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Identification of possible new salivary biomarkers of stress in sheep using a high-resolution quantitative proteomic technique

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Abstract

The aim of this study was to identify biological pathways and proteins differentially expressed in the saliva proteome of sheep after the application of a model of stress, using high-resolution quantitative proteomics. In addition, one of the proteins differently expressed was verified and evaluated as a possible biomarker of stress in this species. Saliva paired samples from eight sheep before and after the application of a model of stress based on shearing were analysed using tandem mass tags (TMT). The TMT analysis allowed for the identification of new stress-related metabolic pathways and revealed 13 proteins differentially expressed between before and after the stress. Six of these proteins pertain to four major metabolic pathways affected, namely: canonical glycolysis, oxygen transport, neural nucleus development, and regulation of actin cytoskeleton reorganization. The rest of proteins were unmapped original proteins such as acyl-coenzyme-A-binding protein; complement C3; alpha-2-macroglobulin isoform-X1; type-II small proline-rich protein; lactoferrin; secretoglobin family-1D-member; and keratin, type-II cytoskeletal 6. Of these proteins, based on its biological significance and specific immunoassay availability, lactoferrin was selected for further validation. The immunoassay intra- and inter-assay coefficients of variation were lower than 13%. The method showed good linearity under dilution and recovery, and the detection limit was low enough to detect salivary lactoferrin levels. A significant decrease ($P < 0.01$) in salivary lactoferrin concentration in the sheep following the application of the model of stress was observed, suggesting that this protein could be a potential salivary biomarker of stress situations in sheep.

Keywords: Ovine, Lactoferrin, Saliva, Stress, TMT analysis
1. Introduction

In recent years, there has been growing interest for the identification of biomarkers in saliva to monitor animal welfare. Saliva sampling is a noninvasive and stress-free procedure and can represent an alternative to blood sampling, which has been demonstrated to be a confounding factor in stress studies of animals (Merlot et al., 2011; Escribano et al., 2013). In addition, the ability to perform multiple saliva procedures without an evident influence on the stress response of animals has been an advantage in trials in which the immediate response of an animal to a stressor is studied (Heintz et al., 2011). Furthermore, saliva collection requires only modest levels of personnel training, facilitating animal monitoring in remote locations (Fuentes et al., 2016).

Proteomic techniques have become widely employed as useful tool for the search of novel biomarkers in biofluids. The comparative quantitative analysis of complex protein samples can be achieved either with two-dimensional gel electrophoresis–based proteomics or gel-free mass spectrometry-based approaches using isobaric tagging options such as isobaric tags for relative and absolute quantification (iTRAQ) and tandem mass tags (TMT). These novel technologies that employ isobaric tags have emerged in the last few years and are becoming widely employed, since they are highly reproducible and sensitive and allow for the relative simultaneous quantification of differentially labelled peptides (Baeumlisberger et al., 2010; Dayon et al., 2011; Giron et al., 2011). In veterinary science, recent investigations using these novel quantitative technologies to detect possible biomarkers of diagnostic and understand the prognosis of diseases in dogs (Martínez-Subiela et al., 2017; Franco-Martínez et al., 2018) have been reported. Also, studies performed in sheep using these techniques to evaluate the alterations in the intestine associated with under weaning stress using jejunum samples (Cui et al., 2018) or to consider age-related changes in the cerebrospinal fluid (Chen et al., 2018) have been published. However, to the best of our knowledge, no reports currently
exist on the use of isobaric tagging technology in saliva samples to detect possible biomarkers of stress in sheep.

Despite this lack of published research, in the last several years, there has been increasing interest in the measurement of saliva biomarkers to evaluate stress in sheep, and analytes such as cortisol, alpha-amylase, and lipase have been assayed to determine their increase in concentration after different stressful stimuli (Fuentes et al., 2016; Mesori et al., 2017; Contreras-Aguilar et al., 2018). The insight in various biological pathways in saliva induced by stress as well the identification of new proteins that can change in stressful situations can help researchers to gain knowledge about the status of salivary secretory proteome components and to identify new possible biomarkers that may be helpful to prevention of the stress situations.

The hypothesis of our study was that the application of TMT proteomic technology in sheep saliva samples could uncover novel pathways and identify new proteins capable of changing in situations of stress that could be considered as novel stress biomarkers. To test this hypothesis, the objectives of the present study were; (1) the identification of the metabolic pathways and proteins differentially represented in the saliva after stress by proteomic approach; (2) the selection of at least one protein, differentially expressed after stressors, and its validation by immunoassay and evaluation as a possible biomarker of stress in sheep.

2. Materials and methods

2.1 Animal and sampling procedures

Details describing sheep model, in which salivary biomarkers of stress were evaluated, was published in a recent paper (Contreras-Aguilar et al., 2018).

Saliva samples of eight Montesina sheep, sub-breed of Manchega, originally from southeast Spain, located at the Education Farm of the University of Murcia (Spain), were
The day of the experiment, the whole flock of 40 sheep was moved to a compound of 26 m² outside in the open air, and the sheep were acclimatized for 30 minutes. A basal saliva sample (baseline or the sample before the application of a stress stimulus) was taken from sheep selected prior to stress induction. The animals were removed from the flock one at a time and the stress stimulus implemented, which consisted of being sheared with a shearing machine for approximately five minutes. The samples collected just after shearing were used in the proteomic study, and the samples were selected on the basis of significant increases of salivary biomarkers (cortisol, alpha-amylase and lipase) showed by Contreras et al. (2018).

Saliva samples were obtained using placing a sponge in the mouth. When sponges were thoroughly moist, they were placed in plastic tubes (Salivette; Sarstedt, Aktiengesellschaft & Co., Nümbrecht, Germany), centrifuged (3,000 g for 10 min, 4°C), and the supernatant was aliquoted and kept at −80°C until analysis. The results of salivary biomarkers from freshly obtained saliva samples were reported in the previous study (Contreras-Aguilar et al., 2018). The aliquots of samples were stored, approximately for a year, at -80°C until proteomic analysis (Internal Disease Clinic, Faculty of Veterinary Medicine, University of Zagreb in Zagreb, Croazia).

2.2 Proteomic study of saliva samples from sheep and Liquid Chromatography Mass Spectrometry (LC–MS/MS)

Two groups were formed in the proteomic study, one containing eight samples obtained prior to the stress induction and another containing eight samples of the same animals obtained after the stress. Saliva proteins were acetone-precipitated (six volumes of ice-cold acetone, overnight), dissolved in 100 mM of triethylammonium bicarbonate (TEAB) (pH 8.5) and protein concentration was obtained by bicinchoninic acid (BCA) assay. A pooled
sample, generated by mixing equal protein quantity of all sixteen samples was employed as an internal standard in all TMT six-plex experiments. For each sample, proteins were submitted to reduction, alkylation and digestion and labelled using six-plex TMT reagents following manufacturer instructions (Thermo Fisher Scientific, Waltham, MA, USA) with some modifications, as reported by Martínez-Subiela et al. (2017). In short, 35 μg of proteins was reduced with 200 mM of 1,4-Dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO, USA), alkylated with 375 mM of iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA) and precipitated with ice-cold acetone (VWR Corp., Radnor, PA, USA) overnight. Saliva samples were then centrifuged and the acetone was eliminated by decantation. Afterward, 50μL of 100 mM TEAB buffer were used to resuspend the pellets and digested with trypsin (Promega Corp., Madison, WI, USA) overnight at 37°C (trypsin-to-protein ratio 1:35, w/w). The reagents of TMT labelling were equilibrated and resuspended with anhydrous acetonitrile LC–MS grade (Thermo Fisher Scientific, Waltham, MA, USA) and added to each saliva sample. For one hour the labelling reaction was incubated (at room temperature) and then 5% hydroxylamine for 15 minutes (Thermo Fisher Scientific, Waltham, MA, USA) was added to each sample for its inactivation. Samples were mixed at same amounts (5 μg) and then were vacuum-dried and kept at -80°C before additional LC–MS/MS analysis. The LC–MS/MS analysis was performed using the Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberley, UK) and the Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as reported by Horvatić et al. (2018). For protein identification and relative quantification Proteome Discoverer (version 2.0., Thermo Fisher Scientific, Waltham, MA, USA) was used for the SEQUEST search against Ovis aries FASTA files downloaded from NCBI database (18/05/2018, 70016 sequences) according to parameters set as follows: precursor and fragment mass tolerances of 10 ppm and 0.02 Da, two trypsin missed cleavage sites, respectively; carbamidomethyl (C),
oxidation (M), fixed peptide modification, deamidation (N,Q) and TMT six-plex (K, peptide N-terminus) dynamic modifications. The false discovery rate (FDR) for peptide identification was calculated using the Percolator algorithm. Proteins with at least two unique peptides and 5% FDR were considered successfully identified. Protein quantification was based on relative intensities of reporter ions representing differentially labelled peptides selected for MS/MS fragmentation. To compare relative quantification data, for each protein between the TMT six-plex experiments, an internal standard was used.

2.3 Validation of lactoferrin in sheep as possible salivary biomarker of stress

2.3.1 Lactoferrin assay

Lactoferrin was measured using a commercial sheep enzyme-linked immunosorbent assay (ELISA) kit specific to lactoferrin (sheep lactotransferrin ELISA kit; Cusabio Biotech., Ltd. Houston, TX, USA), according to manufacturer instructions.

2.3.2 Analytical validation

Sheep saliva samples taken before and after applying the model of stress described above were employed for the validation study of the ELISA assay. The following parameters were evaluated: intra- and inter-assays precision, linearity, recovery and the limit of detection. The intra- and inter-assays precision expressed as coefficients of variation (CVs) were calculated by analysing two pools of saliva samples containing, low and high concentrations of lactoferrin. Each pool was prepared by mixing saliva samples with similar concentrations of lactoferrin (previously quantified by the ELISA method used in our study). Inter-assays CVs were obtained by measuring five time the same pools in different days. Each CV was calculated as the percentage of the standard deviation (SD) of the replicates divided by the
mean. To avoid possible variations due to cycles of thawing and freezing, the saliva samples were aliquots and only was unfrozen the aliquot needed for each assay.

The linearity under dilution was used to evaluate the accuracy of the assay. For it, two sheep saliva samples with high lactoferrin concentrations were serially diluted in varying concentrations (e.g., 1:2, 1:4, 1:8, 1:16, 1:32) with an assay buffer. Afterwards, linear regression between the observed and expected results was performed and the slope, y-intercept, and coefficients of determination ($R^2$) were calculated.

The detection limit, defined as lowest concentration of lactoferrin that assay can distinguished from zero value, was calculated based on mean value of 10 replicate determination of the assay buffer (zero standard) plus three SDs.

2.3.3 Evaluation of lactoferrin changes after stress

The ability of the ELISA assays to distinguish between the stress levels shown by the sheep was investigated by comparing saliva samples from 14 sheep before and after the application of the stress model by shearing for five minutes as previously described for the proteomic study. The samples analysed were from the 8 sheep using the first part related to proteomic approach and from 6 additional sheep. 8 sheep were the same used in the first part related to proteomic approach and another 6 sheep more until completely a total of 14 used before by Contreras-Aguilar et al. (2018).

2.4 Gene ontology pathways

The proteomic results obtained in the study were employed for the gene ontology (GO) analysis. The proteins, encoding in ovine genes, differentially expressed were transformed to their human arthologs employing the Ensembl orthologs database and its tool for data mining BioMart (http://www.ensembl.org/index.html). Obtained genes were used to
add their best known interactors (maximum of 10 per genes) according to the databases STRING-EMBL, IntAct and Reactome by the utilization of the Cytoscape (v3.6.1) plug-in CluePedia (v1.5.2) (Shannon et al., 2003; Bindea et al., 2013). Original proteins (differentially expressed between the two grouping conditions of data, data obtained before vs. data obtained after stress) and enriched proteins (best interactors of the original proteins) were then used to determine the GO terms over-represented in this set of proteins by way of the utilization of the Cytoscape plug-in ClueGO (v2.5.0) (Bindea et al., 2009) on the Homo sapiens GO-biological process (14/08/2018) (GO level from 3 to 8, minimum number of genes = 3, minimum percentage = 4, Kappa score threshold = 0.4, two-sided hypergeometric test with Bonferroni correction). For the REVIGO analysis the GO terms over-represented were submitted to remove redundant GO terms, the similarity allowed was 0.7 SimRel, and their functional description defined the groups related GO terms (Supek et al., 2011). Finally, pathway interactomes were designed in Cytoscape using the radial layout incorporating the GO data generated by ClueGO and ReviGO (e.g., the number of input genes in GO terms, associated p-value, GO/proteins relationships, GO groups).

2.5 Statistical analysis

In order to compare the abundances of proteins identified in the proteomic analysis between two groups of samples (group of samples before versus group of samples after stress stimulus) data, by logarithmic transformation, were normalized and Student’s t-test (two-tailed, paired) was used. A $P < 0.05$ value was considered to be significant. Fold changes (FC) have been obtained with the formula $\text{FC} = \log_2 \left( \frac{\text{Group before}}{\text{Group after}} \right)$ as has been reported before (Franco-Martínez et al., 2018). RStudio (v1.0.143) (R Studio Team. RStudio “RStudio Team. RStudio: Integrated Development Environment for R [Internet]. Boston,
Intra- and interassay CVs of assay were calculated as SD/mean value of repeated measurements and expressed as percentage (multiplied by 100). Detection limits, linearity under dilution, linear regression analyses also were performed with Rstudio. Because of the small sample size, the changes of lactoferrin in sheep between groups for clinical analysis, before and after of stress stimulus, were assessed by a non-parametric Wilcoxon matched-pairs test using a commercial statistics package (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA). A $P < 0.05$ value was considered significant.

3. Results

3.1 Proteomic changes of saliva samples from sheep following the model application

A total of 445 proteins were identified, 35 of which showed significant changes in relation to baseline or to their levels prior to the model application. These proteins appear in Table 1. Five of them showed a decrease after model application, namely: lactoferrin ($P = 0.015$); alpha-2-macroglobulin isoform X1 ($P = 0.034$); keratin, type II cytoskeletal 6A ($P = 0.039$); complement C3 (CC3) ($P = 0.042$); and secretoglobin family 1D member (SCGB1D2) ($P = 0.049$). Conversely, eight showed an increase after model application, namely: triosephosphate isomerase isoform X2 ($P = 0.015$), 14-3-3 protein gamma ($P = 0.039$), phosphoglycerate mutase 1 isoform ($P = 0.015$), acyl-coenzyme-A (CoA)-binding protein ($P = 0.015$), hemoglobin subunit beta isoform X2 (HBB) ($P = 0.023$), 14-3-3 protein zeta/delta isoform X1 ($P = 0.041$), type II small proline-rich protein ($P = 0.039$), and calmodulin-alpha ($P = 0.039$).

3.2 Results of validation of lactoferrin in sheep as possible salivary biomarker of stress
Analytical validation: The intra-assay CVs were 7.1% for the pool with a high lactoferrin concentration and 8.5% for the pool with a low lactoferrin concentration. Additionally, inter-assay CVs were 13% for the pool with a high lactoferrin concentration and 11% for the pool with a low lactoferrin concentration, respectively. A linear regression coefficient of 0.96 was observed when saliva samples with high concentrations diluted 1:2-fold or greater were analyzed. The analytical limit of detection calculated was 0.15 µg/mL.

Differences between lactoferrin levels obtained before and after of the application of the stress model are presented in Figure 1. Sheep showed a significant decrease (P < 0.01) of salivary lactoferrin levels after shearing (median: 0.24 µg/mL; range: 0.21–0.26 µg/mL; 25-75th percentiles) in comparison with the levels observed prior to shearing (median: 0.33 µg/mL; range: 0.27–0.37 µg/mL; 25-75th percentiles).

3.3 Bioinformatics
Although 13 proteins representing unique genes were identified as being differentially expressed between before and after the stress, 38 further proteins were added in the enrichment step in GO analysis. From the GO analysis, four GO groups were defined as follow: canonical glycolysis (11 genes, -log10 P = 18.3), oxygen transport (five genes, -log10 P = 7.6), regulation of actin cytoskeleton reorganization (five genes, -log10 P = 5.7), and neural nucleus development (four genes, -log10 P = 3.2) (Figure 2). Representation of GO terms shows that canonical glycolysis is central and the most relevant pathway in the study (Figure 3). Proteins belonging to the guanine nucleotide exchange factor (RAPGEF3/4), optineurin (OPTN) and enolase-3 (ENO3) were at the crossroads between the most important GO terms despite the fact that they were not identified as differentially expressed in the current proteomic study.
In this study was used for the first time the TMT technology for protein quantification is saliva of sheep. In addition, the main proteins differentially represented in this type of sample and related major metabolic pathways were identified following the application of a model of acute stress. GO analysis showed that four major metabolic pathways were found to be significantly affected in sheep saliva after the stress of shearing, namely: canonical glycolysis, oxygen transport, neural nucleus development, and regulation of actin cytoskeleton reorganization. In addition, thanks to use of this high-resolution quantitative proteomic technique, 13 proteins never before described in the saliva of sheep were observed to change after the experimental stress condition. Six of the proteins identified by TMT technology were related with the metabolic pathways. Phosphoglycerate mutase 1 isoform X1 (PGAM1) and triosephosphate isomerase isoform X2 (TPI1) were found to be upregulated in the canonical glycolysis. PGAM1 catalyses the transfer of phosphate groups from 3-phosphoglycerate to 2-phosphoglycerate, while the TPI1 genes encodes two proteins or enzymes, which catalyses in glycolysis the isomerization of glyceraldehydes 3-phosphate (G3P) and, in the gluconeogenesis, the dihydroxy-acetone phosphate (DHAP). Both proteins, as a part of glycolytic processes, and catalyse the breakdown of a carbohydrate into pyruvate, with the concomitant production of energy (Hitosugi et al., 2012). Therefore, these significant increases in concentration of the above mentioned enzymes might lead to hyperglycaemia in sheep. The hyperglycaemia represents an immediate response to stress in any species, providing rapid energy source to meet the energy requirements of fear and the “fight-or-flight” induced response (Surwit et al., 1992).

Another of the metabolic pathways identified in GO analysis of our study was that for oxygen transport represented by HBB, which was significantly upregulated after the stress of shearing. This protein is involved in transporting oxygen from the lungs to various peripheral
tissues. Therefore, HBB is a protein that could be involved in the “fight-or-flight”-induced response in sheep in a manner intended to improve oxygen delivery and could also be related to the stress response. This increase of expression of hemoglobin genes has been reported before in mice subjected to acute social stress stimulus (Stankiewicz et al., 2014).

Calmodulin-alpha (CaM), which is related with the metabolic pathway of neural nucleus development, was also found to be upregulated in saliva after the stress. CaM is a protein to Ca2+-sensing that is important in many cellular processes (Stevens, 1983) and which regulates a myriad of target proteins, such as G-protein-coupled receptor, ion channels, and kinases. As an example, in the Ca2+-signalling pathway, CaM regulates and activates the calcineurin and myosin light chain kinase IV (Kishi et al., 2018). Therefore, CaM plays part in regulating different biological processes including proliferation, learning and metabolism (Westerlund and Delemotte, 2018). In addition, calcium has an important role in the effects of stress hormones (Malinovská et al., 1991). Regarding actin cytoskeleton reorganization, two upregulated proteins were identified, namely: the 14-3-3 protein gamma (YWHAG) and the 14-3-3 protein zeta/delta isoform X1 (YWHAZ). The 14-3-3 proteins mediate interactions between proteins involved in cell-cycle regulation and signal transduction, being important for protein–protein interactions (Dubois et al., 1997). Therefore, these are adapter proteins implicated in the regulation of a large spectrum of both specialized and general signalling pathways. Stress can be defined as a state of threat to homeostasis (Chrousos and Gold, 1992), and the stress response requires an appropriate coordination of multiple signalling pathways to return to homeostasis. Therefore, these proteins could be related to the stress response, although further studies should be performed to clarify their role.

The rest of the proteins TMT quantified affect by the stress of shearing were unmapped in GO terms. The acyl-CoA-binding protein (DBI) and the type II small proline-rich protein (SPRR2E) were upregulated, whereas CC3; A2M; SCGB1D2; Keratin, Type II
Cytoskeletal 6 (KRTL1); and lactoferrin were downregulated in saliva after the stress response.

DBI can act as an intracellular carrier, binding with high affinity to long- and medium-chain acyl-CoA esters. Furthermore, DBI is able to displace diazepam from its recognition site located on the acid gamma-aminobutyric (GABA) type A receptor. Therefore, it is possible that DBI modulates the function of the GABA receptor, acting also as a neuropeptide. (van Aalten et al., 2001). Acute stressors change the release of different neurotransmitters, such as acetylcholine or GABA in limbic areas of the brain (e.g., hippocampus, prefrontal cortex, nucleus accumbens, and amygdala), which are functionally interconnected, leading to the modulation and promotion of behavioral processes to cope with the stressor (Mora et al., 2012). Notably, in the present investigation, the SPRR2E cross-linked envelope protein of keratinocytes. The encoded protein, along with other family members, is a component of the cornified cell envelope that forms beneath the plasma membrane in terminally differentiated stratified squamous epithelia. This envelope serves as a barrier against extracellular and environmental factors. In humans, it has been shown to have a relationship with problems in skin such as psoriasis (Kainu et al., 2008). In our study, this response could be related with the stress caused by shearing, which is also skin-related, although further studies should be made to clarify this fact.

The downregulated proteins quantified by TMT in our study were associated with different acute reactions. CC3 played a central role in the activation of the complement system in both alternative and classical complement pathways. Derived from the proteolytic degradation of CC3, CC3a anaphylatoxin is a mediator of local inflammatory processes and acts as a chemoattractant for neutrophils (Ricklin et al., 2016). In addition, it induces the contraction of smooth muscle and improves vascular permeability (Ricklin et al., 2016), increasing the cardiac frequency and vascular permeability needed for an acute stress
response. The A2M is a protein that is responsible for the neutralization of proteolytic enzymes and, in humans, it is considered to be a hyperoxia biomarker (Bhattacharya et al., 2014). The SCGB1D2 proteins are widely expressed in endocrine-responsive organs. This protein may bind androgens and other steroids and may be under transcriptional regulation of steroid hormones (Jackson et al., 2011). The gen KRTL1 encoded a protein that belongs of the keratin gene family. These proteins, the type II cytokeratins, interfere during differentiation of simple and stratified epithelial tissues. In general, further studies should be performed to clarify the role of these downregulated proteins in the stress reactions.

Finally, lactoferrin, this secretory glycoprotein is found in body fluids produced by exocrine glands such as the mammary, salivary, and lacrimal (Lodernal and Iers, 1995; Hayashi et al., 2017) and also in neutrophil granules of mammals (Wakabayashi et al., 2006). Numerous physiological roles have been associated to this protein, including in the protection against microbial infection, regulation of iron metabolism, and management of immune functions but also has anti-viral, anti-cancer, antioxidant, and anti-inflammatory activities (Wakabayashi et al., 2006; Hao et al., 2019). In relation to role of the lactoferrin in the ovine physiology has been evaluated previously in several studies, fundamentally, evaluating the effects of the lactoferrin bovine milk administration. For example, Wong et al. (1996 and 1998) showed that bovine milk contains several proteins and peptides having immunomodulatory and/or therapeutic potential effect in sheep. In a recent article, El-Ashker et al. (2018) suggested that, supplemented with Lactobacillus sp., is a possible nutritional supplements to support the immune system in healthy lambs. Due to its role in the protection against microbial infection or its anti-viral activities, it also has been administrated as prebiotic to prevent colonization and excretion Escherichia Coli (Yekta et al., 2011) or against the ovine pulmonary adenomatosis (Sozmen and Beytut, 2012).
In relation to stress studies, its administration after the application of different stressors has been associated with an analgesic effect in rodents (Takeuchi et al., 2003; Zimecki et al., 2005; Tsuchiya et al., 2006). Notably, it seems to exert anxiolytic and analgesic effects that are accompanied by an increase in nitric oxide production or the activation of the μ-opioid system. Due to its possible attenuation effect in the impact against stress stimulus, it has also been used in studies as a prebiotic for stress resistance in rodents (Mika et al., 2016; Peña-Juárez et al., 2016). Maekawa et al. (2017) reported that the lactoferrin ameliorates corticosterone-related acute stress and hyperglycemia in rats, and its effect, which could be attributed to the activation of the HPA axis. In a recent report performed in human, Shinjo et al. (2018) indicated the possible application of lactoferrin in managing psychological stress being able to exert a suppression effect on the changed in parasympathetic and sympathetic activities evoked by the calculation task. Therefore, the pathway of actuation in stress response it could be at both levels (HPA and autonomic nervous system (SNA)). In any way, lactoferrin appears have an important role in stress response acting as an adaptogen during stress (Aleshina et al., 2016).

However, to our knowledge, no studies have been performed in this specie where has been evaluated lactoferrin as stress biomarker, furthermore, few studies about this fact there have being published in veterinary medicine. Kim et al. (2011) found a significant decrease of lactoferrin in the blood levels of calves after stress due to weaning (around of 30% of its levels 315 to 216 μg/mL after 1 day post-weaning). In addition, in the only study performed on saliva in veterinary species, Huang et al. (2017) showed a decrease in saliva lactoferrin expression of pigs after restraint stress stimulus, although the authors did not observe significant changes (P = 0.06 after 30 min of immobilization, measured as fold change in relation to baseline by western blotting). Due to the facts that lactoferrin is one of the most differently expressed proteins after stress, that it has demonstrated stress-related changes in
several other species, and that it can be measured by use of a commercially available immunoassay in sheep, this protein was selected for the validation study. Our analytical results showed that immunoassay used is suitable for the application in sheep saliva samples with an excellent precision, accuracy, and sensitivity. The immunoassay enabled the detection of a significant stress-induced decrease in lactoferrin levels, around of 27% (0.33 to 0.24 µg/mL post stress). In addition, all sheep showed a decrease in lactoferrin concentrations in relation to the levels before the stress was applied, showing hardly any overlap.

In conclusion, four new metabolic pathways and 13 proteins differentially represented in the saliva of sheep after an application of acute stress using TMT quantitative proteomic technique are reported in this study. The new metabolic pathways found include canonical glycolysis, oxygen transport, neural nucleus development, and regulation of actin cytoskeleton reorganization. The new stress-related proteins identified in our study with this high-resolution quantitative proteomic technique could have the potential to be novel biomarkers of diagnostic or prevention of stress situations, specially lactoferrin, which can be measured by use of a commercially available immunoassay and which decreased in the sheep saliva following the application of the stressful stimulus used in our experiment.

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**Declaration of interest**

The authors have declared that no competing interests exist.

**Ethics statement**

In this experiment only were used samples collected before in article Contreras et al., (2018) in accordance with the ethical standards of the Bioethical Commission of Murcia University (CEEA 431/2018).

**Software and data repository resources**

Our data are not deposited in an official repository.

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Statistically significant expression changes of proteins in saliva of sheep with after model of sympathetic activation (differentially expressed between the two grouping conditions of data, data obtained of before vs. data obtained after stress). Ordained based on P value of Paired t-test.

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**Figure legends**

Fig.1. Lactoferrin concentrations in sheep (n = 14) before and after the application of stress model base on shearing. The plot show median (line within box), 25th and 75th percentiles (box) and 10th and 90th percentiles (whiskers). Asterisks indicated significant differences between groups: ***$P < 0.01$.

Fig.2. GO terms over-represented in the pool of significantly expressed saliva proteins before and after stimulus. GO terms are ordered by the number of significant genes/proteins of the study associated with them according to Gene Ontology database (first y-axis). $-\log_{10}$ of p-value for each GO term is represented on the second y-axis. GO terms which define a group of similar GO terms (determined by ReviGO) are in bold.

Fig.3. Network representation of GO terms over-represented in the pool of significant proteins and associated proteins. GO terms are represented by octagon shape, proteins by diamond shape. Proteins are in red with a black border for proteins identified in the experiments, or with a white border for proteins added by the enrichment step. GO nodes are filled by colours corresponding to their GO group (determined by ReviGO). Name of GO terms defining 1 group are in bold. When a protein belongs to 1 GO term, a link is figured between the nodes of protein and GO term. The network representation has been realized under Cytoscape using the radial layout. Acronyms gene and related full name protein of each protein that appears in the figure (Datas obtained of UniProt data base; (http://www.uniprot.org): 1) Mapped original proteins: CALM2 (Calmodulin-2); HBB (Hemoglobin subunit beta); PGAM1 (Phosphoglycerate mutase 1); TPI1 (Triosephosphate isomerase), YWHAG (14-3-3 protein gamma) and YWHAZ (14-3-3 protein zeta/delta). 2) Mapped enriched proteins: ACTRI1A
(Alpha-centractin); ALDOA (Fructose-bisphosphate aldolase A); ALDO B (Fructose-bisphosphate aldolase B); ALDO C (Fructose-bisphosphate aldolase C); CLU (Clusterin); ENO1 (Alpha-enolase); ENO2 (Gamma-enolase); ENO3 (Beta-enolase); GAPDH (Glyceraldehyde-3-phosphate dehydrogenase); GAPDHS (Glyceraldehyde-3-phosphate dehydrogenase, testis-specific); HBA1 (Hemoglobin subunit alpha-1); HBA2 (Hemoglobin subunit alpha-2); HBD (Hemoglobin subunit delta); HBE1 (Hemoglobin subunit epsilon); OPTN (Optineurin); PGK1 (PGK1); RAB10 (Ras-related protein Rab-10); RAPGEF3 (Rap guanine nucleotide exchange factor 3); RAPGEF4 (Rap guanine nucleotide exchange factor 4) and YWHAQ (14-3-3 protein theta). 3) Unmapped original proteins: A2M (Alpha-2-macroglobulin); C3 (Complement C3); DBI (Acyl CoA-binding protein); KRT6B (Keratin, type II cytoskeletal 6B); LTF (Lactoferrin); SCGB1D2 (Secretoglobin family 1D member 2) and SPRR2E (Small proline rich protein 2E). 4) Unmapped enriched proteins: A1BG (Alpha-1B-glycoprotein); AKAP9 (A-kinase anchor protein 9); APCS (Serum amyloid P-component); APP (Amyloid precursor protein); CDKN1B (Cyclin-dependent kinase inhibitor 1B); CXCL13 (C-X-C motif chemokine 13); CXCR6 (C-X-C chemokine receptor type 6); FAM3C (Protein FAM3C); GNB5 (Guanine nucleotide-binding protein subunit beta-5); HSPG2 (Basement membrane-specific heparan sulfate proteoglycan core protein); INSL5 (Insulin-like peptide INSL5); MAFF (Transcription factor MafF); MAGED2 (Melanoma-associated antigen D2); NMUR1 (Neuromedin-U receptor 1); PGK2 (Phosphoglycerate kinase 2); TUBB4A (Tubulin beta-4A chain) and VTI1B (Vesicle transport through interaction with t-SNAREs homolog 1B).
Figure 1

[Diagram showing the effect of stress on salivary lactoferrin concentration. The x-axis represents sampling time (Before stress and After stress), and the y-axis represents the concentration of lactoferrin (μg/mL). The graph shows a significant difference (***).]
Figure 2

[Graph showing GO terms with related biological processes and their counts.]