



Changes in saliva proteins in two conditions of compromised welfare in pigs: An experimental induced stress by nose snaring and lameness

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ABSTRACT

The aim of this study was to identify biological pathways and proteins differentially expressed in saliva of pigs in two conditions of compromised welfare: an acute stress consisting of restraint with a nose snare and in pigs with lameness which is a highly frequent problem in the swine industry. For this purpose, high-resolution quantitative proteomics based on Tandem Mass Tags labelling was used. Four proteins showed significant differences in the conditions of compromised welfare, namely cornulin, the heat shock protein 27 and lactate dehydrogenase (LDH), that showed significant increases, whereas immunoglobulin J chain showed a significant decrease. LDH, which was the protein that showed the highest differences, was selected for validation and clinical evaluation as a diagnostic biomarker. Significant changes in this protein were observed between pigs restrained with a nose snare and pigs with lameness compared with healthy pigs when measured with available commercial assays in a larger population of pigs. In conclusion, this study reports that in situations of compromised welfare on farm, such as acute stress and lameness in pigs, there are changes in proteins and metabolic pathways in saliva, and describes a series of proteins that could potentially be used as biomarkers for both short term acute stress and longer term chronic stress of lameness. These biomarkers would have the advantage of being measured in saliva by a noninvasive and not stressful collection sampling procedure.

1. Introduction

Saliva is a useful fluid to study stress and welfare in pigs (Escribano et al., 2014; Martínez-Miró et al., 2016). It can be collected in a non-invasive way, with the minimum discomfort for the animal, allowing successive sampling without being a confounding factor in welfare studies (Merlot et al., 2011). In addition, saliva can be obtained by personnel after a limited period of training, facilitating sampling on remote farms (Fuentes-Rubio et al., 2016; Martínez-Miró et al., 2016).

In terms of research and diagnostic potential, proteomic studies of saliva have been shown to be an emerging source of new biomarkers (Lamy and Mau, 2012). Novel gel-free mass spectrometry-based proteomic approaches using isobaric tagging options, such as tandem mass

tags (TMT) or isobaric tags for relative and absolute quantification (iTRAQ), are highly sensitive especially when combined with high-resolution MS analysis, and allow simultaneous quantification of differentially labelled peptides (Baeumlisberger et al., 2010; Dayon et al., 2011; Giron et al., 2011). A recent study by Prims et al. (2019) applied the novel gel-free free iTRAQ-based proteomic technologies for the characterization of different salivary gland proteomes in pigs. However, to the authors' knowledge, no studies have been conducted addressing gel-free proteomic technologies applied to whole porcine saliva. Furthermore, this technology has not yet been applied to study possible proteins changes in pig saliva in situations of stress or compromised welfare.

The hypothesis of our study was that high-resolution quantitative

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gel-free proteomic technology could identify proteins and metabolic pathways in saliva of pigs affected by stress or in situations where the welfare of the pigs is compromised. Therefore, our objective was to evaluate the salivary proteome in healthy pigs by gel-free mass spectrometry-based proteomic approach using TMT to explore the possible changes in salivary proteome in two different situations: (1) after an acute stress consisting of restraint with a nose snare, which has been demonstrated as stressor in an experimental model of stress (Escribano et al., 2013, 2014), and (2) in pigs with lameness, which is a highly frequent problem in the swine industry (Anil et al., 2005) and is used as an animal-based welfare measure for commercially farmed pigs (Pandolfi et al., 2017). An additional objective was to determine if a protein, differentially expressed in both situations compared with the healthy pigs, could be validated as a potential diagnostic biomarker of stress or welfare.

2. Materials and methods

2.1. Animal and sampling procedures

All animals used in this study were male crossbred pigs ([*Sus scrofa domestica*] Duroc X [Landrace X Large White]) with the same age (in the mid-fattening period; age = 104.8 ± 10.0 days, mean body weight = 78.3 ± 6.3 kg) and grown in a high health farm, the Higher Education Farm of the University of Murcia (Spain). All animals were vaccinated against *Mycoplasma hyopneumoniae* (Stellamune Mycoplasma, inactivated *Mycoplasma hyopneumoniae* NL 1042, Pfizer Animal Health, Madrid, Spain) and Porcine circovirus type 2 (Porcilis® PCV, MSD Animal Health, Boxmeer, The Netherlands). On this farm, the pigs had ad libitum access to a nutritionally balanced diet (commercial dry diets based in a corn-soybean meal with 15.5% of crude protein -CP-; 0.79% Ileal digestible Lys, %; and 13.5 MJ of metabolizable energy per kg -ME/kg-) and water ad libitum under general commercial housing and husbandry conditions conforming to the European Union Guidelines (Directive 2010/63/EU1). Each pen had an area of 1.139m² per animal, being in concordance to the legislation (Council Directive 2001/88/CE of 23 October 2001 amending Directive 91/630/CEE concerning minimum standards for the protection of pigs). The temperature in the pens was kept between a minimum of 18 °C and a maximum of 23 °C.

Saliva samples were obtained by placing a sponge, made of polyurethane, in the mouth of each pig. When sponges were thoroughly moist, they were placed in plastic tubes (Salivette; Sarstedt, Aktiengesellschaft & Co., Nümbrecht, Germany), centrifuged (3000 g for 10 min, 4 °C), and the supernatant was aliquoted for analysis. Aliquots of samples were stored at -80 °C until proteomic analysis (VetMedZg, Internal Disease Clinic, Faculty of Veterinary Medicine, University of Zagreb in Zagreb, Croatia) and biochemical analysis (Interdisciplinary Laboratory of Clinical Analysis Interlab-UMU, Regional Campus of International Excellence 'Campus Mare Nostrum', University of Murcia).

2.2. Pigs with acute stress induced by restraint with a nose snare

The experimentally induced stress consisted in restraining each pig for 1 min with a nose snare as previously described (Tecles et al., 2017). This is a common veterinary practice used for the immobilization of animals in pig farms and has been demonstrated to produce an increase in salivary biomarkers of stress, such as cortisol or chromogranin A (Escribano et al., 2013, 2014).

This experimental procedure was performed at different time points according to the experimental purpose: (1) for the proteomic study, saliva samples were collected from 6 male pigs before the application of the acute stress stimulus (control group) and further saliva samples were collected from the same animals after 15 min (T15) of the application of the nose snare as stress stimulant; and (2) for the validation

study the saliva samples of 16 male pigs, with the same age and from the same farm as for the proteomic study were sampled before and at T15 after the same stress stimulus by nose snare. All animals were subjected to a clinical examination prior to and throughout the study, and no clinical signs of disease were detected in any animal.

2.3. Pigs with lameness

The presence of lameness in animals was determined based on the observation of the animals according to the score system published by Main et al. (2000). The lameness score is based on behavioural observation, standing posture and gait of pigs while they were undisturbed and during exit into an unfamiliar environment, walking on the aisle. A score from 0 (no abnormality in posture, gait, or behaviour) to 5 (severely lame pig, incapable of standing) was given to each pig. According to scoring system, an animal was considered lame when achieved a score ≥ 1 .

This experimental procedure was performed at different time points according to the experimental purpose: (1) for the proteomic study, the saliva of five male pigs with lameness (one animal with score 2, one with score 3, one with score 4 and two with score 5) was analysed and compared with the saliva of six healthy animals that were those used in the stress model experiment before the application of stimulus (control group) and; (2) for the validation study, saliva from 15 male healthy pigs and 15 male pigs with lameness (five animals with score 2, six with score 3, two with score 4 and two with score 5) was analysed.

According to Spanish National Law for Animal protection (1135/2002), all animals with lameness were located apart from the healthy pigs in a hospital room in pens with the same dimensions and conditions as for healthy animals.

2.4. Proteomic study of saliva samples from pigs using Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS)

Six samples obtained prior to the stress induction (pre-stress or control pigs), six samples of the same animals obtained 15 min after the stress (post-stress) and five samples from pigs with lameness were analysed by LC-MS/MS. 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 seventeen samples, was employed as an internal standard in all TMT sixplex experiments.

For each sample, proteins were submitted to reduction, alkylation and digestion and labelled using TMT sixplex reagents following the manufacturer instructions (Thermo Fisher Scientific, Waltham, MA, USA) with some modifications, as reported before (Martinez-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, 50 µL 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 for TMT labelling were equilibrated and resuspended with anhydrous acetonitrile (LC-MS grade, Thermo Fisher Scientific, Waltham, MA, USA) and added to each sample. The labelling reaction was incubated (at room temperature) for one hour, following by incubation with 5% hydroxylamine for 15 min (Thermo Fisher Scientific, Waltham, MA, USA). Samples were mixed in the same amounts and the aliquots (5 µg) were vacuum-dried and kept at -80 °C for further LC-MS/MS analysis. The LC-MS/MS analysis was performed using the Dionex Ultimate 3000 RSLC nano flow system (Dionex, Camberley, UK) and the Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as reported before

(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 *Sus scrofa* FASTA files downloaded from NCBI database (19/07/2018) 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 sixplex experiments, the pool of all samples was used as the internal standard.

2.5. Validation of lactate dehydrogenase as possible salivary biomarker of welfare in pigs

2.5.1. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) concentration was measured in saliva for validation of proteomic results using a colorimetric commercial kit (Lactate Dehydrogenase (LDH), BioSystems S.A. Costa Brava, 30. 08030, Barcelona, Spain) in an automatic analyser for biochemical assay (Olympus UA600, Olympus Diagnostica GmbH).

2.5.2. Analytical validation

Pigs saliva samples taken in conditions of pre-stress (control pigs), post-stress and pigs with lameness were employed for the validation study of the LDH 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 saliva pools containing low and high concentrations of LDH. Each pool was prepared by mixing saliva samples with similar concentrations of LDH (previously quantified by the method used in our study). Intra-assays CVs were calculated by measuring five times the each pool in the same analytical serie while inter-assays CVs were obtained by measuring five times 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 aliquoted and only the aliquot needed for each assay was thawed.

The linearity under dilution was used to evaluate the accuracy of the assay. For this, two saliva samples with high LDH concentrations were serially diluted in varying concentrations (e.g., 1:2, 1:4, 1:8, 1:16, 1:32) with the 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 LDH that the assay can distinguish from zero value, was calculated based on the mean value of 10 replicate determinations of the assay buffer (zero standard) plus three SDs.

2.5.3. Evaluation of salivary changes in LDH after acute stress (nose snaring) model and in lameness

The ability of the automatic assay to distinguish changes in stress levels shown by the pigs was investigated by comparing saliva samples from 16 male pigs before (pre-stress or baseline) and at T15 after stress stimulus based on nose snaring (described in Section 2.2). In relation to lameness, the activity of LDH in saliva of 15 male pigs with this disease (five animals with score 2, six with score 3, two with score 4 and two with score 5) was compared with the activity of LDH in saliva of 15 male healthy or control pigs.

2.6. Statistical analysis

In order to compare the abundances of proteins identified in the proteomic analysis between three groups of samples (proteins pre-stress (control pigs), post-stress and pigs with lameness) data were normalized by logarithmic transformation and Student's *t*-test (two-tailed, unpaired) was used to determine statistical significance between groups. A $P < .05$ value was considered to be significant. RStudio (v1.0.143) (R Studio Team. RStudio "RStudio Team. RStudio: Integrated Development Environment for R [Internet]. Boston, MA: RStudio, Inc.; 2015. Available from: <http://www.rstudio.com/>," 2015) was employed for statistics.

CVs detection limits, linearity under dilution, linear regression analyses also were performed with Rstudio. The changes between groups were assessed by a non-parametric Wilcoxon matched-pairs test in the case of the model of stress (before vs. after), and a non-parametric Mann-Whitney unpaired test in the case of animals with lameness (Healthy vs. Lameness). A $P < .05$ value was considered significant. Data analyses for clinical analysis were performed using a commercial statistics package (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA).

2.7. Gene ontology pathways

The proteomic results obtained in the study were used for the gene ontology (GO) analysis. The proteins differentially expressed, encoding in porcine genes, were transformed to their human orthologs by 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 three grouping conditions of data, namely, data obtained pre-stress or healthy vs. data obtained post-stress and vs. data obtained with pigs with lameness) 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 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). By utilizing the REVIGO analysis, the over-represented redundant GO terms were removed. 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).

3. Results

3.1. Proteomic changes

A total of 353 proteins were identified. Forty-four of them showed significant differences for both the acute stress of snaring and the situation of lameness. These proteins are represented in Table 1. Three proteins showed significant increases for both comparisons, namely: cornulin (Control vs. Post-stressed $P = .016$; Control vs. Lameness $P = .013$); Heat shock protein beta-1 or 27 kDa (Control vs. Post-stressed $P = .010$; Control vs. Lameness $P = .010$) and L-lactate dehydrogenase A (Control vs. Post-stressed $P = .008$; Control vs. Lameness $P = .001$). Conversely, Immunoglobulin J chain showed a decrease in these two conditions (Control vs. Post-stressed $P = .020$; Control vs. Lameness $P = .0003$).

The following proteins showed significant differences between

Table 1

Proteins with significant differences between groups identified using TMT approach. Asterisks indicate significant post-hoc difference: * $P < .05$; ** $P < .01$; *** $P < .001$. Up (↑) or down (↓) arrow indicates the change in relation to mean \pm standard deviation (SD) of control group.

Protein identification (accession number)	Mean \pm SD Control	Mean \pm SD Post-stressed (PostS)	Mean \pm SD Disease (lameness; L)	Differences between groups
BPI fold-containing family A member 1 isoform X1 (545880122)	1.86 \pm 1.20	1.09 \pm 0.33 ↓	0.81 \pm 0.16↓	L-C**
Calcium-activated chloride channel regulator 1 (75051712)	1.27 \pm 0.23	1.08 \pm 0.14↓	0.99 \pm 0.19↓	L-C*
Calmodulin-like protein 5 (545854020)	0.83 \pm 0.16	0.94 \pm 0.28↑	1.34 \pm 0.11↑	L-C**
CD5 antigen-like precursor (343478265) and isoform X1 (545822519)	0.74 \pm 0.22	0.84 \pm 0.19↑	1.35 \pm 0.29↑	L-C***
Cornulin (227558992) and isoform X1 (927122136)	0.88 \pm 0.18	1.17 \pm 0.21↑	1.20 \pm 0.19↑	C-PostS* L-C*
Cornifin (329299061)	0.68 \pm 0.14	1.30 \pm 0.86↑	0.93 \pm 0.23↑	C-PostS*
Fibronectin (1191840822)	0.78 \pm 0.21	0.90 \pm 0.31↑	1.21 \pm 0.32↑	L-C*
Fructose-bisphosphate aldolase A isoform X1 (1191869592); X2 (1191869594) and X4 (1191869604)	0.82 \pm 0.28	0.94 \pm 0.07↑	1.10 \pm 0.12↑	L-C*
Heat shock cognate 71 kDa protein (345441750)	0.86 \pm 0.19	1.17 \pm 0.16↑	1.12 \pm 0.17↑	C-PostS*
Heat shock protein beta-1 (55926209) and Heat shock protein 27 kDa, partial (50916342)	1.01 \pm 0.16	1.12 \pm 0.15↑	1.25 \pm 0.10↑	C-PostS** L-C**
Hemoglobin Chain D (aquo Met) (beta Chain) (809285)	0.49 \pm 0.06	0.51 \pm 0.05↑	1.52 \pm 0.92↑	L-C*
Hemoglobin subunit alpha (1191866723)	0.38 \pm 0.15	0.45 \pm 0.15↑	1.67 \pm 1.16↑	L-C*
IgA heavy chain constant region, partial (290578621)	1.80 \pm 0.69	1.43 \pm 0.64↓	0.71 \pm 0.16↓	L-C**
Immunoglobulin alpha heavy chain constant region, partial (555827)	1.80 \pm 0.69	1.43 \pm 0.64↓	0.71 \pm 0.16↓	L-C**
Immunoglobulin J chain (335293621)	1.38 \pm 0.24	1.09 \pm 0.20↓	0.87 \pm 0.05↓	C-PostS* L-C***
Immunoglobulin light chain constant region, partial (559775234)	1.33 \pm 0.10	1.29 \pm 0.39↓	0.98 \pm 0.03↓	L-C*
Lactate Dehydrogenase M4, (229621) L-lactate dehydrogenase A chain (1170740), isoform X1 (1191854392) and X2 (1191854394)	0.82 \pm 0.09	1.04 \pm 0.12↑	1.21 \pm 0.20↑	C-PostS** L-C***
Lactoferrin (164525) and Lactotransferrin precursor (1149000932)	1.30 \pm 0.43	1.11 \pm 0.33↓	0.83 \pm 0.06↓	L-C*
Lactotransferrin, partial (41688306)	1.26 \pm 0.30	1.10 \pm 0.25↓	0.83 \pm 0.08↓	L-C**
Lung and nasal epithelium carcinoma associated protein precursor (52352329)	1.86 \pm 1.20	1.09 \pm 0.33↓	0.81 \pm 0.16↓	L-C**
Myeloblastin (927104676)	1.23 \pm 0.14	1.09 \pm 0.21↓	0.94 \pm 0.20↓	L-C*
Neutrophil gelatinase-associated lipocalin isoform X1 (1191803369)	1.16 \pm 0.24	0.91 \pm 0.20↓	0.92 \pm 0.10↓	C-PostS*
Pathogenesis-related protein 2 (1165149)	1.22 \pm 0.35	1.07 \pm 0.30↓	0.72 \pm 0.17↓	L-C*
Peptidylprolyl isomerase A (cyclophilin A) (326368140)	1.03 \pm 0.20	1.01 \pm 0.09↓	0.79 \pm 0.04↓	L-C*
Peptidyl-prolyl cis-trans isomerase A isoform X1 (927217413) and X2 (1191850894)	1.03 \pm 0.20	1.01 \pm 0.09↓	0.79 \pm 0.04↓	L-C*
Peroxiredoxin-2 (347300176)	0.77 \pm 0.14	0.90 \pm 0.22↑	1.39 \pm 0.62↑	L-C*
Plasminogen precursor (113205806)	0.73 \pm 0.21	0.82 \pm 0.21↑	1.36 \pm 0.40↑	L-C*
Protein FAM25A (545870871)	0.55 \pm 0.09	1.62 \pm 1.10↑	1.15 \pm 0.46↑	C-PostS*
Serpin A3-8 (1191899501)	0.80 \pm 0.11	0.99 \pm 0.35↑	1.23 \pm 0.22↑	L-C*
Sulfhydryl oxidase 1 (1191912332)	0.98 \pm 0.20	0.99 \pm 0.18↑	1.30 \pm 0.03↑	L-C**
Tropomyosin 4 (3661527)	0.99 \pm 0.16	1.14 \pm 0.13↑	1.51 \pm 0.22↑	L-C**
Tropomyosin alpha-4 chain isoform X1 (545808822)	0.99 \pm 0.16	1.14 \pm 0.13↑	1.51 \pm 0.22↑	L-C**
y6/PLAUR domain-containing protein 3 (350585274)	0.80 \pm 0.10	1.29 \pm 0.35↑	1.15 \pm 0.36↑	C-PostS*
Zymogen granule protein 16 homolog B (1191866117)	1.68 \pm 0.75	1.02 \pm 0.26↓	0.62 \pm 0.16↓	L-C***

control and post-acute stress group (nose snaring) (Table 1): cornifin ($P = .039$), Heat shock cognate 71 kDa protein ($P = .012$), Protein FAM25A ($P = .027$) and y6/PLAUR domain-containing protein 3 ($P = .013$) showed increases whereas neutrophil gelatinase-associated lipocalin isoform X1 ($P = .045$) showed a decrease in relation to the control group.

The proteins that showed significant differences between controls and pigs with lameness were: BPI fold-containing family A member 1 isoform X1 ($P = .005$), Calcium-activated chloride channel regulator 1 ($P = .026$), IgA heavy chain constant region, Immunoglobulin alpha heavy chain constant region ($P = .007$), Lactoferrin, Lactotransferrin Precursor ($P = .021$) and Lactotransferrin ($P = .003$), Lung and nasal epithelium carcinoma associated protein precursor ($P = .005$), Myeloblastin ($P = .027$), Pathogenesis-related protein 2 ($P = .014$), Peptidylprolyl isomerase A, isoform X and X2 ($P = .015$) and Zymogen granule protein 16 homolog B (0.0004) that showed significant decreases whereas Calmodulin-like protein 5 ($P = 0.002$), CD5 antigen-like precursor and isoform X1 ($P = .001$), Fibronectin ($P = .032$), Fructose-bisphosphate aldolase A isoform X1, X2 and X4 ($P = .028$), Hemoglobin Chain D (aquo Met) (beta Chain) ($P = .038$), Hemoglobin subunit alpha ($P = .032$), Peroxiredoxin-2 ($P = .028$), Plasminogen precursor ($P = .023$), Serpin A3-8 ($P = .029$), Sulfhydryl oxidase 1 ($P = .003$) and tropomyosin 4 and isoform X1 ($P = .003$) showed significant increase in pigs with lameness in relation to the control group.

3.2. Results of validation of LDH

The intra-assay CVs were 6% for the pool with a high LDH concentration (970 U/L) and 5% for the pool with a low LDH concentration (21.1 U/L). Additionally, inter-assay CVs were 10% for the pool with a high LDH concentration and 9% for the pool with a low LDH concentration, respectively. A linear regression coefficient of 0.99 was observed when saliva samples with high concentrations (1001 U/L) diluted 1:2-fold or greater were analysed. The analytical limit of detection calculated was 5 U/L.

Differences between LDH levels obtained before and after of the application of the stress model by nose snaring are presented in Fig. 1A. Pigs showed a significant increase ($P < .01$; $P = .017$) of salivary LDH levels after nose snaring (median: 533 U/L; range: 364–728 U/L; 25–75th percentiles) in comparison with the levels observed before stress (median: 246 U/L; range: 157–415 U/L; 25–75th percentiles). Differences between LDH levels in healthy animals and animals with lameness are presented in Fig. 1B. Pigs with lameness showed a significant increase ($P < .01$; $P = .0086$) of salivary LDH levels (median: 425 U/L; range: 243–593 U/L; 25–75th percentiles) in comparison with healthy pigs (median: 232 U/L; range: 203–322 U/L; 25–75th percentiles).

3.3. Bioinformatics

The peptides of Table 1 related to 29 proteins representing unique genes identified as being differentially expressed between situations of

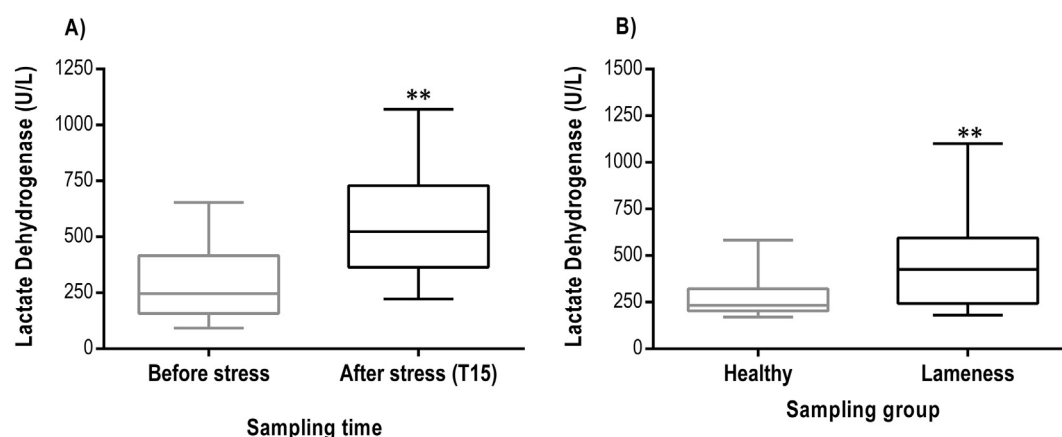


Fig. 1. Lactate dehydrogenase concentrations in A) pigs ($n = 16$) before and at 15 min after (T15) the application of stress model based on snaring and B) pigs with lameness ($n = 15$) in relation to healthy ($n = 15$). The plot shows median (line within box), 25th and 75th percentiles (box) and 10th and 90th percentiles (whiskers). Asterisks indicate significant differences between animal groups: $**P < .01$.

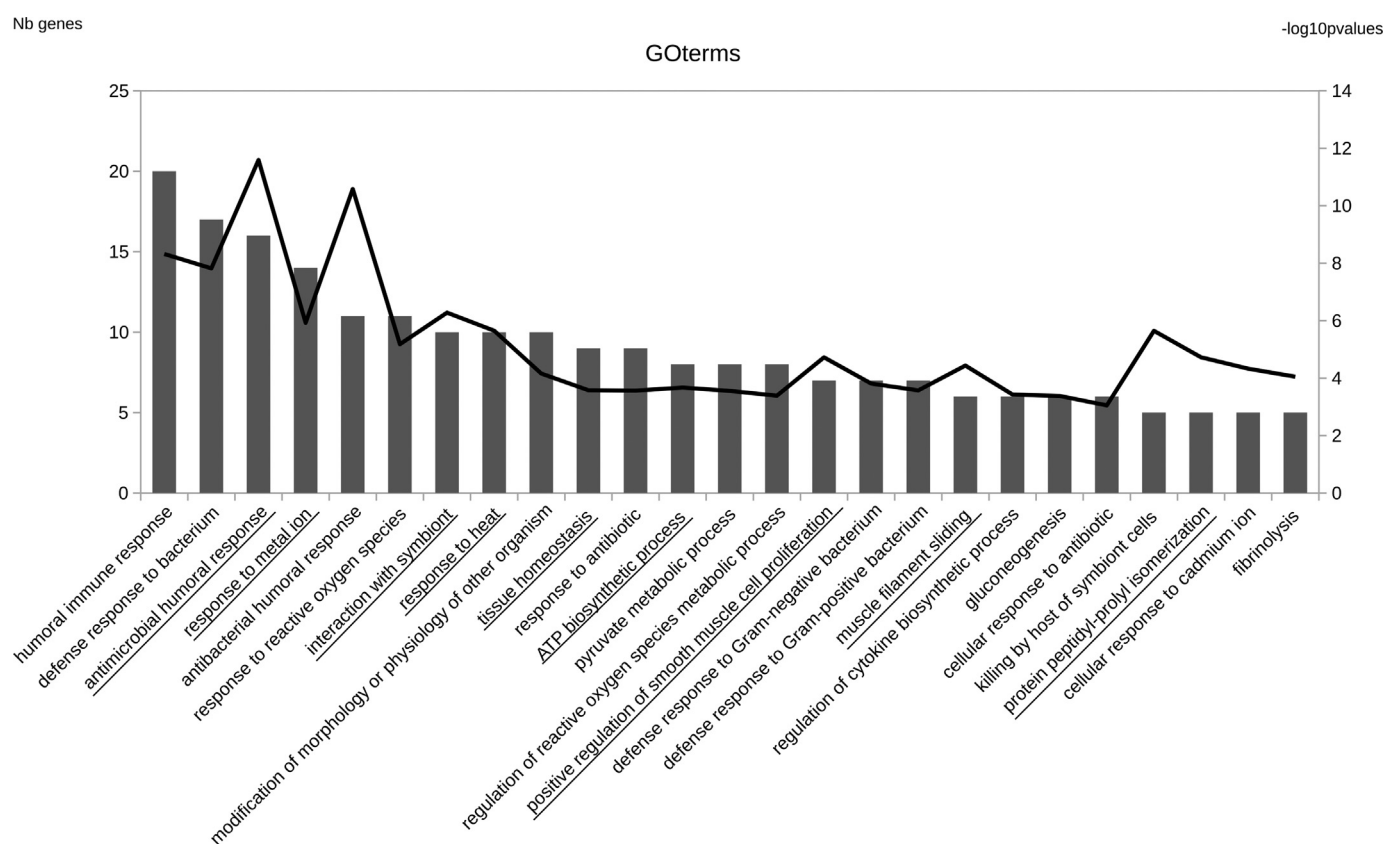


Fig. 2. GO terms over-represented in the pool of significantly expressed saliva proteins pre-stress (healthy pigs), post-stress and pigs with lameness. GO terms are ordered by the number of significant genes/proteins of the study associated with them according to Gene Ontology database (first y-axis). $-\log_{10}$ 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 underlined.

compromised welfare in pigs (induced stress by nose snaring and pigs lameness). The original proteins network have been enriched with 118 best interacting proteins from databases in the enrichment step in GO analysis. From the GO analysis, nine GO groups were defined as leader representative groups in stress and welfare compromised situations, as follow: antimicrobial humoral response (sixteen genes, $-\log_{10} P = 11.6$), response to metal iron (fourteen genes, $-\log_{10} P = 5.9$), interaction with symbiont (ten genes, $-\log_{10} P = 6.3$), response to heat (ten genes, $-\log_{10} P = 5.6$), tissue homeostasis (nine genes, $-\log_{10} P = 3.6$), ATP biosynthesis process (eight genes, $-\log_{10} P = 3.6$), positive regulation of smooth muscle cell proliferation (seven

genes, $-\log_{10} P = 4.7$), muscle filament sliding (six genes, $-\log_{10} P = 4.4$) and protein peptidyl-prolyl isomerization (five genes, $-\log_{10} P = 4.7$) (Fig. 2). Representation of GO terms shows that response to metal iron pathway is central and the rest of most relevant pathways in our study were highlighted in bold (Fig. 3).

4. Discussion

In our study, proteins differentially expressed in saliva and changes in relevant metabolic pathways were found in pigs after application of a model of acute stress and in pigs with lameness, using a high-resolution

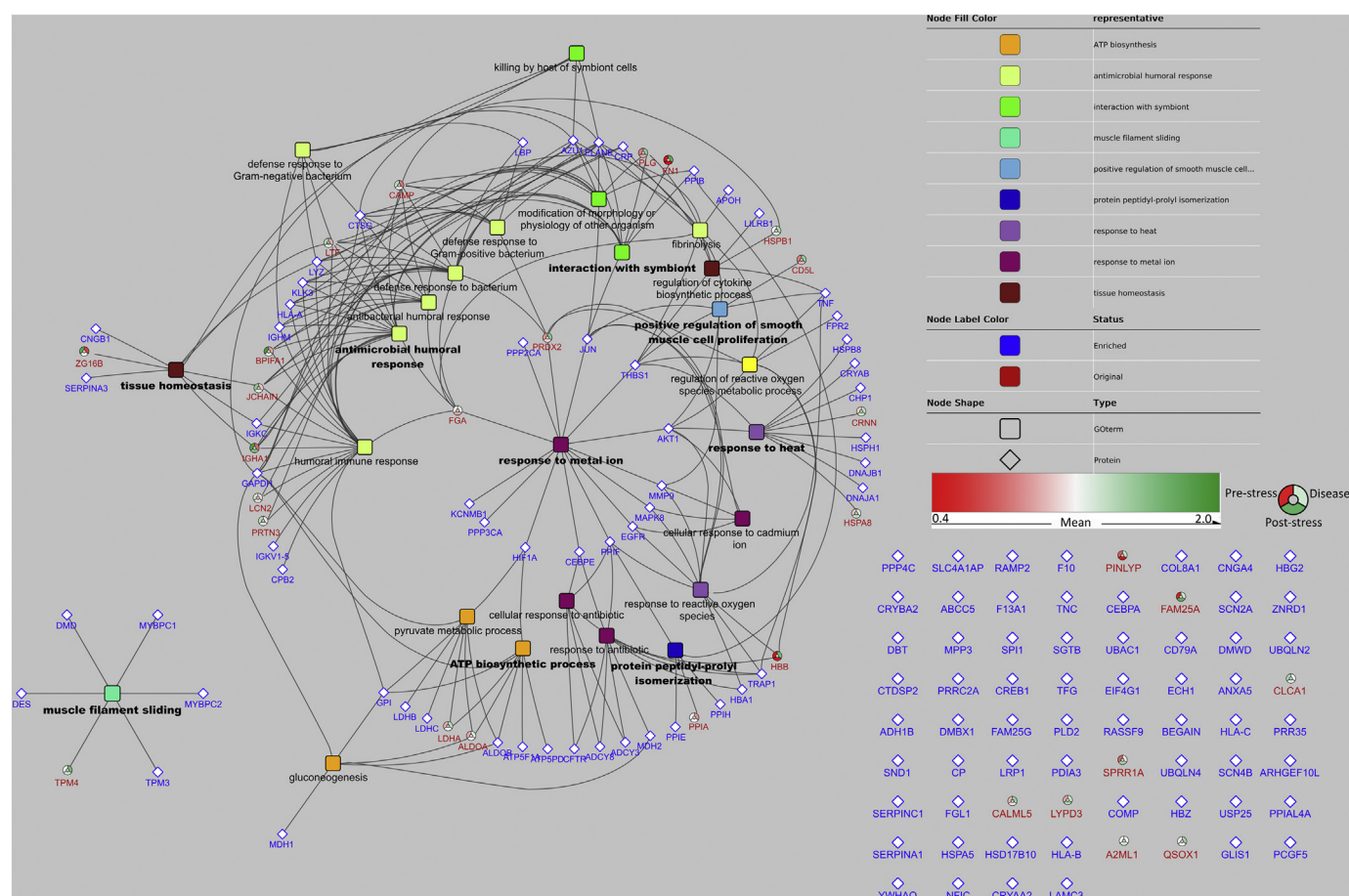


Fig. 3. Network representation of GO terms over-represented in the pool of significant proteins and associated proteins. GO terms are represented by square shape, proteins by diamond shape. Proteins are in red, with wheel shape, for proteins identified in the experiments, or in a diamond form with a blue 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

quantitative proteomic technique based on TMT labelling analysis. Four proteins showed significant differences after both the application of the acute stress model and in lameness compared with the control pigs, namely: cornulin, the heat shock proteins (HSP) HSP27 (also known as HSP β 1) and LDH that showed significant increases whereas immunoglobulin J chain showed a significant decrease. Of these, LDH was the protein that showed the highest difference of these four proteins and was selected for assessment of validation and clinical evaluation.

Cornulin is an intra-cellular epithelia protein fundamentally expressed in the upper layers of differentiated squamous tissues, being a biomarker of late epidermal differentiation (Contzler et al., 2005). In humans it has been associated with the conversion of normal to neoplastic epithelium and it is an important molecule in esophageal pathology (Pawar et al., 2013), being related to esophageal squamous cell carcinoma (Hsu et al., 2014). Furthermore, its expression may contribute to the pathogenesis of psoriasis (Li et al., 2019) and it can have a role in the development of inflammatory processes such as atopic dermatitis (Trzeciak et al., 2017). In pigs, it has been described to be produced by squamous epithelium after tissue damage (Nelson et al., 2008). This protein has not previously been identified in saliva samples. Our results showed a higher expression after the stress response in poor health conditions but further studies should be performed to clarify its role in stress and inflammation.

HSP are ubiquitous in all organisms and they present a high level of conservation across species. They are categorised in different classes according to their monomeric molecular size (about 60, 70, 90 and 100

KDa) and are termed HSP60, HSP70, HSP90 and HSP100, respectively (Fink, 1999). A fifth class of HSP are denominated “small HSP” (HSPs) that are the smallest and most variable in size, having a range of 12 to 42KDa (Haslbeck et al., 2005). These proteins constitute part of the molecular chaperone (protein folding) system of the cell and are expressed, in order to protect the cells, under stress conditions. Although these are intracellular, they have been detected in extracellular fluids in humans with autoimmune, trauma and inflammatory disorders (Khandia et al., 2017).

In this investigation, the HSP 27 KDa was expressed more after acute stress and in pigs with lameness in relation to control animals. In addition, it was related in GO analysis with the regulation of cytokine biosynthetic process pathway but has not previously been detected in porcine saliva. This protein exists as a multimeric complex in the cells and is involved in regulation of cytoskeleton dynamics, refolding of unfolded proteins or cell cycle regulation. HSP 27 KDa binds to a wide range of cellular proteins and also is involved in cellular functions of protection against physical and environmental stressors. In addition, it is implicated in several biological processes, including immunity, therapy, development and diseases (Singh et al., 2017). It increases in different types of cancer (Albany and Hahn, 2014) but also in neurodegenerative diseases, such as Alzheimer's and multiple sclerosis (Nedellec et al., 2002) or neurological disorders (Bakthisaran et al., 2015). In pigs, the mRNA expression levels in the muscle of this protein, together with other HSP, such as HSP70 or 90, are increased after transport stress (Zou et al., 2017).

Immunoglobulin J chain is a small polypeptide, which regulates polymer formation of immunoglobulin (Ig)A and IgM and their incorporation into polymeric IgA (pIgA, mainly dimers) and pentameric IgM. Most importantly, only J-chain-containing polymers show high affinity for the polymeric Ig receptor (pIgR) and therefore J chain has a major role in the mediation of active external transfer and is key in secretory Igs formation (Johansen et al., 2000). The higher production of secretory Igs such as IgA or IgM in situations of stress or loss of welfare that has been reported previously (Escibano et al., 2012, 2015) with a greater consumption of IgJ could explain the decrease obtained in our results. The bioinformatic results showed a relationship of this protein with different metabolic pathways such as tissue homeostasis and antimicrobial humoral response.

LDH was the protein selected for clinical validation since it showed the highest degree of significance among the proteins that changed after stress as well as in pigs with lameness in relation to control animals. LDH can also be measured with an automated assay that was commercially available. Furthermore, the GO analysis showed its relation with the ATP biosynthetic process pathway. During stress condition, a high amount of catecholamines is released to prepare the body for “fight or flight” response (Ranabir and Reetu, 2011). Radaković et al. (2018) showed that adrenaline caused an increase in total activity of LDH, LDH1 and LDH2 isoenzymes in rats. Drouet et al. (2015) showed significant increases (1.9 fold-increases) in plasma levels of LDH in rats after a model of acute restraint stress in relation to control group. In pigs, it is considered as a biomarker of muscle damage (Fàbrega et al., 2002) and increases after muscle fatigue and injury (Averós et al., 2009). In this species there are described increases in LDH activity in serum after different stressor stimuli, such as transport, cold or weaning, (Averós et al., 2009; Faure et al., 2013; Li et al., 2016). However, LDH has not previously been measured in saliva samples after an acute stress model. In relation to lameness, Hogg et al. (1975) showed that LDH values increased in synovia of lame swine and also in other species, such as dairy cows, in which high levels of LDH are related to lameness (Ristevski et al., 2017). Although further studies are needed, our results constitute the first clinical evidence of the possible usefulness of LDH levels in saliva as a biomarker of stress in pigs.

Our results showed that the number of proteins that showed significant changes compared to controls in lameness is higher than those that change in an acute stress. This fact occurs for both increasing and decreasing proteins and is presumably due to lameness being a more severe condition with longer lasting consequences and may involve a higher degree of inflammation. There were five proteins that were only different (increasing and decreasing) when the effect of stress alone was compared to control, and 23 proteins that were only different when lameness was compared to controls. If these proteins could be used as biomarkers to differentiate stress from lameness then this would be a valuable finding.

In relation to the feasibility of using these proteins in commercial facilities in routine, LDH can be measured by spectrophotometric assays that are cheaper, faster and easier to perform than the immunoassays required for measurement of other proteins such as cornulin, Immunoglobulin J chain or HSPs. In relation to these proteins, it would be of interest in the future to consider development of cheaper and faster assays for their measurement in formats that could allow an easier practical application for on farm use.

In conclusion, we have identified that, in situations of compromised welfare on farm such as acute stress and lameness in pigs, there are changes in proteins and metabolic pathways in saliva. These proteins could be potentially used as biomarkers of welfare status in pig farms using a non-invasive and non-stressful sampling procedure.

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Declaration of Competing Interests

The authors have declared that no competing interests exist.

Ethics statement

In the stress experiment the samples used were those collected for the study described in the article Contreras-Aguilar et al. (2018) and in accordance with the ethical standards of the Bioethical Commission of Murcia University (CEEa 431/2018). No animals were acquired for the purpose of this experiment, since the study was conducted at a working commercial farm.

Software and data repository resources

Our data are not deposited in an official repository.

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