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1 **Highlight**

2 - TMT analysis allowed the identification of original welfare proteins in pig saliva.

3 - TMT analysis allowed the identification of new metabolic pathways in welfare of pig.

4 - Salivary lactate dehydrogenase could be a salivary biomarker of welfare in pig.

5

6 **Changes in saliva proteins in two conditions of compromised welfare in pigs: an**

7 **experimental induced stress by nose snaring and lameness**

8

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27

28 **Abstract**

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

48

49

50 *Keywords:* Lameness, Pigs, Saliva, Stress, TMT analysis, Welfare

51 **1. Introduction**

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

58 In terms of research and diagnostic potential, proteomic studies of saliva have been shown
59 to be an emerging source of new biomarkers (Lamy and Mau, 2012). Novel gel-free mass
60 spectrometry-based proteomic approaches using isobaric tagging options, such as tandem
61 mass tags (TMT) or isobaric tags for relative and absolute quantification (iTRAQ), are highly
62 sensitive (especially when combined with high-resolution MS analysis) and allow
63 simultaneous quantification of differentially labelled peptides (Baeumlisberger *et al.*, 2010;
64 Dayon *et al.*, 2011; Giron *et al.*, 2011). A recent study by Prims *et al.* (2019) applied the
65 novel gel-free free iTRAQ-based proteomic technologies for the characterization of different
66 salivary gland proteomes in pigs. However, to the authors' knowledge, no studies have been
67 conducted addressing gel-free proteomic technologies applied to whole porcine saliva.
68 Furthermore, this technology has not yet been applied to study possible proteins changes in
69 pig saliva in situations of stress or compromised welfare.

70 The hypothesis of our study was that high-resolution quantitative gel-free proteomic
71 technology could identify proteins and metabolic pathways in saliva of pigs affected by stress
72 or in situations where the welfare of the pigs is compromised. Therefore, our objective was to
73 evaluate the salivary proteome in healthy pigs by gel-free mass spectrometry-based proteomic
74 approach using TMT to explore the possible changes in salivary proteome in two different
75 situations: (1) after an acute stress consisting of restraint with a nose snare, which has been

76 demonstrated as stressor in an experimental model of stress (Escribano *et al.*, 2013; 2014),
77 and (2) in pigs with lameness, which is a highly frequent problem in the swine industry (Anil
78 *et al.*, 2005) and is used as an animal-based welfare measure for commercially farmed pigs
79 (Pandolfi *et al.*, 2017). An additional objective was to determine if a protein, differentially
80 expressed in both situations compared with the healthy pigs could be validated as a potential
81 diagnostic biomarker of stress or welfare.
82

83 **2. Materials and methods**

84 *2.1. Animal and sampling procedures*

85 All animals used in this study were male crossbred pigs ([Sus scrofa domesticus] Duroc X
86 [Landrace X Large White]) with the same age (in the mid-fattening period; age =104.8 ± 10.0
87 days, mean body weight = 78.3 ± 6.3 kg) and grown in a high health farm, the Higher
88 Education Farm of the University of Murcia (Spain). All animals were vaccinated against
89 Mycoplasma hyopneumoniae (Stellamune Mycoplasma, inactivated Mycoplasma
90 hyopneumoniae NL 1042, Pfizer Animal Health, Madrid, Spain) and Porcine circovirus type
91 2 (Porcilis® PCV, MSD Animal Health, Boxmeer, The Netherlands). On this farm, the pigs
92 had ad libitum access to a nutritionally balanced diet (commercial dry diets based in a corn-
93 soybean meal with 15.5% of crude protein -CP-; 0.79% Ileal digestible Lys, %; and 13.5 MJ
94 of metabolizable energy per kg -ME/kg-) and water *ad libitum* under general commercial
95 housing and husbandry conditions conforming to the European Union Guidelines (Directive
96 2010/63/EU1). Each pen had an area of 1.139m^2 per animal, being in concordance to the
97 legislation (Council Directive 2001/88/CE of 23 October 2001 amending Directive
98 91/630/CEE concerning minimum standards for the protection of pigs). The temperature in
99 the pens was kept between a minimum of 18ºC and a maximum of 23ºC.

100 Saliva samples were obtained by placing a sponge, made of polyurethane, in the mouth of
101 each pig. When sponges were thoroughly moist, they were placed in plastic tubes (Salivette;
102 Sarstedt, Aktiengesellschaft & Co., Nümbrecht, Germany), centrifuged (3,000 *g* for 10 min,
103 4°C), and the supernatant was aliquoted for analysis. Aliquots of samples were stored at -
104 80°C until proteomic analysis (VetMedZg, Internal Disease Clinic, Faculty of Veterinary
105 Medicine, University of Zagreb in Zagreb, Croatia) and biochemical analysis
106 (Interdisciplinary Laboratory of Clinical Analysis Interlab-UMU, Regional Campus of
107 International Excellence 'Campus Mare Nostrum', University of Murcia).
108
109 *2.2. Pigs with acute stress induced by restraint with a nose snare*
110 The experimentally induced stress consisted in restraining each pig for 1 min with a nose
111 snare as previously described (Tecles *et al.*, 2017). This is a common veterinary practice used
112 for the immobilization of animals in pig farms and has been demonstrated to produce an
113 increase in salivary biomarkers of stress, such as cortisol or chromogranin A (Escribano *et al.*,
114 2013; 2014).
115 This experimental procedure was performed at different time points according to the
116 experimental purpose: (1) for the proteomic study, saliva samples were collected from 6 male
117 pigs before the application of the acute stress stimulus (control group) and further saliva
118 samples were collected from the same animals after 15 min (T15) of the application of the
119 nose snare as stress stimulant; and (2) for the validation study the saliva samples of 16 male
120 pigs, with the same age and from the same farm as for the proteomic study were sampled
121 before and at T15 after the same stress stimulus by nose snare. All animals were subjected to
122 a clinical examination prior to and throughout the study, and no clinical signs of disease were
123 detected in any animal.
124

125 *2.3. Pigs with lameness*

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

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

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

143

144 *2.4. Proteomic study of saliva samples from pigs using Liquid Chromatography and Tandem*
145 *Mass Spectrometry (LC–MS/MS)*

146 Six samples obtained prior to the stress induction (pre-stress or control pigs), six samples
147 of the same animals obtained 15 min after the stress (post-stress) and five samples from pigs
148 with lameness were analysed by LC-MS/MS. Saliva proteins were acetone-precipitated (six
149 volumes of ice-cold acetone, overnight), dissolved in 100 mM of triethylammonium

150 bicarbonate (TEAB) (pH 8.5) and protein concentration was obtained by bicinchoninic acid
151 (BCA) assay. A pooled sample, generated by mixing equal protein quantity of all seventeen
152 samples, was employed as an internal standard in all TMT sixplex experiments.
153 For each sample, proteins were submitted to reduction, alkylation and digestion and
154 labelled using TMT sixplex reagents following the manufacturer instructions (Thermo Fisher
155 Scientific, Waltham, MA, USA) with some modifications, as reported before (Martinez-
156 Subiela *et al.*, 2017). In short, 35 μg of proteins was reduced with 200 mM of 1,4-
157 Dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO, USA), alkylated with 375 mM of
158 iodoacetamide (Sigma-Aldrich, S t. Louis, MO, USA) and precipitated with ice-cold acetone
159 (VWR Corp., Radnor, PA, USA) overnight. Saliva samples were then centrifuged and the
160 acetone was eliminated by decantation. Afterward, 50μL of 100 mM TEAB buffer were used
161 to resuspend the pellets and digested with trypsin (Promega Corp., Madison, WI, USA)
162 overnight at 37°C (trypsin-to-protein ratio 1:35, w/w). The reagents for TMT labelling were
163 equilibrated and resuspended with anhydrous acetonitrile (LC–MS grade, Thermo Fisher
164 Scientific, Waltham, MA, USA) and added to each sample. The labelling reaction was
165 incubated (at room temperature) for one hour, following by incubation with 5%
166 hydroxylamine for 15 minutes (Thermo Fisher Scientific, Waltham, MA, USA). Samples
167 were mixed in the same amounts and the aliquots (5 μg) were vacuum-dried and kept at -80°C
168 for further LC–MS/MS analysis. The LC−MS/MS analysis was performed using the Dionex
169 Ultimate 3000 RSLS nano flow system (Dionex, Camberley, UK) and the Orbitrap Q
170 Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as reported
171 before (Horvatić *et al.*, 2018). For protein identification and relative quantification Proteome
172 Discoverer (version 2.0., Thermo Fisher Scientific, Waltham, MA, USA) was used for the
173 SEQUEST search against *Sus scrofa* FASTA files downloaded from NCBI database
174 (19/07/2018) according to parameters set as follows: precursor and fragment mass tolerances

175 of 10 ppm and 0.02 Da, two trypsin missed cleavage sites, respectively; carbamidomethyl (C),
176 oxidation (M), fixed peptide modification, deamidation (N,Q) and TMT six-plex (K, peptide
177 N-terminus) dynamic modifications. The false discovery rate (FDR) for peptide identification
178 was calculated using the Percolator algorithm. Proteins with at least two unique peptides and
179 5% FDR were considered successfully identified. Protein quantification was based on relative
180 intensities of reporter ions representing differentially labelled peptides selected for MS/MS
181 fragmentation. To compare relative quantification data, for each protein between the TMT
182 sixplex experiments, the pool of all samples was used as the internal standard.
183
184 *2.5. Validation of lactate dehydrogenase as possible salivary biomarker of welfare in pigs:*
185 *induced stress by nose snaring and lameness*
186 *2.5.1. Lactate dehydrogenase assay*
187 Lactate dehydrogenase (LDH) concentration was measured in saliva for validation of
188 proteomic results using a colorimetric commercial kit (Lactate Dehydrogenase (LDH),
189 BioSystems S.A. Costa Brava, 30. 08030, Barcelona, Spain) in an automatic analyser for
190 biochemical assay (Olympus UA600, Olympus Diagnostica GmbH).
191
192 *2.5.2. Analytical validation*
193 Pigs saliva samples taken in conditions of pre-stress (control pigs), post-stress and
194 pigs with lameness were employed for the validation study of the LDH assay. The following
195 parameters were evaluated: intra- and inter-assays precision, linearity, recovery and the limit
196 of detection.
197 The intra- and inter-assays precision expressed as coefficients of variation (CVs) were
198 calculated by analysing two saliva pools containing low and high concentrations of LDH.
199 Each pool was prepared by mixing saliva samples with similar concentrations of LDH

200 (previously quantified by the method used in our study). Inter-assays CVs were obtained by
201 measuring five time the same pools in different days. Each CV was calculated as the
202 percentage of the standard deviation (SD) of the replicates divided by the mean. To avoid
203 possible variations due to cycles of thawing and freezing, the saliva samples were aliquoted
204 and only the aliquot needed for each assay was thawed.

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

210 The detection limit, defined as lowest concentration of LDH that the assay can distinguish
211 from zero value, was calculated based on the mean value of 10 replicate determinations of the
212 assay buffer (zero standard) plus three SDs.

213

214 *2.5.3. Evaluation of salivary changes in LDH after acute stress (nose snaring) model and*
215 *poor health condition on farm (lameness)*

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

222

223 *2.6. Statistical analysis*

224 In order to compare the abundances of proteins identified in the proteomic analysis
225 between three groups of samples (proteins pre-stress (control pigs), post-stress and pigs with
226 lameness) data were normalized by logarithmic transformation and Student's t-test (two-
227 tailed, paired) was used to determine statistical significance between groups. A $P < 0.05$ value
228 was considered to be significant. RStudio (v1.0.143) (R Studio Team. RStudio "RStudio
229 Team. RStudio: Integrated Development Environment for R [Internet]. Boston, MA: RStudio,
230 Inc.; 2015. Available from: http://www.rstudio.com/," 2015) was employed for statistics.
231 Intra- and interassay CVs of assay were calculated as SD/mean value of repeated
232 measurements and expressed as percentage (multiplied by 100). Detection limits, linearity
233 under dilution, linear regression analyses also were performed with Rstudio. The changes
234 between groups were assessed by a non-parametric Wilcoxon matched-pairs test in the case of
235 the model of stress (before vs. after), and a non-parametric Mann-Whitney unpaired test in the
236 case of animals with lameness (Healthy vs. Lameness). A $P < 0.05$ value was considered
237 significant. Data analyses for clinical analysis were performed using a commercial statistics
238 package (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA).
239
240 *2.7. Gene ontology pathways*
241 The proteomic results obtained in the study were used for the gene ontology (GO)
242 analysis. The proteins differentially expressed, encoding in porcine genes, were transformed
243 to their human orthologs by employing the Ensembl orthologs database and its tool for data
244 mining BioMart (http://www.ensembl.org/index.html). Obtained genes were used to add their
245 best known interactors (maximum of 10 per genes) according to the databases STRING-
246 EMBL, IntAct and Reactome by the utilization of the Cytoscape (v3.6.1) plug-in CluePedia
247 (v1.5.2) (Shannon *et al.*, 2003; Bindea *et al.*, 2013). Original proteins (differentially expressed
248 between the three grouping conditions of data, namely, data obtained pre-stress or healthy vs.

249 data obtained post-stress and vs. data obtained with pigs with lameness) and enriched proteins
250 (best interactors of the original proteins) were then used to determine the GO terms over-
251 represented in this set of proteins by the utilization of the Cytoscape plug-in ClueGO (v2.5.0)
252 (Bindea *et al.*, 2009) on the *Homo sapiens* GO-biological process (14/08/2018) (GO level
253 from 3 to 8, minimum number of genes = 3, minimum percentage = 4, Kappa score threshold
254 = 0.4, two-sided hypergeometric test with Bonferroni correction). By utilizing the REVIGO
255 analysis, the over-represented redundant GO terms were removed. The similarity allowed was
256 0.7 SimRel, and their functional description defined the groups' related GO terms (Supek *et*
257 *al.*, 2011). Finally, pathway interactomes were designed in Cytoscape using the radial layout
258 incorporating the GO data generated by ClueGO and ReviGO (e.g., the number of input genes
259 in GO terms, associated p-value, GO/proteins relationships, GO groups).
260

261 **3. Results**

262 *3.1. Proteomic changes*

263 A total of 353 proteins were identified. Forty-four of them showed significant differences
264 for both the acute stress of snaring and the long term chronic stress of lameness. These
265 proteins are represented in Table 1. Three proteins showed significant increases for both
266 comparisons, namely: cornulin (Control vs. Post-stressed $P = 0.016$; Control vs. Lameness P
267 = 0.013); Heat shock protein beta-1 or 27 KDa (Control vs. Post-stressed P = 0.010; Control
268 vs. Lameness P = 0.010) and L-lactate dehydrogenase A (Control vs. Post-stressed P = 0.008;
269 Control vs. Lameness P = 0.001). Conversely, Immunoglobulin J chain showed a decrease in
270 these two conditions (Control vs. Post-stressed P = 0.020; Control vs. Lameness P = 0.0003).
271 The following proteins showed significant differences between control and post-stress
272 group (Table 1): cornifin (P = 0.039), Heat shock cognate 71 kDa protein (P = 0.012), Protein
273 FAM25A (P = 0.027) and y6/PLAUR domain-containing protein 3 (P = 0.013) showed

274 increases whereas neutrofil gelatinase-asociated lipocalin isoform X1 (P = 0.045) showed a
275 decrease in relation to the control group.
276 The proteins that showed significant differences between controls and pigs with lameness
277 were: BPI fold-containing family A member 1 isoform X1 (P = 0.005), Calcium-activated
278 chloride channel regulator 1 (P = 0.026), IgA heavy chain constant region, Immunoglobulin
279 alpha heavy chain constant region (P = 0.007), Lactoferrin, Lactotransferrin Precursor (P =
280 0.021) and Lactotransferrin (P = 0.003), Lung and nasal epithelium carcinoma associated
281 protein precursor (P = 0.005), Myeloblastin (P = 0.027), Pathogenesis-related protein 2 (P =
282 0.014), Peptidylprolyl isomerase A, isoform X and X2 (P = 0.015) and Zymogen granule
283 protein 16 homolog B (0.0004) that showed significant decreases whereas Calmodulin-like
284 protein 5 (P = 0,002), CD5 antigen-like precursor and isoform X1 (P = 0.001), Fibronectin
285 (P = 0.032), Fructose-bisphosphate aldolase A isoform X1, X2 and X4 (P = 0.028),
286 Hemoglobin Chain D (aquo Met) (beta Chain) (P = 0.038), Hemoglobin subunit alpha (P =
287 0.032), Peroxiredoxin-2 (P = 0.028), Plasminogen precursor (P = 0.023), Serpin A3-8 (P =
288 0.029), Sulfhydryl oxidase 1 (P = 0.003) and tropomyosin 4 and isoform X1 (P = 0.003)
289 showed significant increase in pigs with lameness in relation to the control group.
290
291 *3.2. Results of validation of LDH*
292 Analytical validation: The intra-assay CVs were 6% for the pool with a high LDH
293 concentration (970 U/L) and 5 % for the pool with a low LDH concentration (21.1 U/L).
294 Additionally, inter-assay CVs were 10 % for the pool with a high LDH concentration and 9%
295 for the pool with a low LDH concentration, respectively. A linear regression coefficient of
296 0.99 was observed when saliva samples with high concentrations (1001 U/L) diluted 1:2-fold
297 or greater were analysed. The analytical limit of detection calculated was 5 U/L.

298 Differences between LDH levels obtained before and after of the application of the stress
299 model are presented in Figure 1A. Pigs showed a significant increase ($P < 0.01$; $P = 0.017$) of
300 salivary LDH levels after nose snaring (median: 533 U/L; range: 364-728 U/L; 25-75th
301 percentiles) in comparison with the levels observed before stress (median: 246 U/L; range:
302 157–415 U/L; 25-75th percentiles). Differences between LDH levels in healthy animals and
303 animals with lameness are presented in Figure 1B. Pigs with lameness showed a significant
304 increase ($P < 0.01$; $P = 0.0086$) of salivary LDH levels (median: 425 U/L; range: 243-593
305 U/L; 25-75th percentiles) in comparison with healthy pigs (median: 232 U/L; range: 203-322
306 U/L; 25-75th percentiles).
307
308 *3.3. Bioinformatics*
309 The peptides of Table 1 related to 29 proteins representing unique genes identified as
310 being differentially expressed between situations of compromised welfare in pigs (induced
311 stress by nose snaring and pigs lameness), The original proteins network have been enriched
312 with 118 best interacting proteins from databases in the enrichment step in GO analysis. From
313 the GO analysis, nine GO groups were defined as leader representative groups in stress and
314 welfare compromised situations, as follow: antimicrobial humoral response (sixteen genes, -
315 log10 $P = 11.6$), response to metal iron (fourteen genes, -log10 $P = 5.9$), interaction with
316 symbiont (ten genes, -log10 $P = 6.3$), response to heat (ten genes, -log10 $P = 5.6$), tissue
317 homeostasis (nine genes, -log10 $P = 3.6$), ATP biosynthesis process (eight genes, -log10 $P =$
318 3.6), positive regulation of smooth muscle cell proliferation (seven genes, -log10 $P = 4.7$),
319 muscle filament sliding (six genes, -log10 $P = 4.4$) and protein peptidyl-prolyl isomerization
320 (five genes, -log10 $P = 4.7$) (Figure 2). Representation of GO terms shows that response to
321 metal iron pathway is central and the rest of most relevant pathways in our study were
322 highlighted in bold (Figure 3).

323

324 **4. Discussion**

325 In our study, proteins differentially expressed in saliva and changes in relevant metabolic
326 pathways were found in pigs after application of a model of acute stress and in pigs with
327 lameness, using a high-resolution quantitative proteomic technique based on TMT labelling
328 analysis. Four proteins showed significant differences after the application of both acute stress
329 model and in lameness compared with the control pigs, namely: cornulin, the heat shock
330 proteins (HSP) HSP27 (also known as HSP β1) and LDH that showed significant increases
331 whereas immunoglobulin J chain showed a significant decrease. Of these, LDH was the
332 protein that showed the highest difference of these four proteins and was selected for
333 assessment of validation and clinical evaluation.

334 Cornulin is an intra-cellular epithelia protein fundamentally expressed in the upper layers
335 of differentiated squamous tissues, being a biomarker of late epidermal differentiation
336 (Contzler *et al.*, 2005). In humans it has been associated with the conversion of normal to
337 neoplastic epithelium and it is an important molecule in esophageal pathology (Pawar *et al.*,
338 2013), being related to esophageal squamous cell carcinoma (Hsu *et al.*, 2014). Furthermore,
339 its expression may contribute to the pathogenesis of psoriasis (Li *et al.*, 2019) and it can have
340 a role in the development of inflammatory processes such as atopic dermatitis (Trzeciak *et al.*,
341 2017). In pigs, it has been described to being produced by squamous epithelium after tissue
342 damage (Nelson *et al.*, 2008). This protein has not previously been identified in saliva
343 samples. Our results showed a higher expression after the stress response in poor health
344 conditions but further studies should be performed to clarify its role in stress and
345 inflammation.

346 Immunoglobulin J chain is a small polypeptide, which regulates polymer formation of
347 immunoglobulin (Ig)A and IgM and their incorporation into polymeric IgA (pIgA, mainly

348 dimers) and pentameric IgM. Most importantly, only J-chain-containing polymers show high
349 affinity for the polymeric Ig receptor (pIgR) and therefore J chain has a major role in the
350 mediation of active external transfer and is key in secretory Igs formation (Johansen *et al.*,
351 2000). The higher production of secretory Igs such as IgA or IgM in situations of stress or
352 loss of welfare that has been reported previously (Escribano *et al*, 2012; 2015) with a greater
353 consumption of IgJ could explain the decrease obtained in our results. The bioinformatic
354 results showed a relationship of this protein with different metabolic pathways such as tissue
355 homeostasis and antimicrobial humoral response.

356 HSP are ubiquitous in all organisms and they present a high level of conservation across
357 species. They are categorised in different classes according to their monomeric molecular size
358 (about 60, 70, 90 and 100 KDa) and are termed HSP60, HSP70, HSP90 and HSP100,
359 respectively (Fink, 1999). A fifth class of HSP are denominated "small HSP" (HSPs) that are
360 the smallest and most variable in size, having a range of 12 to 42KDa (Haslbeck *et al.*, 2005).
361 These proteins constitute part of the molecular chaperone (protein folding) system of the cell
362 and are expressed, in order to protect the cells, under stress conditions. Although these are
363 intracellular, they have been detected in extracellular fluids in humans with autoimmune,
364 trauma and inflammatory disorders (Khandia *et al.*, 2017).

365 In this investigation, the HSP 27 KDa was expressed more after stress and in pigs with
366 lameness in relation to control animals. In addition, it was related in GO analysis with the
367 regulation of cytokine biosynthetic process pathway but has not previously been detected in
368 porcine saliva. This protein exists as a multimeric complex in the cells and is involved in
369 regulation of cytoskeleton dynamics, refolding of unfolded proteins or cell cycle regulation.
370 HSP 27 KDa binds to a wide range of cellular proteins and also is involved in cellular
371 functions of protection against physical and environmental stressors. In addition, it is
372 implicated in several biological processes, including immunity, therapy, development and

373 diseases (Singh *et al.*, 2017). It increases in different types of cancer (Albany and Hahn, 2014)
374 but also in neurodegenerative diseases, such as Alzheimer and multiple sclerosis (Nedellec *et*
375 *al.*, 2002) or neurological disorders (Bakthisaran *et al.*, 2015). In pigs, the mRNA expression
376 levels in the muscle of this protein, together with other HSP, such as HSP70 or 90, are
377 increased after transport stress (Zou *et al.*, 2017).

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

397 Our results showed that the number of proteins that showed significant changes compared
398 to controls in lameness is higher than those that change in an acute stress. This fact occurs for
399 both increasing and decreasing proteins and is presumably due to lameness being a more
400 severe condition with longer lasting consequences and may involve a higher degree of
401 inflammation. There were five proteins that were only different (increasing and decreasing)
402 when the effect of stress alone was compared to control and 23 proteins that were only
403 different when lameness was compared to controls. If these proteins could be used as
404 biomarkers to differentiate stress from lameness then this would be a valuable finding.
405 In relation to the feasibility of using these proteins in commercial facilities in routine,
406 LDH can be measured by spectrophotometric assays that are cheaper, faster and easier to
407 perform that the immunoassays required for measurement of other proteins such as cornulin,
408 Immunoglobulin J chain or HSPs. In relation to these proteins it would be of interest in the
409 future to consider development of cheaper and faster assays for their measurement in formats
410 that could allow an easier practical application for on farm use.
411 In conclusion, we have identified that, in situations of compromised welfare on farm such
412 as acute stress and lameness in pigs, there are changes in proteins and metabolic pathways in
413 saliva. These proteins could be potentially used as biomarkers of welfare status in pig farms
414 using a non-invasive and non-stressful sampling procedure.
415

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425

426 **Declaration of interest**

427 The authors have declared that no competing interests exist.

428

429 **Ethics statement**

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

435

436 **Software and data repository resources**

437 Our data are not deposited in an official repository.

438

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654 **Table 1**
655
656 Proteins with significant differences between groups identified using TMT approach.

657 Asterisks indicate significant post-hoc difference: * P < 0.05 ;** P < 0.01; *** P < 0.001. Up

658 (↑) or down (↓) arrow indicates the change in relation to mean ± standard deviation (SD) of

659 control group.

660

Protein identification (accession number)	Mean±SD Control	Mean±SD Post-stressed (PostS)	Mean±SD Disease (lameness; L)	Differences between groups
BPI fold-containing family A member 1 isoform X1 (545880122)	1.86±1.20	1.09±0.33 ↓	0.81±0.16↓	L-C**
Calcium-activated chloride channel regulator 1 (75051712)	1.27±0.23	1.08±0.14↓	0.99±0.19↓	L-C*
Calmodulin-like protein 5 (545854020)	0.83±0.16	0.94±0.28↑	1.34±0.11↑	L-C**
CD5 antigen-like precursor (343478265) and isoform X1 (545822519)	0.74±0.22	0.84±0.19↑	1.35±0.29↑	L-C***
Cornulin (227558992) and isoform X1 (927122136)	0.88±0.18	1.17±0.21↑	1.20±0.19↑	C-PostS* L-C*
Cornifin (329299061)	0.68±0.14	1.30±0.86↑	0.93±0.23↑	C-PostS*
Fibronectin (1191840822)	0.78±0.21	0.90±0.31↑	1.21±0.32↑	L-C*
Fructose-bisphosphate aldolase A isoform X1 (1191869592); X2 (1191869594) and X4 (1191869604)	0.82±0.28	0.94±0.07↑	1.10±0.12↑	L-C*
Heat shock cognate 71 kDa protein (345441750)	0.86±0.19	1.17±0.16↑	1.12±0.17↑	C-PostS*
Heat shock protein beta-1 (55926209) and Heat shock protein 27kDa, partial (50916342)	1.01±0.16	1.12±0.15↑	1.25±0.10↑	C-PostS** L-C**
Hemoglobin Chain D (aquo Met) (beta Chain) (809285)	0.49±0.06	0.51±0.05↑	1.52±0.92↑	L-C*
Hemoglobin subunit alpha (1191866723)	0.38±0.15	0.45±0.15↑	1.67±1.16↑	L-C*
IgA heavy chain constant region, partial (290578621)	1.80±0.69	1.43±0.64↓	0.71±0.16↓	L-C**
Immunoglobulin alpha heavy chain constant region, partial (555827)	1.80±0.69	1.43±0.64↓	0.71±0.16↓	L-C**
Immunoglobulin J chain (335293621)	1.38±0.24	1.09±0.20↓	0.87±0.05↓	C-PostS* L-C***
Immunoglobulin light chain constant region, partial (559775234)	1.33±0.10	1.29±0.39↓	0.98±0.03↓	L-C*

Lactate Dehydrogenase M4, (229621) L-lactate dehydrogenase A chain (1170740), isoform X1 (1191854392) and X2 (1191854394)	0.82±0.09	1.04±0.12↑	1.21±0.20↑	C-PostS*; L-C***
Lactoferrin (164525) and Lactotransferrin precursor (1149000932)	1.30±0.43	1.11±0.33↓	0.83±0.06↓	L-C*
Lactotransferrin, partial (41688306)	1.26±0.30	1.10±0.25↓	0.83±0.08↓	L-C**
Lung and nasal epithelium carcinoma associated protein precursor (52352329)	1.86±1.20	1.09±0.33↓	0.81±0.16↓	L-C**
Myeloblastin (927104676)	1.23±0.14	1.09±0.21↓	0.94±0.20↓	L-C*
Neutrophil gelatinase-associated lipocalin isoform X1 (1191803369)	1.16±0.24	0.91±0.20↓	0.92±0.10↓	C-PostS*
Pathogenesis-related protein 2 (1165149)	1.22±0.35	1.07±0.30↓	0.72±0.17↓	L-C*
Peptidylprolyl isomerase A (cyclophilin A) (326368140)	1.03±0.20	1.01±0.09↓	0.79±0.04↓	L-C*
Peptidyl-prolyl cis-trans isomerase A isoform X1 (927217413) and X2 (1191850894)	1.03±0.20	1.01±0.09↓	0.79±0.04↓	L-C*
Peroxiredoxin-2 (347300176)	0.77±0.14	0.90±0.22↑	1.39±0.62↑	L-C*
Plasminogen precursor (113205806)	0.73±0.21	0.82±0.21↑	1.36±0.40↑	L-C*
Protein FAM25A (545870871)	0.55±0.09	1.62±1.10↑	1.15±0.46↑	C-PostS*
Serpin A3-8 (1191899501)	0.80±0.11	0.99±0.35↑	1.23±0.22↑	L-C*
Sulfhydryl oxidase 1 (1191912332)	0.98±0.20	0.99±0.18↑	1.30±0.03↑	L-C**
Tropomyosin 4 (3661527)	0.99±0.16	1.14±0.13↑	1.51±0.22↑	L-C**
Tropomyosin alpha-4 chain isoform X1 (545808822)	0.99±0.16	1.14±0.13↑	1.51±0.22↑	L-C**
y6/PLAUR domain-containing protein 3 (350585274)	0.80±0.10	1.29±0.35↑	1.15±0.36↑	C-PostS*
Zymogen granule protein 16 homolog B (1191866117)	1.68±0.75	1.02±0.26↓	0.62±0.16↓	L-C***

686 **Figure legends**

687 Fig.1. Lactate dehydrogenase concentrations in A) pigs (n = 16) before and at 15 min after
688 (T15) the application of stress model based on snaring and B) pigs with lameness (n = 15) in
689 relation to healthy (n = 15). The plot shows median (line within box), 25th and 75th
690 percentiles (box) and 10th and 90th percentiles (whiskers). Asterisks indicate significant
691 differences between animal groups: **P < 0.01.

692

693 Fig.2. GO terms over-represented in the pool of significantly expressed saliva proteins pre-
694 stress (healthy pigs), post-stress and pigs with lameness. GO terms are ordered by the number
695 of significant genes/proteins of the study associated with them according to Gene Ontology
696 database (first y-axis). - log10 of p-value for each GO term is represented on the second y-
697 axis. GO terms which define a group of similar GO terms (determined by ReviGO) are
698 underlined.

699

700 Fig.3. Network representation of GO terms over-represented in the pool of significant proteins
701 and associated proteins. GO terms are represented by square shape, proteins by diamond
702 shape. Proteins are in red, with wheel shape, for proteins identified in the experiments, or in a
703 diamond form with a blue border for proteins added by the enrichment step. GO nodes are
704 filled by colours corresponding to their GO group (determined by ReviGO). Name of GO
705 terms defining 1 group are in bold. When a protein belongs to 1 GO term, a link is figured
706 between the nodes of protein and GO term. The network representation has been realized
707 under Cytoscape using the radial layout.

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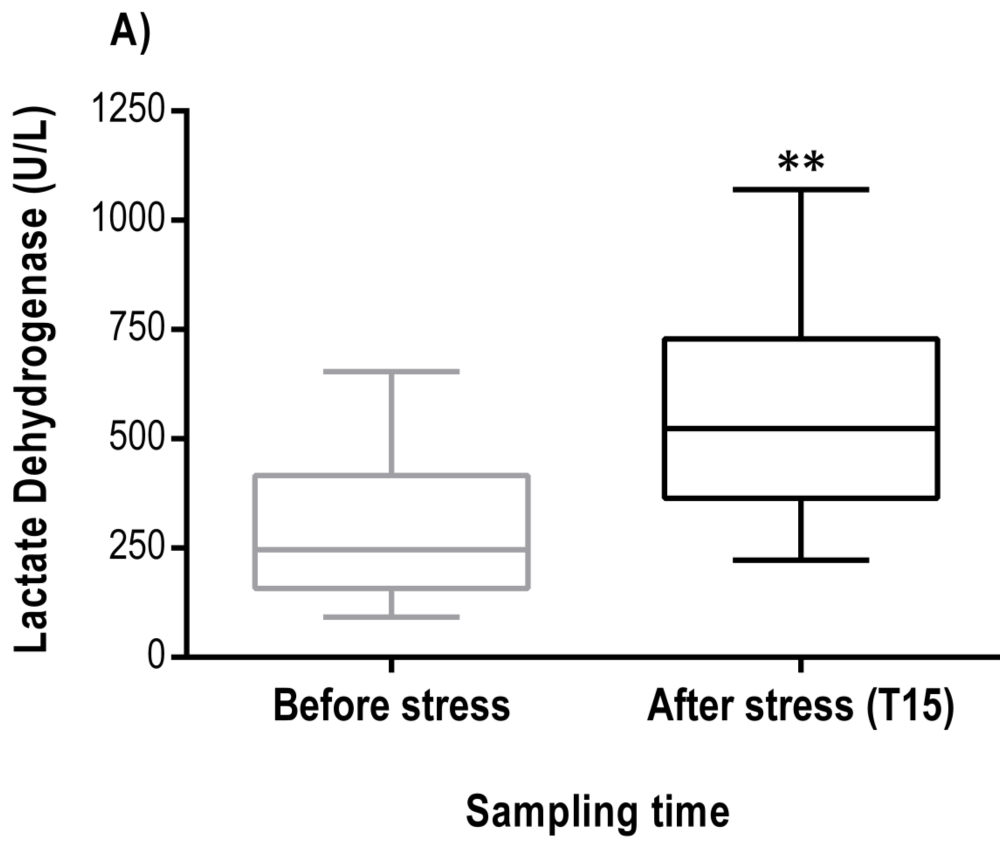

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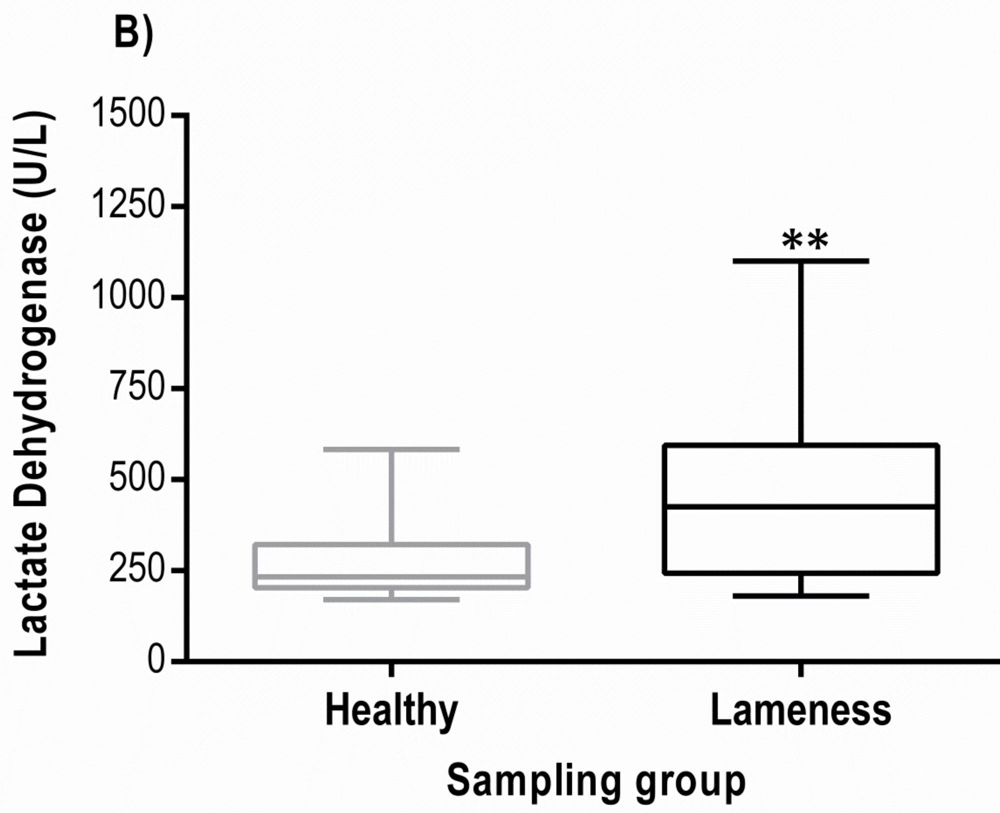

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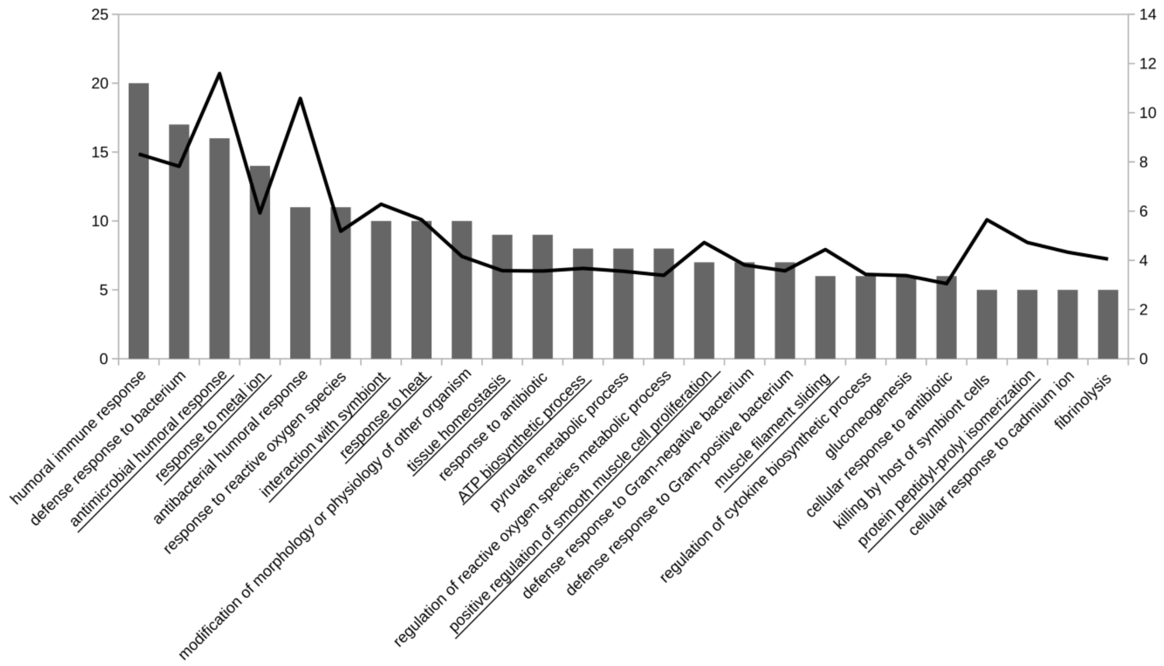

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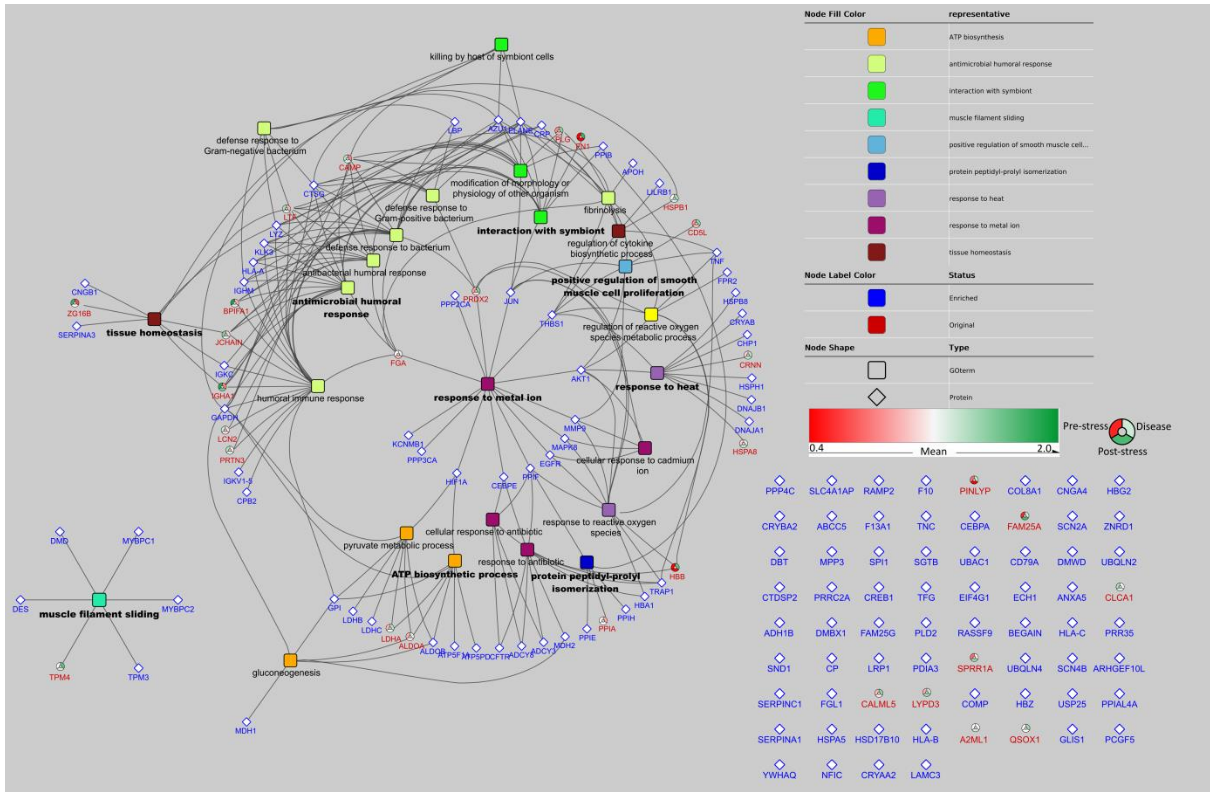

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