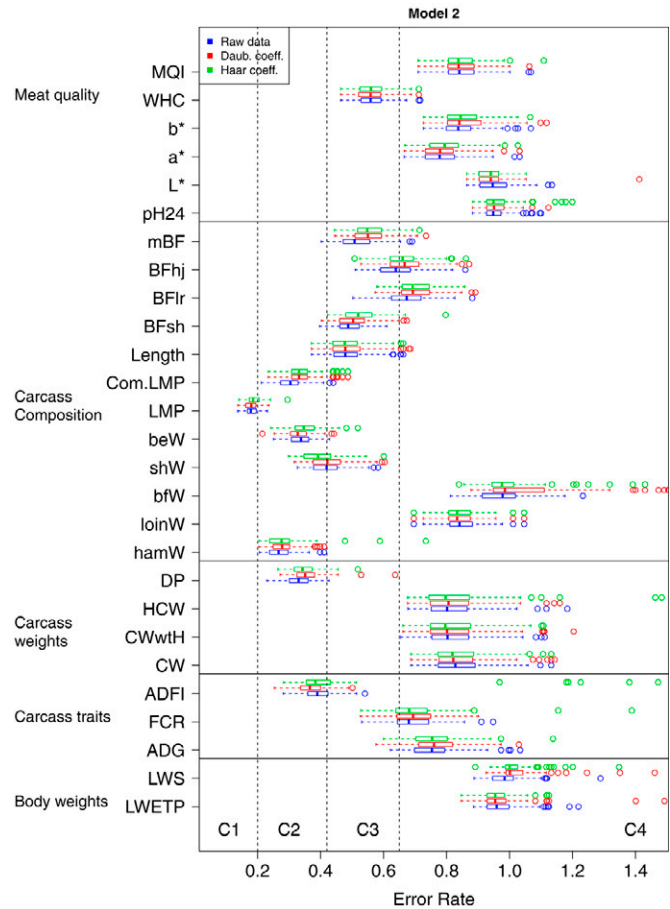


Figure 4. Mean square error of prediction for all the considered phenotypes on the raw metabolomic data with breed information expressed in phenotypic variance units. Variables C1, C2, C3, and C4 define 4 classes of prediction accuracies. The 3 preprocessing methods are displayed [raw data, wavelet transformation with Daubechies basis (Daub. coeff.), and with Haar (Haar coeff.)]. The phenotypes: MQI = meat quality index; WHC = water holding capacity of the gluteus superficialis muscle; b^* , a^* , and L^* = color; pH_{24} = ultimate pH of the semimembranosus muscle; mBF = mean backfat thickness; BFhj = backfat thickness at the hip joint; BFlr = backfat thickness at the last rib; BFsh = backfat thickness at the shoulder; Length = length of the carcass from the pubis to the atlas; Com.LMP = commercial lean meat percentage; LMP = lean meat percentage; beW = belly weight; shW = shoulder weight; bfW = backfat weight; loinW = loin weight; hamW = ham weight; DP = dressing percentage; HCW = weight of the right half-carcass; CWwth = carcass weight without the head; CW = carcass weight with the head; FCR = feed conversion ratio; LWS = weight before departure to the slaughterhouse; LWETP = weight at the end of the test period. See online version for figure in color.

model (Supplemental Table S2; Figure 3; Supplemental Figures S8, S9, and S10).

Prediction Using Breed, Batch, and Metabolomic Information (Model 3). The batch variable does not appear to be a key parameter in the prediction of phenotypes (Supplemental Table S2; Figure 3; Supplemental Figures S8, S9, and S10). Indeed, MSE/P values were almost always slightly greater when batch was taken into account (except shW and DP for phenotypes of classes C1 and C2).

Selected Variables

As shown in Figure 2B for the ADFI phenotype, the

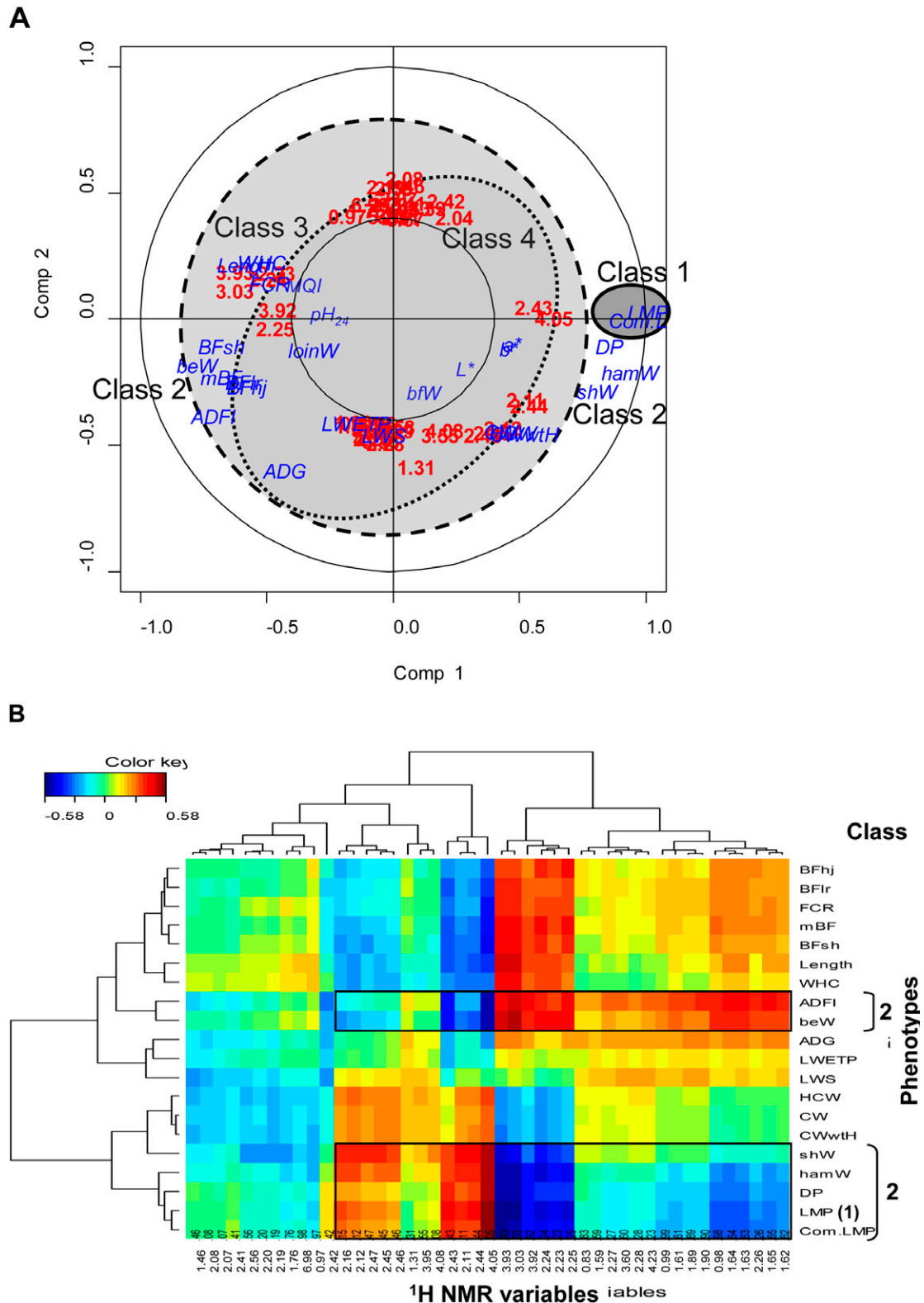


Figure 5. Canonical analysis between the proton nuclear magnetic resonance (^1H NMR) dataset (X) and the phenotype dataset (Y). A. Projection of variables. The ^1H NMR variables with correlation <0.4 were not plotted. B. Correlation heatmap between variables belonging to the 2 datasets (X and Y). Classes of variables refer to the prediction levels in Figure 3. The phenotypes: MQI = meat quality index; WHC = water holding capacity of the gluteus superficialis muscle; L^* , a^* , and b^* = CIELAB color scale; pH_{24} = ultimate pH of the semimembranous muscle; mBF = mean backfat thickness; BFhj = backfat thickness at the hip joint; BFlr = backfat thickness at the last rib; BFsh = backfat thickness at the shoulder; Length = length of the carcass from the pubis to the atlas; ComLMP = commercial lean meat percentage; LMP = lean meat percentage; beW = belly weight; shW = shoulder weight; bFW = backfat weight; loinW = loin weight; hamW = ham weight; DP = dressing percentage; HCW = weight of the right half-carcass; CWwTH = carcass weight without the head; CW = carcass weight with the head; FCR = feed conversion ratio; LWS = weight before departure to slaughterhouse; LWETP = BW at the end of the test period. See online version for figure in color.

Table 2. Variable selection for lean meat percentage (LMP) using the raw data for the three models: metabolomic data alone (Model 1), metabolomic + breed (Model 2), and metabolomic + breed + batch (Model 3)¹

Model 1		Model 2		Model 3	
δ , ppm (n)	Assignment	δ (ppm) (n)	Assignment	δ (ppm) (n)	Assignment
4.05 (100) PL	Creatinine	4.05 (100) PL	Creatinine	4.05 (100) PL	Creatinine
3.93 (100) NL	Creatine	1.04 (92) NL	Valine	2.25 (97) NL	Valine
2.43 (100) PL	Glutamine	2.54 (88) PL	Citrate, β -alanine, and unknown	1.04 (84) NL	Valine
1.33 (100) PL	Lactate	2.40 (78) PL	Glutamine	2.54 (83) PL	Citrate, β -alanine, and unknown
3.20 (97) NL	Choline, P-choline, and glycerol-P-choline	2.25 (78) NL	Valine		
1.45 (89) PL	Alanine				
2.15 (82) PL	Glutamine				
7.67 (80) NL	Unknown				
2.51 (74) NL	Citrate				
0.99 (74) NL	Isoleucine				

¹Chemical shifts (δ) in parts per million (ppm) and putative assignments are given. The appearance of the variable over the 100 replications is given in parentheses, with the threshold at 70. Metabolites that are positively linked with LMP are denoted by PL (positively linked), and negatively linked metabolites are denoted by NL (negatively linked).

number of selected coefficients was always smaller for preprocessed data using a wavelet transform than for raw data. Such transformed datasets gave more parsimonious models with smaller numbers of explanatory variables.

Concerning Model 2, it should be recalled that the breed effect did not undergo feature selection; in this setting, the minimum number of selected variables is 3. A nonempty set of metabolites is still of predictive importance in addition to the breed effect. For Model 3, breed and batch did not undergo selection; in this setting, the minimum number of selected variables is 11. The number of selected variables (metabolites and interactions; i.e., breed \times metabolome and batch \times metabolome) is less when the batch variable is not considered. It is to be noted that no interaction term between metabolite or wavelet coefficients and breed (or batch) was selected in Model 2 (or in Model 3).

A few of the explanatory variables obtained for the prediction of the LMP phenotype (Table 2) were the same when using raw data (Model 1) as when using Models 2 or 3. However, their number was significantly reduced when the breed factor was taken into account in Models 2 and 3 compared with Model 1. When using the bootstrap process, some variables were either mostly positively linked (**PL**; i.e., δ 4.05, 2.43, 2.15, 1.33, and 1.45 ppm), negatively linked (**NL**; i.e., δ 3.93, 3.20, 7.67, 2.51, and 0.99 ppm), or both NL and PL (δ 1.03, 2.25, and 1.47 ppm) to LMP (not shown). Only variables that are steadily linked, either positively or negatively, such as creatinine (δ 4.05 ppm, PL), creatine (δ 3.93 ppm, NL), choline or phosphocholine or glycerophosphocholine (δ 3.20 ppm, NL), glutamine (δ 2.43 and 2.15 ppm, PL), lactate (δ 1.33 ppm, PL), alanine (δ 1.45 ppm, PL), and isoleucine (δ 0.99 ppm, NL) can be considered for

the elaboration of the functional hypotheses that could explain how the LMP phenotype can be predicted from these serum biomarkers. Interestingly, as displayed in Figure 5A, canonical analysis performed on all the variables present in the 2 datasets (i.e., ¹H NMR and phenotype) demonstrated that the phenotypic variables belonging to the classes C1 and C2 were also those that were steadily selected in Models 1, 2, and 3. So, the positive correlation underlined by the LASSO-based regression between LMP and creatinine (δ 4.05 ppm) or glutamine (δ 2.43 ppm) is again well evidenced, as is the negative link between LMP and creatine detected at δ 3.93, 3.92, and 3.03 ppm (Figure 5B). This significant correlation between LMP and creatine is also well evidenced for class 2 phenotypes such as ComLMP, DP, shW, hamW, beW, and ADFI (Figure 5B). Citrate also would be found as NL regressor of LMP when considering the chemical shift at δ 2.51 ppm in Model 1 but would be found as PL regressor of LMP if we consider the variable at δ 2.54 ppm. The 2D ¹H-¹H COSY and ¹H-¹³C HSQC NMR spectra showed that signals at 2.51 and 2.54 ppm belong to citrate. Indeed, HSQC NMR spectra showed correlation between ¹³C chemical shift at 48.6 ppm and ¹H chemical shift at 2.51 and 2.54 ppm. Chemical shift at δ 2.51 and 2.54 ppm have been assigned to citrate and correspond to a doublet even though the chemical signal recorded at δ 2.54 ppm may contain also a low intensity signal attributable to β -alanine (correlation between the signals at 3.17 and 2.54 ppm in the COSY spectrum) and an unknown compound (correlation between the signals at 2.39 and 2.54 ppm in the COSY spectrum). Quantitative information measured at these 2 chemical shifts are correlated ($\rho = 0.35$) and would be in favor of an assignment to citrate even though the correlations with LMP

are of different signs but based on different models involving very different numbers of regressors (Table 2).

Reasoning at Constant BW

There was some variability in the development status of the pigs included in the dataset, both at the time of blood sampling and at the time of slaughter. To be able to compare samples, the BW of the animal before departure to the slaughterhouse (**LWS**) was added as a covariable in the 3 models described previously. Then the phenotype prediction could be considered as being at constant weight. Focusing on the LMP phenotype, the results obtained with these 3 modified models were similar in nature to those presented previously; knowledge of the breed improved the prediction of phenotype and decreased the number of explanatory variables selected. Moreover, the relation between LMP and the few variables referred to above (PL or NL) was preserved. More precisely, the lists of important metabolites were larger and included those already highlighted in the model that did not take into account the animal BW. However, the prediction power was slightly less when the BW at slaughter time was considered (not shown).

DISCUSSION

In this article, we showed that it is possible to use metabolomic data from a plasma sample to better predict some production phenotypes in growing pigs. Metabolomic data alone are sufficient to predict these phenotypes. Additional information and predictive power are provided by the metabolome when the breed of the animal is known. For data from a test farm, small variations in a breeding environment, which are classically summarized in a batch effect, did not disrupt phenotype predictions. Additionally, although this work was centered on prediction accuracy, we supplied supplementary information on a limited number of metabolites that have, as valuable biomarkers, a high predictive power. The biological coherence of the list of biomarkers validated the whole data analysis. In addition, a methodological aspect of the statistical treatment was related to the specificity of ^1H NMR metabolomic data: a pretreatment of the signal based on the use of wavelets.

Justification of the Statistical Treatment

Metabolomic profiles are continuous by essence. Discretization is performed routinely (bucket steps). The bucket size was rather large with 0.01 ppm, to avoid a possible misalignment between spectra, due to shifts of signals, a rather rare phenomenon but still occurring. Actually, small shifts at 2 to 3 regions of the spectrum re-

corded in plasma samples were locally observed for some samples that were reanalyzed with the same spectrometer at 2 different times (not shown). This motivated the choice of a relatively large bucket size (0.01 ppm) even though a consequence is that some buckets could contain more than 1 compound. However, the primary goal of this work was prediction and not biological interpretation.

To recover the continuity of the signal, which is moreover nonregular, we proposed the use of wavelet decomposition, which is one of the most commonly used signal transformation approaches. The underlying idea is to decompose a complex signal into elementary forms (orthogonal functions or basis). Unlike Fourier transformation, the wavelet approach is particularly suited for uneven and chaotic signals, making it a method of choice for NMR profiles, and it has already been applied in such a context by Davis et al. (2007) and Xia et al. (2007). An improvement due to the use of wavelet transformation was observed on our data but in a limited manner. Depending on the tissue (blood, urine, and other) and the stability of the baseline on the spectra, the wavelet approach could lead to a dramatic improvement of the signal (P. Martin, P. Besse, and S. Déjean, personal communication); approximations of the signal at the lowest levels (see Supplemental Material) correct rough fluctuations of the baseline. Results depended only slightly on the chosen wavelet basis in this study. When the signal is continuous, Daubechies wavelets are usually a better choice than Haar ones (step functions). The dependency on the basis is generally observed (e.g., Luisier et al., 2005, for image denoising; Mahmoud et al., 2007, for audio data).

Predictive Power: Valuable Aspects for All Phenotypes

An important methodological question arose before the global prediction analysis concerning the choice of preprocessing the ^1H NMR metabolomic spectra. When considering metabolomic data only as predictive variables of highly functionally integrated phenotypic variables, as shown here, the wavelet transformation of original data led to best performances.

Adding information concerning the breed led to fewer errors of prediction whereas adding batch information did not really improve the prediction results. Moreover, the batch even seemed to constitute a noisy endogenous variable because the predictive power in Model 3 was slightly less than in Model 2. Interestingly, in the breeding conditions encountered here, this meant that we could put aside the possible microenvironmental effect, which may vary from batch to batch, for a phenotype prediction objective. The environmental effect on the phenotype, particularly diet variation, is probably captured by the metabolomic information (Yde et al., 2010). Thus, given the

fact that data are obtained in a control farm that ensures standardized breeding conditions, some phenotypes of interest such as LMP can be well predicted without having to characterize more precisely the microenvironment of a given batch of growing individuals. The same phenomenon seems to be encountered for the slight variations of animal weight or age that were observed in the dataset; the metabolome carries some information pertaining to developmental differences so that the prediction of some phenotypes such as LMP is better without the weight information than with it.

Yet this conclusion is based on a large dataset issued from 3 breeds. Indeed, when similar analysis was undertaken within a given breed, predictions of phenotypes were disastrous (not shown). This can be explained by the smaller number of observations and by less variability of the within-breed phenotype.

Prediction Power among Phenotypes and Practical Implications

The prediction accuracy is very dependent on the phenotype being studied and surprisingly even within a group of related phenotypes. Canonical analysis confirmed that the LASSO-based predictions and the same 4 classes of prediction of the different phenotypes were identified. Two groups of phenotypes were poorly predicted (class C4 of prediction). They correspond to the values of some weights (LWETP, LWS, and CWwtH) that depend directly on the decision to send animals to the slaughterhouse or not. Therefore, these phenotypes can be considered as negative controls, because they should be poorly predicted by essence and not worth predicting. Meat quality measurements (pH₂₄, L*, a*, b*, WHC, and MQI) were poorly predicted possibly because meat quality is highly influenced by preslaughter conditions and the blood sample was collected at the test farm during the growing period between 60 and 70 kg BW. Indeed, pH is known to be very sensitive to the duration of feed deprivation and transportation. Moreover, evidence of stress conditions has been observed on NMR metabolomics in pigs (Bertram et al., 2010) near slaughter or in sheep (Li et al., 2011). Meat quality, even though it does not represent a direct objective for the selection because it is difficult to measure, could be potentially considered as a prime objective if reliable predictions were available. Metabolomic data from a single blood sample, taken approximately 3 wk before slaughter, are clearly not sufficient for such an ambitious task for this complex trait.

Backfat measurements (BFsh, BFlr, BFhj, and their average mBF) all showed a medium degree of predictability (class 3 of prediction), potentially linked to the dynamics of fat deposition during growth, which essentially occurs after 70 kg BW. However, the metabo-

lome-based prediction of these phenotypes is not crucial because they are easily measured on the living animal. Carcass length (Length) displayed also a limited prediction level but is of no economic interest to date.

In the last groups of phenotypes, 1 phenotype within each group was accurately predicted but the others were not. Concerning traits recorded during growth (ADG, FCR, and ADFI), we observed that ADFI was better predicted than ADG and FCR separately. Individual measurements of ADFI require specific and expensive equipment and hence are performed rarely. However, it represents a very important criterion from an economic perspective and presents a moderate to acceptable level of prediction here. As regards carcass efficiency, DP was actually quite well predicted (class 2 of prediction) even though individual weights (CW and LWS) were not. The lean meat content estimated from cut weights (LMP) displayed the greatest prediction accuracy (class 1 of prediction). The prediction of weights for separate cuts varied from poor to acceptable but was always worse than LMP. Lean meat content is a crucial trait for breeders because it directly influences the payment for carcasses. Two measurements were available and ComLMP and LMP are highly correlated. The latter measurement is time consuming and requires half of a carcass for the cutting of the various pieces. The LMP impacts the income of the breeder and the slaughterhouse and displayed the highest predictability level among the phenotypes considered here as well as among those included in the current selection objective (i.e., MQI, ADG, FCR, and LMP).

A Possible Biological Interpretation of the Prediction Performance of Lean Meat Percentage

The purpose of this work was not to dissect the metabolic mechanisms linked to the measured traits but to quantify the power of prediction of NMR metabolomic spectra for production and quality traits. Discussing biological aspects of the most predictive metabolites can be proposed but only to check biological coherence of the whole statistical process. Because of a risk of over-interpretation, we chose to limit the discussion on that point. Thus, the results described above can be validated considering the coherent biological significance of the metabolites selected to predict LMP. Indeed, a connection between the phenotype LMP and some metabolites found in plasma has been highlighted. It involves 1) 3 AA: valine, alanine, and glutamine; 2) an energetic intermediate of the Krebs cycle, citrate; 3) an end metabolite of AA, creatinine, and its precursor creatine; and 4) choline, a quaternary ammonium derivative, involved in the biosynthesis of the choline-containing phospholipids, acetylcholine and betaine.

In Model 1, the LMP measured at slaughter is posi-

tively linked to circulating creatinine and negatively linked to creatine measured between 60 and 70 kg BW. Creatinine is directly linked to the muscular mass and as such is correlated to the total AA catabolism in muscle, which may depend on gender and hormonally based anabolic treatment (Dumas et al., 2005). Interestingly, when no qualitative covariate such as breed (Model 2) or batch (Model 3) was used in the prediction model, creatine was found in plasma as an independent variable negatively linked to LMP. This may imply that the energetic requirements needed to sustain muscular metabolism are adjusted in a coordinated manner according to the relative potential to increase muscle mass and result in different circulating concentrations of creatine. When breed or batch covariates are introduced in the models, creatine is not found as a main independent variable. Probably, creatine as precursor of phosphocreatine, this phosphagen represents the greater part of the total P-bonded energy in muscle instantaneously available to regenerate ATP (Hochachka, 1994; Brosnan and Brosnan, 2007), is metabolized at different levels in the different breeds because it seems to be linked to a final LMP phenotype, which is strikingly differentiated between breeds and probably between genders. Glutamine, detected at δ 2.43 ppm, and lactate, detected at δ 1.33 ppm, also displayed a differential pattern of energy supply to muscle, which was positively correlated to LMP between breeds (and genders). Glutamine, as a functional AA is involved in multiple metabolic pathways and regulates gene expression and signal transduction pathways (Wu, 2010; Wu et al., 2011). Among its different physiological functions, it is an important energy substrate, more particularly for rapidly dividing cells such as enterocytes. Within-breed (and -gender) variations in LMP also are positively correlated to citrate. As for phosphagen P-creatine, a greater potential in muscle accretion seems to be coordinately sustained by systemic bioenergetic adaptation observed at the level of the citric acid cycle and lactate metabolism. Unfortunately, complementary observations are lacking so it is difficult to provide, at this stage, sound physiological interpretation concerning the relative involvement of factors related either to the genetic background or to a gender-adjusted physiology of such energetic homeostatic adjustments. Indeed, there are here 2 confounded factors leading to LW or LR castrates on one side and PI females on the other.

Because the data (raw, Haar transformed, or Daubechies transformed) may have some influence on the selected metabolites, we displayed on the mean spectrum the regions corresponding to the selected variables, on the particular case of Model 2 for the LMP phenotype as a matter of example. These results showed that the use of raw data is the best approach if one is interested in a biological interpretation whereas the preprocessing using the Daubechies basis is overall the best approach in the case of prediction even though its effect is not tremendous on our dataset. The pre-

processing with the Haar basis appeared as a trade-off between the 2 goals: biological interpretation and phenotype prediction.

The 3 approaches all pointed out the fine region of the spectrum corresponding to creatinine (4.05 ppm). The selected points of the raw data were included in the larger regions pointed out by Daubechies, which displayed regions too large to be interpretable.

The purpose of this article was to predict a phenotype with NMR metabolomic profiles. This is different from an analysis aiming at dissecting the phenotype and discovering metabolites underlying the trait. We only proposed a discussion on the selected metabolites (i.e., those with the greatest predictive value) for the sake of biological coherence. In this context, it is not a problem that the same metabolites are selected for 2 highly correlated phenotypes. This could be due (or not) to a common set of metabolic mechanisms.

Metabolomic profiles are now relatively cheap. One may use them in practice to obtain targeted metabolic information for identified biomarkers or to predict phenotypes of economic interest. Several samples could be considered during an animal's life, depending on the phenotypes desired (i.e., linked to growth during the breeding period or to meat quality near slaughter time). Generally speaking, metabolomic-based prediction of production phenotypes would be of practical interest in animal selection, especially when phenotypes cannot be measured directly on selection candidates because the measurements require slaughter (carcass efficiency traits or meat quality traits) or are too expensive (feed efficiency). The current solution is to measure these traits on relatives of selection candidates, and this information is used to predict the genetic value of the candidates. However, phenotypic measurements performed on the animal itself rather than on its relatives would provide more accurate predictions of the genetic value. If individual meat quality traits could be predicted by accurate indirect measures (based on metabolome profiles), selection would be more efficient than when based on the performances of relatives, which is, moreover, more expensive. The first results obtained in this study need further validation before any practical use in selection schemes.

In conclusion, metabolomic data can be used to predict a phenotype without any further knowledge of the individual. Nevertheless, this prediction ability is again improved when the breed information is available as additional data. For prediction purposes in general, a well-adapted method of reducing noise in data coupled with a sparse prediction approach is to be recommended. This is the first time to our knowledge that breeding and production traits on growing pig have been predicted on the basis of a single blood sample collected on the living animal during its breeding period. The prediction accuracies varied considerably among the traits, and some of them showed an accurate prediction.

We are enthusiastic about the finding that some economically important traits can be predicted from a simple NMR metabolomic profile obtained from blood.

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Supplementary Material

Supplementary material can be found at:

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