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Proteomic and animal health

PROTEOMICS: THE MAGIC WORLD OF PROTEINS

The term proteomics refers to the large-scale study of proteins, including their structures and functions. The proteome defines the set of proteins expressed by the genetic material of an organism under given environmental conditions (Schlieben et al., 2012). Proteomic science emerged as a distinct field of research during the last twenty years (Thanomsridetchai et al., 2011) and, following a slow start, it has developed rapidly, driven by improvements in electrophoresis techniques and mass spectrometry analyses. Being a proteome more complex than its encoding genome (Corthals et al., 2000) the complete characterization of the proteins that compose even simple biological systems is hardly achievable, as opposed to the determination of full genomes (Burgess and Burchmore, 2012), on the background that the proteins are also present across a broad dynamic range. These issues are compounded by regulation of protein expression, in response to developmental and environmental stimuli, which results in a dynamic proteome. Nevertheless, the importance of proteins as the primary effector molecules of biology, which are also the major drug targets and antigens, has triggered strong interests and investments in proteomics, and the field continues to develop rapidly.

Proteomic techniques

Proteomics involves the resolution of a complex mixture of proteins into com-

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ponents that can then be identified, matching protein to encoding gene, and possibly quantified. As opposite to genomics or transcriptomics techniques, proteomics is unique in providing detection of post-translational protein modifications, such as phosphorylation or glycosylation. Protein characterization is carried out by mass spectrometry, which is generally performed after initial fractionation, on the background that even simple prokaryote proteomes comprise thousands of proteins and multicellular species may comprise greater than 100.000 proteins. The type of fractionation depends on the complexity of the proteome and the specific research question but must be compatible with the downstream mass spectrometry (MS). Major MS platforms employed for proteomics differs by the mechanism through which ions are generated and include matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). MALDI instruments receive analytes in the solid state, while the sample is delivered to ESI instruments in a volatile solvent.

The protein fractionation systems can be either electrophoretic (usually applied to intact proteins) or chromatographic (usually applied to peptides generated by protein cleavage). Orthogonal separation approaches are often utilized to enhance resolution, and the archetypal orthogonal separation in proteomics is 2-dimensional electrophoresis (2DE) (Gorg et al., 2004). Conventional 2DE involves separation by isoelectric focusing in the first dimension, followed by sodium dodecyl sulphate electrophoresis in the second, both dimensions being performed in a polyacrylamide gel matrix, and the proteins migrate on 2-dimensional gels as spots according to isoelectric point and apparent molecular weight. The resulting spot map are visualized by protein staining and are able to resolve several thousand protein species. Spots can then be excised directly from the gel and identified by means of mass spectrometry.

2DE remains the highest-resolution protein separation approach and is inherently quantitative. The separation of intact proteins by charge and mass provide information about post-translational modifications that would not be evident in the lower resolution 1-dimensional electrophoresis or in peptide-based separations (Rogowska-Wrzesinska et al., 2013). Yet, 2DE tends to under-represent those proteins that are of relatively low abundance, very large, or highly charged. Prefractionation to enrich proteins of interest or by focusing 2DE on specific charge and/or mass ranges can circumvent some of these issues. Hydrophobic proteins may be refractory to solubilization in the nonionic conditions that are required for isoelectric focusing and alternative detergents or 2-dimensional separations, such as the BAC/SDS-PAGE system (Bridges et al., 2008; Hinz et al., 2012) come useful in this context.

The heterogeneity of intact proteins provides a serious challenge the resolution of chromatography for proteomic workflows. Proteins can also be fragmented to peptides and then separated by chromatography. Reversed-phase chromatographic separation of peptides is ideally suited to proteomics because peptides can be trapped and desalted before elution and because the mobile phase comprises volatile solvents that can be evaporated in the ESI source. Chromatography can thus be directly coupled to ESI-MS, Multidimensional chromatographic separation is increasingly employed (Yates et al., 2009), being automated and because even highly charged or hydrophobic proteins will likely generate some peptides that can be identified on MS analysis. Ion exchange is mostly used as a first dimension. Chromatographic approaches can be more sensitive than electrophoresis because there is no requirement to recover proteins or peptides from a gel matrix. Combinations of electrophoresis and chromatography are among the most efficient fractionation systems (Xie et al., 2011). Although targeted approaches such as subcellular fractionation or affinity purification of protein complexes can result in greatly enhanced coverage of a subproteome.

Proteins obtained from a biological source are fractionated by electrophoresis, peptides are generated after trypsinization and further fractionated by high-performance liquid chromatography (LC) before analysis by an ESI-MS which allows the identification of the peptides (Burgess and Burchmore 2012). The resulting data are analyzed with the support of dedicated softwares or search engines, such as for example Mascot (Matrix Science Ltd), which generates in silico MS data for the specified genome sequence database and looks for statistically significant matches with the experimentally generated MS data. The data output provides a list of potential matches, ranked by confidence, to proteins that may be components of the sample.

Proteomics can also provide protein relative quantitation, which can be achieved by a diversity of comparative approaches. Relative quantitation of intact proteins can be carried by gel-based methods, such as 2DE using semi-quantitative protein stains, or protein-labeling strategies, such as difference gel electrophoresis (DiGE) (Alban et al., 2003). DiGE techniques have increased the utility of 2DE for quantitative proteomic analysis, allowing the direct comparison on a single gel of samples that are differentially labeled by fluorophores that are mass and charged matched but spectrally discrete.

Quantitation at the peptide level can be achieved by stable isotope-labeling approaches or by label-free comparison. Isolated proteins or tryptic peptides are chemically labeled before separation [iTRAQ], dimethyl labeling (Hsu et al., 2006) or proteins can be metabolically labeled with heavy and light ami-

no acids (stable isotope labeling with amino acids in culture [SILAC]) (Ong et al., 2002). An appropriate software deconvolutes from the resulting MS data the relative abundance of specific proteins in each sample. Of the protein-labeling approaches, SILAC is incorporated during growth, avoiding the possibility of introducing artifactual changes in protein abundance during the sample preparation step, although chemical labeling of proteins can be performed with proteins from any source.

Label-free approaches involve serial LC-MS analysis of multiple unlabeled samples and are becoming commonplace as the robustness of chromatographic separation improves and facilitates the alignment of data sets that is essential for comparison. Label-free approaches are more costly in instrument time, as unlabeled samples cannot be multiplexed – an important consideration, as LC-MS instrumentation is costly to maintain. Regardless of the quantitation approach, comparative proteomics experiments have the potential to highlight key proteins in phenotypes of interest and thus have tremendous potential to highlight drug targets and biomarkers and elucidate biological mechanisms (Burchmore, 2006). A brief summary of the two proteomics workflows is provided in figure 1.

PROTEOMICS IN VETERINARY AND ANIMAL SCIENCES

Several proteomic techniques have been applied to the understanding of protein pathways involved in host and pathogen interactions during diseases. Pathogens and immune defenses adapt to each other, due to the regulation of the expression of several genes of both sides, to cope with changing stimuli. The fine-tune of gene expression has been studied by Next Generation Sequencing techniques, in particular for what concerns host-pathogen relationship (Ojha and Kostrzynska, 2008). However, since the correlation between DNA levels and actual protein expression is poor (Griffin et al., 2002) and integration between the two techniques, genomics and proteomics, is required.

Given the background of economic needs, most of proteomics investigation explored the pathogenesis of mastitis, in bovine milk in particular, because of the relative ease of sample collection (Bohemer, 2011).

Proteomics was very useful to describe the modification of milk proteins during mastitis in cows with naturally occurring infection (Smolenski et al., 2007) as well as in experimentally induced coliform mastitis (Danielsen et al., 2010), providing a list of milk proteins that can be found in milk and that

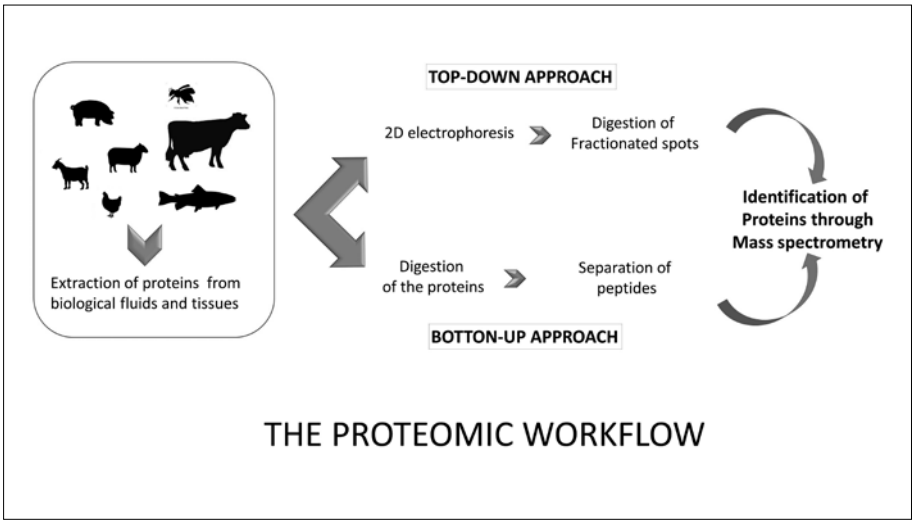

Fig. 1

can be possibly used as biomarkers for early diagnosis of mastitis.

Proteomic techniques have been widely applied to the pathogenic mechanisms of bacterial infection in farm animal diseases, focusing, again, on pathogen responses during clinical intramammary infections (Tedeschi et al., 2009), providing the identification of immunogenic proteins in bovine mastitis *S. aureus* isolates involved in virulence. *M. avium subsp. paratuberculosis* was also investigated, identifying among the others a set of 10 proteins whose expressions are upregulated during natural infection (Hughes et al., 2007), as well as the proteome of pathogenic leptospires, the causative agent of leptospirosis, expressed during urinary excretion from reservoir hosts of infection (Nally et al., 2007).

Beside bovine species, a significant amount of proteomic studies has been performed on other farm animals, such as porcine and caprine species (de Almeida and Bendixen, 2012; Ceciliani et al., 2014). In addition to its role in meat production, the porcine species is an important animal model for the study of disease in humans.

An interesting field of application of proteomics is the study of the pathogenesis of infectious disease of poultry, which has an impact on both the production and the need to study avian diseases as zoonosis, such as for example avian flu, where human host adaptation signatures have been identified (Miotto et al., 2010).

Fish diseases are responsible for the main economic losses in aquaculture.

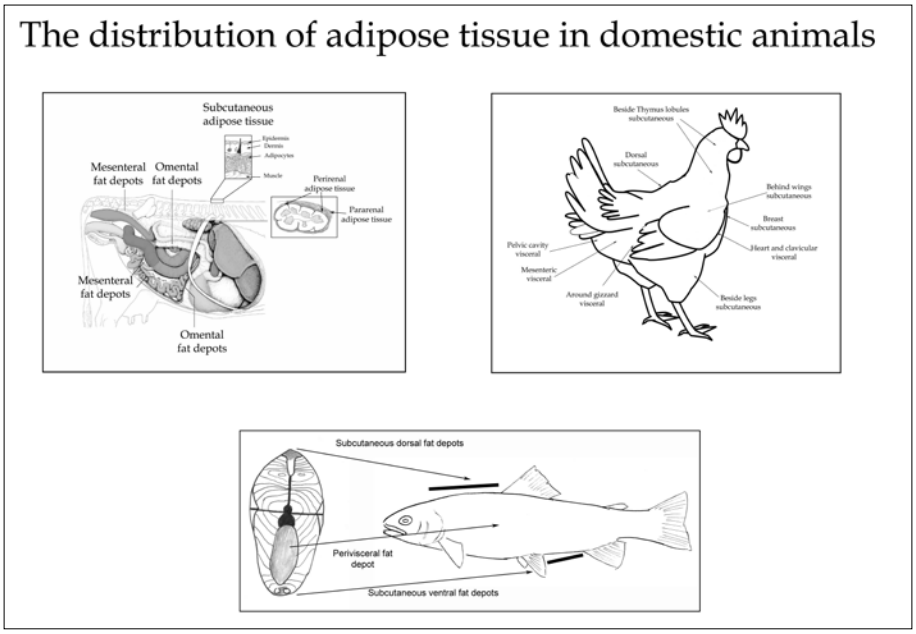

Fig. 2

Proteomics techniques have been used to address this issue, in particular for the development of new vaccines and disease diagnostics. Finally, the complete proteomic atlas of bee has been recently published (Chan et al., 2013), providing a deep insight into organ-level resolution of protein expression.

Due to its involvement in complex processes like reproduction, inflammation and immune response, also adipose tissue has been analyzed by applying proteomic techniques (Sauerwein et al., 2014), presenting the evidence that different adipose tissue depots express a different protein pattern.

THE ADIPOSE TISSUE: NEW CONCEPTS FOR AN OLD TISSUE

The importance of adipose tissue as energy store was already well established many centuries ago. In farm animal physiology, most of the studies on adipose tissue were aiming to understand the mechanisms underlying the mobilization of lipids in lactating animals to fulfill the energy requirements of milk production or the deposition of fat in different depots, in particular those associated with muscle. Since the discovery of leptin in 1994 (Zhang et al., 1994), adipose tissue was no longer considered as a mere source of energy

but was increasingly recognized to be actively involved in complex regulatory processes, e.g. regulating appetite, energy expenditure, body weight, inflammation and reproduction via its synthesis and secretion of messenger molecules which are now collectively referred to as "adipokines". There are several types of adipose tissue depots (fig. 2).

Priorities in adipose tissue research in farm animals are different from the focus of human biomedical research. Most of the information available in mammalian adipose tissue biology has been linked with obesity and metabolic diseases in humans, where the specific term "obesidomics" was coined to define proteome and secretome in pathological obesity (Pardo et al., 2012). Although several animal models of obesity were developed (Lutz et al., 2012), obesity is hardly an issue in farm animals due to their controlled feeding according to well defined needs.

Transcriptomics has provided important advancement in understanding the functions of adipose tissue. Yet, the major limit of transcriptomics is that it does not provide any hint about the effective expression of the proteins, the correlation between mRNA level and actual protein expression being poor (Griffin et al., 2002). Therefore, integration between genomics and techniques focused on protein expression is required. Within the past decade proteomics has emerged as an accessory technique to transcriptomics. Hereby are provided examples of how adipose tissue features can be explored by means of applying proteomic techniques, focusing on goat adipose tissue.

ADIPOSE TISSUE PROTEOMICS

Adipose tissue is distributed as fat depots throughout the whole body, and is classified mainly as subcutaneous (SAT) and visceral (VAT) adipose tissue (fig. 2). In ruminants, SAT includes depots located beneath the skin, e.g. the armpit cavity, the subcutaneous areas over the sternum and the withers and the base of the tail, while VAT is located in the intra-abdominal cavity, surrounding specific organs, such as kidney and heart, or distributed among peritoneum layers, such as mesenteric and omental fat. Therefore, adipose tissue should not be considered a single endocrine organ located in different region of the body, but a group of endocrine organs with specialised and location specific endocrine function (Kershaw and Flier, 2004). In fact, VAT AND SAT are distinguished according to their different metabolic characteristics and by their ability to release inflammatory cytokines. In farm animals, and particularly in ruminants, the regulation of lipid

metabolism is of key importance not only for animal health, but also for production of meat and milk. Nevertheless, a systematic investigation of the molecular mechanisms of adipose tissue has not yet been undertaken in these species. Transcriptome studies of adipose tissues from mice, rat, sheep and cow have shown that visceral and subcutaneous adipose tissue depots differ in mRNA abundance, highlighting the importance of sampling site in studies of e.g. metabolic pathways in AT (Mukesh et al., 2010).

Proteomic studies have been limited to bovine species, and include a comparative study of adipogenic differentiation of preadipocytes in the omental, subcutaneous and intramuscular tissues (Rajesh et al., 2010), a time resolved investigation of embryonic fat deposition (Taga et al., 2012) and of intramuscular fat depots of Korean steers (Zhang et al., 2010). LC-MS/MS was applied to characterize and compare the proteome composition of SAT (base tail and sternum) and VAT (perirenal and omentum) of young goats (Restelli et al., 2014) providing the first adipose tissue proteome of goat. The proteomic analysis of different SAT AND VAT deposits showed protein expression's differences, confirming also in goat-kids the importance of sampling site when studying adipose tissue's metabolic roles. The protein expression characteristics of adipose tissues was determined by quantitative RT-PCR and confirmed that adipose tissues seems to play a central role in control of inflammation, detoxification and coagulation pathways, as well as for regulation of body fat mobilization in dairy animals. These findings were of particular interest in farm animals where health and production traits are important for animal welfare and for economic gain. A number of 761 proteins were found to be uniquely produced by adipose tissue. Of them, most were involved in metabolic processes, dominating both VAT and SAT tissues, although significantly more in VAT (73.5%) than in SAT (54.5%). Structural proteins represented 20. 4 % of the observed adipose tissue proteome, being more abundant in visceral AT than subcutaneous AT (30.1% and 19.6%, respectively), and particularly so for sternum deposits (48.5%). In our studies, 71 out of 761 adipose tissue proteins relate to this group (9.3%) (Restelli et al., 2014). Other proteins were involved in proteins involved in toxic response and folding, including families such as HSP, chaperons and peroxiredoxins. The last group of proteins were involved in immune and inflammatory response, and represented 6.5% of the total.

In a second experiment, the impact of different diet was studied (Restelli et al., manuscript in preparation). The comparative investigation of visceral adipose tissue proteomes of goat-kids with different high-fat fed mothers was perfomed. Periparturient goats ere fed with fish oil and stearic

acid enriched diets, and a quantitative 2D-LC-MS/MS analysis was carried out, using iTRAQ labelling, in order to evaluate the possible influence of different diets on kids' omentum protein expression. The involvement of adipose tissue (AT) in several physiological and pathological processes, such as appetite regulation, reproduction, and inflammatory and immune response, is well recognized. In humans, adipose tissue has a key role in obesity, while in farm animals, where obesity is not an issue due to the controlled environment in which they live, particular focus has been given to adipose tissue's influence on animal health and meat quality. Indeed, it has been demonstrated that adipose tissue within the muscle (i.e. marbling fat) strongly influence meat quality and composition, by affecting parameters such as tenderness, juiciness and taste (Wood et al., 2008). On the other hand, in dairy animals AT metabolism gained particular interest for its essential role in the transition period when a hormonally-controlled lipid mobilization is established in order to support milk synthesis (Shirley et al., 1973; Contreras and Sordillo, 2011). The active role of adipose tissue in regulating the wide range of body functions is explicated by its ability to produce and secrete adipokines. Adipokines are signalling molecules with endocrine, autocrine or paracrine functions, secreted in response to stimulus coming from the hormone system and the central nervous system (Harwood, 2012). Taking into account the profound relationship between body fat reserves and food, it is not surprising that adipose tissue's transcriptomic profile can be modified by diets or feed deprivation. Indeed, as demonstrated in goats, 48 h of feed deprivation alter the expression profile of several genes in omental and perirenal AT deposits, with omentum more sensitive to feed deprivation than perirenal areas (Faulconnier et al., 2011). In addition, Ebrahimi et al. (2013) demonstrated that linseed oil supplementation to Boer goats' diet, leads to changes in fatty acid profile of subcutaneous adipose tissue and expression of genes related to fat metabolism such as PPARα, PPARγ and stearoyl-CoA desaturase. Few proteomic studies are regrettably available.

Another aspect to consider when studying the relationship between diets and adipose tissue, is the different impact that distinct fat sources have on adipose tissue itself, as demonstrated by Thering et al. (2009), which investigated the effect of fish and soybean oils or saturated lipids enriched diets on lipogenic and adipogenic gene expression in cow's tailhead adipose tissue. Fish oil is particularly rich in eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3) that can positively influence animal health due to their involvement in innate

immune pathways (Pisani et al., 2009; Lecchi et al., 2011). On the other hand, Bueno et al. (2010) demonstrated that diets enriched with coconut oil or lard, both rich in saturated fatty acids, can modify the pro-inflammatory environment of white adipose tissue in rats, by upregulating haptoglobin expression.

Adipose tissues originate during early life from mesenchymal stem cells, which can differentiate into adipocytes, osteoblasts, chondrocytes, and myoblasts (Lee et al., 2013). It has been shown that a diet based on milk or milk replacer can influence meat quality and fat composition of suckling kids (Bañon et al., 2006), therefore the next step to be investigated was the influence that the maternal diet has on kid's adipose tissue characteristics. UCP1 expression and thermogenesis can be modulated by high fat diets, in perirenal adipose tissue of newborn lambs (Chen et al., 2007), while overfeeding sheep during late gestation, enhances adipogenesis in lamb's fetal muscles (Tong et al., 2008). In addition, fish oil enriched diets increases the amount of n-3 PUFAs in colostrum and mature milk in pregnant dairy goats (Cattaneo et al., 2006), and a specific involvement in fetal and neonatal development has been recognized for DHA (Innis, 2000). Although proteomics could be of great help in understanding the relationship between maternal diet and kids' adipose tissues' characteristics, mainly transcriptomic studies have been carried out. The few existing proteomic experiments have been performed in rodents, aimed to evaluate the effect of high fat diets on the protein expression of insulin target tissues in mice (Schmid et al., 2004) or on the expression of adipose tissue proteins between obesity-susceptible and obese-resistant rats (Joo et al., 2011). In farm animals, few information are available, among which, the demonstration of the influence of maternal diets on adipose tissue proteome in newborn pigs (Sarr et al., 2010).

The results demonstrated that at least 30 proteins were differentially expressed as a consequence of different diets administered to the mothers, belonging to different families, including immune related proteins, fatty acid related metabolism and oxidative stress.

HEADING TOWARD NEW HORIZONS: THE FUTURE OF PROTEOMICS

Better biomarkers are urgently needed in both veterinary medicine and animal sciences for diagnosis and prognosis of diseases, and for phenotyping of QTL needed to provide breeds more resilient and resistant to diseases. The

research world is entering a postgenomic era, which provides great opportunities in the pursuit of new biomarkers. Despite its importance in animal health, the application of proteomics in animal and veterinary science is still lagging, if compared with proteomics in humans and mice. There is an evident need for proteomics to be included in future investigation of animal health, welfare and production. It is clear that valuable information on the molecular mechanism of diseases of animals of veterinary interest is being and will be generated in the future as the technology becomes more applicable in studies designed to explore and explain the pathology of veterinary diseases and animal productions. Initial proteomic studies, when applied to novel areas, have tended to focus at first on describing the proteome of a particular tissue or fluid. Then the power of the techniques is recognized, and experiments to compare and quantify protein changes in experimental procedure or in comparison of disease to healthy samples, or to different farming conditions, become more common.

One of the reasons why proteomics has played a limited role in veterinary medicine and diagnostics, and animal sciences, beside the economic one, is the scarce genomic and proteomic data available as compared with rodents and humans. The recent publication of genomes from pig and cow as well as the growing availability of proteomic reference maps of companion animal tissues and biological fluids will probably overcome these technical barriers. The cost of proteomics experiments is decreasing as well. Given these premises, the still-limited number of proteomic maps is expected to increase, providing new opportunities to utilize proteomic information for diagnosis of animal diseases and better identification of animal productive traits. Technological advances in proteomics have expanded the dynamic range of detection for low-abundance proteins, allowing the detection of disease-specific proteins to be used as potential biomarkers in veterinary medicine as well. The ultimate goal should be to develop diagnostic protocols on clinical samples at multiple levels, including transcriptome (RNA) and proteome (proteins).

RIASSUNTO

La proteomica è quell'insieme di tecniche che permettono di studiare il proteoma, ovvero l'intero insieme di proteine che costituiscono un dato organismo o un sistema biologico (cellula/tessuto/organo). Il potenziale applicativo delle tecniche proteomiche nel campo delle scienze zootecniche è enorme. Le tecniche proteomiche sono complementari alle tecniche genomiche, con il valore aggiunto che sono le uniche che permettano la

caratterizzazione delle modificazioni post traduzionali delle proteine, come per esempio quelle che intercorrono nella maturazione del formaggio, oppure nella trasformazione da muscolo a carne.

Nella presente relazione viene presentata in una prima parte una ampia descrizione dello stato dell'arte sulla applicazione delle tecniche proteomiche che vengono comunemente utilizzate nel campo delle scienze zootecniche. La seconda parte è invece focalizzata su alcuni esempi di come le tecniche proteomiche possano essere applicate alle scienze animali. In modo particolare vengono evidenziati i risultati di un esperimento sugli effetti di differenti diete, arricchite di acidi grassi a catena lunga saturi e polinsaturi, sulla modificazione della espressione di proteine presenti nel tessuto adiposo della capra da latte.

Vengono inoltre presentati alcuni i risultati ottenuti applicando tecniche proteomiche alla caratterizzazione delle modificazioni post-traduzionali (glicosilazioni e fosforilazioni) di proteine coinvolte nella immunità innata del bovino e nella capra.

ABSTRACT

Proteomics allows the study of proteins present in a given tissue or fluid (the proteome). Proteomics is of significant importance to several scientific areas, including veterinary and animal sciences. Application of proteomics to animal sciences has been limited due to the cost and lack of genomic data from livestock. The present report provide examples of successful applications of proteomics in animal production and health with insights into adipose tissue.

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