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Application of a non-invasive tool and identification of genetic markers in swine to enhance ham quality: a preliminary study

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ABSTRACT

A genome-wide association study (**GWAS**) was performed as a preliminary step to identify regions potentially related to ham quality traits. In this research, genomic information was obtained from 238 commercial hybrid pigs utilising the GeneSeek® Genomic Profiler genome-wide porcine genotyping array. Carcasses were tested for hot weight, the thickness of backfat and loin, and lean meat percentage. The corresponding fresh hams were assayed for weight and ultimate pH; the activities of Cathepsin B and Ferrochelatase of *Semimembranosus* muscle were determined through fluorimetric methods. The lean meat percentage of fresh ham (**LMPH**), salt absorbed after first (**SALT1**) and overall salting stages (**SALT**) were estimated online by the Ham Inspector™ apparatus. Hams were processed in compliance with the procedures established for Protected Designation of Origin Parma ham, and ham weight losses were measured at the main processing stages. Hot carcass weights showed a significant negative correlation with their lean meat percentage and LMPH, while LMPH was correlated positively with carcass lean meat, SALT1, SALT, and weight losses. The GWAS detected genome-wide association for 12 single nucleotide polymorphisms with Ferrochelatase activity. The results obtained in this preliminary study were achieved by combining innovative and non-destructive technologies for screening hams under processing, measures of enzymatic muscle properties relevant to dry-cured ham quality, and genomic information obtained through a GWAS. Additional studies carried out in a larger number of pigs have been planned to investigate the effect of gene variants of Ferrochelatase activity in the dry-cured ham's quality with main reference to colour development and to confirm the GWAS results obtained in this study.

Implications

In this study, we observed that ham proneness to salt absorption and weight loss are related to fresh ham traits measured with an online control system. This would imply the possibility to adapt the dry-cured ham process management to thigh characteristics. We also detected strong associations between mutations in the pig genome and the muscle Ferrochelatase activity involved in the synthesis of Zinc Protoporphyrin, the natural purple-red pigment occurring in dry-cured hams. This achievement provides information to enhance the aptitude of thighs to produce no-nitrite dry-cured hams with adequate colour.

Introduction

Among the Italian Protected Designation of Origin (**PDO**) meat products, Parma and San Daniele hams play a role of primary importance in the Italian pig production chain, with an economic value in the year 2020 of 687 and 309 million euros, respectively (Qualivita-ISMEA, 2021). In addition to processing conditions, final product quality relies on fresh ham properties, which in turn are dependent on pig genetics, growth efficiencies, and diet composition. To meet required quality features, Parma and San Daniele hams can be exclusively obtained from thighs originating from pigs fed with feedstuffs included in the product regulations (Parma ham – General Rules and Dossier, 1992; San Daniele ham – General Rules and Dossier, 2007) and subsequent revisions (Official Journal of the European Union, L 68/2 of 6/3/2023; Official Journal of the European Union, L 68/94 of 6/3/2023). Moreover, Italian heavy pigs intended for PDO dry-cured hams were derived from a selection programme from or in agreement with the Italian

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herd book maintained by the National Association of Pig Breeders (ANAS, 2020), and only specified breeds or approved hybrids are allowed. Heavy pigs and single carcasses must meet specific requirements in terms of age at slaughter, weight range, and classification in the EUROP grid; pigs intended for the PDO chain are reared for at least 9 months up to a live weight of 160 kg (average weight of the batch at slaughter). Pigs meeting the above requirements are classified as "heavy". Hams must fall within an established weight range, have a minimum subcutaneous fat thickness, and specified limitations in the trimming shape. Further, the composition of ham covering fat cannot exceed established values of iodine number and linoleic acid (Parma ham – General Rules and Dossier, 1992; San Daniele ham – General Rules and Dossier, 2007).

In the last years, there has been a growing interest in evaluating fresh hams by non-invasive tools working online and in developing analytical and genomic markers predicting the quality of the final product. Recently, non-destructive techniques were tested in ham traits detection (Pérez-Santaescolástica et al., 2019). The single-frequency magnetic induction Ham Inspector™ system (Lenz, Barcelona, Spain) has been able to predict the lean meat percentage of fresh ham and monitor salt intake in a fast, simple, and reproducible way (Zappaterra et al., 2021). The signal generated by the Ham Inspector™ system is based on the measurement of perturbation caused by an alternating excitation magnetic field on a conductive sample as in the case of meat.

Cathepsin B was thoroughly investigated in research on ham texture and flavour development (Toldrá, 2006), and the correlation of its activity with muscle protein degradation was confirmed for Italian PDO dry-cured hams (Piasentier et al., 2021). In addition to proteolysis, colour is a key quality marker of dry-cured ham. The typical colour of Parma ham is due to the formation of the purple-red pigment Zinc Protoporphyrin (**ZnPP**) in which Zn(II) is inserted in Protoporphyrin IX. The synthesis of ZnPP has been suggested to be totally or partially enzymatic, and a role was assigned to the endogenous Ferrochelatase. Ferrochelatase activity, meat pH, and haem pigment content have been reported as the main endogenous factor affecting ZnPP formation in nitrite-free ripened meat products (Wakamatsu, 2022).

Some studies were carried out to identify candidate genes for green hams to be processed into high-value dry-cured hams (Škrlep et al., 2010; Davoli et al., 2017). Identifying genetic markers associated with the activity of endogenous enzymes involved in proteolysis and ZnPP formation may be of primary importance in selecting pigs suitable for producing high-quality dry-cured hams. However, as far as the authors' knowledge, scientific literature lacks studies associating variations in Ferrochelatase activity in fresh hams with DNA markers.

In this preliminary study, 238 commercial hybrid heavy pigs intended for PDO dry-cured ham production were tested with the following objectives:

(i) to widen the assayed ham traits predictive of the final quality of dry-cured hams;
(ii) to evaluate the relationships between phenotypic traits of carcass and hams under processing;
(iii) to apply a genome-wide association study (**GWAS**) to identify regions related to ham quality variability.

Material and methods

Animals

In this study, commercial hybrid pigs belonging to three Italian finishing pig farms were provided for the trial. No pedigree data nor litter information were available, as commercial pigs are reared collectively after weaning and piglets from different litters are mixed. The heavy pigs included in this study were reared in pens containing a variable number of animals until at least 9 months of age, according to the management rules decided by each farmer. Pigs were fed following the constraints indicated by the Parma ham regulation (Parma ham – General Rules and Dossier, 1992): the diet provided to the animals was mainly based on cereals (corn and barley) and soybean, according to the DM content, independently from the growth phase. Animals were bred up to an average of 160 kg live weight per slaughtering batch, in agreement with the PDO Parma ham specifications. Each farmer delivered the pigs for slaughter twice, once during the autumn (September–October 2018) and once during the winter (December 2018–January 2019), in numerically similar batches per replica. The animals were slaughtered in two slaughterhouses on five slaughter days. Pigs were electrically stunned, and slaughter procedures were monitored by the veterinary team appointed by the Italian Ministry of Health. Animal care and slaughter of the pigs used in this study were performed in compliance with the European rules (Council Regulation (EC) No. 1/2005 and Council Regulation (EC) No. 1099/2009) on the protection of animals during transport and related operations, and at the time of slaughter, respectively.

Carcass traits

Hot carcass weight and thickness of backfat and loin were measured at the slaughterhouse. The Fat-O-Meat'er optical probe (Frontmatec A/S, Kolding, Denmark) was used to assess the thickness of the backfat and loin between the 3rd and 4th last ribs, 8 cm off the carcass midline. The carcass lean meat was calculated in compliance with European and Italian legislation (Commission Implementing Decision authorising methods for grading pig carcasses in Italy, 2014/38/EU – https://eur-lex.europa.eu). All carcasses have been uniquely numbered on the thighs to be able to trace them individually in the subsequent steps. Left thighs were dissected from the hot carcasses, stored at 0–2 °C for 24 h, shaped according to Parma ham regulation, and then delivered to a processing plant licensed for Parma ham production. Fresh hams with visible defects were not included in the trial.

Ham selection and processing

The weight of the left ham (**HW**) was measured after the trimming made in the processing plant, and the ultimate pH of the visible section of *Semimembranosus* muscle was measured at 48 h *postmortem* using a portable pH meter (WTW pH3110, Weilheim, Germany). A total of 238 fresh hams (from 114 barrows and 124 gilts) were selected (Table 1), to be sampled for analyses and monitored until full maturation, according to the weight range of 13.0–15.0 kg, required by the ham producer for a dry-cured ham process lasting 15 months, and to the ultimate pH range 5.5–6.0. Ham with low final pH is more prone to excessive muscle proteolysis, which can load excessive softness and bitter taste in aged hams (Virgili and Schivazappa, 2002). Conversely, high pH values and/or Dark Firm Dry meat prevent the normal moisture release from ham muscles, resulting in an inadequate adhesive texture (Guerrero et al., 1999).

All 238 fresh hams were processed in five batches according to the slaughtering days, following the standard procedure of the plant and in compliance with the procedures established for PDO Parma ham. The procedure applied for processing is summarised in Supplementary Table S1.

Phenotypic analyses

The parameter lean meat percentage of fresh hams (**LMPH**) was estimated at the processing plant by a Ham Inspector™ system

Table 1
Details of sampled fresh hams: the pig genetic types utilised, slaughter periods, slaughter plants, and slaughter days, and the number of animals from each farm are reported.

Farm	Crossbred	Slaughter Period	Slaughter Plant	Slaughter Day	n
F1	D × LW	P1	SP1	d1	35
F2	(D × LW) × (LW × L)	P1	SP1	d2	44
F3	D × LW	P1	SP2	d3	38
F1	D × LW	P2	SP1	d4	41
F2	(D × LW) × (LW × L)	P2	SP1	d4	38
F3	D × LW	P2	SP2	d5	42

Abbreviations: F1 to F3 = finishing farms; D = Duroc; LW = Large White; L = Landrace; P1 = September-October 2018; P2 = December 2018-January 2019; SP1 = Slaughter Plant 1, SP2 = Slaughter Plant 2; d1 to d5 = slaughter days.

working online (Zappaterra et al., 2021) according to the following Multiple Linear Regression Analysis model (*Stepwise* method) SPSS Statistical package (SPSS Inc., Chicago, USA) version 22.0:

$$LMPH = 25.87 + 782.57 \times fS/HW + 181.76 \times HW^{-1} \ (R^2_{adj} = 0.92;\ RMSE = 1.23\%).$$

where LMPH is the fat-free lean meat (%); *f*S is the signal generated by the Ham Inspector™ system during fresh ham scanning, and HW is the fresh ham weight (kg).

The accuracy of the system was checked by the dissection of additional fresh hams (10% of the hams scanned for the trial).

A sample (about 50 g) of *Semimembranosus* muscle was taken from the external part of the ham muscle of the 238 carcasses, put in plastic bags, and stored at −80 °C until the activities of Cathepsin B and Ferrochelatase were assayed. An additional portion of *Semimembranosus* muscle (about 5 g) was sampled from each ham and stored in a plastic vial at −20 °C until genotyping. Cathepsin B activity was assayed with Z-Arg-Arg-7-amido-4-methyl coumarin hydrochloride as substrate according to Barrett and Kirschke (1981). The release of 7-amido-4-methyl coumarin (**AMC**) from the enzymatic reaction at 37 °C was measured using a spectrofluorometer (Perkin Elmer LS30) with λex = 380 nm and λem = 460 nm. Free AMC (Sigma-Merck) was used as standard. The activity was expressed as nmol of AMC released min^{-1} g $muscle^{-1}$.

Ferrochelatase activity was determined by measuring the insertion of Zn(II) into Protoporphyrin IX to form ZnPP (forward reaction) as described by Parolari et al. (2016) with slight changes. The sample (1.0 g) was homogenised (Ultra-Turrax® T25, IKA®-WERKE) at 5 000 rpm for 2 min in 15 mL of ice-cold Tris-HCl buffer 20 mmol L^{-1} (pH 8.0) containing glycerol 20% (w/v), Triton X-100 1% (w/v), and KCl 0.8% (w/v). The homogenate was subsequently sonicated for 5 min and filtered through filter paper Whatman n. 40 (Whatman Intl. Ltd, Maidstone, UK). For the activity assay, the crude extract (675 µL) was incubated at 37 °C for 45 min in the dark with 100 µL of $ZnCl_2$ 2 mmol L^{-1}, 25 µL of Protoporphyrin IX 1 mol L^{-1}, and 200 µL of ATP dipotassium salt 25 mmol L^{-1} in NaCl 3.42 mol L^{-1}. After incubation, 35 µL of EDTA 100 mmol L^{-1} was added to stop the reaction then the tubes were cooled on ice. Then, 3.0 mL of acetone was added and the resulting solution was centrifuged at 10 000*g* for 15 min at 4 °C. The formation of ZnPP was measured using a spectrofluorometer (FluoroMax-4 HORIBA Scientific) with λex = 420 nm and λem = 590 nm. ZnPP (Sigma-Merck) was used as standard. Each extract was assayed against a blank obtained by adding 35 µL of EDTA 100 mmol L^{-1} to the reaction mixture. The activity was expressed as nmol of ZnPP formed min^{-1} 100 g^{-1} DM.

After salting, the hams deprived of the unabsorbed salt were scanned with the Ham Inspector™ system. The percent of salt content in the lean part of hams after the first (**SALT1**) and overall salting stages (**SALT**) were estimated by the Ham Inspector™ system (Zappaterra et al., 2021), according to the following predictive models (Multiple Linear Regression Analysis – *Stepwise* method – SPSS Statistical package version 22.0).

$$SALT1 = -0.24 + 18.91 \times s1S/SHW + 8.76 \times s1HW^{-1} \ (R^2_{adj} = 0.72;\ RMSE = 0.12\%).$$
$$SALT = -0.49 + 25.70 \times sS/SHW + 15.63 \times sHW^{-1} \ (R^2_{adj} = 0.86;\ RMSE = 0.13\%).$$

where SALT1 and SALT are the salt contents in the lean part of hams after first salting and at the salting end (%), respectively, *s1*S *and s*S are the signals generated by the Ham Inspector™ system during scanning of hams after first salting and at the salting end, *s1*HW and *s*HW the ham weight after the first salting and at the salting end (kg).

The accuracy of the system was checked by the dissection of additional salted hams equal to 10% of the hams scanned after SALT1 and SALT.

Ham weight losses were calculated as cumulative losses after the first salting step, at the end of salting, cold drying, and after 15 months of processing time.

Genotyping

First, DNA was extracted from *Semimembranosus* muscle by means of the Wizard Genomic DNA Purification Kit (Promega Corp, Madison, WI, USA) applying the standard protocol provided by the company. Then, the extracted DNA samples were analysed with the GeneSeek® Genomic Profiler porcine HD genotyping array (Illumina-NeoGen, Illumina Inc, San Diego, CA, USA, https://www.illumina.com/products/by-type/microarray-kits/ggp-porcine.html), which contains 68 516 markers evenly distributed on all porcine autosomes. The procedures utilised for the genotyping were those indicated by the company and were performed by an external service (https://www.lgscr.it/). Before association analyses, the localisation of all markers of the array was checked and updated according to their position on the ENSEMBL *Sus scrofa* 11.1 genome assembly (https://www.ensembl.org/Sus_scrofa/Info/Annotation) using an updated version of the "map" file kindly provided by the Illumina research team.

The sex of the animals was assayed by identifying the genotypes at the markers located on the Y chromosome: only the animals showing a genotype for these markers were considered males.

Statistical analyses

Descriptive statistics and Pearson correlations

All statistics on phenotypic traits related to pig carcass, fresh and salted hams, and processing weight losses were obtained by SPSS ver. 28 statistical package (SPSS Inc., Chicago, USA); normal distribution of data was inspected before statistical analysis. Descriptive statistics were given for the traits related to pig carcass, fresh and salted hams, and processing weight losses. Pearson phe-

notypic correlations were evaluated between the ham-assayed quality traits and the technological traits measured over processing.

Genome-wide association study

After the genotyping, the markers mapped on X and Y sex chromosomes, on the mitochondrial genome, or unmapped, were removed. On the whole 5 497 markers were excluded from subsequent analyses. Quality control of the 63 019 markers was performed using gPLINK 1.9 (Chang et al., 2015), based on PLINK v1.07 (Purcell et al., 2007), and the markers that did not satisfy the set criteria were excluded: call rate for single nucleotide polymorphisms (**SNP**s) < 90%, minor allele frequency < 0.05, and Hardy-Weinberg equilibrium with *P*-value < 0.001. The call rate was also computed for the sampled animals, and individuals with more than 10% of missing data were removed.

Following the filtering carried out using PLINK, genetic distances calculation, and vector extrapolation were performed using the same software. A Principal Component Analysis was elaborated with R environment using *cmdscale* and *cbind* functions. To visualise the results, a three-dimensional scatterplot of the first, second, and third principal components was produced with the R package gg3D, which is an extension of the R package ggplot2.

Furthermore, following the filtering carried out with PLINK, another cleaning of the samples was performed using the function *check.marker* of the GenABEL package (version 1.8–0; Aulchenko et al., 2007) in the R environment (R Version 3.4.4; https://cran.r-project.org/), the samples showing an identity by state > 0.9 were removed. After the filtering procedures, 51 166 SNPs and 237 individuals were retained.

A GWAS was performed with the GenABEL package (version 1.8–0) using *polygenic* (grammar-gamma) and *qtscore* (Svischeva et al., 2012), according to a modification of the procedure described in Nicolazzi et al. (2015). The statistical model used to perform the association analyses between the genotypic data and each phenotypic trait included sex, the day of slaughter, and the farm (each farm was characterised by a genetic type) as fixed effects. A genomic kinship matrix was calculated using the *ibs* function included in the GenABEL package and considering the employed SNPs included in the statistical model to infer the pedigree relationships among the pigs.

The following additive polygenic model was fitted with a genomic relationship matrix in GenABEL:

$$Y_i = X_i\beta_i + Z_i a_i + e_i.$$

where Y_i is the observation vector for the ith trait; β is the vector of effects for three factors (sex: two levels for barrows and gilts; slaughtering date: five levels; farm: three levels). The random factors in the model were animal (a) and residuals (e). They were assumed to be normally distributed as $a_i \sim N(0, G\sigma^2_a)$ and $e_i \sim N(0, I\sigma^2_e)$, where G is the genomic relationship matrix and σ^2_a and σ^2_e the additive genomic and residual variances, respectively.

Samples were not included in the analysis when phenotypic traits were missing.

Markers were considered significant with *P*-values (*P*-value for the 1 degree of freedom test corrected for the genomic inflation factor lambda) below the genome or chromosome-wide significant threshold (Supplementary Table S2). Manhattan and Quantile-Quantile plots were drawn in the R environment (R 4.1.2) by using the GenAbel package (R 3.4.4). The lambda estimated for all traits was always close to 1 with a minimum value of 0.9999805, indicating a good correction for the stratification of the samples carried out in this experiment. The False Discovery Rate (**FDR**; Benjamini and Hochberg, 1995) adjusted *P*-values were calculated in the R environment using the *p.adjust* function included in the *graphics* 4.1.2 package to correct for false positives due to multiple tests. The significance of the test was considered for FDR < 0.01.

Detection of the nearest genes and isoform characterisation

The genes located in the region flanking the significant markers for the analysed traits, within ±500 kilobases from each associated marker, were used for the identification of candidate genes mapping in the same regions. The list of the flanking genes was obtained visually by checking the genomic regions where the markers are located using the ENSEMBL pig genome viewer (https://www.ensembl.org/Sus_scrofa/Location/Genome).

To characterise the two swine *Ferrochelatase* (***FECH***) messenger RNA isoforms so far detected in pigs and to define where the mutant SNP was located within the two mRNAs, the alignment of the two transcripts (FECH-201: ENSSSCT00000005016; FECH-202: ENSSSCT00000053748) of *FECH* gene (ENSSSCG00000004541) was performed using the *blastn* online tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Postgenome-wide association study

For the FDR significant markers (i.e., FDR < 0.01), an association study was performed in R 4.1.2 with a linear model (*lm* function from *stats* package) to obtain the Estimated Marginal Means (formerly Estimated Least Squares Means) considering the parameters utilised for the GenABEL analysis (sex, day of slaughter, and farm as fixed effects).

$$y_{ijkl} = \mu + G_i + sex_j + slau_k + farm_l + e_{ijkl}.$$

where *y* is the observation vector for the ith trait; μ is the mean of the population; G is the fixed effect of each SNP (i = 1, 2, 3); sex: two levels for barrows and gilts; slau is the slaughter day (five levels); farm (three levels); e, error represents random effects of residues.

Additional functions used for the Estimated Marginal Means analysis were *Anova* (*car* 3.0-10 package), *emmeans* (*emmeans* 1.5.2-1 package), and *pairs* (*graphics* 4.1.0 package).

Linkage disequilibrium analysis was performed using Haploview 4.2 software (Broad Institute, Cambridge MA, USA; https://www.broadinstitute.org/haploview/haploview) with default settings (Barrett et al., 2005) and using the most significant markers (i.e. the markers showing the three genotypes or having a *P*-value and/or the contrasts significant) located on *Sus scrofa* chromosome 1.

Results

Carcass, ham, and processing parameters

In this study, we explored carcass and fresh ham traits associated with ham performance in dry-curing. Descriptive statistics for the investigated traits are reported in Table 2. The HW must comply with the range required by the PDO Parma ham producer, therefore, a low CV was observed for this trait. In this study, the LMPH was estimated by the Ham Inspector™ system. The obtained LMPH values ranged from 57.8% to 67.9%.

Cathepsin B and Ferrochelatase activities of the fresh ham were assayed respectively as markers of the endogenous proteolytic activity and potential to generate ZnPP, the pigment associated with the red colour development in nitrite-free dry-cured hams. The obtained values of Ferrochelatse showed to be largely variable (CV = 68.6%).

The salt values estimated by the Ham Inspector™ system indicate the percent of salt absorbed in the lean part of the ham during the first and the overall salting treatment. Ham processing lasted

Table 2
Descriptive statistics for the traits related to pig carcasses, fresh and salted hams, and processing weight losses.

Phenotypic traits	n	Mean	SD	CV
Carcass				
Weight, kg	238	144.4	8.3	5.7
Backfat thickness[1], mm	238	36.6	6.8	18.5
Loin thickness[1], mm	238	61.8	8.1	13.1
Lean meat[2], %	238	48.8	3.1	6.4
Fresh ham				
Weight, kg	238	13.94	0.47	3.37
Lean meat[3], %	237	62.8	2.1	3.4
Ultimate pH[4]	238	5.66	0.10	1.77
Cathepsin B activity, nmol AMC min^{-1} g^{-1}	237	1.47	0.30	20.40
Ferrochelatase activity, nmol ZnPP min^{-1} 100 g^{-1} DM	233	34.0	23.3	68.6
Salted ham				
NaCl content at the end of 1st salting[5], %	236	1.22	0.10	7.95
NaCl content at the end of salting[5], %	235	2.65	0.24	9.02
Weight loss[6]				
At the end of 1st salting, %	238	1.41	0.31	21.98
At the end of salting, %	236	2.68	0.41	15.30
At the end of cold drying, %	231	16.02	1.04	6.49
After 15 months of processing time, %	231	27.83	1.79	6.43

Abbreviations: AMC = 7-amino-4-methylcoumarin; ZnPP = Zinc Protoporphyrin.
[1] Measured with Fat-O-Meat'er optical probe.
[2] Calculated according to the equation specified by Commission Implementing Decision 2014/38/UE.
[3] Percentage of fat-free lean meat in fresh ham predicted by Ham Inspector™.
[4] Measured in *Semimembranosus* muscle.
[5] Percentage of salt content in the lean part of salted hams predicted by Ham Inspector™.
[6] Expressed as a percentage of fresh ham weight.

15 months and weight losses were measured at the main processing stages. In the present study, a higher CV was observed for salting weight losses compared to those measured at the end of cold drying and after the overall processing time.

Pearson correlations were used for testing the linear relationship between carcass and ham traits. Hot carcass weight (measured at the slaughterhouse) and HW (provided by the Ham Inspector™ system at the manufacturing plant) showed a positive correlation ($r = 0.41$; $P \leq 0.01$). The negative correlations between carcass weight and the two parameters carcass lean meat ($r = -0.45$; $P \leq 0.01$) and LMPH ($r = -0.44$; $P \leq 0.01$) indicated that the leanness of carcass and ham was moderately associated with a lighter carcass weight. The correlation coefficient of carcass lean meat with LMPH was positive ($r = 0.47$; $P \leq 0.01$). Correlation between ham traits, absorbed salt, and processing weight losses was reported in Table 3: positive and significant correlations were found between LMPH, salt absorbed in the lean, and processing weight losses. The salt absorbed in the lean was negatively correlated with ultimate pH.

Table 3
Pearson phenotypic correlation coefficients between the pork ham quality traits, technological traits for the absorbed salt, and ham weight losses measured at the main processing stages.

Item	Fresh ham weight	Fresh ham lean meat	Ultimate pH[1]
NaCl content at the end of 1st salting	−0.19**	0.67**	−0.15*
NaCl content at the end of salting	−0.09	0.72**	−0.25**
WL at the end of 1st salting	−0.07	0.41**	0.13*
WL at the end of salting	−0.10	0.60**	−0.02
WL at the end of cold drying	−0.15*	0.56**	−0.17**
WL after 15 months of processing time	−0.08	0.77**	−0.06

Abbreviation: WL = weight loss.
[1] Measured in *Semimembranosus* muscle.
* $P < 0.05$.
** $P < 0.01$.

Genome-wide association study for the markers associated with the phenotypic traits

The Principal Component Analysis indicated a clear separation between the three populations reared in the three different farms (Fig. 1), in agreement with the fact that pigs belonging to different crossbred lines were bred by the different farms considered in the present study.

As a result of the GWAS, a total of 33 SNPs were found associated with 12 out of the 15 phenotypic traits. The 19 SNPs showing chromosome-wide associations with 11 phenotypic traits are listed in Supplementary Table S3 and represented in Supplementary Fig. S1. Supplementary Table S3 reports also the allele and genotype frequencies for the chromosome-wide significant markers while Supplementary Table S4 lists the nearest coding genes for the same markers.

Considering the genome-wide associated markers, 14 associated with the Ferrochelatase activity were located on a limited region of porcine chromosome 1 (105.75–116.36 Mb), displayed in Supplementary Fig. S2. The FDR adjustment of the *P*-value for the 1 degree of freedom test corrected for the genomic inflation factor lambda obtained by GWAS indicated that 13 out of 14 SNPs had FDR below 0.01 (Table 4). Finally, three SNPs presented a very high level of significance (FDR = 4.02E−11). The SNPs associated with Ferrochelatase activity were adjacent to each other in the narrow region, 106.87–106.96 Mb of *Sus scrofa* chromosome 1. The Manhattan plot and Quantile-Quantile plot graphically showing the markers associated with Ferrochelatase activity are displayed in Fig. 2A and 2B. The genome map of the three most significant markers on *Sus scrofa* chromosome 1 is shown in Fig. 2C. One of these markers (ASGA0004152) is located in an exon of *FECH* and is indicated with a red arrow. The exon structure of the two swine transcripts for *FECH* is also displayed in Fig. 2C.

The other two highly significant SNPs found associated with Ferrochelatase activity, namely DIAS0002366 and ALGA0005395, do not map within *FECH* locus. The former is located in an exon of the gene *Asparaginyl-tRNA synthetase 1* (*NARS1*) producing a

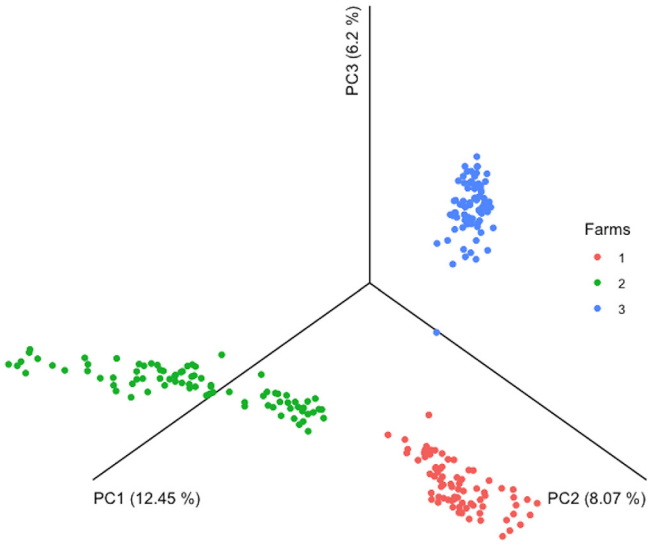

Fig. 1. Diagram showing the Principal Component Analysis results obtained among the three pig genetic types (=farm of origin) analysed. Abbreviation: PC = Principal Component.

synonymous variant, and the latter maps in an intergenic region between the genes *NARS1* and ENSSSCG00000033063. Supplementary Table S5 reported the nearest coding genes mapping in the region where the 14 SNPs associated with Ferrochelatase activity were detected. Allele and genotype frequencies were calculated for the 14 SNPs obtained by GWAS (Table 4). In general, the allele frequencies noticed for the SNPs associated with Ferrochelatase activity match a balanced distribution, with the minor allele frequency between 0.41 and 0.49.

To estimate the effect of the alleles of the 13 SNPs, an association analysis for each SNP was carried out using a linear model (Table 5): 7 SNPs showed a significant *P*-value for both the Estimated Marginal Means of each allele and the contrasts between pairs of alleles. The linkage analysis results evidenced three linkage blocks in the region 106.6–107.3 Mb of porcine chromosome 1 (Fig. 3) where the nine most adjacent markers for Ferrochelatase activity were located. GWAS analysis detected the highest significant associations with the three markers ASGA0004152, DIAS0002366, and ALGA0005395 of the third linkage block, further suggesting that the mutation influencing Ferrochelatase activity is located in the genomic region delimited by block 3. The same markers included on the Haploview linkage block 3 presented an Estimated Marginal Mean effect of about 49–50 nmol ZnPP min^{-1} 100 g^{-1} DM for one of the two homozygous genotypes (Table 5), the highest values observed; conversely, the Estimated Marginal Means of the opposite homozygous genotype are between 14 and 16 nmol ZnPP min^{-1} 100 g^{-1} DM, demonstrating the effect exerted by the alleles originated from the three cited SNPs on Ferrochelatase activity.

Table 4
Significant markers located on porcine chromosome 1 associated with Ferrochelatase activity. The markers are listed in decreasing order of FDR. Allele and genotype frequencies are also listed.

SNP markers	rs#	nt[1]	A1	A2	effB (SEB)[2]	effAB[3]	effBB[4]	FDR	Allele frequencies	Genotype frequencies[5]	SNP markers		
									1	2	11	12	22
ALGA0005395[6]	rs81355515	106 952 113	G	C	−4.85 (0.75)	−5.61	−9.44	4.02E−11	0.51	0.49	0.27 (65)	0.48 (113)	0.25 (59)
ASGA0004152[6]	rs81216562	106 877 209	A	G	−4.92 (0.76)	−5.46	−9.73	4.02E−11	0.55	0.45	0.32 (77)	0.46 (108)	0.22 (52)
DIAS0002366[6]	rs81216057	106 917 692	A	G	−4.92 (0.76)	−5.46	−9.73	4.02E−11	0.41	0.59	0.20 (48)	0.41 (98)	0.38 (91)
H3GA0002488[6]	rs81355534	107 100 243	G	A	−4.49 (0.77)	−4.19	−8.99	1.39E−08	0.49	0.51	0.22 (53)	0.53 (126)	0.24 (58)
INRA0003647[6]	rs321031460	106 714 123	C	A	−4.74 (0.84)	−5.63	−8.51	3.51E−08	0.07	0.93	0.00 (0)	0.15 (35)	0.85 (202)
ASGA0095614[6]	rs81474204	106 683 184	A	G	−4.50 (0.81)	−5.05	−8.55	6.03E−08	0.66	0.34	0.45 (106)	0.43 (102)	0.12 (29)
ASGA0004144[6]	rs81355510	106 697 548	A	C	−4.50 (0.81)	−5.05	−8.55	6.03E−08	0.32	0.68	0.10 (24)	0.43 (102)	0.47 (111)
ASGA0004161[6]	rs81355527	106 823 633	G	A	−4.85 (0.94)	−5.79	−7.17	1.57E−06	0.25	0.75	0.06 (14)	0.38 (90)	0.56 (133)
ALGA0005414[6]	rs80808933	107 261 641	A	G	−4.18 (0.82)	−3.21	−8.45	2.21E−06	0.42	0.58	0.13 (30)	0.58 (137)	0.30 (70)
ALGA0005524[6]	rs80820778	110 947 652	G	A	4.24 (0.93)	4.68	7.51	9.42E−05	0.70	0.30	0.48 (113)	0.46 (108)	0.07 (16)
INRA0003923[6]	rs318548067	116 359 904	G	A	−3.39 (0.80)	−3.01	−7.09	8.39E−04	0.70	0.30	0.51 (121)	0.39 (92)	0.10 (24)
INRA0003610[7]	rs319996306	105 751 534	A	C	−3.88 (0.95)	−4.61	−5.83	2.23E−03	0.85	0.15	0.71 (168)	0.28 (66)	0.01 (3)
H3GA0002483[8]	rs80929711	106 679 629	G	A	2.96 (0.74)	5.44	5.66	4.33E−03	0.34	0.66	0.12 (29)	0.43 (102)	0.45 (106)
ALGA0005538[9]	rs80800059	111 184 327	G	A	3.13 (0.86)	4.50	4.64	2.85E−02	0.79	0.21	0.61 (144)	0.37 (87)	0.03 (6)

Abbreviations: FDR: False Discovery Rate; SNP = Single Nucleotide Polymorphism; rs# = the reference number for SNPs; A1 = Allele 1; A2 = Allele 2.
[1] Nucleotide on *Sus scrofa* chromosome 1.
[2] Effect of the B allele in the allelic test, SEB: SE of effB reported in brackets.
[3] Effect of the AB genotype in the genotypic test.
[4] Effect of the BB genotype in the genotypic test.
[5] The number of samples is reported in brackets.
[6] 1% genome-wide significant markers.
[7] 5% genome-wide significant marker.
[8] 1% chromosome-wide significant marker.
[9] 5% chromosome-wide significant marker.

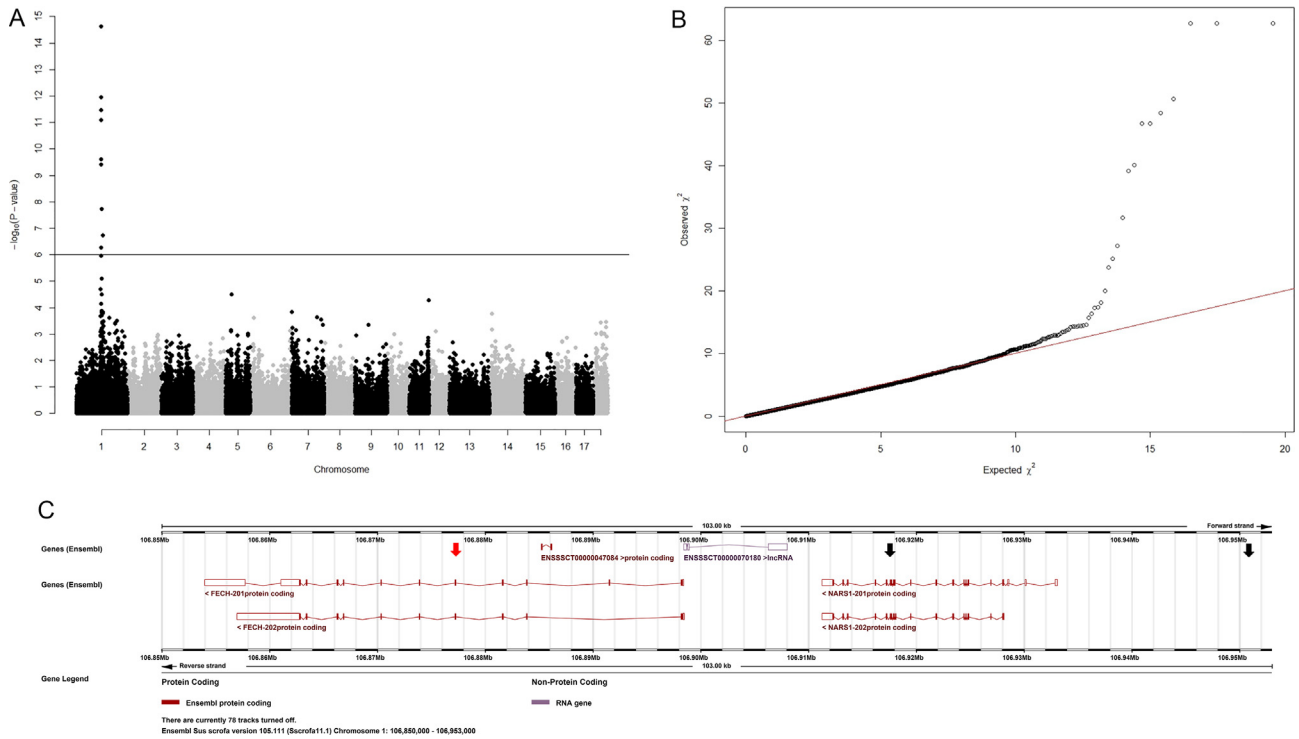

Fig. 2. **(A)** Manhattan plot and **(B)** Quantile-Quantile plots were obtained considering Ferrochelatase activity (λ = 1). **(C)** The genomic region of porcine chromosome 1 where the gene *Ferrochelatase* (*FECH*), and its two transcripts, are displayed. The position of the most significant SNP detected in an exon of the *FECH* gene where the missense variant (Val > Ala) is present is indicated by a red arrow at the top of the figure. The other genes than *FECH* displayed in the figure are *asparaginyl-tRNA synthetase 1* (*NARS1*), ENSSSCT00000070180 (gene: *ENSSSCG00000046224*), ENSSSCT00000047084 (gene: *ENSSSCG00000033063*). https://Dec2021.archive.ensembl.org/Sus_scrofa/Location/View?r=1%3A106850000-106953000. Abbreviation: SNP = Single Nucleotide Polymorphism.

Table 5
Estimated marginal means (least-square means). The regression models were carried out for the FDR significant markers detected on the pig genome for Ferrochelatase activity (nmol ZnPP min^{-1} 100 g^{-1} DM).

SNP markers	*P*-value	Estimated Marginal Means[1]			Contrasts[2]			Additive effect[3]	Dominance effect[4]
		11	12	22	11–12	11–22	12–22		
ALGA0005395	<2.2E−16	49.70	34.00	16.10	15.70***	33.50***	17.80***	***	0.65
ASGA0004152	<2.2E−16	50.20	31.60	14.50	18.60***	35.80***	17.10***	***	0.74
DIAS0002366	<2.2E−16	48.90	29.80	14.10	15.80***	34.80***	19.00***	***	0.49
H3GA0002488	2.36E−15	46.70	35.20	15.70	19.50***	31.00***	11.60***	***	0.11
INRA0003647[d]	3.67E−05	-	20.00	36.10	-	-	−16.10***	-	-
ASGA0095614	5.62E−13	43.00	29.90	12.40	13.10***	30.50***	17.50***	***	0.42
ASGA0004144	1.21E−12	42.80	28.60	12.60	16.10***	30.30***	14.20***	***	0.75
ASGA0004161	0.09	43.70	30.50	34.70	13.22	9.00	−4.22	0.17	0.03
ALGA0005414	1.71E−05	51.20	33.50	26.80	17.66***	24.36***	6.69***	***	0.07
ALGA0005524	0.10	31.90	34.20	44.60	−2.36	−12.77	−10.40	0.03	0.26
INRA0003923	0.57	33.40	35.10	30.00	−1.68	3.40	5.08	0.50	0.30
INRA0003610	0.14	39.50	38.30	31.80	6.55	7.78	1.23	0.61	0.74
H3GA0002483	3.61E−13	12.40	29.90	43.10	−17.50	−30.70	−13.20	***	0.44

Abbreviations: FDR = False Discovery Rate; ZnPP = Zinc Protoporphyrin; SNP = Single Nucleotide Polymorphism.
[1] All significant Estimated Marginal Means; adjusted means of the considered trait according to the statistical model used effects are significant for $P < 0.001$.
[2] *** means that the contrasts are significant for $P \leq 0.001$.
[3] *** means that the effects are significant for $P < 0.0001$.
[4] Only two genotypes were detected on the analysed samples for INRA0003647.

Close or in correspondence with the other two SNPs belonging to Haploview linkage block 3, there is a not characterised protein-coding gene (gene *ENSSSCG00000033063*, transcript ENSSSCT00000047084, located on an intron of *FECH*), one long non-coding RNA (gene *ENSSSCG00000046224*; transcript ENSSSCT00000070180), and the protein-coding gene *NARS1* (Fig. 1C). These three genes can be also considered positional candidates for Ferrochelatase activity considering their proximity to each other and with *FECH*. Nevertheless, no functional connections with the enzyme activity were available for *ENSSSCG00000033063*, *ENSSSCG00000046224*, and *NARS1* in the literature.

Discussion

Carcass, ham, and processing parameters

As displayed in Table 3, LMPH affects salt uptake and weight losses, contributing to an increase in the variability of dry-cured

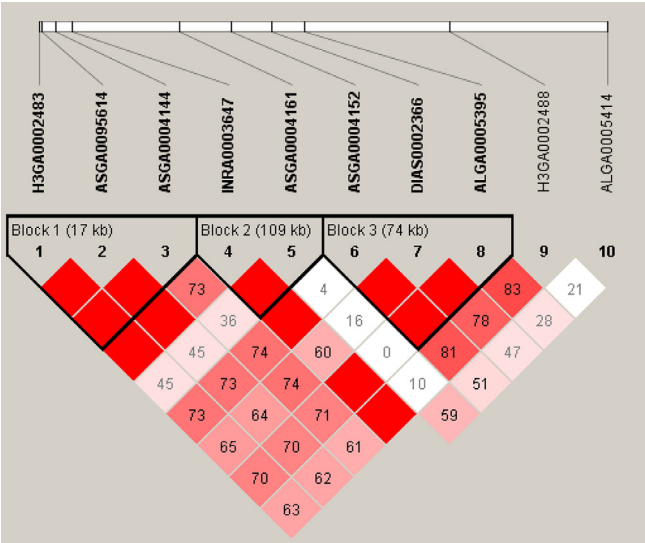

Fig. 3. Haploview representation of the region of *Sus scrofa* chromosome 1 where the FDR most significant markers for Ferrochelatase activity are present. Eight markers are grouped on three linkage blocks delimited by thick black lines. Abbreviation: FDR = False Discovery Rate.

ham quality. Currently, this issue is faced by tutelary regulations with the exclusion from the PDO chain of carcasses graded as too lean or too fat (Parma ham – General Rules and Dossier, 1992; San Daniele ham – General Rules and Dossier, 2007). A close relationship between carcass classification and ham leanness would be useful for process setting, as it would allow the ham to be identified by a reliable score. Conversely, the moderate positive correlation between lean meat percentage of the carcass and LMPH indicates that carcass grading for leanness partly predicts the lean percentage of the ham, even maintaining a similar trend between carcasses and thighs. To counteract this mismatch, the Italian breeding scheme of boar selection included the parameter weight loss after first salting, similar to the values reported in this study (Table 2), to obtain a balance between growth performances, affecting carcass leanness, and processing yield, related to ham suitability for dry-curing. Under standard processing, weight loss after first salting was found to be affected by pig genetic type (Schivazappa et al., 2002), added salt (Pinna et al., 2020), duration and conditions of the salting phase (Martuscelli et al., 2015).

The variability shown by Cathepsin B activity in fresh hams partly explains the variations found in the proteolysis of dry-cured ham. The variability of Cathepsin B activity detected in this study (CV = 20.4%) may be influenced by the slaughter season, autumn and winter, in the present research, the breeding system, and the genetic type of the pigs (Russo et al., 2002; Virgili and Schivazappa, 2002; Sturaro et al., 2008). Russo et al. (2000) reported a moderate heritability (h^2 = 0.23–0.28) for Cathepsin B activity measured on Large White pigs. The latter result and the absence of specific DNA markers found in the present study to be associated with Cathepsin B activity could be due to the combined effect of several genes and the fact that variations in the proteolytic activity may also be ascribed to environmental factors. Ferrochelatase activity has been postulated as a key parameter in the generation of ZnPP in nitrite-free cured meat products (Wakamatsu, 2022), although the relationship between its activity in fresh ham and ZnPP in the final product has not yet been confirmed. The obtained CV values agree with the results obtained by Parolari et al. (2009), which measured Ferrochelatase activity in representative muscles of fresh and processed hams. Based on the authors' knowledge, screenings performed on large ham batches are not available to compare the average value reported in Table 2.

Although the study was carried out under standard processing conditions, salted hams showed positive correlations between SALT1, SALT, and LMPH, linking the increased absorption of salt in lean to the lean meat content of the hams. During the salting phase, salt concentration gradients between the outside and inside of the ham occurred, and the absorption and diffusion of added salt are favoured in lean meat. Therefore, the non-invasive grading of LMPH is useful to evaluate potential and actual salt absorption and plan corrective actions to improve the homogeneity of the ham batches. As shown by the negative correlation between absorbed salt and ultimate pH, a positive contribution to salt uptake from a low ultimate pH of the hams is expected. The obtained values agree with salt contents obtained by other non-destructive technologies (Fulladosa et al., 2015) or using traditional methods of analysis (Pinna et al., 2020).

Previous studies have shown no or very low correlations between HW and weight losses (Ramos et al., 2007), confirmed by the present study, despite being affected by the restricted weight range of the hams. As expected, all processing weight losses were positively correlated with LMPH, confirming that ham weight loss control needs the containment of LMPH. This is due to the large water content gap between adipose and muscle tissue (5–15% vs. 70–75%) and the positive barrier effect of fat on water that drips out from the hams through diffusion and evaporation during processing (Bosi and Russo, 2004).

Genome-wide association study for the markers associated with Ferrochelatase activity and in silico characterisation of the Ferrochelatase gene

Ferrochelatase activity, despite being involved in the formation of the red colour in nitrite-free ripened pork meat products, is a trait that has not yet been considered for GWAS or other association analyses. For this reason, the results obtained in the present research indicating an association between DNA markers and Ferrochelatase activity may be of particular interest to the field of meat science. Interestingly, the ten animals showing the highest and lowest Ferrochelatase activity, respectively, were homozygous for the three markers most significantly associated with this parameter (ALGA0005395, ASGA0004152, and DIAS0002366). This evidence indicates that the opposite and extreme values of the activity of this enzyme agree with the associated DNA markers, hypothesising a specific role of the DNA markers located on this portion of *Sus scrofa* chromosome1 on Ferrochelatase activity. The nuclear *FECH* codes for the Ferrochelatase protein (protoheme ferrolyase, EC 4.99.1.1) located in the inner mitochondrial membrane, the terminal enzyme of the haem biosynthetic pathway, also involved in the pathway leading to the formation of the red pigment ZnPP characteristic of nitrite-free Parma ham (Wakamatsu et al., 2004). Although the research carried out has not yet totally elucidated the mechanisms leading to the formation of ZnPP, Ferrochelatase appears to play a key role.

At the time of writing this paper, two transcripts of *FECH* have been found in pigs (https://www.ensembl.org/Sus_scrofa/Gene/Summary?db=core;g=ENSSSCG00000004541;r=1:106854002-106898418, last accessed: 01/03/2023). The first transcript (FECH-201) includes 10 exons, and the mutation recognised by the SNP ASGA0004152 is in exon 4. The other transcript (FECH-202) is longer and includes 12 exons, with ASGA0004152 being located in exon 5. The two transcripts differ in length due to an alternative splicing event, the shorter transcript FECH-201 lacks exon 2 and has a different organisation of the 3′-UTR compared to FECH-202. On both transcripts, the same missense variant Val > Ala (p.Val207Ala, p.Val234Ala), corresponding to the SNP ASGA0004152 (c.620 T > C, c.701 T > C), is present. To the best of our knowledge, differences in the activity of the proteins coded by the two porcine

transcript variants have not yet been reported in the literature. Searching on the EMBL website, 22 missense variants are present in the porcine *FECH*; among these variants, only four, including the variant rs81216562 (ASGA0004152), showed higher evidence as they were found by multiple studies (as indicated in the EMBL DNA variants of porcine *FECH*, https://www.ensembl.org/Sus_scrofa/Gene/Variation_Gene/Table?db=core;g=ENSSSCG00000004541;r=1:106853114106899306;v=rs81216562;vdb=variation;vf=202791). The Sorting Intolerant From Tolerant (SIFT, https://sift.bii.a-star.edu.sg/) values for the variant rs81216562 (ASGA0004152) in the two transcripts FECH-201 and FECH-202 were 0.22 and 0.16, respectively. These values are commonly categorised as "tolerated", meaning that the variant rs81216562 (ASGA0004152) seems to be non-deleterious for both transcripts and the mutant aminoacidic sequence is expected to be compatible with the function of the coded proteins. Up to now, there is no indication whether the two transcripts detected for this porcine gene may be responsible for a different Ferrochelatase activity related to the formation of ZnPP. In the present study, based on the large difference noticed between the estimated means for the two opposite homozygotes, it is possible to hypothesise an influence exerted by rs81216562 on the activity of the translated peptide and, as a consequence, the efficiency of the enzymatic reaction catalysed by Ferrochelatase activity. Nevertheless, the rs81216562 SNP could be either the causal mutation itself or a marker associated with the causal mutation. Apart from that, the association found between this marker and the variability in the amount of ZnPP in nitrate-free dry-cured hams is of extreme interest and needs further investigation.

Considering the region outside the linkage block 3 (Fig. 2C, Fig. 3), other protein-coding genes are mapped there. The closest genes are *One cut homeobox 2* (*ONECUT2*), and *ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 3* (*ST8SIA3*) on the left side of the genome with respect to the location of *FECH*. On its right side is located *Ankyrin repeat and death domain-containing 1A* (*ANKDD1A*). To the best of our knowledge, functional connections with Ferrochelatase activity for these genes have not been described in the literature. Interestingly, *ANKDD1A* maps to human chromosome 15 while the other cited genes, including *FECH*, are located on human chromosome 18. In pigs, all the seven cited genes map on *Sus scrofa* chromosome 1, indicating that there is a syntenic break differentiating humans and pigs in the genomic region between genes *NARS1* and *ANKDD1A* that corresponds to the division in the observed linkage blocks.

So far, this is the first study to find a strong association between a genetic marker and Ferrochelatase activity. Nevertheless, the molecular mechanisms underlying this phenotypic trait deserve further investigation to elucidate whether other genes besides *FECH* can be involved in determining changes in the Ferrochelatase activity. Future studies should investigate how the alleles detected in the SNPs associated with Ferrochelatase activity could affect ZnPP formation in the corresponding dry-cured hams. Moreover, a validation of the obtained results on a larger number of pigs, with a known pedigree, would be highly advisable before including the marker in selection schemes aimed at improving dry-cured ham quality.

Conclusion

The application of a fast and non-invasive system suitable for the online acquisition of ham technological traits allowed an accurate prediction of the individual differences in salt absorption and weight losses existing among hams. In particular, the increase in LMPH and, even if to a lesser extent, the decrease in ultimate pH, enhance ham tendency to salt absorption and to weight loss. The online prediction of LMPH, SALT1, and SALT allows the early identification of hams that require an adaptation of processing steps (i.e., amount of added salt) to counteract the variability of dry-cured ham quality. The variability observed for the ham traits showed to be significantly associated with 33 markers. Among them, 12 SNPs were related to Ferrochelatase activity, the enzyme involved in the synthesis of the natural purple-red pigment ZnPP. In particular, the mutation ASGA0004152 (rs81216562) can be considered an interesting candidate for further studies due to its strong association with Ferrochelatase activity. In perspective, these findings could be integrated into the ham production chain, to select pig genotypes more prone to develop red colour in dry-cured ham and to evaluate fresh hams according to their potential in salt uptake and weight loss.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.100864.

Ethics approval

All animals used in this study were kept according to the Italian and European legislations for pig production. All procedures described followed Italian and European Union regulations for animal care and slaughter. An ethics approval was not required for this study as data were collected at ham processing plants on cuts (hams) intended for human consumption.

Data and model availability statement

None of the data was deposited in an official repository. Data reported in this paper can be shared after the signature of an agreement on their use by contacting the corresponding author.

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PZ, RV, RD: Conceptualisation; **PZ, JV, MZ, AC, CS, NS:** Data curation; **PZ, JV, MZ, CS, NS:** Formal analysis; **PZ, RV, RD:** Funding acquisition; **JV, PZ, CS, RV:** original draft writing; all authors: review and editing.

Declaration of interest

None.

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