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Evaluation of gene expression profiles of pig skeletal muscle in response to energy content of the diets using human microarrays

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ABSTRACT

The aim of the research was to compare gene transcription profiles of Musculus longissimus dorsi (MLD) between pigs fed diets with high (HED) or low (LED) energy contents. Two groups of 4 Casertana pigs were reared from 3 to 12 months of age in the same environmental conditions and fed HED or LED. In the HED, the average daily gain and back fat thickness were significantly (P<0.05) higher than in LED pigs. Differential expression of genes in MLD of pigs fed diets with different energy density was assessed by a human high-density complementary DNA (cDNA) muscle microarray consisting of 4670 probes and further confirmed by quantitative real time RT-PCR analysis. Seven of the genes up-regulated in MLD of HED pigs were involved in the glycolytic and oxidative metabolism (phosphoglycerate mutase 2, glyceraldehyde-3-phosphate dehydrogenase, NADH dehydrogenase ubiquinone1 beta 9, muscle pyruvate kinase, enolase 3, muscle creatine kinase, isocitrate dehydrogenase 3 (NAD+) gamma) and four in the contractile apparatus (troponymosin 1 alpha, troponin C2, fast, fast skeletal myosin light chain 2, troponin T3, skeletal, fast). Instead, HED diet reduced the level of expression of muscle proteins associated with slow fibre type (troponin T1, skeletal, slow, supervillin, myosin binding protein C, slow type, titin, myosin, heavy polypeptide 7, beta, calponin homology-associated smooth muscle) and signal transduction (SH3-binding domain protein 5-like, hypothetical protein FLJ12143B, protein kinase CAMP-dependent, catalytic, rho guanine nucleotide exchange factor 15). The down-regulation of CTSB was also observed for HED group. From the results it can be assumed that high energy content of the diet influence physiological processes in the muscle tissue by switching slow fibres into fast reacting fibres and thus enhancing meat quality.

Key Words: Dietary energy, Gene transcription, Microarray, Skeletal muscle, Pig.
RIASSUNTO
USO DI MICROARRAY PER LO STUDIO DELL’ESPRESSIONE GENICA NEL MUSCOLO SCHELLETRICO DI SUINI ALIMENTATI CON DITELE A DIVERSA DENSITÀ ENERGETICA

Nella presente ricerca si è voluto confrontare il profilo di espressione genica del muscolo MLD (M. longis - simus dorsii) di due gruppi di suini alimentati rispettivamente con una dieta ad alta (HED) e a bassa (LED) densità energetica. A tal fine sono stati impiegati due gruppi di 4 suini ciascuno, omogenei per età e peso vivo iniziale, che sono stati allevati da 3 a 12 mesi di età e alimentati con le diete sperimentali. L’accrescimento medio giornaliero e lo spessore del grasso dorsale sono risultati significativamente più elevati (P<0,05) nei suini del gruppo HED rispetto al gruppo LED (0,436 vs 0,417 g/d; 49,25 vs 35,75 mm). L’espressione genica differenziale è stata valutata mediante l’utilizzo di un microarray umano cdNA e ad alta densità comprese 4670 sonde; i risultati sono stati validati mediante analisi in real time RT-PCR. Tra i geni sopra regolati in MLD del gruppo di suini HED, la maggior parte è risultata appar tenere al metabolismo glicolitico e ossidativo (phosphoglycerate mutase 2, glyceraldehyde-3-phosphate dehydrogenase, NADH dehydrogenase ubiquinone1 beta 9, pyruvate kinase muscle, enolase 3, creatinine kinase muscle, isocitrate dehydrogenase 3 NAD+ gamma) e sistema contrattile (tropomyosin 1 alpha, tropomin C2, fast, fast skeletal myosin light chain 2, troponin T3, skeletal, fast). Al contrario, HED ha portato a una riduzione del livello di espressione delle proteine muscolari associate alle fibre di tipo lento (tropomin T1, skeletal, slow, supervillin, myosin binding protein C, slow type, titin, myosin, heavy poly peptide 7, beta, calponin homology-associated smooth muscle) e a quelle legate ai segnali di trasduzione (SH3-binding domain protein 5-like, hypothetical protein FLJ21438, protein kinase CAM-dependent, catalytic, rho guanine nucleotide exchange factor 15). Inoltre nel gruppo HED la CTBS è risultata sotto-regolata. L’analisi dei dati con Gene Ontology ha indicato che il contenuto energetico della dieta comporta un adattamento dei processi fisiologici e delle funzioni molecolari e può causare una variazione delle fibre dal tipo lento a quello veloce.

Parole chiave: Energia, Trascrizione genica, Microarray, Muscolo, Suino.

Introduction

The understanding of molecular mechanisms regulating muscle metabolism and structure is of major importance for technological and nutritional management of improved meat quality. Meat sensory quality is affected by productive factors, such as nutrition and environment, which exert biological characteristics of muscle tissue (collagen and lipids contents) and, in particular, muscle fibres (Klont et al., 1998).

The variation of the nutrition plan during the initial process of growth can affect tissue development in later phases of life. The effect of compensatory energy regimen has been demonstrated for mammary gland development and lactation potential in rats and cattle (Park et al., 1998; Kim and Park, 2004) and for muscle growth in pigs (Chiba et al., 2002). In swine, the relationship between caloric restriction and molecular changes in muscles was recently reported (Da Costa et al., 2004), and indicated that energy availability plays a role in the regulation of specific genes, also affecting muscle functions and compositions (Chiba et al., 1999).

The application of genomic technology to nutrition (nutrigenomics) will allow the identification of transcriptional profile in response to nutrients, enabling the development of much stronger theoretical and molecular bases for nutrient responses (Bryden, 2004). The limited knowledge of livestock-specific muscle expressed sequence data has delayed the development of genomic studies in meat-producing animals and this research was performed in a heterologous system using a human muscle repertoire (Lanfranchi et al., 1996). A comparable approach was recently published, and microarray-based gene transcription was used to compare transcription profiles of muscle between two differ-
Table 1. Ingredients, chemical composition (as fed) and nutritive value of high energy (HED) and low energy (LED) diets used in the experiment.

| Item                                | From 3 to 6 months | From 6 to 12 months |
|-------------------------------------|-------------------|---------------------|
|                                     | HED   | LED   | HED   | LED   |
| Feed                               |       |       |       |       |
| Corn, meal                         | %     |       |       |       |
|                                    | 39.7  | 17.7  | 55.0  | 11.3  |
| Barley                             |       |       |       |       |
|                                    | 10.0  | 17.3  | 11.7  | 15.5  |
| Flour by-products                  | "     |       |       |       |
|                                    | 29.2  | 40.1  | 16.4  | 48.8  |
| Beet pulp, dehy                    | "     |       |       |       |
|                                    | 0.0   | 6.8   | 0.0   | 13.5  |
| Soybean meal, s.e.                 | "     |       |       |       |
|                                    | 12.7  | 7.9   | 12.5  | 3.2   |
| Soybean, roasted                   | "     |       |       |       |
|                                    | 4.0   | 0.0   | 0.0   | 0.0   |
| Sunflower meal, s.e.               | "     |       |       |       |
|                                    | 0.0   | 5.0   | 0.0   | 2.0   |
| Potato protein                     | "     |       |       |       |
|                                    | 0.5   | 0.1   | 0.3   | 0.2   |
| Oil, coconut                       | "     |       |       |       |
|                                    | 0.8   | 0.3   | 0.0   | 0.0   |
| Mineral-vitamin                    | "     |       |       |       |
|                                    | 3.2   | 5.0   | 4.1   | 5.5   |
| Composition :                      |       |       |       |       |
| Dry matter                         | %     |       |       |       |
|                                    | 90.4  | 91.4  | 89.7  | 91.9  |
| Crude protein                      | "     |       |       |       |
|                                    | 16.4  | 15.9  | 14.5  | 14.1  |
| Lipids                             | "     |       |       |       |
|                                    | 4.4   | 3.5   | 3.1   | 2.8   |
| Crude fibre                        | "     |       |       |       |
|                                    | 3.1   | 4.6   | 3.0   | 5.3   |
| Ash                                | "     |       |       |       |
|                                    | 4.4   | 5.2   | 4.8   | 5.7   |
| NDF                                | "     |       |       |       |
|                                    | 11.3  | 15.5  | 10.1  | 18.2  |
| Digestible Energy, Kcal/kg         |       |       |       |       |
|                                    | 3290  | 3020  | 3190  | 2890  |

Different pig breeds which differ for growth potential (Lin and Hsu, 2005).

The aim of the presented study is to refer to differential expressions of genes in MLD of pigs fed diets with different energy density, assessed by a human muscle microarray.

Material and methods

Animals and sample collections

Eight male pigs of the same age belonging to a local breed (Casertana) reared from 3 to 12 months of age in the same environmental conditions were used. Animals were divided in two groups and fed diets differing for digestible energy content. Four pigs were allotted to a low energy diet (LED group) starting from 3 months of age until slaughter and the remaining four pigs were fed a high energy diet (HED group) for the same period (Table 1). Animals were offered an amount of feed according to their weight and were kept in separate boxes with free access to water and to external paddock. Individual live weights were measured at 3, 6 and 12 months of age and feed refusal, if any, recorded per group on weekly basis. Animals were slaughtered at 12 months of age in a local abattoir using conventional procedures. The eviscerated carcass was split longitudinally and the midline backfat thickness was measured at the last rib. The Musculus longissimus dorsi (MLD) area was traced using acetate paper. Immediately after
Table 2. Live weights and average daily gains of pigs fed high energy (HED) and low energy (LED) diets.

|                        | HED          | LED          | Significance of t |
|------------------------|--------------|--------------|------------------|
| Average daily intake   | kg           | kg           |                  |
| Feed conversion unit   | kg/kg        | 5.36 ± 0.45  | 5.31 ± 0.44      | 0.874            |
| Live weight:           |              |              |                  |
| 3 months               | kg           | 34.6 ± 3.1   | 33.30 ± 7.2      | 0.171            |
| 6 months               | "           | 83.1 ± 4.6   | 84.20 ± 9.6      | 0.131            |
| 12 months              | "           | 155.0 ± 6.6  | 148.40 ± 5.4     | 0.111            |
| 3–12 months            | "           | 120.4 ± 6.8  | 115.15 ± 6.2     | 0.043            |
| Average daily gain     | g/d          |              |                  |
| 3–6 months             | 0.522 ± 0.019| 0.545 ± 0.025| 0.228            |
| 6–12 months            | 0.393 ± 0.042| 0.352 ± 0.046| 0.046            |
| 3–12 months            | 0.436 ± 0.025| 0.417 ± 0.023| 0.043            |
| MLD area               | mm²          | 45.00 ± 7.2  | 46.55 ± 5.4      | 0.743            |
| Back fat thickness     | mm           | 49.25 ± 4.7  | 35.75 ± 5.6      | 0.010            |

MLD: Musculus longissimus dorsi.
SD: standard deviation.

slaughter, samples of MLD were collected at the level of the 3-4th rib and frozen in liquid nitrogen. All the procedures were carried out in accordance with state and local laws and ethical regulations.

*Microarray hybridisation*

Total RNA was isolated from 50 mg of MLD homogenized in 1 ml Trizol Reagent (Invitrogen, Milan, Italy) according to the instructions. Total RNA quality was assessed by formaldehyde agarose gel electrophoresis. mRNA was directly reverse transcribed to cDNA from 25 µg of total RNA using the Superscript indirect cDNA labeling Core kit (Invitrogen, Milan, Italy). Due to the poor quality of total RNA extracted from some samples, microarray analysis was restricted to 3 pigs of the LED group and 2 pigs of HED group.

To assess the diet effect on the MLD expression profiles, co-hybridization experiments were performed using individual RNA extracts (pig #1-LED Vs pig #1-HED; pig #2-LED Vs pig #2-HED; pig #3-LED Vs pig #1-HED). For each microarray experiment hybridizations were performed in duplicate dye swap. A total of 6 microarray hybridizations were performed.

The microarray consisted of a set of 4670 clones of cDNA obtained by sequencing 3′-terminal libraries from muscle, heart and bone marrow human mRNA. Spotted cDNA inserts lengths ranged from 300 to 500 bp. The array used for the present study was a custom array that was derived from the release 2.0 of the human CRIBI microarray (CRIBI, 2005). The
array contains 3777 clones of genes expressed in muscle and heart, 913 in lymphocytes and 24 negative controls. The clones amplified by PCR were deposited in duplicate glass slides (MICROMAX Glass Slides SuperChip 1, PerkinElmer Life Sciences, Inc) using the roboticising genpakARRAY 21 (Shalon et al., 1996). The quality was checked by GSI Lumonics scanner, with a scan of 10 μm, using a CRIBI proprietary protocol, based on the reading of salts added to the cDNA solution.

The muscle cDNA was purified and labelled with Cy3 and Cy5 fluorochromes using the cDNA labeling purification module kit (Invitrogen, Milan, Italy). The HED samples were analysed in comparison to LED samples. The labelled cDNA was appropriately coupled and used for competitive hybridisation on the same microarray at 42°C for 16h. The relative intensity of labelled cDNA in HED and LED was acquired with ScanArray LITE scanner (PerkinElmer Life Sciences, Inc).

**Microarray analysis**

Microarray images were analysed with ScanArray software (PerkinElmer Life Sciences, Inc). The raw data acquired from the two channels were normalised using the statistical analysis of microarray data normalization program of the Institute for Genomic Research (TIGR, 2005) according to the LOWESS and SD normalization (Quackenbush, 2002; Yang et al., 2002). Normalization was done using all data in the beginning, and then excluding the negative controls and positive controls containing mitochondrial genes to reduce the number of differential expressed genes since the aerobic metabolism is principally affected by caloric restriction. The normalization was carried out for each slide separately and we obtained 4 logarithmic values for each spot, considering the spot duplication in each slide and the dye swap. This 4 logarithmic transformation of the dual channel intensity ratio was used to identify genes that significantly differed from a null value using the Significance Analysis of Microarrays (SAM, 2005). SAM works using the spots and representing them by their M- and A-values: M= log2 (HED/LED); thus, the M-value indicates the differential expression between the HED and LED labeled RNA samples; and A = (log2 [HED * LED])/2. Therefore, the A-value indicates a weighted mean expression level of the Cy5- and Cy3-labeled RNA samples (Yang et al., 2002). Additionally, the significance of the difference is indicated by the 2-sided P value tested on a logistical distribution. The false discovery rate (FDR) method presented in Storey (2002) was applied to identify up and down regulated genes. Data shown in Table 3 and 4 were extracted from analysis with a total FDR of 17% including in the SAM analysis mitochondrial genes, negative and positive controls. Parallel analysis (data not shown), not considering the controls, reduce the FDR of the same dataset to 8%.

All data collected and analysed in these experiments adhere to the “Minimum Information about a Microarray Experiment” (MIAME) (Brazma et al., 2001) guidelines and are submitted to Gene Expression Omnibus (GEO, 2006; Edgard et al., 2002) under sample accession numbers GSM52686 - GSM52693 and series accession number GSE2785. After annotation and association with the National Center for Biotechnology Information (NCBI) Gene ID nomenclature, each gene was linked to the GO ID by a free flat file database from the NCBI (2006) ftp download server. To properly know the function of each gene, Gene Ontology (GO) Name, GO Definition and GO Term Type were downloaded from GO site (Gene Ontology, 2006). Annotations were performed by local BLAST searches against the free NCBI non-redundant database. For this aim, a number of PERL scripts that automatically launches BLAST queries for every gene, extract the best match and link to the GO ID were constructed, and a link to the Gene ID was added manually. After these steps, FatiGO (Al-Shahrouf et al., 2004) was used to extract GO categories. To assess the
Table 3. Genes up-regulated in MLD of pigs fed with high energy diet (HED) compared to those of low energy diet (LED)*.

| Gene description                        | Gene name | Ref. Seq.  | Identity % | Q-valuea (%) | n-foldb |
|-----------------------------------------|-----------|------------|------------|--------------|---------|
| phosphoglycerate mutase 2 (muscle)      | PGAM2     | NM_002090  | 90         | 1.79         | 1.4     |
| glyceraldehyde-3-phosphate dehydrogenase| GAPDH     | NM_002046  | 91         | 1.79         | 1.3     |
| WW domain binding protein 1             | WBP1      | NM_012477  | 87         | 1.79         | 1.4     |
| NADH dehydrogenase (ubiquinone) 1 beta, 9| NDUFB9    | NM_005005  | 90         | 1.79         | 1.4     |
| pyruvate kinase, muscle                 | PKM2      | NM_002654  | 94         | 1.79         | 1.3     |
| hypothetical protein MGC14151           | MGC14151  | NM_032356  | 90         | 1.79         | 1.5     |
| U6 snRNA-associated Sm-like protein LSm7| LSM7      | NM_016199  | 90         | 1.79         | 1.4     |
| enolase 3                               | ENO3      | NM_053013  | 92         | 1.79         | 1.2     |
| RAD23 homolog A                         | RAD23A    | NM_005053  | 87         | 1.79         | 1.2     |
| tropomysin 1 (alpha)                    | TPM1      | NM_003666  | 98         | 1.79         | 1.2     |
| troponin C2, fast                       | TNNC2     | NM_003279  | 89         | 1.79         | 1.1     |
| creatine kinase, muscle                 | CKM       | NM_001824  | 96         | 1.79         | 1.2     |
| isocitrate dehydrogenase 3 (NAD+) gamma | IDH3G     | NM_174869  | 87         | 2.94         | 1.1     |
| eukaryotic translation initiation factor 3| EIF34     | NM_00375   | 91         | 2.94         | 1.2     |
| skeletal muscle, embryonic nuclear protein 1| MUSTN1   | NM_205853  | 85         | 2.94         | 1.3     |
| fast skeletal myosin light chain 2      | MLYPF     | NM_013292  | 92         | 5.00         | 1.3     |
| eukaryotic translation elongation factor 1 alpha 1| EEF1A1 | NM_001402  | 90         | 8.38         | 1.2     |
| hypothetical protein MGC13275           | MGC13275  | XM_372194  | 8.38       | 8.38         | 1.2     |
| ribosomal protein S29                   | RPS29     | NM_001032  | 95         | 8.38         | 1.2     |
| troponin T3, skeletal, fast             | TNNT3     | NM_006757  | 92         | 16.58        | 1.3     |

* Genes HED-related are reported in this table. Homology percentages between human and porcine sequences are given in relation to the published sequences. Q-value represents the probability that the gene is not differentially expressed and is related to FDR (False Discovery Rate, Storey 2002). Genes with a relatively high Q-value (>10) are printed because in analysis without mitochondrial genes their Q-value was significantly lower.

b Q-value is the lowest False Discovery Rate (FDR) at which the gene is called significant.

c The n-fold represents the 2 exponential of the median value [log2(HED/LED)], where HED and LED are intensity values after normalisation.

MLD: Musculus longissimus dorsi.

Statistical significance, p-value adjustments were applied, using the step-down min p proposed by Westfall and Young (1993), that adjusts p-values referring to the result of re-sampling and a permutation test of unadjusted ones.

Real time PCR

On the basis of microarray analysis, two genes up-regulated (phosphoglycerate mutase 2, PGAM2, and WW domain binding protein 1, WBPI) and two genes with no differences in the level of expression (telethonin TCAP, and heat shock 27kDa protein 1, HSPB1) were randomly selected between the most interesting skeletal muscle genes (Lin and Hsu, 2005) for quantitative real time PCR. Individual levels of expression were normalised to TATA box binding protein (TBP, housekeeping gene, Radonic et
Table 4. Genes down-regulated in MLD of pigs fed with high energy diet (HED) compared to those of low energy diet (LED)\(^a\).

| Gene description                                                                 | Gene name  | Ref. Seq. | Identity % | Q-value\(^a\) (%) | n-fold\(^a\) |
|---------------------------------------------------------------------------------|------------|-----------|-------------|-------------------|--------------|
| mitochondrial ribosomal protein S18B                                            | MRPS18B    | NM_014046 | 89          | 1.79              | 0.7          |
| von Willebrand factor A domain-related protein                                   | WARP       | NM_199121 | 89          | 1.79              | 0.6          |
| troponin T1, skeletal, slow                                                      | TNNT1      | NM_003283 | 89          | 1.79              | 0.7          |
| KIAA1961 gene                                                                    | KIAA1961   | NM_133372 | 89          | 1.79              | 0.8          |
| cathepsin B                                                                     | CTSB       | NM_001908 | 89          | 1.79              | 0.6          |
| supervillin                                                                     | SVIL       | NM_003174 | 89          | 1.79              | 0.8          |
| hypothetical protein FLJ21438                                                   | FLJ21438   | XM_029084 | 89          | 1.79              | 0.8          |
| myosin binding protein C, slow type                                              | MYBPC1     | NM_002465 | 89          | 1.79              | 0.7          |
| protein kinase (cAMP-dependent, catalytic)                                      | PKIG       | NM_007066 | 89          | 1.79              | 0.8          |
| glyceronephosphate O-acyltransferase                                            | GNPAT      | NM_014236 | 89          | 1.79              | 0.8          |
| translocase of inner mitochondrial membrane                                     | TIMM13     | NM_012458 | 89          | 1.79              | 0.9          |
| hepatitis delta antigen-interacting protein A                                   | DIPA       | NM_006848 | 89          | 2.94              | 0.8          |
| myoglobin (MB)                                                                  | MB         | NM_203377 | 89          | 3.83              | 0.6          |
| hypothetical protein MGC4692                                                    | MGC4692    | NM_003174 | 89          | 3.83              | 0.8          |
| calponin homology-associated smooth muscle                                       | CHASM      | XM_166203 | 89          | 3.83              | 0.9          |
| titin                                                                            | TTN        | NM_133437 | 90          | 3.83              | 0.8          |
| myosin, heavy polypeptide 7, beta                                                | MYH7       | NM_000257 | 90          | 10.03             | 0.8          |
| ATP synthase, H+ transporting, beta polypeptide                                  | ATP5B      | NM_001686 | 90          | 10.03             | 0.9          |
| microtubule-associated protein, member 2                                         | MAPRE2     | NM_014268 | 90          | 13.88             | 0.9          |
| SH3-binding domain protein 5-like                                               | SH3BP5L    | NM_030645 | 90          | 16.58             | 0.9          |
| rho guanine nucleotide exchange factor 15                                        | ARHGEF15   | NM_173728 | 91          | 16.58             | 0.8          |

\(^a\) Genes HED-related are reported in this table. Homology percentages between human and porcine sequences are given in relation to the published sequences. Q-value represents the probability that the gene is not differentially expressed and is related to FDR (False Discovery Rate, Storey 2002). Genes with a relatively high Q-value (>10) are printed because in analysis without mitochondrial genes their Q-value was significantly lower.

\(^b\) Q-value is the lowest False Discovery Rate (FDR) at which the gene is called significant.

\(^c\) The n-fold represents the 2 exponential of the median value [\log(\text{HED/LED})], where HED and LED are intensity values after normalisation.

MLD: Musculus longissimus dorsi.

..., 2004) because other reference genes like glyceraldehydes-3-phosphate dehydrogenase (GAPDH) did not show an equal expression level in muscle tissue under the experimental conditions. For the analysis, all 4 pigs of the HED and LED groups were used.

Primer design was performed with the help of Primer3 software (Rozen and Skaltsky, 2000). Constant amounts of 1000 ng of total RNA were reverse transcribed to cDNA with ImPromII (Promega corp., Madison USA) according to the manufacturer’s instructions. Real-time PCR was conducted by amplifying cDNA with the qPCR Platinum SYBR green qPCR Supermix UDG kit (Invitrogen, Milan, Italy) in triplicate. Melting curve analysis of
amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected.

To examine the sensitivity and linearity of the assay, a 10-fold serial dilution of a positive sample was used. The correlation between cDNA concentration and the Ct value of real-time PCR was determined. The initial cDNA concentration was 50 ng/μl, after which the samples were serially diluted 10-fold for the real-time PCR assay and the linearity of cDNA concentration vs. Ct values was calculated by linear regression analysis. Reactions were performed in a 25 ml volume using a 96-well spectrofluorometric thermal cycler (DNA Engine Opticon 2; MJ Research, Inc., Waltham, MA, USA).

Real-time PCR efficiencies were calculated from the slopes obtained from the Opticon 2 software of MJ Research. The PCR efficiency (E) was calculated according to the following equation: \( E = 10^{-\frac{1}{S_{\text{mp}}}} \). Results of the real-time PCR data were represented as Ct values, where Ct was defined as the threshold cycle of PCR at which amplified product was first detected (Johnson et al., 2000). For the relative quantification of gene expression (n-fold), the delta-delta Ct method was applied (Livak and Schmittgen, 2001). Significance levels between HED and LED groups were determined by pair-wise fixed reallocation randomisation test (Pfaffl et al., 2002).

Results

The average daily gain of pigs fed the LED during the whole experimental period diet was significantly lower (P< 0.05) than that of the HED pigs (Table 2). The reduction of daily gain in the LED pigs was mostly due to the reduction of growth in the second period of the trial, i.e. from 6 to 12 months of age, when the difference in energy content between diets was higher. The variation of dietary energy density was also associated to a decrease of back fat thickness in the LED pigs (P< 0.05), but not for the MLD area.

Microarray analysis (Tables 3 and 4), resulted in 42 genes differentially regulated in the MLD of HED group compared to LED group (20 genes up-regulated and 22 genes down-regulated). The median Q-value was 1.79% indicating that we probably have less than 1 gene erroneously considered differentially expressed. In order to determine if the low n-fold values of differentially expressed genes in pigs is caused by the degree of conservation between the considered orthologous gene sequences, the spotted human sequences in the microarray were compared to pig EST sequences. Blasting the spotted DNA sequences of the genes differentially expressed (42 sequences) vs. the dbEST database, Sus scrofa subset, 28 homologous pig transcripts (18 and 10 over and under-expressed respectively, median Blast “e value” 3.5e-5) were found. The average identity level was fairly high (91% over a total of 6214 bp of sequence), so probably the low n-fold values of the differentially expressed transcripts depict the fine tuning of the same type skeletal muscle of the same pig variety living in the same environment with the diet as the only variable.

The different pattern of transcriptome between the LED and HED groups found with the human microarray was validated using the quantitative real time PCR analysis on 6 randomly selected genes, using SybrGreen assays (Table 5) in all the pigs. The specific product of melting curves showed only single peaks and no primer-dimer peaks or artefacts. Results of real time PCR were in line with those obtained with heterologous microarray (Figure 1), although the numerical values (n-fold) were always higher for the former (Tables 3 and 4).

Most of the up-regulated genes in MLD of HED pigs are involved in the glycolytic and oxidative metabolism (PGAM2, GAPD, NDUFB9, PKM2, ENO3, CKM, IDH3G) and in the contractile apparatus (TPM1, TNNC2, MYLPF, TNNT3), with minor genes involved in signal transduction and ribosomal protein. The administration of HED diet reduced the level of expressions of CTSB,
Figure 1. Relative expressions (n-fold, ± SE) of the 6 randomly selected genes for pigs fed with high energy diet (HED) compared to those of low energy diet (LED) measured by real time PCR (solid bar). Dashed bars refer to the n-fold measured with microarray and the line refers to a n-fold equal to 1.

* Significance levels between HED and LED groups in real time PCR were determined by pair-wise fixed reallocation randomisation test (Pfaffl et al., 2002) and are indicated as follows: * P<0.05; **P<0.01; ns = not significant.

muscle proteins associated with slow fibre type (TNNT1, SVIL, MYBPC1, TTN, MYH7) and signal transduction (KIAA1961, FLJ21438, PKIG, ARHGEF15).

The classifications of differentially expressed genes were calculated for the three categories reported by FATIGO: “cellular component”, “biological process” and “molecular function”. Table 6 reports the best clusters of transcripts in the skeletal muscle of pigs in relation to dietary energy. Of the 22 down-regulated and 20 up-regulated genes, 7 and 6, respectively, genes were excluded due to the lack of function annotation in the GO database.

For the “cellular component” category the number of down and up-regulated genes was 10 and 12, respectively. The best cluster for this category was found for the “membrane” sub-category (GO:0016020, n.s.), which included 3 down-regulated genes (30.0%, TIMM13, ATP5B and GPNAT) and 1 up-regulated gene (8.3%, NDUFB9). Within the “biological process”, 14 and 13 genes were identified for the down and up-regulated genes in the HED group and the number of genes included in the “cellular and physiological process” (GO:0050875) were 10 (71.4%) and 4 (30.8%), respectively. These sets of genes identified clusters significant at a level of P< 0.10 (P= 0.056). The third GO category, “molecular function” included 15 and 14 genes, which were down and up-regulated in HED group. Sub-category “nucleic acid binding” (GO:000376, P<0.05) and “oxidoreductase activity” (GO:0016491, P<0.05) included 4 (28.6%) and 3 (21.4%) over-expressed genes.

Discussion

Animal performances

The Casertana pig that is the subject of this study is a local breed from Southern Italy characterized by slow growth and a massive accumulation of backfat. Considering that the breed did not undergo selective programmes, Casertana retains the traits of a slow growing and high fat depositing pig comparing to the genetic lines actually exploited in the pig industry. Very few data are reported in the literature for the Casertana breed; the only recent paper reports live weight at 1 year of age of 150 kg, with an average daily gain of 0.4 kg, a dressing percentage over 81% and a back fat thickness ranging from 35 to 45 mm (Pietrolà et al., 2006). The typ-
Table 5. Oligonucleotide sequences used for the real time PCR and reaction efficiency (E).

| Oligonucleotides | forward / reverse | E  |
|-----------------|------------------|----|
| TBP             | 5’- ACACGACAGGCAACAGT-3’ | 1.87 |
|                 | 5’- GAAGAGCTGTGGGATCTGG-3’ |    |
| CTSB            | 5’- GACTGCATGGTGTAAGT-3’ | 1.99 |
|                 | 5’- TCCACTTTGTAGGGAAAA-3’ |    |
| MYBPC1          | 5’- CTCTCAGCTCTTCTTG-3’ | 1.85 |
|                 | 5’- CTTCAAGGATTGGCTGAGTGT-3’ |    |
| TCAP            | 5’- GTCCGAGGAGAACKTGAGC-3’ | 1.83 |
|                 | 5’- GCCTGACAGGCTGAGT-3’ |    |
| HSPB1           | 5’- CCGGTGTTTACTCGAAAT-3’ | 1.84 |
|                 | 5’- CTCAAGGGCAAGGGAGG-3’ |    |
| PGAM2           | 5’- ATTTGCAAGCAGCTGAA-3’ | 1.81 |
|                 | 5’- AGCCTCCTTGTAGCTGATTA-3’ |    |
| WBP1            | 5’- TCCAGTGAATGGTATCTGTC-3’ | 2.00 |
|                 | 5’- GGAGAAACACCTTCCACAT-3’ |    |

Validation of microarray findings with real-time PCR

A human microarray was used in the experiment and data were generated in a very stringent conditions, although the average identity level was 91% over 6214 bp of sequence, a value significantly higher than the 84% identity found on a similar analysis of a pig-human cross-hybridisation experiment on filter microarray (Moody et al., 2002) and than the 70% identity found on other human cDNA microarray (Lin and Hsu, 2005). Using a human microarray dedicated only for muscle, the cross-specie hybridisation problems, expected in a heterologous system, were limited (Gladney et al., 2004; Lin and Hsu, 2005). Levels of expression (n-fold) of the selected genes measured by real time PCR followed the trend detected by microarray analysis (Figure 1). The comparison of methods also indicated that this technique led to a higher sensitivity, as can be seen from the n-fold values from real time PCR. It must be emphasised that whilst the primers for real time PCR were specifically designed on pig sequences, the human microarray cDNA probes were specific for the 3’-end, which is the most variable interspecies region. As reported by Bai et al. (2003), the cDNA microarray is intrinsically less effective in...
| Gene Ontology (GO) and annotation | Item | Down regulated | Up regulated | Sign. |
|---|---|---|---|---|
| Total number of genes | | 22 | 20 | |
| Genes without GO annotation | | 7 | 6 | |
| Cellular component Membrane | Number of genes | 10 | 12 | 0.293 |
| GO:0016020 | Genes included, % | 30.0 | 8.3 | |
| | List of genes included | TIMM13, ATP5B, GNPAT | NDUFB9 | |
| Biological process Cellular physiological process | Number of genes | 14 | 13 | 0.056 |
| GO:0050875 | Genes included, % | 71.4 | 30.8 | |
| | List of genes included | TIMM13, MAPRE2, SVIL, TBCA, MYBPC1, TPM1, MB, PGAM2, TNCC2, TNNT3 | |
| Molecular function Nucleic acid binding | Number of genes | 15 | 14 | 0.042 |
| GO:0003676 | Genes included, % | 0.0 | 28.6 | |
| | List of genes included | EIF3S4, LSM7, RAD23A, MGC14151 | | |
| Oxidoreductase activity | Genes included, % | 0.0 | 21.4 | 0.099 |
| GO:00016491 | List of genes included | NDUFB9, GAPDH, IDH3G | | |

HED: cluster query; LED: cluster reference. GO was performed at level 3 of annotation. Sign. is the significance of step-down min p adjusted p-value.
differentiating between highly homologous members of the same gene family. Therefore, quantitative real time PCR on selected representative genes had demonstrated functional integrity of the human constructed cDNA microarray in the appropriately identification of differentially expressed genes of pig.

Classification and categorization of co-regulated genes in the microarray analysis

In order to get useful information from the microarray, data mining with gene ontology was carried out, extracting categories with the GOA (Camon et al., 2004). GO (Harris et al., 2004) is widely accepted as the standard for the vocabulary, which consists of 3 categories, “biological process”, “molecular function”, and “cellular component”. Fatigo (Al-Shahrour et al., 2004) is a GO tool that extracts relevant GO categories for a given gene set with respect to a reference set using Fisher’s exact test, which considers the multiple-testing nature of the statistical contrast performed. Al-Shahrour et al. (2004) indicated that GO level 3 constitutes a suitable compromise between the information quality and the number of genes annotated in each category.

We use the genes differentially expressed in MLD muscle of pigs fed with high energy diet (HED, cluster query) in comparison to genes differentially expressed with low energy diet (LED, reference query; Tables 3 and 4). The most significant GO sub-categories, representing genes differentially expressed between HED and LED groups within the 3 main categories, are reported in Table 6. The higher number of differentially expressed transcripts in the skeletal muscle of pigs that were fed with HED diet was found for cellular physiological process (GO:00050875) within biological process ($P = 0.056$), that is, in agreement with the GO definition, “the process pertinent to the integrated function of a cell” like cell growth and/or maintenance. For pigs fed with HED diet, up-regulation of TPM1, TNCC2 and TNNT3, and down-regulation of MYBP1, TTN, TNNT1 and MYH7 were observed. Troponin (TNNs) and tropomyosin (TPMs) isoforms determine the variable sensitivity to calcium, whereas titin (TTN) isoforms dictate the elastic properties of muscle fibres at rest. Both myosin and troponin isoforms contribute to the differences in the resistance to fatigue of muscle fibres. Myofibrillar protein multiple isogenes are differentially expressed in various muscle types and fibre types, can be coexpressed within the same fibre (Kim et al., 2004) and can be affected by energy metabolism (Schiaffino and Reggiani, 1996). The MLD muscle has a mixed fibre composition, with a prevalence of white fibre (Lefaucheur et al., 2002), and energy availability may further modify the structural components of muscle increasing gene expressions of fast isoforms of sarcomeric proteins that are already associated to white fibres (Bai et al., 2003). The modification of fast and slow protein isoforms also affects meat quality (Bee et al., 2004). Our data clearly show that different diet can change the muscle fibre type composition. For example, typical fast twitch markers (troponin C2 and T3 fast type) are over expressed in HED animals while troponin T1 and the myosin binding protein C slow type tagged LED animals (Tables 3 and 4).

Fatigo analysis indicated an increase of oxidoreductase activity (GO:00016491), with a cluster of NDUFB9, GADP and IDH3G (Table 6). The increased availability of glucose would have stimulated insulin secretion and the utilisation for acetate production and the fatty acid synthesis in skeletal muscle (Ikemoto et al., 1995). The up-regulation of genes involved in glycolytic and oxidative metabolisms can also be observed in animals experiencing energy restriction, as reported in trial with mice (Lee et al., 1999) and pigs (Da Costa et al., 2004). In these conditions, the shortage of caloric intake induces a reprogramming of energy metabolism and protein turnover, to deal with the lower availability of energy substrates (Lee et al., 1999).

A significant and intriguing sub-category within molecular function, the nucleic acid bind-
ing (GO:0003676), was up-regulated in the HED pigs. It is likely that the high-energy diet triggers a set of genes involved in signal transduction and mRNA splicing in order to deal with the different metabolism.

Among the other differentially expressed genes, not included at level 3 of FatiGO analysis, CTSB is of interest, since it is involved in the post-mortem proteolysis and has practical implications in meat quality. The enhancement of enzyme activity in raw ham is related to a decrease in pH after 24 hours from slaughter, enhancement of weight loss during storage and an increase in proteolysis (Schivazappa et al., 2002).

Another transcript that attracts our attention is the calponin homology-associated smooth muscle (CHASM), a novel member of the smoothelin protein family (Borman et al., 2004), that elicits relaxation of muscle by activating the Ca2+ desensitisation pathway. The primary expression of this gene on slow-twitch fibre of skeletal muscle is not reported, but the tissue specificity and the physiological activity turns CHASM into an intriguing transcript to be further studied not only for meat quality purposes.

Conclusions

The application of microarray technology to investigate patterns of gene expression in pig muscle opens new insight in the understanding of cellular processes which might regulate phenotype and metabolism. From the results, a list of candidate regulatory genes that could influence muscle phenotype was identified, such as those involved in protein isoform-specific expressions. On the basis of gene ontology analysis, the modification of gene expression profile in MLD with dietary energy was more related to the adaptation of physiological process and molecular function than to cellular constituents. Whether these differential gene expressions could result in a differential translation process and protein compositions of muscles requires further investigation, as does their effects on meat quality.

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