

Molecular Biomarkers, Near Infra-Red Spectroscopy and Computed Tomography as New Methodologies Applied in TREASURE Project to Predict the Quality of Pork and Pork Products from Local Pig Breeds

Bénédicte LEBRET¹(✉), Carolina PUGLIESE², Riccardo BOZZI², Maria FONT-I-FURNOLS³, Maja PREVOLNIK POVŠE^{4,5}, Urška TOMAŽIN⁴, Marjeta ČANDEK-POTOKAR^{4,5}

Summary

Emerging non-destructive technologies are of interest in meat sector science and industry since they allow the characterization of products and quality control throughout processing. Three different new technologies described in this paper will be considered in the TREASURE project for the evaluation and prediction of quality of pork and processed products: molecular biomarkers, near-infra red spectroscopy (NIRS), and computed tomography (CT). Molecular biomarkers are single genes, or a set of few genes, whose expression level determined in muscle few minutes after slaughter are associated to technological or sensory pork traits. External validation of biomarkers of pork quality, available from previous studies, will be undertaken. NIRS shows a great potential to predict composition of muscle and fat tissues, in particular their lipid content and fatty acid profiles. These novel techniques will be assessed in the project using a wide variety of loin and subcutaneous fat samples from various European breeds. NIRS will also be used to determine chemical composition (water, salt, etc) and physical traits (rheology) of fresh meat and processed products. CT, which corresponds to 3D images constructed using X-ray technology, allows determining the quantity and repartition of lean, fat and bone tissues in living animals and in carcass or cuts. CT will be used to study the distribution of fat and muscle in carcasses and in loin from breeds exhibiting various adiposity levels.

Key words

molecular biomarkers, near infra-red spectroscopy, computed tomography, carcass composition, meat quality

¹ PEGASE, Agrocampus Ouest, INRA, 35590 Saint-Gilles, France

✉ e-mail: Benedicte.Lebret@inra.fr

² UNIFI, DISPAA, Via delle Cascine 5, 50144, Firenze, Italy

³ IRTA, Monells, Finca Camps i Armet, 17121 Monells (Girona), Spain

⁴ Agricultural Institute of Slovenia, Hacquetova ulica 17, 1000 Ljubljana, Slovenia

⁵ University of Maribor, Faculty of Agriculture and Life Sciences, Pivola 10, SI-2311 Hoče, Slovenia

Received: April 28, 2017 | Accepted: August 22, 2017

ACKNOWLEDGEMENTS

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 634476 (Project acronym: TREASURE). The content of this paper reflects only the author's view and the European Union Agency is not responsible for any use that may be made of the information it contains.

Introduction

One of the main objectives of the multidisciplinary and multi-actor TREASURE project (<http://treasure.kis.si/>) is to evaluate the intrinsic quality attributes of a variety of European traditional pork products according to pig breed, production system, animal feeding and management practices. The aim is to determine the average and variability of quality of pork and pork products from various local pig breeds and pork chains, and to identify quality attributes that could be improved by animal management practices (e.g. feeding) or processing techniques. This involves the determination of carcass, meat and product quality traits using objective techniques. In addition to 'conventional' methodologies, new and non-invasive methodologies and techniques for evaluation of carcass composition and meat quality traits, known from improved breeds but which have not yet (or hardly ever) been applied on products of local pig breeds, will be considered: 1) molecular biomarkers to predict at slaughter stage the sensory and technological quality of pork, 2) Near Infra-Red Spectroscopy (NIRS) to predict chemical and physical traits of pork and pork products, and 3) an imaging technique: the computed tomography (CT) to assess the proportions and distribution of lean, fat and bone tissues in carcasses and main cuts (loin, ham) from local pig breeds. These techniques and their applications in local breeds within TREASURE project are presented.

Molecular biomarkers to predict pork quality

Eating and technological qualities of pork result from interactions between pig genetic background, rearing system, slaughtering conditions and carcass and meat processing. To date, many factors influencing pig meat quality (MQ) have been identified but its variability remains high and the muscle properties underlying high eating quality are still unclear (Listrat et al., 2016). Therefore, the identification of biomarkers and the further development of rapid tests would be helpful for the control and improvement of MQ in pork industries.

Functional genomics (transcriptomics, proteomics, metabolomics) enables high throughput screening of expressed genes, proteins or of their metabolites in a given tissue, and is thus of utmost interest to study the development of complex phenotypic traits determined by interactions between genotype and environment such as in the case of MQ. These techniques have also been recently used in various animal species to identify early post-mortem (p.m.) biomarkers of MQ i.e. genes whose expression level is associated to MQ traits (Te pas et al., 2011; Picard et al., 2015 for review). Biomarkers could thus allow assessment of traits whose measurement is difficult or expensive, like shear force or sensory tenderness, or late compared with duration of relevant production phase in meat industry, for example ultimate pH (pHu) and drip loss. Some studies have been conducted to identify markers of pork traits such as pHu, color parameters, drip loss, shear force, intramuscular fat (IMF) content, however most of them compared contrasted groups for a given trait instead of considering a range of MQ values (Lobjois et al., 2008; Ponsuksili et al., 2009). Moreover, they did not include a validation of potential markers on different samples as those used for identification. More recently, an experimental design including contrasted pig breeds and various production systems

inducing a high variability in MQ was developed to identify and validate biomarkers of technological and sensory traits of pork (Damon et al., 2013). Muscle transcriptome profiles were established on $n=50$ Longissimus muscle (LM) samples taken 30 min after slaughter using a specific 15 K pig skeletal muscle microarray (around 9000 unique genes), and associated to MQ traits determined on LM one up to few days after slaughter to identify new biomarkers of MQ: pHu, drip loss, lightness (L^*), redness (a^*), hue angle (h°), IMF, shear force and sensory tenderness and juiciness. The wide range of MQ traits and of LM expression patterns allowed to establish thousands of correlations between microarray gene expression and MQ traits (140 for a^* up to 2892 for tenderness, adjusted Benjamini-Hochberg P -value < 0.10). Then, considering 40 genes selected for high correlation coefficient value or relevant biological process terms for MQ development, these associations were confirmed on the same samples by RT-PCR technique which is more accurate for the further development of molecular tools. Thus, 113 transcript-trait associations were confirmed ($R^2 \leq 0.52$, $P < 0.05$), out of which 60 were validated ($R^2 \leq 0.46$, $P < 0.05$) for 8 MQ traits on complementary experimental data ($n=50$): in other words, expression level of one gene could explain up to 46% of the variability of one MQ trait (Damon et al., 2013). External validation on 100 commercial crossbreds from different genetic origin allowed validating 19 transcript-trait associations ($R^2 \leq 0.24$, $P < 0.05$), i.e. biomarkers for pHu (6), drip loss (4), L^* (5), h° (2), IMF content (1) and tenderness (1) (Lebret et al., 2013a). Moreover, within the pure local Basque breed of pig, muscle transcriptomic studies highlighted that extensive production system compared to the conventional induced over-expression of genes involved in the control of muscle structure and thermal response (small Hsp) (Lebret et al., 2013b). Combining MQ data with transcriptomic profiles of this local breed allowed the identification of biomarkers of high quality pork: 90 transcript-trait correlations were confirmed by RT-PCR ($|r| \leq 0.74$, $P < 0.05$) among which 27 were validated ($|r| \leq 0.73$, $P < 0.05$) on complementary data ($n=30$).

These results are promising but highlight that predictive capacity of biomarkers should be improved to foresee the development of control tools for pork industry. We thus considered another approach based on the identification and validation of biomarkers of sensory and technological MQ classes, i.e. low, acceptable or extra pork quality levels, with final aim to propose molecular tools to classify carcasses or primary cuts early after slaughter in meat industries, according to their predicted quality level. To this aim, the previous data set including individual sensory, technological and gene expression (RT-PCR) data ($n=100$ pigs) was used. Scientific expertise and statistical approaches were combined to select indicators and their thresholds to specify quality classes differing in sensory and technological attributes. Among 14 MQ traits, the most discriminant ones (pH 30 min pm, pHu, drip loss, IMF) were used to define 3 classes: low (impaired) quality (L), acceptable (A) and extra (E). Expression level of 40 genes were used as predictive variables in a generalized linear model to discriminate quality classes (stepwise selection with chosen probability cut-point to predict class L as 0.3, giving thus more 'weight' to upgrading than downgrading errors). The best model included expression levels of 12 genes and allowed good classification of 88% of L and 82% of E samples at cross-validation step (Lebret et al., 2015).

Within the TREASURE project, the availability of a wide quantity of muscle and pork samples from various local breeds that will be assessed for MQ using common methodologies is a great opportunity to carry out an external validation of the discriminant model of pork quality classes. This will be undertaken on LM samples collected on Krškopolje (SL, n=24), Turropolje (HR, n=20), Iberian (ES, n=52) and Gascon (Fr, n=77) pig breeds. Biomarkers of individual sensory and technological quality traits previously validated will be also tested on these samples for further validation / prediction of MQ traits.

NIRS to predict chemical and physical traits of pork and pork products

Non-destructive technologies that allow the characterization of food product and quality control throughout processing are of high interest in food industry - including meat sector, especially when they can be used under industrial conditions (Font-i-Furnols et al., 2015a). Among these non-invasive technologies, NIRS shows a great potential to predict composition of muscle and other tissues, in particular lipid and fatty acid profile, but moderate to low ability for technological meat quality traits (Weeranantanaphan et al., 2011).

NIRS functions on the principle of energy absorption of organic molecules (bonds) at specific wavelengths depending on the characteristics of the matrix. Applications of NIRS in muscle foods in the majority of reported studies work with the visible (400-700 nm) and NIR range of the spectrum (700-2500 nm). Measurements can be performed in transmittance, reflectance or transreflectance mode on intact, minced or homogenized samples. NIRS has been repeatedly shown to have high accuracy to assess the chemical composition (fat, protein, water, dry matter, etc.) of muscle foods. For instance, the maximum intramuscular fat prediction accuracy has been reported as an $R^2 = 1.00$ and RPD = 28.46 (Viljoen et al., 2007) with more typical accuracy values of R^2 between 0.34 and 0.94 and RPD between 1.09 and 4.1 depending on the species (Cozzolino and Murray, 2002; Prevolnik et al., 2005). The technology has also been shown to have a satisfactory ability to determine major fatty acids composition in all species, e.g. in case of oleic acid R^2 between 0.77 and 0.963 and RPD higher than 2 were obtained (Pla et al., 2007; Pérez-Juan et al., 2010). Compared with high predictive ability for chemical constituents, where the accuracy of calibrations can even approach the accuracy of analytical methods, the ability of NIRS to determine pork physical quality characteristics (e.g. pH, colour, water holding capacity, texture) is low to moderately reliable (Čandek-Potokar et al., 2006; Prieto et al., 2009). Namely, in this case the predictive ability of NIRS is limited by the accuracy of the reference methods which are less repeatable and reproducible (Prevolnik et al., 2010), and also because physical measurements (for instance shear force) are only partly related to chemical composition of the meat - NIRS technology relying especially on chemical bonds. NIRS has also been tested for applications in classifications like discriminating genotypes, feeding regimes, meat quality classes, and results showed comparatively good accuracy for such purposes (reviews by Prevolnik et al., 2004; Prieto et al., 2009 and Weeranantanaphan et al., 2011).

Within the TREASURE project, NIRS technology will be used both on fresh tissues and pork processed products. Lipid

content and fatty acid (FA) composition of both subcutaneous fat and LM muscle will be analyzed by NIRS as well as according to reference (chemical and chromatographic) methods to calculate an equation of prediction of these components in muscle and fat tissues collected on 17 different local pig breeds. Regarding processed products, NIRS analyses will be undertaken to develop and test calibrations for chemical composition of processed products (e.g., dry cured ham, salami, sausages, dry neck, dry belly/pancetta) and for quality traits of dry-cured hams: proteolysis index, salt content, rheology.

Computed Tomography to evaluate carcass composition and distribution of lean and fat tissue in primary cuts

CT is a non-invasive technique that is used in animal and food science to predict its composition and characteristics. X-rays go through the body and they are attenuated in more or less degree depending on the density of the tissues of the irradiated body. A matrix of attenuation values (expressed as Hounsfield units) is created, which is used to construct 3D grey tones images (tomograms), by means of a reconstruction algorithm. Grey tones are related with the density and, due to the different density between lean, fat and bone, it is possible to separate qualitatively these tissues and to quantify them.

CT has been proved to be very accurate to evaluate live animals body composition, to develop growth curves of the different tissues (Kolstad et al., 1996; Carabús et al., 2015; Font-i-Furnols et al. 2015b), to evaluate carcass composition (Font-i-Furnols et al., 2009) and also ossification and articular osteochondrosis (Olstad et al., 2014). Intramuscular fat has also been evaluated in live pigs and carcass cuts (Font-i-Furnols et al., 2013; Kongsro and Gjerlaug-Enger, 2013) and in dry cured ham (Santos-Garcés et al., 2014) but the accuracy was not always good enough. Salt content, salt diffusion, water activity and water content (Frøystein et al., 1989; Fulladosa et al., 2010; Picouet et al., 2013) can be accurately predicted with CT, as well as fatty acid composition (Prieto et al., 2010). Nevertheless, CT was not accurate enough to determine texture, colour and sensory characteristics in beef (Prieto et al., 2010). Thus, CT can be a good technology to be used in animal science for breeding purposes, for nutritional studies, for animal health determination and an industrial CT could also be used in slaughter, and cutting and processing plants to optimize the production.

Within TREASURE project, CT has been used to scan carcasses from Iberian pigs and the distribution of fat and muscle has been compared with those from commercial white pigs, both qualitatively and quantitatively in the whole carcass. Also in CT images from selected anatomical locations of the loin and the ham, some linear and area measurements of fat and muscle were determined and compared between breeds. This technology allowed highlighting strong breed differences in the quantity and repartition of these tissues in the carcass.

Conclusion

This paper describes update of knowledge on three different recent methodologies for the evaluation and prediction of quality traits of pork and processed products: molecular biomarkers, NIRS, and CT. Recent studies conducted in "conventional" pig

breeds and pork chains have highlighted the interest of these methodologies in the meat sector. Some data have also been obtained in local/non selected pig breeds which generally exhibit high carcass fatness but also high sensory and technological meat quality as compared to conventional breeds. Therefore, these methodologies have to be validated in local breeds before foresee their implementation as quality control tools. This validation will be undertaken within the TREASURE project on meat samples and products from various local breeds and pork chains.

References

- Čandek-Potokar M., Prevolnik M., Škrlep M. (2006). Ability of near infrared spectroscopy to predict pork technological traits. *J Near Infrared Spec* 14: 269-277.
- Carabús A., Sainz R.D., Oltjen J.W., Gispert M., Font-i-Furnols M. (2015). Predicting fat, lean and the weights of primal cuts for growing pigs of different genotypes and sexes using computed tomography. *J Animal Sci* 93: 1388-1397.
- Cozzolino D., Murray I. (2002). Effect of sample presentation and animal muscle species on the analysis of meat by near infrared reflectance spectroscopy. *J Near Infrared Spec* 10: 37-44.
- Damon M., Denieul K., Vincent A., Bonhomme N., Wyszynska-Koko J., Lebre B. (2013). Associations between muscle gene expression pattern and technological and sensory meat traits highlight new biomarkers for pork quality assessment. *Meat Sci* 95: 744-754.
- Font i Furnols M., Teran F., Gispert M. (2009). Estimation of lean meat percentage of pig carcasses with Computer Tomography images by means of PLS regression. *Chemom Intell Lab Syst* 98: 31-37.
- Font-i-Furnols M., Brun A., Tous N., Gispert M. (2013). Use of linear regression and partial least square regression to predict intramuscular fat of pig loin computed tomography images. *Chemom Intell Lab Syst* 122: 58-64.
- Font-i-Furnols M., Fulladosa M., Prevolnik Povše M., Čandek-Potokar M. (2015a). Future trends in non-invasive technologies suitable for meat quality determinations. In: *A handbook of reference methods for meat quality assessment - (M Font-i-Furnols, M Čandek-Potokar, C Maltin, M Prevolnik Povše, eds), SRUC, Edinburgh, 90-103.*
- Font-i-Furnols M., Carabús A., Pomar C., Gispert M. (2015b). Estimation of carcass and cuts composition from computed tomography images of growing live pigs of different genotypes. *Animal* 9: 166-178.
- Frøystein T., Sørheim O., Berg S.A., Dalen K. (1989). Salt distribution in cured hams, studied by computer X-ray tomography. *Fleischwirtschaft* 69: 220-222.
- Fulladosa E., Santos-Garcés E., Picouet P., Gou P. (2010). Prediction of salt and water content in dry-cured hams by computed tomography. *J Food Eng* 96: 80-85.
- Kolstad K., Jopson N.B., Vangen O. (1996). Breed and sex differences in fat distribution and mobilization in growing pigs fed at maintenance. *Livest Prod Sci* 47: 33-41.
- Kongsro J., Gjerlaug-Enger E. (2013). In vivo prediction of intramuscular fat in pigs using computed tomography. *Open J Anim Sci* 3: 321-325.
- Lebre B., Denieul K., Vincent A., Bonhomme N., Wyszynska-Koko J., Kristensen L., Young J.F., Damon M. (2013a). Identification by transcriptomics of biomarkers of pork quality. In: *Proc 45th Journées Rech Porcine*, pp 97-102.
- Lebre B., Denieul K., Damon M. (2013b). Muscle transcriptome profiles highlight biomarkers of pig production system and high meat quality. In: *Proc 59th Int Cong Meat Sci Technol, Izmir, Turkey, p 33 - n°O13.*
- Lebre B., Castellano R., Vincent A., Faure A., Kloareg M. (2015b). Molecular biomarkers to discriminate pork quality classes based on sensory and technological attributes. In: *Proc 61st Int Cong Meat Sci Technol, Clermont-Ferrand, France, p 99 - n° 4.13.*
- Listrat A., Lebre B., Louveau I., Astruc T., Bonnet M., Lefaucheur L., Picard B., Bugeon J. (2016). How do muscle structure and composition determine the meat and flesh quality? *Scientific World J* 2016 : article ID3182746
- Lobjois V., Liaubet L., SanCristobal M., Glénisson J., Fève K., Rallières J., Le Roy P., Milan D., Chel P., Hatay F. (2008). A muscle transcriptome analysis identifies positional candidate genes for a complex trait in pig. *Anim Genet* 39: 147-162.
- Olstad K., Kongsro J., Grindflek E., Dolvik N.I. (2014). Consequences of the natural course of articular osteochondrosis in pigs for the suitability of computed tomography as a screening tool. *BMC Vet Res* 10, 212. <http://www.biomedcentral.com/1746-6148/10/212>
- Pérez-Juan M., Afseth N.K., González J., Gispert M., Font i Furnols M., Diaz I., Oliver M.A., Realini C.E. (2010). Prediction of fatty acid composition using a NIRS fiber optic probe at two different locations of ham subcutaneous fat. *Food Res Int* 43: 1416-1422.
- Picard B., Lebre B., Cassar-Malek I., Liaubet L., Berri C., Le Bihan-Duval E., Hocquette J.F., Renand G. (2015). Recent advances omic technologies for meat quality management. *Meat Sci* 109: 18-26.
- Picouet P.A., Gou P., Fulladosa E., Santos-Garcés E., Arnau J. (2013). Estimating of NaCl diffusivity by computed tomography in the Semimembranosus muscle during salting of fresh and frozen/thawed hams. *LWT-Food Sci Technol* 51: 275-280.
- Pla M., Hernández P., Ariño B., Ramírez J.A., Díaz I. (2007). Prediction of fatty acid content in rabbit meat and discrimination between conventional and organic production systems by NIRS methodology. *Food Chem* 100: 165-170.
- Ponsuskili S., Murani E., Phatsara C., Schwerin M., Schellander K., Wimmers K. (2009). Porcine muscle sensory attributes associate with major changes in gene networks involving CAPZB, ANKRD1, and CTBP2. *Funct Int Genomics* 9: 455-471.
- Prevolnik M., Čandek-Potokar M., Škorjanc D. (2004). Ability of NIR spectroscopy to predict meat chemical composition and quality – a review. *Czech J Animal Sci* 49: 500-510.
- Prevolnik M., Čandek-Potokar M., Škorjanc D., Velikonja-Bolta Š., Škrlep M., Žnidaršič T., Babnik D. (2005). Predicting intramuscular fat content in pork and beef by near infrared spectroscopy. *J Near Infrared Spec* 13: 77-85.
- Prevolnik M., Čandek-Potokar M., Škorjanc D. (2010). Predicting pork water-holding capacity with NIR spectroscopy in relation to different reference methods. *J Food Eng* 3: 347-352.
- Prieto N., Roehe R., Lavín P., Batten G., Andrés S. (2009). Application of near infrared reflectance spectroscopy to predict meat and meat products quality: A review. *Meat Sci* 83: 175-186.
- Prieto N., Navajas E.A., Richardson R.I., Ross D.W., Hyslop J.J., Simm G., Roehe R. (2010). Predicting beef cuts composition, fatty acids and meat quality characteristics by spiral computed tomography. *Meat Sci* 86: 770-779.
- Santos-Garcés E., Muñoz I., Gou P., Garcia-Gil N., Fulladosa E. (2014). Estimation of the intramuscular fat content in dry-cured ham from computed tomography images and its effect on the accuracy of aw, salt and water content estimates. *Meat Sci* 96: 943-947.
- Te pas M. F. W., Hoekman A. J. W., Smits M. A. (2011). Biomarkers as management tools for industries in the pork production chain. *J Chain Network Sci* 11: 155-166.
- Viljoen M., Hoffman L.C., Brand T.S. (2007). Prediction of the chemical composition of mutton with near infrared reflectance spectroscopy. *Small Ruminant Res* 69: 88-94.
- Weeranantapan J., Downey G., Allen P., Sun D. (2011). A review of near infrared spectroscopy in muscle food analysis: 2005-2010. *J Near Infrared Spec* 19: 61-104.