Transcriptomic analysis of two sheep breeds during lactation, using a new custom microarray platform

Silvia Bongiorni[1], Giovanni Chillemi[2], Gianluca Prosperini[2], Susana Bueno[2], Federica Signorelli[3], Bianca Moioli[3], Lorraine Pariset[1]

[1]Dipartimento di Produzioni Animali, Università della Tuscia, Viterbo, Italy
[2]CASPUR - Consorzio per le Applicazioni di Supercalcolo Per Università e Ricerca, Roma, Italy
[3]CRA - Centro per le Produzioni delle carni e il Miglioramento genetico, Monterotondo, Italy

Corresponding author: Lorraine Pariset. Dipartimento di Produzioni Animali, Università della Tuscia. Via S. Camillo de Lellis, 01100 Viterbo, Italy - Tel. +39 0761 357354 - Fax: +39 0761 357434 - Email: pariset@unitus.it

Abstract - We aim at understanding the genomic influence on milk quality and synthesis by comparing two sheep breeds using sheep-specific microarray technology. From sheep ESTs deposited at NCBI we generated a chip carrying about 22,000 non-redundant features in quadruplicate, achieving very good technical outcomes. Oligos were *in situ* generated on chip using the Combimatrix equipment. We analysed the mammary transcriptome in individuals of two sheep breeds at two lactation stages, to identify genes controlling milk production and metabolic pathways in which these genes are involved. With $|FC|>1.4$, and p-value≤ 0.05, 142 and 14 genes resulted differentially expressed in stages 01 and 02, respectively.

Key words: Sheep, Microarray, Lactation, EST

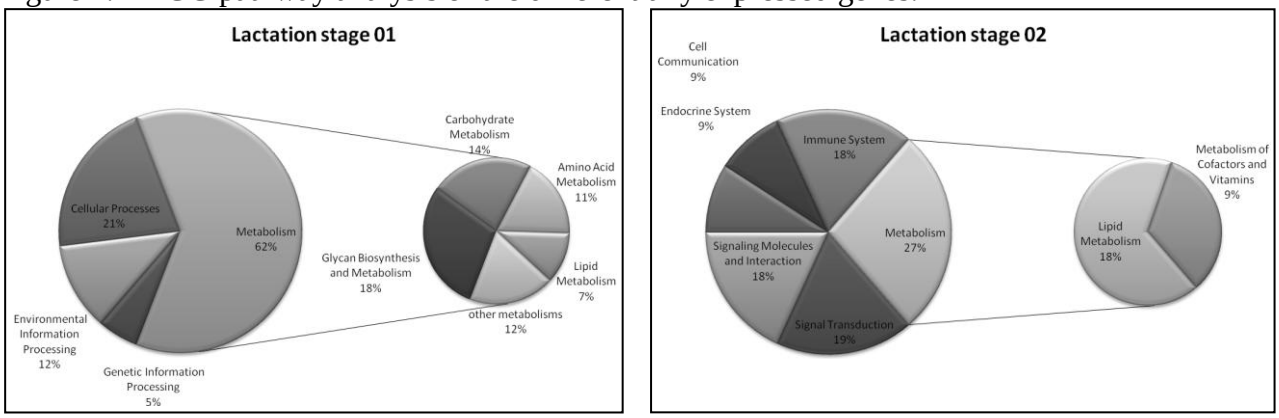
Introduction - In dairy animals, mammary gland undergoes huge functional and metabolic adaptation to prepare to lactogenesis. After parturition, the metabolic activity of mammary epithelial cells increases dramatically to carry out high levels of milk production. Milk yield significantly increases during the first few weeks of lactation. During this period a well-studied set of genes, which are involved in milk synthesis, also increases their expression. After lactation peak, milk synthesis and specific gene expression gradually decrease (Sorensen *et al.*, 2006). Sheep milk yield, composition and lactation persistency can fluctuate across and within breeds. Candidate genes were intensively analyzed to identify the molecular mechanisms affecting sheep milk quality (Moioli *et al.*, 2006). Among milk protein genes, the major effects were assessed for α_{s1}-casein and β-lactoglobulin. The bovine B variant of κ-casein is considered favorable for milk quality and quantity of cheese, and it is included in breeding strategies of dairy cattle (Aliphan *et al.*, 2008). Other important genes are those directly involved in fatty acid metabolism. Microarray analysis of total mRNA from mammary gland is a valuable tool allowing deeply investigation of involved genes and identification of new candidate genes. Moreover, the comparative analysis of some sheep breeds with different attitude to milk production could demonstrate the association between genetic variants and milk quality (Kirov *et al.*, 2006). Among the several thousands of experiments on microarrays conducted so far and accumulating in Gene Expression Omnibus (GEO) at NCBI, only 3% regard agricultural species. One of the major reasons because microarray analyses on livestock breeds are still scarce is the unavailability of specific platforms. We have developed a pipeline of software instruments that starting from unannotated redundant sequences as those found in public databases or generated by parallel sequencing, yields oligonucleotides suitable for *in situ* generation on chip (Chillemi *et al.*, 2009). Using this pipeline we generated a chip from sheep ESTs deposited at NCBI, carrying 21,743 non-redundant features in quadruplicate, 73.4% of which are fully annotated and corresponding to 10,190 genes, thus representing a good coverage of the sheep genome (Pariset *et al.*, 2008). We used this novel chip to investigate differences in gene expression in two sheep breeds with different milking attitude at two lactation stages.

Material and methods - *Microarray design* - Sequences were retrieved from NCBI sheep dbEST. Each single sequence was BLASTed against the others and similar sequences were tagged and excluded from the set. Criteria to filter BLAST outputs were alignment length≥100bp, maximum score and identity≥75%. Each non redundant sequence was BLASTed on NCBI RefSeq, when available, or on DNA database of human, cattle or other model species to retrieve full and unique annotation of the gene. Oligos, formed by two short sequences spaced by a random DNA for achieving a better annealing of the probe, were designed using the GoArrays software and *in situ* generated using the Combimatrix equipment. *Microarray experiment* - Two individuals per breed with different milking attitude, Sarda (S) and Gentile di Puglia (G), fed and housed under the same conditions, were sampled immediately after slaughtering at two different lactation stages (stage 01: 6 days after lambing; stage 02: 44 days after lambing). Udder samples were preserved in RNAlater (Sigma) and stored at –80°C. RNA was extracted using the RNeasy midi kit (Qiagen), amplified and Cy3 and Cy5 labelled using the ULS technology (Kreatech Diagnostics). The labelled and fragmented aRNA was then hybridized onto the slide according to Combimatrix instructions. A dye-swap experiment was performed, labelling each sample independently with each fluorescent dye. Images were obtained by a ScanArray Lite (Perkin Elmer) laser scanner and Microarray Imager 5.8.0 software was used to extract feature data from microarray fluorescence images.

Results and conclusions - Starting from unannotated redundant EST sequences deposited in dbEST we generated a sheep microarray which yields reproducible patterns of differentially expressed genes (in slide replicates show a coefficient of variation<0.25 for differentially expressed genes with P<0.01). We analysed udders at two lactation stages of individuals of two sheep breeds (S *vs* G). Using a cutoff of |FC|<1.4 with P≤0.05, 142 genes in stage 01 and 14 in stage 02 resulted differentially expressed in the two breeds. At stage 01, a total of 49 genes were upregulated in G and 93 in S, while at stage 02, only 3 genes were upregulated in G, the other 11 resulted upregulated in S. A Kegg pathway analysis was performed, to show the most represented pathways among the differentially expressed genes (Figure 1). At stage 01 genes encoding caseins αS2, β and k are upregulated in S dairy breed. In addition to the milk protein genes, we identified genes involved in apoptosis, cell cycle control, energy metabolism, oncogenes and genes involved in several cellular processes like cell communication (focal adhesion adherens junction), endocrine system (insulin signaling pathway, adipocytokine signaling pathway). A differential expression of genes involved in the ubiquitination pathway, like USP9X, can be observed. This pathway is the most significantly enriched during both lactation and involution (Lemay *et al.*, 2007). Udder development is spatially regulated by the interaction of the mammary epithelium with the extracellular matrix (ECM) through a family of adhesion receptors called integrins, required for proliferation, accurate morphological organisation, as well as milk secretion. Integrins assist cells in sensing their appropriate developmental context in response to both hormones and growth factors. Cell adhesion to the ECM plays a key role in alveolar survival, morphogenesis and function. Downstream, small Rho GTPases mediate cellular polarisation and differentiation (Katz and Streuli, 2007). A significant difference in expression of genes involved in ECM formation and cell adhesion (TJP1 upregulated in G; CDH5 and TNXB in S) can be observed between the two breeds. Also the expression of one apoptosis initiator (Caspase 8) and of the oncogene VAV3 increases during stage 01 in S. Apoptosis acts during involution of mammary gland in cattle (Wilde *et al.*, 1997), and recently an amplified expression of many apoptosis-related genes during lactation was reported (Suchyta *et al.*, 2003). Another category of genes differentially expressed between the two breeds includes cytochrome c oxidase, NADH dehydrogenase, and ferritin, all genes involved in oxidoreductase activity. The activity of cytochrome oxidase increases from late pregnancy to the first days of lactation (Rosano and Jones, 1976). The overall expansion of oxidative metabolism mirrors the increased energy demands of the lactation period. At stage 01, we observed an upregulation of cytochrome c oxidase and NADH dehydrogenase in G. This may reflect a difference in lactation persistence (60-150 days in G, up to 210 days in S). Finally, several studies

have demonstrated that the fatty acid (FA) profile of raw milk influences cheese characteristics. Lipolysis is particularly important in sheep cheese due to the high fat content and lipase activity (Fontecha *et al.*, 2006). We observed an increased expression of PLD3, a phospholipase, in S breed selected for milk characteristics. At stage 02 only 8 genes are upregulated in G and 27 in S.

Figure 1. KEGG pathway analysis of the differentially expressed genes.



We can remark the overexpression in S of some interesting genes as those encoding casein k, lipase (DAGLB) and proteins involved in oxidoreductase activity (like TGOLN2 and FTH1), and in ECM-interaction (like COL1A2). We have proven that our chip is a valuable tool which can be employed in gene expression analysis in sheep. Moreover, this is the first approach by far aimed to analyse differential gene expression at various lactation stages by microarray technology, using an homologous chip generated from sheep ESTs.

References – Alipanah, M., Alexandrovna Kalashnikova, L., Veladimirovich Rodionov, G., 2008. Kappa-casein and PRL-Rsa I genotypic frequencies in two Russian cattle breeds. Archivos de Zootecnia 57:131-138. **Chillemi,** G., Pariset, L., Bueno, S., Prosperini, G., Bongiorni, S., Valentini, A., 2009. Aristaeus chip 1.0: an *in situ* synthesized oligonucleotide microarray for *Ovis aries*. Submitted. **Fontecha**, J., Mayo, I., Toledano, G., Juárez, M., 2006. Triacylglycerol composition of protected designation of origin cheeses during ripening. J. Dairy Sci. 89:882-887. **Hadsell**, D.L., 2004. Genetic manipulation of mammary gland development and lactation. Adv. Exp. Med. Biol. 554:229-251. **Katz,** E., Streuli, C.H., 2007. The extracellular matrix as an adhesion checkpoint for mammary epithelial function. Int. J. Biochem. Cell Biol. 39:715-726. **Kirov,** G., Nikolov, I., Georgieva, L., Moskvina, V., Owen, M.J., O'Donovan, M.C., 2006. Pooled DNA genotyping on Affymetrix SNP genotyping arrays. BMC Genomics 7:27. **Lemay**, D.G., Neville, M.C., Rudolph, M.C., Pollard, K.S., German, J.B., 2007. Gene regulatory networks in lactation: identification of global principles using bioinformatics. BMC Syst. Biol. 1:56. **Moioli**, B., D'Andrea, M., Pilla, F., 2006. Candidate genes affecting sheep and goat milk quality. Small Rumin. Res. 68:179-192. **Pariset,** L., Chillemi, G., Bueno, S., Prosperini, G., Bongiorni, S., Valentini, A., 2008. From public sequences to custom microarrays: an easy and affordable tool. XXXI ISAG Conference, Amsterdam, The Netherlands. **Rosano**, T.G., Jones, D.H., 1976. Developmental changes in mitochondria during the transition into lactation in the mouse mammary gland. J. Cell Biol. 69:573-580. **Sorensen**, M.T., Nørgaard, J.V., Theil, P.K., Vestergaard, M., Sejrsen, K., 2006. Cell turnover and activity in mammary tissue during lactation and the dry period in dairy cows. J. Dairy Sci. 89:4632-4639. **Suchyta**, S.P., Sipkovsky, S., Halgren, R.G., Kruska, R., Elftman, M., Weber-Nielsen, M., Vandehaar, M.J. Xiao, L., Robert, J., Tempelman, R.J., Coussens, P.M., 2003. Bovine mammary gene expression profiling using a cDNA microarray enhanced for mammary-specific transcripts. Physiol. Gen. 16:8-18. **Wilde**, C.J., Addey, C.V.P., Li, P., Fernig, D.G., 1997. Programmed cell death in bovine mammary tissue during lactation and involution. Exp. Physiol. 82:943-953.