International Journal of Meat Science

ISSN 2071-7113

science alert

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International Journal of Meat Science 1 (2): 93-107, 2011
ISSN 2071-7113 / DOI: 10.3923/ijmeat.2011.93.107

Ex-vivo Nuclear Magnetic Resonance Characterization of Pig Muscles

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ABSTRACT

In the last several years Nuclear Magnetic Resonance (NMR) has been widely used to assess muscle structure and function and its potential in meat research is being validated. In this study a unique panel of NMR techniques and morphological procedures was used in order to characterize and discriminate muscles from finishing pigs (Large white hybrid) fed on a standard industrial feed (n = 10) or a 0-miles feed of similar composition (n = 10). The *Supraspinatus*, *Biceps femoris* and *Longissimus dorsi* muscles were dissected out at slaughtering and analyzed. Results showed that muscles from the two groups of pigs differed in connective/fat tissue amount as assessed by NMR imaging and histochemistry; instead, no difference was found in T2 values and fiber type composition. Moreover, some differences in fatty acid mean chain length and molar percentage of 18C fatty acids were revealed by high-resolution NMR spectroscopy. Principal component analysis of NMR spectra discriminated the two groups according to certain metabolites and localized NMR spectroscopy showed several well resolved peaks attributable to creatine, taurine, choline, intra- and extramyocellular lipids suggesting additional discriminant parameters. In conclusion, these data showed the ability of a multimodal NMR approach to detect diet-related features in muscles *ex-vivo*; further study is needed to transfer this approach to meat technology.

Key words: Nuclear magnetic resonance, magnetic resonance imaging, magnetic resonance spectroscopy, skeletal muscle, pig

INTRODUCTION

In the last several years, Nuclear Magnetic Resonance (NMR) has been qualifying as a reliable and powerful technology in the analysis and characterization of food products (Webb *et al*., 2001; Bertocchi and Paci, 2008; Ogrinc *et al*., 2003); NMR procedures are in principle noninvasive and several specific techniques have been proposed to investigate both chemical composition and texture of foods (Dixon *et al*., 2006; Falcone *et al*., 2006). NMR has been widely used to assess muscle structure and function (Prompers *et al*., 2006) and its potential in meat research has been envisaged (Laurent *et al*., 2000). A number of studies thereafter, investigated meat and meat derivatives from different animal sources using NMR techniques (Bertram and Andersen, 2004, 2007), albeit diet is a relevant factor in meat quality (Apple, 2007) the ability of NMR techniques to discriminate meat from animals fed different diets has been not evaluated yet.

The globalization of the swine industry has caused major changes to national and international swine production over the past decade and these changes are likely to continue. Maintaining and increasing the demand for pork, in both domestic and international markets, will depend heavily on such factors as the assurances of food safety, environmental sustainability, animal welfare and the final quality of the product, principally through its effects on the amount and type of fat in meat. Diet components can influence more than one parameter of pork quality, at times in an antagonistic fashion: the goal in feeding pigs for meat quality is finding a balance between the optimization of producer returns, the needs of the packing and retailing industry and the demands of consumers (Buhr, 2004; McCarthy *et al*., 2004; Honeyman *et al*., 2006; Singh and Neelam, 2011).

Since the 1990s several initiatives led by groups of producers and consumers or by local institutions arose with the objective of re-appropriating food at the local level e.g., the Community Supported Agriculture (CSA), the farmer' s markets movement (USA, UK, Ireland, Scotland), local food-buying groups and farm direct selling (Hendrickson and Heffernan, 2002) or food policy councils (Friedmann, 2007). This model of food re-localisation may take place in a context of long-standing industrial agriculture (Morgan *et al*., 2006; Wrigley, 2002); a key factor in this process is the local production of animal feeds (0-miles feed).

In this study a unique panel of NMR techniques, supported by control morphological procedures, was used in order to discriminate muscles from pigs fed on a standard industrial feed or a 0-miles feed of similar composition.

MATERIALS AND METHODS

Animals and housing: Twenty five-month-old pigs (Large white hybrid, Latorre *et al*., 2004) weighing about 90 kg were purchased from one local supplier and grown (finished) at a farm in the Cremona province (Northern Italy). Pigs were randomly assigned to two experimental groups housed in separate pens; the first group (Group A, n = 10) was fed on a standard commercial feed (FA.MA.VIT S.p.A., Pompiano, BS, Italy), the second group (Group B, n = 10) was fed on a locally produced (0-mile) feed made of vegetables cultivated with an inorganic fertilizer with a very low content of N, P and K (Bio-Pre s.r.l., Vicomoscano, CR, Italy). The row composition of the two feeds is reported in Table 1. Both feeds were made of maize, soy, wheat (these components were genetically modified in the standard commercial feed), barley and bran and were supplemented with minerals and vitamins; in addition, the standard commercial feed contained palm oil and sunflower while the 0-miles feed contained protein pea. Field peas (*Pisum sativum* L.) are extensively grown in Italy but they are usually not included in diets for swine. However, field peas may be a substitute for corn and soybean meal in diets for pigs without any alteration in pork quality (Stein *et al*., 2006).

Table 1: Row composition (%) of the feeds Group A and B were fed on

Parameters	Group A	Group B
Moisture	12.50	12.50
Protein	15.37	13.80
Fat	3.49	5.40
Fibre	2.53	3.12
Ash	5.18	6.20
Lysine	0.68	0.44
Methionine	0.46	0.16

Pigs had free access to feed and water and were maintained on the two diets until month 9 of age, when they were slaughtered. The study was conducted between June 2008 and December 2009.

Sample collection: Slaughtering was done according to E.U. regulations at the same slaughterhouse in the morning. The slaughterhouse was within the farm where pigs were grown. An equal number of pigs from Group A and B were slaughtered on three different days. After slaughtering, the following muscles, *Supraspinatus* (chuck tender), *Biceps* (*B.*) *femoris* (topside muscle) and *Longissimus* (*L.*) *dorsi* (boneless loin), were immediately isolated and prepared for subsequent analyses.

***Ex-vivo* Magnetic resonance imaging (MRI):** Specimens were enclosed under vacuum in a plastic bag, stored at 4°C and imaged within 48 h. All experiments were carried out using a Biospec Tomograph System (Bruker, Germany) equipped with a 4.7 T, 33 cm bore horizontal magnet (Oxford Ltd, UK), a 20 G cm^{-1} gradient insert and a Linux computer. A 154 mm i.d. birdcage volume coil and a 50 mm surface coil were used. Contiguous, transversal, T2-weighted (T2W), 2 mm-thick slices were acquired using a RARE (Rapid Acquisition with Refocused Echoes) sequence with the following parameters: repetition time (TR) = 5126 msec, echo time (TE) = 70 msec, RARE factor = 8, field of view and matrix size were optimized depending on the sample size as a compromise between space resolution and signal to noise ratio. Afterwards, transversal multi-echo (number of echoes = 10) T2W SE images were acquired for quantitative T2 mapping with the following parameters: TR = 2000 msec, TE = 20 msec.

T2 values were measured on 10 areas of identical surface per specimen taking care to avoid connective tissue and fat.

Morphometrical analysis was carried out on 10 consecutive slices per specimen by using the software Image J (NIH, USA): the total slice area, corresponding to the total muscle sectional surface was measured (= 100%) and the percentage occupied by connective and fat tissue was calculated.

High-resolution magnetic resonance spectroscopy (MRS): About 1 g of muscle tissue from each specimen of *B. femoris* and *L. dorsi* was extracted according to Folch *et al.* (1957) with modifications of Zancanaro *et al.* (1994).

Spectra were obtained in a Bruker DRX 500 spectrometer, operating at 500.13 MHz proton Larmor frequency at 298 K. Pulse programs from the standard Bruker library (Topspin 1.3) were used according to previously published procedures of Zancanaro *et al.* (1994). Where necessary, confirmation of assignments was obtained from two-dimensional (2D) NMR sequences, as J-resolved, correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY) and 2D-heterocorrelated spectroscopy (HSQC).

Metabolite concentrations in aqueous solution were calculated by comparing their peak areas to the area of TSP, scaled according the number of the corresponding protons. Areas were determined using the integration software implemented in the instrument. Calculations of chain length and mean instauration of fatty acids in the organic fractions were calculated from proton spectra as previously described by Zancanaro *et al.* (1994). In carbon spectra the peak areas between 127 and 132 ppm were used to calculate the molar percentages of the different fatty acids as previously described by Wollenberg (1991).

***Ex-vivo* Localized proton MRS:** Proton-MRS experiments were carried out on selected *L. dorsi* specimens in a 4.7 T Bruker Biospec System equipped with a birdcage coil (72 mm of diameter) and flat surface coil (15 mm of diameter). Spectra were obtained using a PRESS sequence with TR/TE = 2500/20 msec, NEX = 128 , BW = 20.03 ppm, VOX = 64 mm^3 with and without VAPOR water suppression. Temperature was approximately 22°C. Before PRESS acquisition, the magnetic field was shimmed using FASTMAP protocol. LCModel (for spectrum type 'muscle-1') was utilized for water suppressed spectra quantification relatively to the Creatine signal. Integrals were calculated by using TOPSPIN 1.3 Version provided by PARAVISION 4.0 acquisition software.

Light microscopy: Immediately after slaughtering, muscle tissue samples dissected out from *Supraspinatus*, *B. femoris* and *L. dorsi* muscles were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 5 h at 4°C, dehydrated with ethanol and embedded in paraffin wax. Five-µm-thick transversally sectioned muscle samples were either stained with the Mallory technique for evidencing connective tissue or submitted to immunohistochemical procedures for fibre typing as previously described by Malatesta *et al.* (2009). Briefly, sections were incubated with a mouse monoclonal antibody recognizing the skeletal fast fibre myosin (clone MY-32, Sigma-Aldrich, Buchs, Switzerland), then revealed with an Alexa 488 conjugated antibody (Molecular Probes, Invitrogen, Milan). Micrographs were taken with an Olympus BX51 microscope and recorded with an Olympus Camedia C-5050 digital camera.

Morphometrical evaluation of the percentage of connective tissue was carried out on Mallory-stained sections: the area occupied by the connective structures was measured at 20X magnification on ten randomly selected areas (15,000 μm^2 each) per muscle sample by using the software Image J (NIH). Morphometrical evaluation of fast and slow fibre size was performed on immunolabelled samples: the cross-sectional area of 100 muscle fibres per sample was measured by using the software Image J (NIH). Moreover, the percentage of fast and slow muscle fibres was calculated on a total of 500 fibres per sample.

Results for each variable were pooled according to the experimental groups.

Statistical analysis: Data are presented as Mean±SE. Comparisons of variables were performed by one-way ANOVA. High-resolution NMR spectra were analysed using Principal Component Analysis (PCA), an unsupervised pattern recognition method, to examine the intrinsic variations of the dataset and to discriminate between the main metabolites responsible for them; the free HiRes 1.5 software (Zhao *et al.*, 2006) developed at Columbia University was used. The water peak was excluded from analysis to remove variations in the suppression of the signals; the signal from lactate was not considered as well because of the great variance. Peak alignment and normalization by multiple regions were performed before data analysis. Four Principal Components (PCs) were adopted for each dataset in order to obtain satisfactory score plots. For all analyses the level of significance was set at $p \leq 0.05$.

RESULTS

At the end of the 4-month experimental period, pigs had similar mean body weight in Group A (152.1±2.00 kg) and Group B (153.7±2.79 kg). Complete dataset (MRI and morphological data) were available for seven and eight pigs in groups A and B, respectively.

MRI and Light microscopy: Representative serial MRI sections of *L. dorsi* from Group A and B pigs are presented in Fig. 1. T2 values of muscle tissue were not significantly different in the two groups of pigs for all the considered muscles (Table 2).

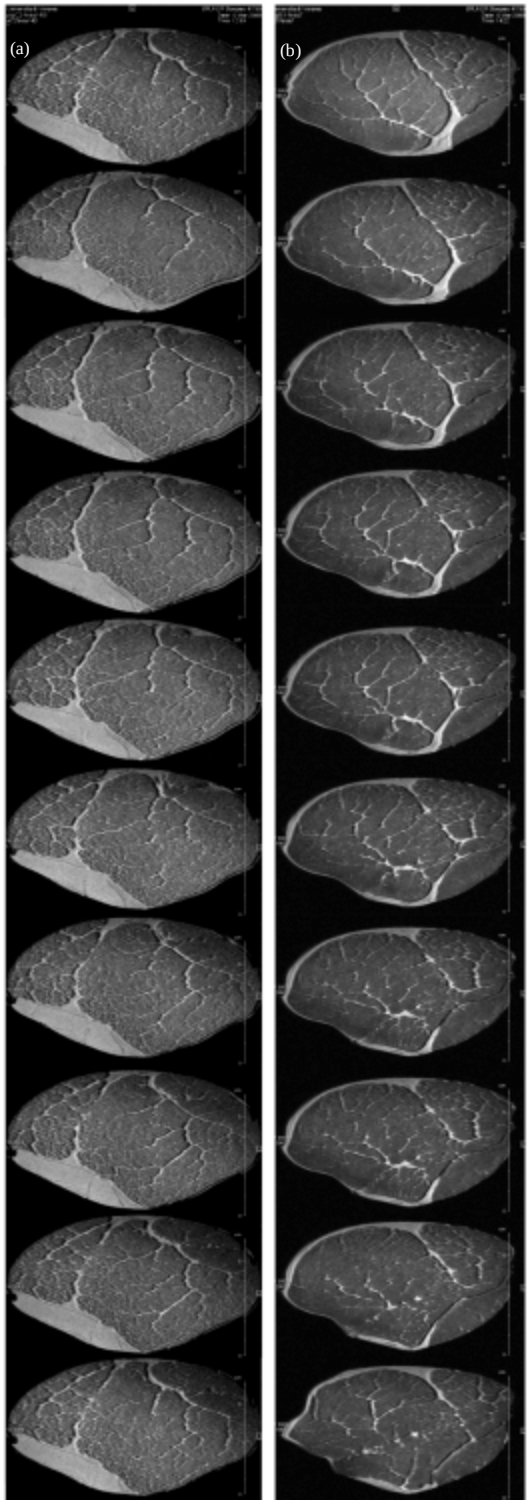

Fig. 1: Serial MRI sectioning of *L. dorsi* muscles from group A (a) and group B (b) pigs. The internal texture of the meat is clearly depicted. Connective and fat (bright areas) and muscle tissue are easily identified

Table 2: T2 values (Mean±SE) in muscles from group A and B pigs

Groups	*Supraspinatus* (msec)	*B. femoris* (msec)	*L. dorsi* (msec)
A (n = 7)	51.57±1.49	53.21±0.46	51.18±1.40
B (n = 8)	50.37±1.20	53.59±1.02	50.42±1.52

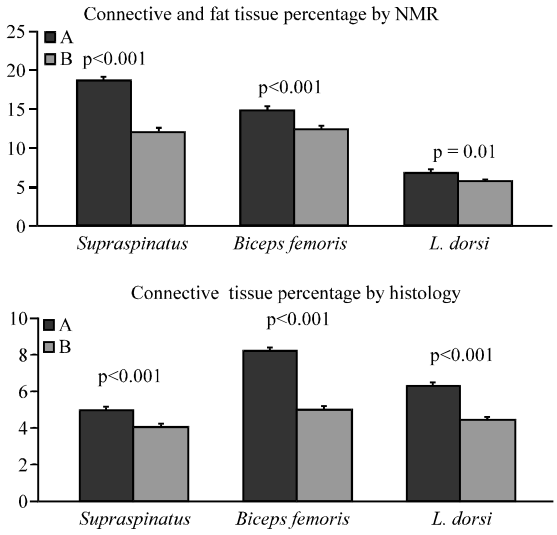

Fig. 2: Morphometry of three muscles from Group A (n = 7) and B (n = 8) pigs. Value are Mean±SE

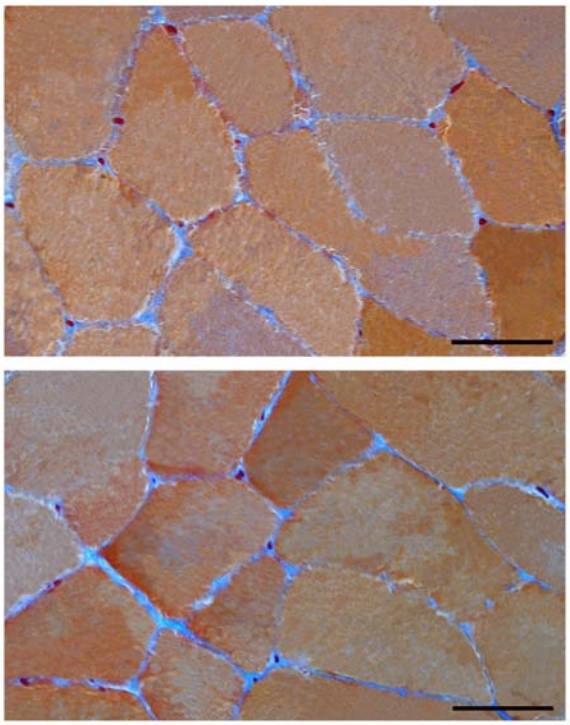

Fig. 3: Representative light micrographs of pig muscles from Group A (upper panel) and B (lower panel). Mallory staining for connective tissue (azure). Bar, 50 μm

Morphometrical analyses on MRI sections (Fig. 2) showed significantly lower percentages of connective and fat tissue in *Supraspinatus*, *B. femoris* and *L. dorsi* muscle of Group B pigs.

Light microscopy did not reveal differences in the general features of any muscles from the two groups. Representative pictures of Mallory-stained sections are presented in Fig. 3; muscle sections immunostained for fast and slow fibres are presented in Fig. 4. Morphometrical analyses on histological sections (Fig. 2) showed significantly lower percentages of connective tissue in *Supraspinatus*, *B. femoris* and *L. dorsi* muscle of Group B pigs. The percentage of slow and fast fibres (Fig. 5) was similar in all the muscles studied, without difference between Group A and B pigs.

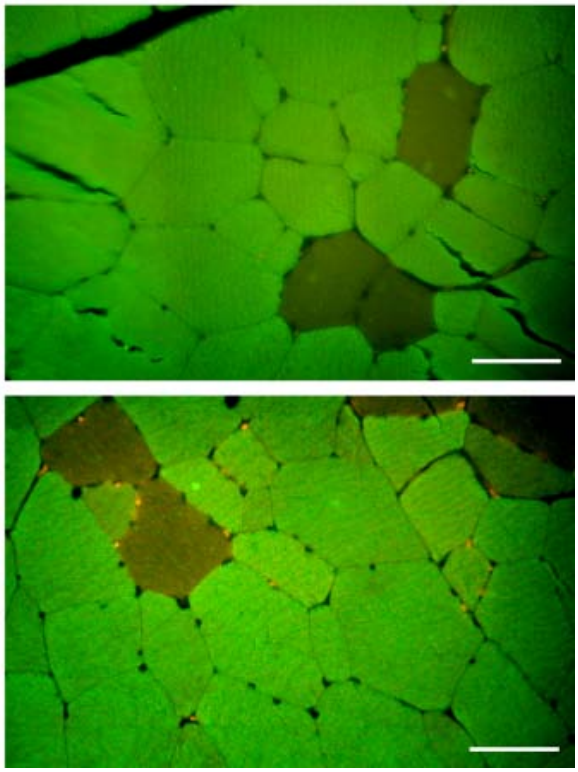

Fig. 4: Representative fluorescence micrographs of pig muscles from Group A and B after immunolabeling for fast, type II fibres (green); the few slow, type I muscle fibres are unstained. Bar, 50 µm

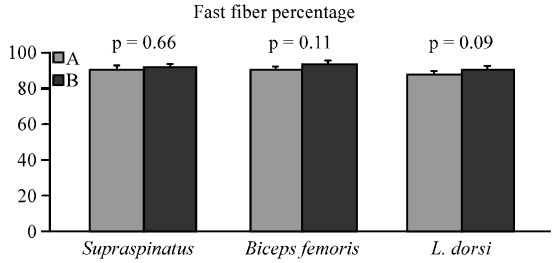

Fig. 5: Morphometry of muscle sections from Group A (n = 7) and B (n = 8) after immunolabeling of fast type II fibres. Value are Mean±SE

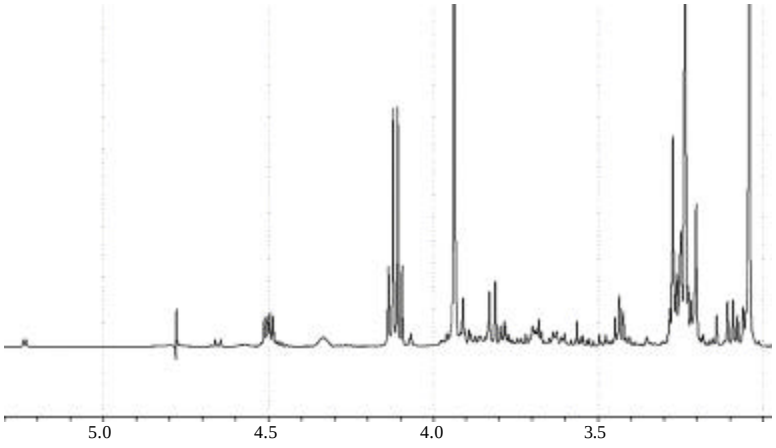

Fig. 6: Midfield region of a representative proton NMR spectrum at 500 MHz of the water fraction of Folch extracted pig skeletal muscle

MRS: A representative proton NMR spectrum of the aqueous fraction of extracted pig muscle is presented in Fig. 6. Several chemical constituents of the meat are clearly identifiable. A list of the main NMR-visible metabolites in pig muscle and their correspondent chemical shifts are showed in Table 3.

Quantitative analysis of proton spectra is summarized in Table 4. The mean unsaturation of carbon chains in *B. femoris* and *L. dorsi* was similar in Group A and B pigs; mean chain polyunsaturation showed an opposite pattern in the two muscles, its mean value being higher in *B. femoris* of Group A pigs and in *L. dorsi* of Group B pigs but these differences were not statistically significant. Mean chain length was longer in *L. dorsi* of Group B pigs ($p<0.001$) and similar in *B. femoris* of A and B group.

Quantitative analysis of carbon spectra (Table 5) showed significant difference in the molar percentages of 18C fatty acids in *B. femoris* of the two groups of pigs ($p<0.0001$ for all) but not in *L. dorsi*, although a similar pattern was present in the two muscles i.e., a higher molar percentage of 18:1 and 18:3 fatty acids in Group B pigs.

Results of PCA analysis are presented in Fig. 7. The two first principal components (1 and 2), which explained 93.62% of variance did not discriminate *L. dorsi* muscles from the two groups of pigs (Fig. 7, upper panel); however, principal components 1 and 4 (explaining 86.74% of variance) allowed good discrimination of the two groups (Fig. 7, middle panel), a finding confirmed when principal components 3 and 4 (explaining 3.61% of variance) were considered (Fig. 7, lower panel). Metabolites discriminating Group A muscles were cystein, taurin, choline and glycerophosphorylcholine; metabolites discriminating Group B muscles were alanine, creatine/phosphocreatine, carnitine and lysine.

Ex vivo proton MRS of *L. dorsi* (Fig. 8) showed several well resolved peaks attributable to creatine, taurine, choline, intra and extramyocellular lipids. Data were available for four pigs in Group A and five pigs in Group B. When expressed as creatine ratio, choline showed a tendency to be lower in Group B pigs (0.2±0.05 vs. 0.4±0.07) as well as taurine (0.3±0.17 vs. 0.4±0.29) and intramyocellular lipid (5.0±1.65 vs. 8.6±2.61), whereas extramyocellular lipid was similar (2.81±.1.17 vs. 2.8±1.31). The fat/water ratio was lower in Group B pigs (0.043±0.0130 vs 0.064±0.0280).

Table 3: Metabolites found in the aqueous extract of pig muscle (left column) and chemical shifts of the corresponding molecular component (in parentheses)

Metabolite	Chemical shift (ppm)
Hystidine	8.37 (imidazole), 7.20 (imidazole), 4.50 (α-CH), 3.14-3.05 (β-CH)
Alanine	1.48 (β-CH_3), 3.7 (α-CH)
Glutamine	3.77 (α-CH), 2.48-2.43 (γ-CH_2), 2.14-2.12 (β-CH_2)
Glutamate	3.74 (α-CH), 2.48-2.43 (γ-CH_2), 2.14-2.12 (β-CH_2)
Carnitine	4.57 (OH), 3.44 (CH_2), 3.27 (NCH_3), 2.42 (CH_2)
Leucine	3.69 (α-CH), 1.74-1.69 (β-CH_2, γ-CH), 1.06-0.93 (δ-CH_3)
Isoleucine	3.64 (α-CH), 2.06-1.99 (β-CH), 1.26-1.24 (γ-CH), 1.06-0.93 (δ-CH_3)
Glycine	3.55 (CH_2)
Valine	3.60 (CH), 2.31-2.25 (CH) ,1.06-0.93 (CH_3)
Cysteine	3.97 (α-CH), 3.22 (β-CH), 3.05 ((β-CH)
Lysine	3.76 (α-CH), 3.03 (ε-CH), 1.92 (β-CH), 1.47 (γ-CH) 1.74-1.70 (δ-CH)
Lactate	1.33 (CH_3), 4.11(CH)
Formiate	8.46 (CH)
Succinate	2.41 (CH_2)
Pyruvate	2.38 (CH_3)
α-chetoglutarate	2.47 (CH_2), 3.19 (CH_2)
Malate	4.29-4.24 (α-CH), 2.43 (β-CH_2), 2.65 (β'-CH_2)
Acetate	1.92 (CH_3)
Citrate	2.65 (CH_2), 2.51 (CH_2)
IMP	7.15 (H1), 8.27
ATP	8.45 (CH(8)), 8.27 (CH(2)), 6.11 (CH(1'))
ADP	8.45 (CH(8)), 8.27 (CH(2)), 6.11 (CH(1'))
Creatine	3.04 (CH_3), 3.93 (CH_2)
Phosphocreatine	3.04 (CH_3), 3.93 (CH_2)
Coline	3.20 ($N(CH_3)_3$), 3.52 (NCH_2), 3.97 (CH_2OH)
Phosphorylcoline	4.33 (CH_2), 3.71-3.67 (CH_2), 3.23 ($N(CH_3)$)
GPC	4.33 (CH_2), 3.91 (CH_2, glyceril), 3.84-3.76 (CH, CH_2 glyceril) 3.71-3.67 (CH_2), 3.23 ($N(CH_3)$)
γ-OHB (butirric acid)	0.96 (γ-CH_3), 1.68-1.63 (β-CH_2), 4.08 (α-CH)
β-OHB (butirric acid)	2.06-2.02 (γ-CH_3), 2.48 (β-CH_2), 4.14 (α-CH)
Taurine	3.28 (CH_2NH), 3.43 (CH_3SO_3)
Acetone	2.15 (CH_3)
α-glucose	5.24 (α-H1), 3.80-3.52 (H2, H3, H4, H5, H6, H6')
β-glucose	4.65 (α-H1), 3.72-3.33 (H2, H3, H4, H5, H6, H6')
myo-inositol	4.07 (H1, H3), 3.62 (H4, H6), 3.54 (H1, H3), 3.27 (H5)

Table 4: Quantitative analysis of high-resolution proton spectra of the aqueous fraction of extracted pig muscles. The characteristics of carbon chains in Group A and B are presented

	Chemical parameter		
Muscle	Mean chain unsaturation	Mean chain polyunsaturation	Mean chain length
B. femoris			
Group A (n = 6)	0.79±0.007	0.17±0.011	19.42±0.370
Group B (n = 7)	0.79±0.008	0.14±0.011	19.36±0.379
F value/significance	F = 0.023, p = NS	F = 4.627, p = NS	F = 0.010, p = NS
L. dorsi			
Group A (n = 7)	0.73±0.010	0.14±0.011	19.15±0.230
Group B (n = 7)	0.75±0.014	0.16±0.011	21.02±0.285
F value/significance	F = 1.666, p = NS	F = 1.680, p = NS	F = 25.950, p<0.001

NS: Not significant

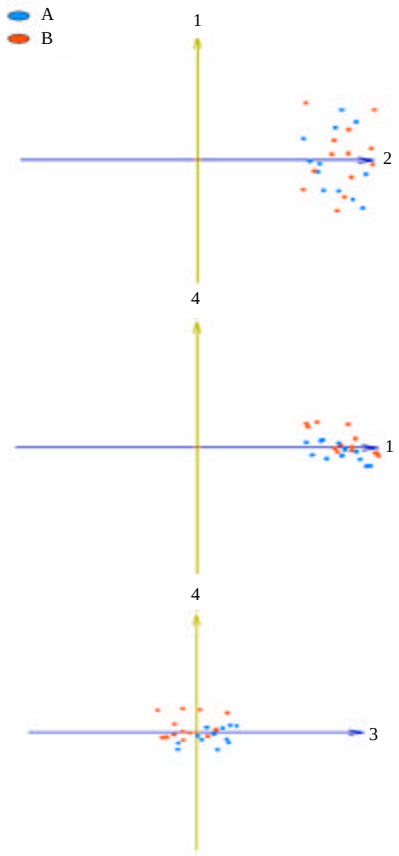

Fig. 7: PCA analysis of high-resolution proton NMR spectra of *B. femoris* and *L. dorsi* muscles from group A and B pigs. The first two principal components (upper panel) do not discriminate between groups. Partial discrimination is achieved using principal components 1 and 4 (middle panel), and 3 and 4 (lower panel)

Table 5: Quantitative analysis of high-resolution carbon spectra of the organic fraction of extracted pig muscles. Molar percentages of 18C fatty acids in Group A and B

	18C Fatty acid		
Muscle	18:1	18:2	18:3
B. femoris			
Group A (n = 5)	63.60±1.291	35.79±1.315	0.61±0.028
Group B (n = 5)	84.09±1.756	15.07±1.775	0.84±0.019
F value/significance	F = 88.316, p<0.0001	F = 87.890, p<0.0001	F = 43.405, p<0.0001
L. dorsi			
Group A (n = 5)	73.79±3.922	25.44±3.999	0.74±0.039
Group B (n = 6)	77.01±2.504	22.17±2.483	0.77±0.024
F value/significance	F = 0.513, p = NS	F = 0.526, p = NS	F = 0.522, p = NS

NS: Not significant

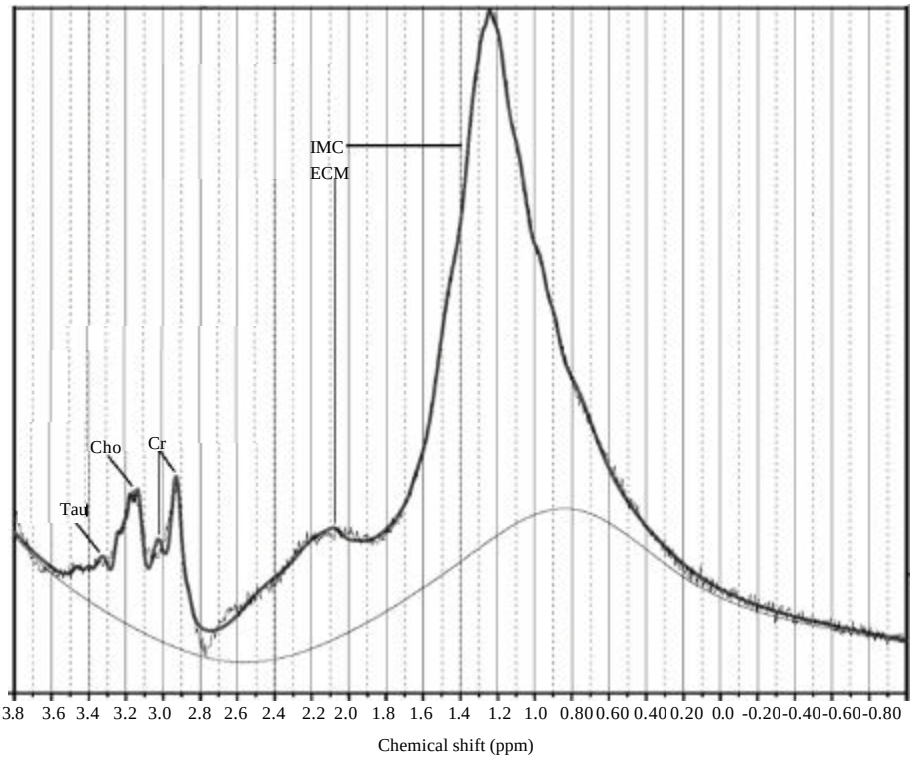

Fig. 8: Representative *ex vivo* proton MRS spectrum of pig *L. dorsi* muscle. Peak assignment is as follows: Tau, taurine; Cho, coline compounds; Cr, creatine compounds; IMC, intramyocellular lipids; EMC, extramyocellular lipids

DISCUSSION

Results of this study show that a panel of NMR techniques is able to evidence some structural and compositional differences in muscles of pigs fed standard industrial (Group A) or 0-miles (Group B) feed. In particular, the three considered muscles (*Supraspinatus*, *B. femoris*, *L. dorsi*) showed lower amounts of fat and connective in Group B by MRI, a finding confirmed by histochemical analysis (Fig. 1-3). The connective tissue is mainly composed by collagen. Previous studies performed on pig muscles demonstrated that the distribution of collagen types I and II and the characteristics of the three dimensional network formed by the collagen fibers influence meat tenderness (McCormick, 1999). These data suggest that the 0-mile feed can affect pork quality by acting upon the intramuscular amount of connective tissue.

On the contrary, no difference was found in T2 values and fiber type composition in muscles from the two groups of pigs (Table 2; Fig. 4 and 5). The NMR signal arises from magnetic activity of protons, mainly in tissue water and fat. Longitudinal (T1) and transverse (T2) relaxation times are the main signal components used to generate NMR images; T1 and T2 have no direct biological significance but are sensitive to changes in tissue structure and/or water content (Hazlewood, 1974) as well as the water holding capacity of meat (Brondum *et al*., 2000; Bertram and Andersen, 2007). Interestingly, it has been demonstrated that T2 values give information about fiber type composition of skeletal muscles, the increase in fast, type II fibers being associated with decreased T2 values and the increase in slow, type I fibers with increased T2 values (Hatakenaka *et al*.,

2001); in turn, muscle composition in fiber type represents an important parameter for meat evaluation. In fact, fiber types I and II are characterized by different metabolism (oxidative or glycolytic/anaerobic, respectively) and play an important role in determining the organoleptic features of the meat (Andersen *et al*., 2005; Bee *et al*., 2007). Muscles mainly composed of type I fiber, characterized by a high oxidative activity, tend to show a high pH value and the meat can become dry, firm and dark (Hambrecht *et al*., 2005; Franck *et al*., 2007). On the other hand, muscles rich in type II fibers, characterized by a high glycolytic activity, can give origin to pale, soft and exudative meat due to the excessive lowering of the tissue pH (Solomon *et al*., 1998; Hambrecht *et al*., 2005; Maltin *et al*., 2003). Moreover, it is known that diet can influence muscle fiber distribution in rodents when the protein (Fruhbeck *et al*., 1999) or fat (Shortreed *et al*., 2009) content are altered. In the present study we found similar T2 values in Group A and Group B pigs indicating similar muscle fiber composition; accordingly, immunohistochemical data (Fig. 4 and 5) showed no difference in type II fibers percentages. It may be concluded that the two feeds pigs were fed on did not affect these parameters. This was expected because the two feeds were not markedly different in their protein or fat content; in addition, it is worth recalling that the pigs used in this study started to be fed on different feeds when they were 5 months old and it has been shown that different diets can influence muscle fiber distribution in pigs mainly during the early postnatal period (Picard *et al*., 2002).

Proton and carbon MRS of Folch-extracted pig muscle is able to identify a number of chemical constituents therein (Fig. 6; Table 3), also enabling quantitative analysis of selected items (Table 4 and 5). Despite the limited number of analyzed samples, quantitative data show a general tendency for chemical parameters to differentiate between both muscles (*B. femoris* vs. *L. dorsi*) and diets (Group A vs. Group B) with a clear-cut effect for C18 fatty acids in *B. femoris* (Table 5). It is known that the effect of dietary fat content is muscle fiber type dependent (Janovska *et al*., 2010) and it is therefore, likely that different muscles are differently affected; the fact that the diets used in our study slightly differed in percent fat content further emphasizes the sensitivity of MRS analyses. Besides allowing identification of a limited number of chemical constituents in tissue (Table 3), NMR spectra (Fig. 6) yields a wealth of information which can be exploited by chemometric statistical methods independently of individual peak assignment (Weljie *et al*., 2006). PCA analysis of pig muscle spectra (Fig. 7) revealed clear, albeit limited, ability to discriminate Group A and B animals, the main difference being related to some aminoacids and phosphorylated compounds. While a larger number of samples are required to substantiate this finding, the NMR chemometric approach confirms to be useful for food analysis (Ogrinc *et al*., 2003).

Finally, results of *ex-vivo* localized proton MRS deserve comment. To the best of our knowledge it is the first time this NMR procedure is used in meat analysis. Localized proton MRS has been used to assess the presence and concentration of chemical substances in living tissue and the detection of biochemical changes associated with certain diseases (Weber *et al*., 1997) as well as measure intramuscular fat content in pig (Ville *et al*., 1997); localized proton MRS is, however, limited by its relatively low sensitivity and spectral resolution. Albeit preliminary, due to low number of samples from one muscle (*L. dorsi*) and the relatively large inter-sample variability, the localized proton MRS approach was able to clearly, semi-quantitatively identify some relevant metabolites in *L. dorsi* samples (Fig. 8). This may reveal practical in pork evaluation; for example, the amount of creatine in the muscle affects the formation of lactic acid and, in turn, the pH decline post-mortem and thus the color and water-holding capacity of meat; moreover, quantifying the

and extramyocellular amount of lipid could help better understanding of the important role of marbling in the eating quality and composition of meat (Moloney *et al*., 2001).

CONCLUSION

In this study, we used a panel of complementary NMR and morphological approaches to confirm and extend the suitability of this technique in evaluating *ex-vivo* the compositional characteristics of skeletal muscle intended for alimentary use. Results allowed identifying several meat structural and chemical constituents; further discrimination of muscle from pigs fed on a standard industrial feed or a 0-miles feed of similar composition was obtained for the first time. Further work is needed to transfer this approach to meat technology.

ACKNOWLEDGMENTS

This work was supported by the University of Verona (Joint Projects 2007). EM is a PhD student in receipt of a fellowship from the Dottorato di ricerca in Imaging Multimodale in Biomedicina (University of Verona).

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