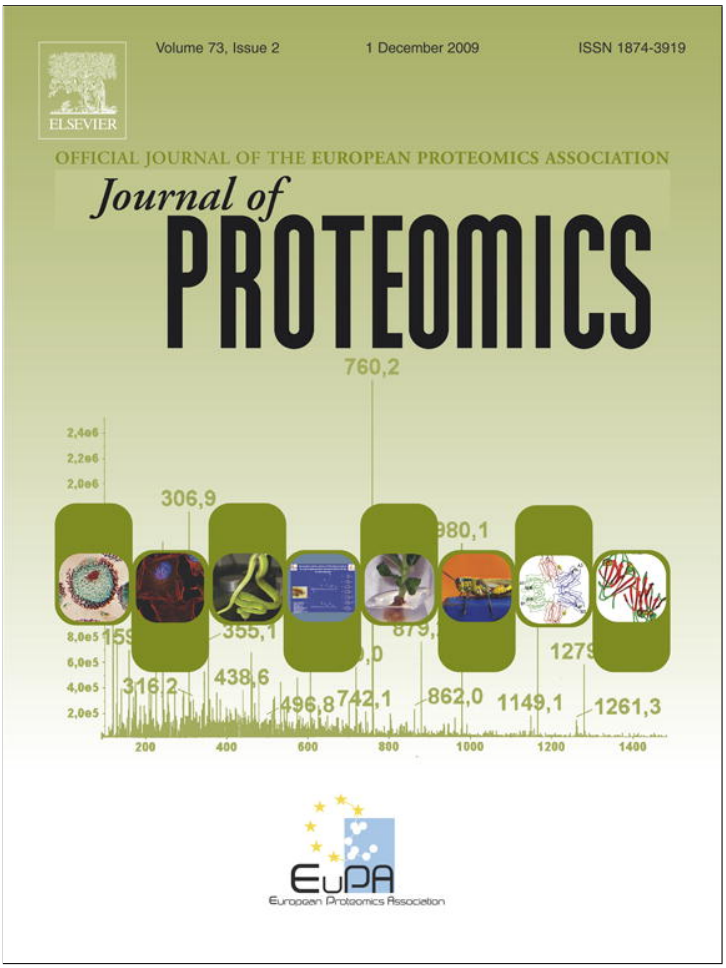

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Comparative proteomics and transcriptomics analyses of livers from two different *Bos taurus* breeds: "Chianina and Holstein Friesian"

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ABSTRACT

The Holstein Friesian and Chianina cattle breeds are representative of extreme selection for milk and meat traits, respectively, with significant changes in metabolism resulting from human selection over the past centuries. In the present study, we wanted to assess whether selection for different purposes has had a measurable effect on liver metabolism through a comparison of the protein and gene expression profiles of the two breeds. We applied 2-DE in order to identify proteins which were differentially expressed in the livers of the two breeds and relate them to different liver functions. We expected to find that only a small number of proteins would be differentially expressed, due to the relatively short phylogenetic distance between these cattle breeds. Nonetheless, thirty nine differentially-expressed proteins were characterized between Chianina and Holstein Friesian, out of a total of 560±57 spots that matched.

Microarray analyses evidenced the differential expression of 167 genes (148 for the Holstein Friesian and 19 for the Chianina). Despite being closely related at the genetic level, the disparity of the proteomic and transcriptomic profiles of these two breeds allows us to perform pathway analysis thus to pinpoint proteins whose expression might render the latter capable of greater milk production, or proteins involved in altered thermoregulatory ability or hormone production. On the other hand, we found proteins and gene transcripts in Chianina, not expressed in Holstein, which, upon interaction pathway analysis, were mainly involved in anabolic pathways. In brief, our integrated study provides molecular evidences to support the physiological differences between Holstein and Chianina cattle breeds.

1. Introduction

There is a huge amount of work currently underway on the human proteome, but little attention has been given to most animals of importance to humans. However, the importance of *Bos taurus* for the entire agricultural economy has prompted investigations into the fundamental mechanisms controlling animal health and productivity, including genetic analysis,

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animal physiology and susceptibility to microbial infections [1]. A combined effort of different genomic sequencing resources has now been coordinated to definitively sequence and annotate the entire bovine genome [2]. An updated bovine gene database is now available on the web at http://www.ncbi.nlm.nih.gov/genome/guide/cow [3]. The bovine genome contains about 35,000 type I coding genes arranged as 30 haploid chromosomes [4]. Almost 5000 genes have been sequenced to date, which have shown a high degree of homology with human and mouse counterparts. As a consequence, preliminary genome projects on this species have already yielded powerful tools for the assessment of genes that specify hereditary disorders, infectious disease resistance, breed-specific quantitative loci and phenotypes of agricultural relevance [5,6]. Alongside the genetic investigation, a series of structural studies have been performed on isolated bovine proteins, depending on their easy availability or their importance as main constituents of food consumed in the human diet. In classical veterinary studies, single enzymatic activities or genetic anomalies have been associated with specific bovine physiological and pathological conditions. Limited systematic studies have been hitherto performed to evaluate the entire protein repertoire in bovine tissues and fluids during certain specific phenomena, or to detect novel markers for specific pathologies. Meanwhile, a small number of proteomic studies have been performed on bovine tissues and biological fluids, mainly focused on chondrocytes, mammary glands, cerebrospinal fluid, pulmonary endothelial cells, seminal plasma, milk and the corneal lens [7–10]. The liver has received special attention, containing as it does, enzymes involved in energy generation, carbohydrate, lipid, amino acid and xenobiotic metabolism, as well as proteins involved in polypeptide synthesis, folding and cell structure. Xu and Wang [11] carried out a comparative study on proteomic investigations of livers from ketotic cows. They found thirty-eight different proteins between groups. Our interest in liver proteins differs, in that we are exploring the potential of this type of analysis to reveal unique profiles for different cattle breeds, despite the relatively short phylogenetic distance between Holstein Friesian and Chianina [12,13]. In this way, we purported to reveal important protein actors underlying those extreme traits that have been selected for in these breeds. Hereby we will present data about separation and identification of the differentially-expressed proteins and gene products through an integrated proteomics and transcriptomics (microarray) approach. Discussions will follow on how those changes in liver metabolism may be associated with different biological and productive aptitudes.

2. Materials and methods

2.1. Sample collection

All animals used in this study were treated according to International Guiding Principles for Biomedical Research Involving Animals. In a commercial dairy farm located in Viterbo (Italy), we selected 12 animals (6 per breed) which were of the same breed, age, and body score condition. Cows were fed the same diet.

2.2. Sample preparation and 2-DE analysis

Sample preparation and solubilization was performed by slight modification of the SWISS-2D PAGE sample preparation procedure. Frozen samples of liver tissue from the 6 Chianina and 6 Holstein Friesian (approximately 20 mg per sample) were crushed in a mortar containing liquid nitrogen, and to remove lipids, proteins were precipitated from a desired volume of each sample with a cold mix of tri-n-butyl phosphate/acetone/methanol (1:12:1). After incubation at 4 °C for 90 min, the precipitate was pelleted by centrifugation at 2800 g, for 20 min at 4 °C. After washing with the same solution, the pellet was air-dried and then resuspended in the focusing solution containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.8% (w/v) pH 3–10 carrier ampholyte, 40 mM Tris, 5 mM TBP, 10 mM acrylamide, 0.1 mMEDTA (pH 8.5), 2% (v/v) protease inhibitor cocktail (Sigma-Aldrich), and 2 mM PMSF. Before focusing, the sample was incubated in this solution for 3 h at room temperature, under strong agitation. To prevent over-alkylation, acrylamide was destroyed by adding an equimolar amount of DTE. The protein concentration of each group was determined according to Bradford [14] using BSA as a standard curve. A total of 250 μL of the resulting protein solution was then used to rehydrate 13 cm long IPG 3-10 NL (Amersham Biosciences) for 8 h. IEF was carried out on a Multiphor II (Amersham Biosciences) with a maximum current setting of 50 μA/strip at 20 °C [15]. The total product time voltage applied was 50,000 Vh for each strip. For the second dimension, the IPG strips were equilibrated for 30 min in a solution containing 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, and 375 mM Tris–HCl (pH 8.8), with gentle agitation. The IPG strips were then laid on a 5–16% T gradient SDS-PAGE gel with 0.5% (w/v) agarose in the cathode buffer (192 mM glycine, 0.1% w/v SDS and Tris to pH 8.3). The anode buffer was 375 mM Tris–HCl, pH 8.8. The electrophoretic run was performed at a constant current (10 mA for 60 min, followed by 40 mA until the run was completed). During the whole run, the temperature was set at 13 °C. Proteins were visualized by a staining procedure: sensitive Coomassie Brilliant Blue G-250 stain [16]. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity can be achieved using Coomassie Brilliant Blue G-250 and R-250. Three technical replicates were performed for each of the 12 samples (6 Chianina and 6 Holstein Friesian) for a total of 36 2-DE maps. There was good reproducibility between the technical replicates of each sample.

2.3. Image analysis

Thirty-six stained gels were digitalized using an ImageScanner and LabScan software 3.01 (Bio-Rad Hercules, CA). The 2-DE image analysis was carried out and spots were detected and quantified using the Progenesis SameSpots software v.2.0.2733.19819 software package (Nonlinear Dynamics, New Castle UK). Each gel was analyzed for spot detection and background subtraction. Within-group comparison of protein spot numbers was determined by repeated measures analysis. Among-group comparisons were determined by ANOVA (Analysis of Variance) procedure in order to classify sets of

proteins that showed a statistically significant difference with a confidence level of 0.05. Spots which were significantly different between groups (Chianina vs Holstein Friesian) and not significantly different in the three technical replicate and six biological replicate samples were identified by qTOF-MS/MS. All statistical analyses were performed with the Progenesis SameSpots software v.2.0.2733.19819 software package [17]. After the background subtraction, spot detection and match, one standard gel was obtained for each group (Fig. 1), Chianina and Holstein Friesian. These standard gels were then matched to yield information about the spots of differentially expressed proteins. Differential protein expression was considered significant at $P<0.05$ and the change in the photodensity of protein spots between Chianina and Holstein Friesian samples had to be more than 2 fold.

2.4. *In-gel digestion*

Spots from 2-DE maps were carefully excised from the gel and subjected to in-gel trypsin digestion according to Shevchenko et al. [18] with minor modifications. The gel pieces were swollen in a digestion buffer containing 50 mM NH4HCO3 and 12.5 ng/mL trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) in an ice bath. After 30 min, the supernatant was removed and discarded; then 20 mL of 50 mM NH4HCO3 was added to the gel pieces, and digestion was allowed to proceed overnight, at 37 °C. The supernatant containing tryptic peptides was dried by vacuum centrifugation. Prior to mass spectrometric analysis, the peptide mixtures were redissolved in 10 μL of 5% FA (formic acid).

2.5. *Peptide sequencing by nano-RP-HPLC-ESI-MS/MS*

Mass spectrometric procedures were performed as previously described [19]. Peptide mixtures were separated using a nano flow-HPLC system (Ultimate; Switchos; Famos; LC Packings, Amsterdam, The Netherlands). A sample volume of 10 μL was loaded by the autosampler onto a homemade 2 cm fused silica precolumn (75 μm I.D.; 375 μm O.D.; Resprosil C18-AQ, 3 μm, Ammerbuch-Entringen, Germany) at a flow rate of 2 μL/min. Sequential elution of peptides was accomplished using a flow rate of 200 nL/min and a linear gradient from solution A (2% acetonitrile; 0.1% formic acid) to 50% of solution B (98% acetonitrile; 0.1% formic acid) in 40 min over the precolumn in-line with a homemade 10–15 cm resolving column (75 μm I.D.; 375 μm O.D.; Resprosil C18-AQ, 3 μm, Ammerbuch-Entringen).

Peptides were eluted directly into a high-capacity ion trap (model HCTplus Bruker-Daltonik, Germany). The capillary voltage was 1.5–2 kV, and a dry gas flow rate of 10 L/min was used with a temperature of 200 °C. The scan range used was from 300 to 1800 m/z.

Protein identification was performed by searching in the National Center for Biotechnology Information nonredundant (NCBInr) database using the MASCOT program (http://www.matrixscience.com). The following parameters were adopted for database searches: complete carbamidomethylation of cysteines and partial oxidation of methionines, peptide mass tolerance ± 1.2 Da, fragment mass tolerance ± 0.9 Da, missed cleavages 2. For positive identification, the score of the result of [−10 × log(P)] had to be over the significance threshold level (P, 0.05). The last update of NCBInr was on July, 2007 when keratins and trypsin were added as contaminants and the program used for converting instrument files to the mascot generic files for the database search was Data Analysis Version 3.3 (Built 146) Bruker Daltonics esquire 5.3. Even though high MASCOT scores are obtained with values between 40 and 80, when proteins were identified with a few peptides, a combination of an automated database search and manual interpretation of peptide fragmentation spectra

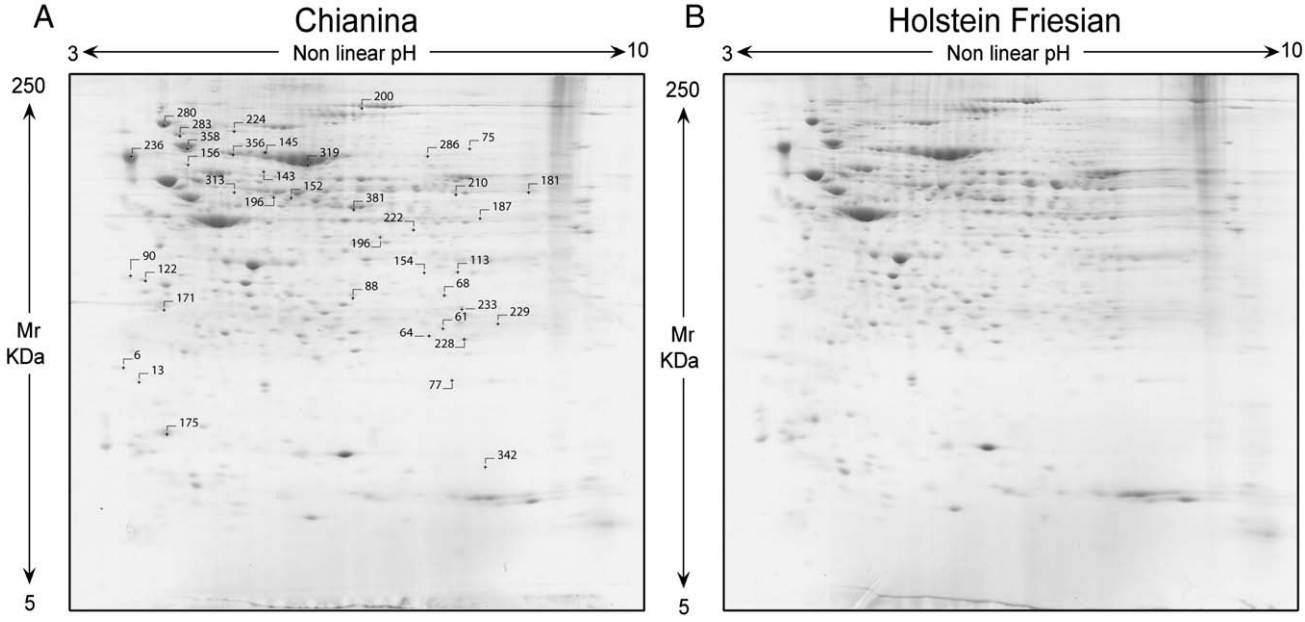

Fig. 1 – 2-DE of liver extracts from Chianina and Holstein Friesian breeds. Each gel image has been elaborated with Progenesis SameSpots (Nonlinear Dynamics, NewCastle, UK) and represent an average of 18 gels (3 technical replicate for 6 biological replicate samples), upon background subtraction.

was used to validate protein assignments. In this manual verification, the mass error, the presence of fragment ion series, and the expected prevalence of the C-terminus containing (Y-type ions) in the high mass range were all taken into account. Moreover, replicate measurements have confirmed the identity of these protein hits.

2.6. RNA samples

Liver samples of Frisona and Chianina individuals (see above) were collected immediately after slaughtering. Samples were preserved in RNA later (Sigma-Aldrich) and stored at −80 °C. Total RNA of was extracted using the RNA easy midi kit (Quiagen).

2.7. Microarray experiments

Inverse transcription was realized by SuperScript Indirect cDNA Labelling System (Invitrogen). cDNAs from liver samples were hybridised onto bovine cDNA slides (ARK genomics, UK) containing 14250 genes spotted in replicate, using 3DNA Array 900 MPX Cy3/Cy5 Kit (Genisphere), according to manufacturer's instructions. A dye-swap experiment was performed [20], labelling each sample independently with each fluorescent dye.

2.8. Microarray data analysis

Images were obtained by a ScanArray Lite (Perkin Elmer) laser scanner and Spotfinder software (TIGR) was used to extract feature data from microarray fluorescence images. cDNA spots were automatically segmented, total foreground and background intensities of the two dyes were calculated for each spot. We filtered for poor or saturated hybridization signals, then removed systematic bias in the data by applying the dye-swap normalization [21] that makes use of the reverse labelling in the two microarray replicates and the Lowess normalization. To establish the significance of observed regulation for each gene, *t*-test with Welsh's correction was performed. Finally, only genes with Fold-Change over |1.5|) were considered.

2.9. Cluster analysis

Quantitative data from image analysis elaboration (Progenesis SameSpots; Nonlinear Dynamic, New Castle UK) were used to perform a two-way hierarchical cluster analysis of the 39 differentially-expressed protein spots, either up or on-regulated in Chianina versus Holstein Friesian liver samples (Fig. 2A). In parallel, cluster analysis was performed on microarray data involving 167 gene transcripts which displayed differential expression with Fold-Change over |1.5| between liver samples from Chianina (19) and Holstein Friesian (148) (Fig. 2B). The clustering analysis was performed with PermutMatrix graphical interface [22], upon *Z*-score normalization of the averages of relative spot values. Pearson's distance and Ward's algorithm were used for the analysis. Each coloured cell represents the average of the relative spot value, according to the colour scale reported in Fig. 2.

2.10. Pathway analysis

A preliminary analysis was carried out using the Pathway Studio Enterprise software Edition 5.0 (Ariadne Genomics). Differentially-expressed proteins shown in Table 1 and gene products from Supplementary material 1 have been used to perform the pathway analysis. Groups shown in Table 1 (Chianina ON, Chianina UP, Holstein Friesian ON, Holstein Friesian UP) and Supplementary material 1 (Chianina, Holstein Friesian) have been maintained. Protein and gene groups have then been inserted in the Pathway Genomics Built Pathway function to evaluate their connectivity, finding all the shortest paths between them and finding all the entities directly connected to the inserted proteins. Results are shown in Fig. 3.

The networks (Figs. 4 and 5) were generated through the use of Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com) [23]. A data set containing gene identifiers and corresponding expression values was uploaded into in the application, basing on either proteomics or transcriptomics results. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) a ratio of the number of proteins from the data set that map to the pathway divided by the total number of proteins that map to the canonical pathway is displayed. 2) Fischer's exact test was used to calculate a *P*-value determining the probability that the association between the proteins in the dataset and the canonical pathway is explained by chance alone. Proteins/gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Nodes are displayed using various shapes that represent the functional class of the gene product. Grey nodes represent the proteins/genes from the submitted dataset which have a match in the canonical pathway from the database, while white nodes represent gene products that the software attributed to the same networks, although they were not present in the submitted dataset. Continuous lines (edges) represent direct interactions, while indirect ones are represented by interrupted lines. Circular lines around one node describe a feed-back loop of activity of that node on itself (e.g. by self-modulating its activity or expression). Grey edges represent interactions within a single network, while orange edges cross-link nodes from multiple interacting networks. The program could either graph single networks alone or merged together to stress their interactions.

It is worthwhile to recall that the network analysis only relied on those proteins and gene transcripts which were differentially-expressed between Chianina and Holstein Friesian breeds. Therefore, some proteins/gene products which are shown in the networks as "white nodes" could be likely individuated during the experimental phase as well, although this was not the goal of this study. Pathway analyses have been performed either for independent (Fig. 4B and C, Fig. 5B and C) or merged (Figs. 4A and 5A) datasets from both transcriptomics and proteomics approaches.

The Ingenuity Pathway Analysis software allows performing an unbiased elaboration of the experimental data, in order to

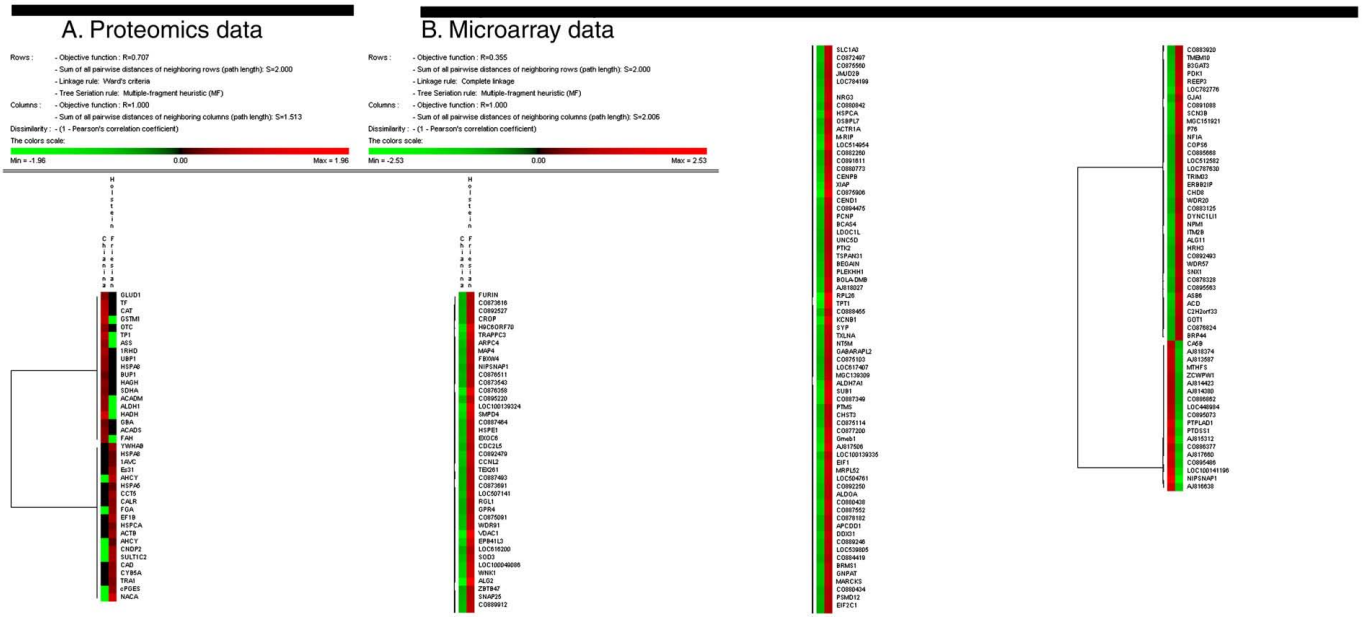

Fig. 2 – Two-way hierarchical clustering analysis of the 39 differentially-expressed proteins (up/on-regulated) (A) and 167 differentially-expressed gene transcripts (B) in Chianina versus Holstein Friesian livers. The clustering analysis was performed with PermutMatrix graphical interface upon Z-score normalization of the averages of relative spot values. Pearson's distance and Ward's algorithm were used for the analysis. Each coloured cell represents the average of the relative spot value, according to the color scale above.

Table 1 – Proteins differentially expressed from "Chianina and Holstein Friesian" breeds.

N	SSP	MwkDa	pI	No. of peptides identified	Mascot Score	NCBI Accession Number	Protein ID [Bos taurus]	Protein Function	Gene
Chianina Up									
1.	210	61992	8.03	22	1040	gi|74354891	GLUD1 protein	Dehydrogenase	GLUD1
2.	113	33115	6.78	10	407	gi|230290	Chain rhodanese	Transferase	1RHD
3.	286	61774	7.55	6	313	gi|89574195	Succinate dehydrogenase subunit A	Dehydrogenase	SDHA
4.	75	79870	6.75	13	562	gi|2501351	Serotransferrin precursor (Transferrin) (siderophylin) (beta-1-metal-binding globulin)	Metal Binding Globulin	TF
5.	77	57664	8.45	4	165	gi|229299	Catalase	Oxidoreductase	CAT
6.	313	53959	5.50	3	94	XP_001787596.1	Similar to cytosolic beta-glucosidase, partial	Hydrolase	GBA
7.	233	34410	7.71	10	429	gi|78369248	Hypothetical protein LOC509274	Hydrolase	HAGH
8.	222	43302	6.44	22	1152	gi|76639590	Similar to beta-ureidopropionase	Hydrolase	UBP1
9.	224	71423	5.49	6	109	gi|123644	Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	Chaperonine	HSPA8
10.	228	40045	8.20	3	98	gi|29135271	Ornithine carbamoyltransferase	Transferase	OTC
11.	229	44809	8.82	3	63	gi|77735757	Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	Dehydrogenase	ACADS
12.	342	43302	6.44	9	206	gi|76639590	Similar to beta-ureidopropionase	Hydrolase	BUP1
Chianina On									
13.	61	26901	6.45	12	677	gi|62888856	Triosephosphate isomerase	Isomerase	TP1
14.	64	25810	6.45	12	574	gi|76613071	Similar to class mu glutathione S-transferase isoform 1	Transferase	GSTM1
15.	68	27294	8.45	3	169	gi|27805907	Hydroxyacyl-Coenzyme A dehydrogenase, type II hydroxyacil-Coenzyme A	Oxydoreductase	HADH
16.	187	46673	7.14	13	629	gi|27806925	Argininosuccinate synthetase	Ligase	ASS
17.	181	46943	8.31	6	258	gi|115497690	Acyl-coenzyme A dehydrogenase, C-4 to C-12 straight chain	Dehydrogenase	ACADM
18.	196	46491	6.49	3	122	gi|119913765	Similar to Fumarylacetoacetate (FAA) Hydrolase	Hydrolase	FAH
19.	154	55426	6.24	7	280	gi|27806321	aldehyde dehydrogenase 1 family, member A1	Oxydoreductase	ALDH1
Holstein Friesian Up									
1.	358	72470	5.07	23	977	gi|115495027	Heat shock 70kDa protein 5	Chaperonine	HSPA5
2.	143	33287	5.39	8	391	gi|77736137	Chaperonin containing TCP1, subunit 5 (Epsilon)	Chaperonine	CCT5

Table 1 (continued)

N	SSP	MwkDa	pI	No. of peptides identified	Mascot Score	NCBI Accession Number	Protein ID [Bos taurus]	Protein Function	Gene
Holstein Friesian Up									
3.	145	62861	5.62	5	172	gi|119910189	Similar to esterase 31	Chaperonine	Es31
4.	236	46524	4.31	14	532	gi|631545	Calreticulin, brain isoform 1	Chaperonine	CALR
5.	280	92654	4.76	38	1770	gi|27807263	Tumor rejection antigen (gp96) 1	Chaperonine	TRA1
6.	175	11044	5.15	5	209	gi|353817	Cytochrome b5	Hemoprotein	CYB5A
7.	200	165833	6.28	33	1668	gi|76659916	Similar to carbamoyl phosphate synthetase 1 isoform 1	Transferase	CAD
8.	156	42084	5.22	9	370	gi|116242946	Actin, cytoplasmic 1 (Beta-actin)	Structural Protein	ACTB
9.	319	76197	5.60	8	342	gi|2914360	Chain, Bovine Annexin Vi (Calcium Bound)	Lipid Binding	1AVC
10.	122	24902	4.54	2	75	gi|58760396	Eukaryotic translation elongation factor 1 beta 2-like	Transferase	EF1B
11.	356	71424	5.37	26	1051	gi|146231704	Heat shock 70kDa protein 8	Chaperonine	HSPA8
12.	171	27946	4.80	18	764	gi|2852383	14-3-3 protein beta	Oxydoreductase	YWHAB
13.	283	85077	4.93	28	668	gi|60592792	Heat shock 90kD protein 1 , alpha	Chaperonine	HSPCA
Holstein Friesian On									
14.	152	53078	5.60	3	94	gi|78042564	Hypotetical protein LOC512626	Peptidase	CNDP2
15.	90	48120	5.88	5	223	gi|77735583	S-Adenosylhomocysteine hydrolase	Hydrolase	AHCY
16.	196	47116	5.49	6	197	gi|6980816	Chain C, the Crystal structure of modified bovine fibrinogen	Structural Protein	FGA
17.	6	18813	4.33	2	87	gi|51493666	Cytosolic Prostaglandin E synthase	Chaperonine	cPGES
18.	381	48120	5.88	17	843	gi|77735583	s-Adenosylhomocysteine hydrolase	Hydrolase	AHCY
19.	88	35062	6.00	13	583	gi|124249242	Hypothetical Protein LOC783020	Transferase	SULT1C4
20.	13	23356	4.51	4	257	gi|62460528	Hypothetical Protein LOC513312	Structural Protein	NACA

Proteins were identified on the basis of Fig. 1. Proteins highlighted in green have been confirmed to be up-regulated through microarray analysis.

focus subsequent analyses and discussions on the pivotal networks which are revealed upon the experimental phase (in this case: differentially-expressed liver proteins and gene transcripts).

3. Results and discussion

3.1. *Proteomics results*

On the basis of Talamo et al. [15], who compared proteomic analysis of bovine liver, kidney, skeletal, plasma and red blood cells, we analyzed differential proteome profiles of livers from two bovine breeds, Chianina and Holstein Friesian. For the analysis a sampling of 12 animals (six Chianina and six Holstein Friesian) was examined using immobilized pH gradient-based 2-DE and ESI-TOF-MS. The 2-DE protein extraction step was performed separately for each animal. To reduce the technical variance, each sample was analyzed in 3 technical replicates. Fig. 1 shows the well-resolved and reproducible 2-DE maps of liver tissue obtained from Chianina and Holstein Friesian. The maps show similar 2-DE patterns and only high-quality protein spots that were present in at least 24 out of 36 (both biological and technical) replicates were considered for quantification. A total of 560±57 protein spots were found to be commonly expressed in both cattle

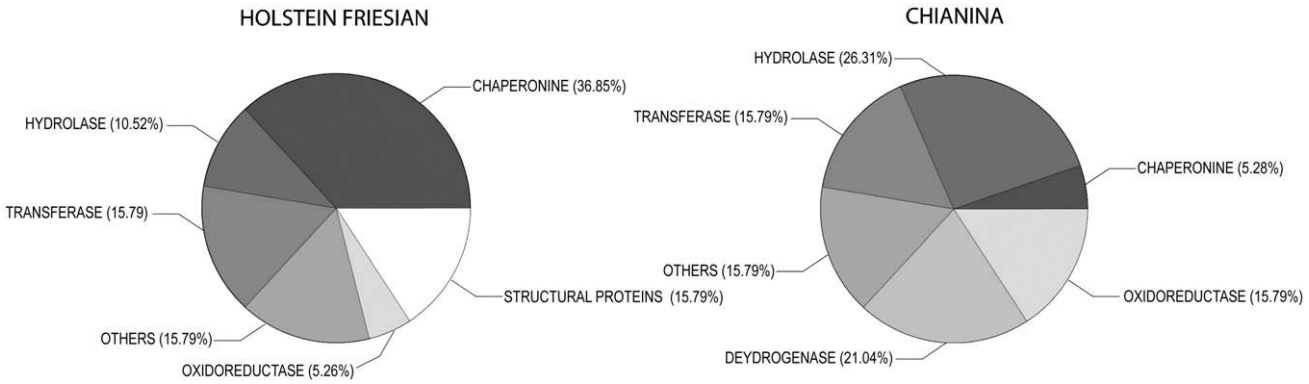

Fig. 3 – Elaboration of differentially-expressed proteins between Holstein Friesian and Chianina livers by means of Pathway Studio Enterprise software Edition 5.0 (Ariadne Genomics). The former (left side) cattle breed was characterized by an increase in chaperonines, mainly heat shock proteins, in line with its reduced thermoregulatory capacity. The latter (right side) was characterized by an increase in metabolic and anabolic enzymes, such as hydrolases, which are involved in fatty acid metabolism.

breed liver samples, without any significant quantitative difference. On the other hand, 39 different spots were found to be differentially expressed between Chianina and Holstein Friesian (Table 1).

In detail, 12 spots were found to be up-regulated in Chianina (Chianina UP in Table 1), 13 spots were up-regulated in Holstein Friesian (Friesian UP) (ratio>2), 7 spots were only expressed in Chianina (Chianina ON), while 7 in Holstein Friesian only (Friesian ON). Data were statistically elaborated with cluster analysis, which stressed the up/on-regulation trend of these 39 proteins (Fig. 2). No problems of reproducibility or calibration of the gels occurred during image processing, thus confirming the suitability of these 2-DE gels as reference standards. The aim of this study was to provide molecular evidences of the physiological differences in liver metabolism in these two beef (Chianina) and dairy (Holstein Friesian) cattle breeds. Therefore, differentially-expressed rather than commonly-detected proteins intuitively reflect the molecular basis of such differences. Hence, only modulated spots were cut out of the second dimension gel, digested with trypsin and analysed with MS tools, as described in the Materials and methods section.

In Table 1 the identities are listed of the successfully identified proteins, together with the standard spot number (SSP), the identification parameters, and the indication of their gene ontology (GO) annotation (molecular function).

3.2. *Microarray results*

In order to complement and validate proteomics results, we performed a thorough microarray analysis of the same liver samples (see the Materials and methods section). After filtering, 167 genes were found significantly ($P<0.05$) over/under expressed in the samples with Fold-Change>|1.5| (Supplementary material 1). 85% of the differentially expressed genes were up-regulated in the Frisona breed. Data confirming proteomics analyses are highlighted in green in Table 1.

HSP90AA1, HACY and CYB5A were found to be over-expressed in Friesian, thus confirming proteomics observations. However, proteomics and transcriptomics data seldom overlapped. This is mainly due to a series of technical caveats.

First of all, the 14 K ARK Genomics slide used did not include many of the genes encoding for the proteins identified by the proteomic analysis and thus little overlap between the obtained datasets was expected.

However, our aim was to outline a molecular trend paralleling the physiological differences between the analyzed dairy and cattle breeds. Although we could not manage to find a perfect match among proteins and gene products from the proteomics or transcriptomics approach, respectively, we purported to outline that this integrated approach could evidence whole pathways, which were turned on in livers from Chianina beef and Holstein Friesian dairy cattle breeds. To this end, data were statistically elaborated with cluster analysis (Fig. 2) and Ingenuity Pathway Analysis was performed (Figs. 4C and 5C) to assess whether the differentially-expressed gene transcripts could be included in specific networks to be compared (and merged) with the ones obtained from the elaboration of proteomics data.

3.3. *Delving into network complexity*

Pathway analyses have been performed on proteomics and microarray data, either merged or alone, for Chianina and Holstein Friesian cattle breeds. Top 3 pathways and the relative scores for each pathway analysis are reported in Table 2.

Most of the up/on-regulated proteins (Fig. 4B) and gene transcripts (Fig. 4C) in Chianina (with respect to Holstein Friesian) were found to be involved in anabolic and catabolic pathways. Pathway analysis of the Chianina-related differentially-expressed proteins and gene transcripts revealed their connection with lipid metabolism, aminoacid metabolism and molecular transport (Fig. 4). Notably, although transcriptomics and proteomics data did not match, pathway analyses of either differentially-expressed gene transcripts or proteins for Chianina samples delivered identification of a central network, namely the “Lipid Metabolism, Amino acid metabolism and Molecular Transport” network.

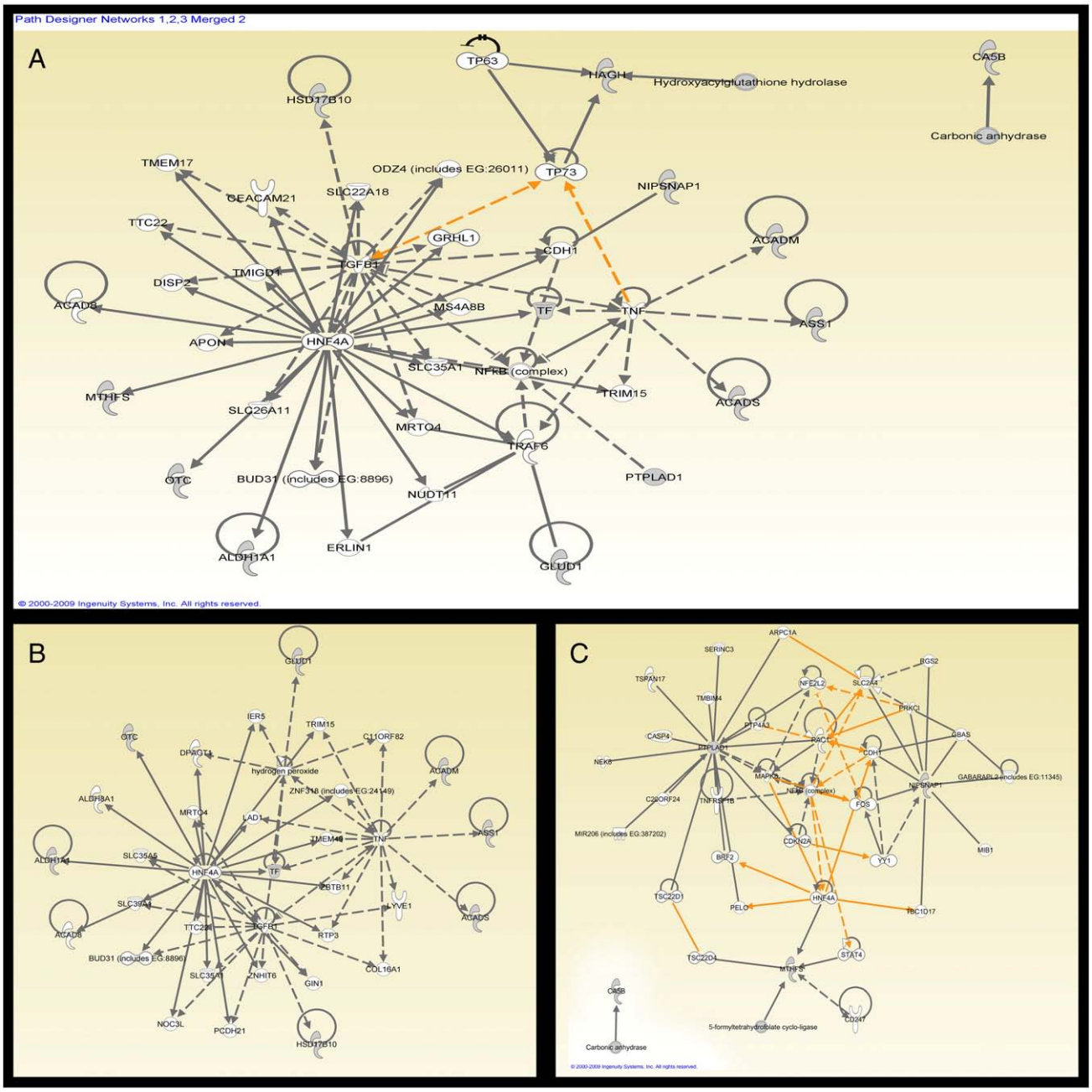

Fig. 4 – Ingenuity Pathway Analysis of the on/up-regulated proteins (B) and gene transcripts (C) alone or merged together (A) in Chianina livers only. Upon interrogation of the internal database, the software indicated that the "Lipid Metabolism, Amino acid metabolism and Molecular Transport" pathway represented the central core network either when observing elaboration of proteomics or transcriptomics data alone or together. Grey nodes are the proteins individuated during the experimental phase having a match in the network. It is worthwhile to recall that the utterly identified proteins and gene transcripts were eligible for identification and network analysis only if differentially-expressed. Therefore, some proteins/gene products which are shown in the networks as "white nodes" could be likely individuated during the experimental phase as well, although this was not the goal of this study. Altogether, proteins and gene-products individuated in the network may account for the increased metabolic and anabolic capacity of beef cattle (in this case, Chianina). Graph elaboration software: Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). Grey nodes: proteins from the dataset having a match in the database; White nodes: proteins from the database which were not identified (if present) upon the experimental phase; Continuous edge: direct interaction; Interrupted edge: indirect interaction; Grey edges: interactions within a network; Orange edges: interactions between networks.

Indeed, hydroxiacyl-Coenzyme A dehydrogenase (HADH, spot 68), Acyl Co A dehydrogenase straight chain, C-4 to C-12 straight chain (ACADM, spot 181) and Acyl-Coenzyme A dehydrogenase C-2 to C-3 short chain (ACADS, spot 229) from the differential proteomics analysis, and protein tyrosine phosphatase-like A domain containing 1 (PTPLAD1), 5,10-methenyltetrahydrofolate synthetase (MTHFS) from the microarray analysis are all proteins involved in fatty acid catabolism, in valine, leucine and isoleucine and carbon metabolism, respectively. In general these enzymes catalyze the oxidative dehydrogenation of different substrates present in the mitochondrial matrix and thus exert a key role in the fatty acid beta-oxidation pathway, converting fatty acid reserves to energy, especially during periods without food (fasting). ACADS, for example, is an essential enzyme for fatty acid metabolism (lipid metabolism) because it catalyzes the alpha, beta-dehydrogenation of acyl-CoA. Its inhibition causes low blood sugar (hypoglycemia), a lack of energy (lethargy), poor feeding, and failure to gain weight and grow at the expected rate (failure to thrive). HADH and ACADS are also involved together with in the degradation of isoleucine and valine, together with ACADM, a homotetramer, which also catalyzes the key step in leucine degradation. MTHFS had already been observed in human liver as well and it catalyzes the ATP- and Mg(2+)-dependent conversion of 5-formyltetrahydrofolate (5-FTHF) to 5,10-methenyltetrahydrofolate (5,10-MTHF), which is subsequently interconverted into other reduced folates involved in carbon metabolism [24].

Other up-regulated enzymes in Chianina take part in the amino acid metabolism and map on the network (Fig. 4), such as Glutamate dehydrogenase (GLUD1, spot 210), Arginine Succinate Synthetase (ASS, spot 187) and Ornithine Carbamoyltransferase (OTC, spot 228). These basic enzymes act in the arginine and proline biosynthesis. In particular ASS and OTC are involved in the urea cycles. ASS is responsible for the third step of the urea cycle and one of the reactions of the Citrulline-NO cycle and is a precursor to fumarate in the citric acid cycle via argininosuccinate lyase. OTC is an enzyme that catalyzes the reaction between carbamoyl phosphate and ornithine to form citrulline and phosphate. GLUD1 (spot 210, Table 1; Fig. 4) represents a key link between catabolic and anabolic pathways: ammonia incorporation in animals occurs through the actions of glutamate dehydrogenase and glutamine synthetase. The product α-Ketoglutarate can be exploited to provide energy through the citric acid cycle, in order to ultimately produce ATP.

Catalase (CAT, spot 77) and Oxydoreductase (HADH, or HSD17B1 in Fig. 4) are enzymes involved in the final step of tryptophan degradative metabolism to acetil-CoA. HADH in this case catalyzes the dehydrogenation of 3-hydroxy-butanoyl-CoA to acetoacetil-CoA and its over-expression can improve the Glycolitic pathway. Finally ACADAM and Beta-Ureidopropionase (UBP1, spot 222) are proteins that link the metabolism of beta-alanine to pyrimidine and propanoate metabolism, respectively. In fact Beta-ureidopropionase, also known as beta-alanine synthase, catalyzes the last step in pyrimidine degradation and its depletion causes muscular hypotonia, dystonic movements. Degradation of uracil is the only pathway to provide b-alanine in mammals [25]. The net result of the alanine cycle is the transport of nitrogen from muscle to liver.

In conclusion, the metabolic functions of the up/on-regulated proteins in the Chianina breed with respect to Holstein Friesian seem to indicate a mobilisation of non-conventional energy supplies (proteins, fatty acids) regarding the use of glycogen. In parallel, the increased levels of PTPLAD1 from microarray observations, which is known to take part in the NRF2-mediated oxidative stress response pathway, and of transferrin (TF), a pivotal iron transporter, are consistent with catalase up-regulation in Chianina livers [26], and may account for an increased resistance to oxidative stress [27] also in consequence to fatty acid oxidation [28].

All this information has been retrieved from the: KEGG PATHWAY Database Kanehisa Laboratory, Bioinformatics Center, Institute for Chemical Research, Kyoto University (http://www.genome.jp/kegg/pathway.html) [29,30] and the Ingenuity Pathways Knowledge Base (Ingenuity® Systems, www.ingenuity.com).

In contrast a wider series of proteins and transcripts were expressed in Holstein Friesian which were absent in Chianina. These gene and proteins were mainly related to protein folding and degradation, hormone homeostasis and ability to thermoregulate (Fig. 5). Indeed, in a recent paper, Sevi and colleagues [31] suggested that high ambient temperature may markedly modify the lipid composition of ewe's milk and that provision of shade, but not feeding management, can improve the milk fatty acid profile in dairy sheep raised in hot climates. Therefore, it is worthwhile to underline the identification of the hypothetical protein LOC78302 in Holstein Friesian (SULT1C4 in Fig. 5), but not in the Chianina. LOC783020 is a protein belonging to the sulfotransferase (SULT1C4, spot 88) family that plays a key role in the biosynthesis and homeostasis of a number of hormones, including estrogens and iodothyronines. The gene structure (number and length of exons) is similar among family members. This gene encodes a protein that belongs to the SULT1 subfamily, responsible for transferring a sulfo moiety from PAPS to phenol-containing compounds. Two alternatively spliced transcript variants, encoding different isoforms, have been described for this gene. The final protein enzymes catalyze the sulfate conjugation of many neurotransmitters, drugs, xenobiotic compounds and, above all, hormones. Involvement of the thyroid hormones in adapting metabolism postpartum in calves is now a generally accepted idea: changes in the blood levels of T3 and T4 are detectable in calves suffering from ketosis [11]. For example blood levels of thyroid hormones were reduced in milk from post-partum cows [32]. Furthermore, several papers have reported studies on bovine milk investigating the relationship between the presence of iodine, thyroid metabolism and fertility problems [33]. For example, Wemheuer found that TRH increased the production of milk and lactose slightly (as well as milk enzymes) [33]. However Whitaker and co-workers [34] concluded that, although several texts have reported that iodine deficiency in bovine milk has an influence on fertility, more research is required to establish a firm link [35,36].

Over-expression of Calreticulin (HSP90AA1 in Fig. 5), a protein that binds calcium, has been observed in Holstein Friesian both with the proteomics and transcriptomics approach. HSP90AA1 is probably in correlation with SULT1C4. HSP90AA1 has a chaperonin-like activity and binds transcription

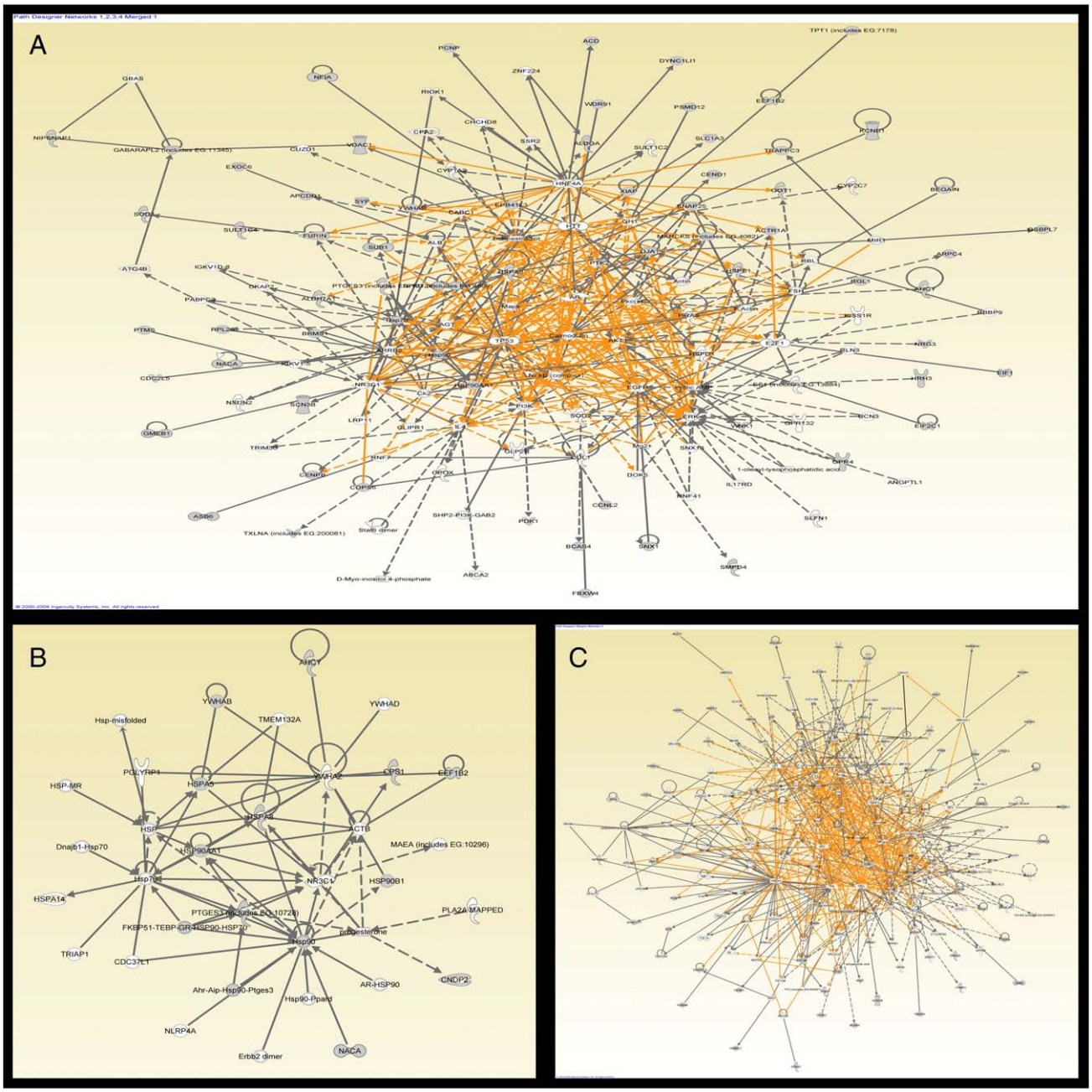

Fig. 5 – Ingenuity Pathway Analysis of the on/up-regulated proteins (B) and gene transcripts (C) alone or merged together (A) in Holstein Friesian livers only. The networks retrieved upon software elaboration of proteomics (B) and transcriptomics (C) data, either alone or merged together, display a constant central core of heat shock proteins and proteins involved in stress responses, such as HSP90AA1, which was individuated via both approaches. Grey nodes are the proteins individuated during the experimental phase having a match in the network. It is worthwhile to recall that the utterly identified proteins and gene transcripts were eligible for identification and network analysis only if differentially-expressed. Therefore, some proteins/gene products which are shown in the networks as "white nodes" could be likely individuated during the experimental phase as well, although this was not the goal of this study. The complexity of the network is increased by the increased number of the overall gene transcripts individuated in Holstein Friesian livers only (85%). Proteins individuated in the network may account for the compromised thermoregulatory capacity of dairy cattle (in this case, Holstein Friesian). Graph elaboration software: Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). Grey nodes: proteins from the dataset having a match in the database; White nodes: proteins from the database which were not identified (if present) upon the experimental phase; Continuous edge: direct interaction; Interrupted edge: indirect interaction; Grey edges: interactions within a network; Orange edges: interactions between networks.

Table 2 – Top 3 networks — scores.

Pathway analysis	Chianina		Holstein Friesian	
Proteomics data	Aminoacid metabolism	7.85 E–06 – 2.59 E–03	Protein degradation	6.86 E–06 – 6.86 E–06
	Molecular transport	7.85 E–06 – 2.63 E–02	Post-translational modification	1.92 E–05 – 3.50 E–05
	Lipid metabolism	3.03 E–04 – 1.10 E–02	Protein folding	1.92 E–05 – 3.50 E–05
Microarray data	One carbon pool by folate	8.43 E–03 – 4.68E–03	Cellular compromise	2.52E–04 – 4.44E–02
	Nitrogen metabolism	1.78 E–02 – 2.20E–02	Cell-to-cell signaling and interaction	1.15E–03 – 4.50E–02
	Skeletal and muscle physiology	1.21 E–02 – 2.36E–02	Protein synthesis	5.52E–03 – 4.44E–02
Merged data	Amino acid metabolism	1.69E–05 – 3.74E–03	Cellular compromise	4.48E–04 – 2.73E–02
	Small molecule biochemistry	1.69E–05 – 3.95E–02	Post-translational modification	8.30E–04 – 4.35E–02
	Post-translational modification	9.35E–04 – 1.30E–02	Protein folding	8.30E–04 – 8.91E–03

factors of the thyroid (TTF-1), a homeodomain-containing protein implicated in the differentiation of lung and thyroid. HSP90AA1 mRNA levels in thyroid cells are under strict control by the thyroid-stimulating hormone, thus implicating calreticulin in the modulation of thyroid gene expression by thyroid-stimulating hormone. HSP90AA1 is a ubiquitously expressed and multifunctional Ca^{2+}-binding protein that regulates diverse vital cell functions, including Ca^{2+} storage in the ER and protein folding. Calreticulin deficiency in mice is lethal in utero due to defects in heart development and function. Interestingly, HSP90AA1 is closely located on the same map of SULT1C4 and takes part in a sub-pathway involving casein kinase 2 (ck2) and Furin, the latter known to cleave the proparathyroid hormone [37], for an indirect control on the thyroid hormone, and superoxide dismutase (SOD3 in the map, Fig. 5A), thus reducing the capacity to protect against oxidative stress [38].

The presence of Heat shock 70 kDa protein 5 (HSAPS, spot 358 in Table 1), as well as of HSP90, and HSPE1 were also observed in Holstein Friesian. It is worthwhile to underline that HSPs represented the central network individuated upon pathway analysis of either proteomics/transcriptomics data alone (Fig. 5B and C) or taken together (Fig. 5A). Increased levels of HSPs occur in response to environmental stresses, infection, normal physiological processes, and gene transfer. Stress can disrupt the physiology and productive performance of an animal. The increase in body temperature caused by heat stress has direct, adverse consequences on cellular function, such as an increased glucose and amino acid oxidation and a reduced fatty acid metabolism [39]. This unbalance might favour trygliceryd deposition and, finally cause fatty liver. Fatty liver (i.e., hepatic lipidosis) is a major metabolic disorder of many dairy cows in early lactation and is associated with decreased health status and reproductive performance. In severe cases, milk production and feed intake are decreased. Therefore, a practical preventative or an efficacious treatment of fatty liver could help dairy farmers yearly save millions of dollars in treatment, replacement, and production losses. Since even mild fatty liver is associated with decreased health status and reproductive performance of dairy cows, prevention of fatty liver by supplying cows with sufficient nutrients and a clean and health-promoting environment in the peripartal period would reduce production losses of cows more than any subsequent treatment of fatty liver. This, however, might not be enough for those cows suffering from additional complications such as obesity, poor appetite, metabolic disorders, infectious diseases, calving difficulty or twin birth, or severe negative energy balance because of high milk production immediately after calving. Changes in the genetics and physiology of food animals for increased production are making these animals less able to regulate body temperature, i.e., less adapted to warm environments. This is especially true for dairy cattle. Selection for milk yield reduces the ability to thermoregulate in the face of heat stress [40] and magnifies the seasonal depression in fertility caused by heat stress [41]. Besides, use of bovine somatotropin (BST) as a lactational promotant can increase body temperatures during heat stress [42–45]. Moreover, adaptation of both beef and dairy cattle is reduced when native, genetically adapted cattle in tropical or semitropical regions are replaced by higher producing, maladapted breeds. Chronic elevation of uterine temperature has long been known to increase embryo mortality in dairy cattle. Short-term elevation in temperature of mouse embryos to 43 °C (acute) has been shown to induce intracellular production of heat-shock proteins (HSPs).

HSP 90 kDa beta member (HSPA8, spot 356 in Table 1 and Fig. 5) is important for converting certain fats to energy with a shortage (deficiency) of functional medium-chain acyl-CoA dehydrogenase. This causes that medium-chain fatty acids are not metabolized properly. As a result, these fats are not converted to energy, which can lead to several symptoms such as lack of energy (lethargy) and low blood sugar.

Fig. 3 summarizes the nature of the identified polypeptide species and the differences in expression profiles between up/on proteins from Holstein Friesian and Chianina. In general, the livers of both breeds have revealed a large number of protein species, in accordance with the numerous specialized biochemical and physiological functions of this organ. However, relative abundances vary between the two: in Holstein Friesian the largest group of proteins are chaperonines (36.85% of total proteins), whilst in Chianina they only represent 5.28%; hydrolases follow the opposite trend representing 10.52% and 26.31% respectively. Hydrolases perform a wide range of different digestive functions, though they are most prevalent in fatty acid metabolism, in agreement with pathway analysis elaborations for Chianina cattle livers; in contrast chaperonines are most involved in protein folding and consequently in the synthesis of new molecules or protection from denaturing stress (e.g. heat or oxidative stress) of the yet existing ones (as stressed by the pathway analysis elaboration for Holstein Friesian cattle livers).

Although current proteomic methodologies applied to mammalian cell analysis still suffer from several technical

limitations, which prohibit a systematic comprehensive description of the entire protein expression profile and result in non-detection of many important low soluble or less abundant components, these preliminary tissue analyses by conventional 2-DE procedures are still able to yield useful data on poorly characterized animals.

Relevant information could be retrieved when performing both proteomics and transcriptomics analyses simultaneously, even when the experimental datasets display low to absent overlap, due to a series of intrinsic technical limitations — last but not least, the inadequacy of available statistical tools to compensate for biases in the data collection methodologies, as it has already been pointed out before [46]. Even in this case, however, interaction pathway analysis of those datasets allows the individuation of central networks sharing the same biological meaning, but not necessarily the same molecular actors. Hereby, we pinpointed at two main networks which undergo primary modifications in cattle breeds with different productive aptitudes, such as Chianina and Holstein Friesian. This kind of approach definitely eases data analysis and final discussions, other than bridging the gap between the arid experimental phase and its biological interpretation (in this case, bovine liver).

This study contributes to a more detailed understanding of important biological processes which have moulded Holstein Friesian and Chianina into two distinct populations. Moreover, this study provides evidence of how direct measures of gene expression, that is to say, protein- and transcript-oriented analyses, can still provide information on the global dynamic changes in the protein repertoire associated with the two distinct populations, and elucidate the important metabolic differences that have arisen upon modification of relatively few genes/proteins (or gene/protein networks), over thousand years of human breeding selection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jprot.2009.09.015.

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