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1 **Comparative proteomic profiling of myofibrillar**  
2 **proteins in dry-cured ham with different**  
3 **proteolysis index and adhesiveness**

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21           **Abstract**

22           Excessive proteolysis during the dry-cured ham processing may lead to high  
23 adhesiveness and consumer dissatisfaction. The aim of this research is to identify  
24 biomarkers for proteolysis and adhesiveness. Two hundred *biceps femoris* porcine  
25 muscle samples from Spanish dry-cured ham were firstly evaluated for various physico-  
26 chemical parameters, including the proteolytic index and instrumental adhesiveness.  
27 Proteins of samples with extreme **proteolytic index** were subsequently separated by two-  
28 dimensional electrophoresis and identified by tandem mass spectrometry (MALDI-  
29 TOF/TOF). We found that hams of higher **proteolytic index** had significantly ( $P<0.05$ )  
30 increased adhesiveness. Proteomic analysis revealed marked **proteolytic index**  
31 dependent qualitative and quantitative differences. Thus, protein fragments increased  
32 remarkably in samples with higher **proteolytic index** scores. In addition, a total of five  
33 non-redundant myofibrillar and sarcoplasmic proteins showed increased degradation in  
34 hams of higher **proteolytic index**. However, myosin-1,  $\alpha$ -actin and myosin-4 proteins  
35 seem to be the most reliable biomarkers for proteolysis and adhesiveness because they  
36 underwent the most intense response to proteolysis.

37

38           **Keywords:** Defective textures; Instrumental adhesiveness; Meat proteomics;  
39 Protein degradation; Relative change measure

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41           **1. Introduction**

42           Dry-cured ham is a high-quality food product traditionally consumed in Europe. A  
43 wide variety of physico-chemical changes during the elaboration process influence the  
44 final product characteristics such as flavor and texture (Bermudez, Franco, Carballo, &  
45 Lorenzo, 2014a). Salting and ripening are the two main steps in the elaboration process  
46 of dry-cured ham. The curing processing requires salt as preserving agent. The amount  
47 and type of salt have a significant influence on flavor, texture, color and overall quality  
48 of the final product (Paredi, Sentandreu, Mozzarelli, Fadda, Hollung, & Almeida, 2013;  
49 Toldrá, Flores, & Sanz, 1997). The proteins undergo an intense proteolysis during the  
50 ripening process, which constitutes the most important enzymatic reactions regarding  
51 muscle proteins (Bermúdez, Franco, Carballo, Sentandreu, & Lorenzo, 2014b; Lorenzo,  
52 Cittadini, Bermúdez, Munekata, & Domínguez, 2015). Salt content together with other  
53 many factors, such as rearing conditions (e.g. feeding, sex and slaughter age), pig line,  
54 features of raw product (initial weigh, fat level and pH), type of muscle and the ripening  
55 process, have a recognized impact on protein denaturation of dry-cured hams (Škrlep *et*  
56 *al.*, 2011; Théron, Sayd, Pinguet, Chambon, Robert, & Santé-Lhoutellier, 2011).

57           The intensity of proteolysis during dry-cured ham processing is often measured by  
58 the proteolysis index. It is defined as the percentage of non-protein nitrogen accounting  
59 for total nitrogen. The relationship between **proteolytic index** and texture throughout the  
60 dry-cured ham process has been previously studied under a variety of variables  
61 including pH, water and NaCl content and lipid oxidation (**García-Garrido, Quiles-**  
62 **Zafra, Tapiador, & Luque de Castro, 1999, 2000; Harkouss *et al.*, 2015; Ruiz-Ramírez,**  
63 **Arnau, Serra, & Gou, 2006; Virgili, Parolari, Schivazappa, Bordini, & Borri, 1995**). The

64 **proteolytic index** of good quality Spanish dry-cured ham is considered to be between 33  
65 and 36%, whereas in Italian is between 22 and 30% (Careri, Mangia, Barbieri, Bouoni,  
66 Virgili, & Parolari, 1993). Myofibrillar and sarcoplasmic proteins are intensively  
67 degraded during the ripening process contributing to dry-cured ham texture and ultimate  
68 quality (Bermúdez *et al.*, 2014b). But myofibrillar proteins are the major fraction of the  
69 total, accounting for **around** 65-70% muscle proteins (Lana, & Zolla, 2016).  
70 Accordingly, proteolytic changes in this protein fraction are important for the  
71 development of texture and sensorial characteristics. In particular, myosin and actin are  
72 two main targets of proteolysis (Mora, Sentandreu, & Toldrá, 2011; Théron *et al.*,  
73 2011). However, excessive proteolysis may generate the pastiness defect characterized  
74 by excessive softness, mushy texture and unpleasant flavors (Škrlep *et al.*, 2011). In this  
75 regard, Morales, Arnau, Serra, Guerrero, and Gou (2008) showed that there is a close  
76 relationship between pastiness and adhesiveness. Therefore, the determination of  
77 instrumental adhesiveness could be good indicator of pastiness level in dry-cured ham.

78 Proteomics has great potential to enhance our knowledge on the biochemical  
79 processes underlying the conversion of muscle into meat and identify biomarkers for  
80 meat quality traits (Lana, & Zolla, 2016; Paredi, Raboni, Bendixen, Almeida, &  
81 Mozzarelli, 2012; Paredi *et al.*, 2013). In dry-cured ham, proteomic studies, generally  
82 based on one- or two-dimensional electrophoresis coupled to mass spectrometry, have  
83 tackled a wide diversity of topics. For instance, variations in quality traits, evolution of  
84 proteolysis during its processing, comparative proteomics profiling of *biceps femoris*  
85 and *semimembranosus* muscles and identification of antioxidant peptides (Di Luccia *et*  
86 *al.*, 2005; Mora, Escudero, Fraser, Aristoy, & Toldrá, 2014; Petrova, Tolstorebrov,

87 Mora, Toldrá, & Eikevik, 2016, Škrlep *et al.*, 2011; Théron *et al.*, 2011). To the best of  
88 our knowledge, however, proteome changes linked to differential adhesiveness have not  
89 been previously reported.

90 In this study, we undertook a comparative proteomic profiling in *biceps femoris*  
91 muscle from dry-cured hams with different proteolysis index to identify biomarkers for  
92 differential proteolytic activity and adhesiveness, using two-dimensional electrophoresis  
93 and tandem mass spectrometry (MALDI-TOF/TOF MS).

## 94 **2. Materials and methods**

### 95 *2.1. Dry-cured ham samples*

96 Two hundred raw hams (average weight of  $11.72 \pm 1.06$  kg) obtained from a  
97 commercial slaughterhouse resulting from Large White  $\times$  Landrace breed crosses were  
98 elaborated according to the traditional system with some modifications regarding the  
99 temperature at specific steps in order to ensure hams with high proteolysis. At the end  
100 of process, hams were cut and boned and the cushion part containing *biceps femoris*  
101 muscle was excised and sampled. Six biological replicates of low proteolysis and high  
102 proteolysis dry-cured hams were selected for texture, chemical and proteomic analysis  
103 according to their proteolytic index scores: low proteolysis, proteolytic index  $< 33\%$ ;  
104 and high proteolysis, proteolytic index  $> 36\%$ . Ten slices from each dry-cured ham were  
105 vacuum packed and stored at room temperature for no longer than 4 weeks for analysis.

### 106 *2.2. Instrumental texture*

107 Textural analysis was performed using a texture analyzer (Stable Micro Systems,  
108 TA-XT Plus, London, UK) by carrying out a separation test using different load cells  
109 with a specific probe. Instrumental adhesiveness was measured in sliced ham samples (1

110 mm) by applying probe tests and calculating the negative area of a force-time curve in  
111 tension tests with a single-cycle. The texturometer was equipped with a probe connected  
112 to a special device that enables horizontal probe displacement. After the separation of  
113 the slices, the probe returned to the initial position. The conditions for the measurement  
114 of adhesiveness of dry cured ham slices were: load cell = 5 N; speed = 0.5 mm/s and  
115 distance = 100 mm. From the obtained graph force vs. distance, the adhesiveness was  
116 calculated. All the measurements were made in triplicate, at room temperature.

### 117 2.3. Chemical analysis

118 After instrumental adhesiveness determination, *biceps femoris* samples were  
119 minced and subjected to chemical analysis in triplicate. Water content was analysed by  
120 drying at  $103 \pm 2$  °C until reaching a constant weight (AOAC, 1990); whereas the  
121 chloride content was analysed according to ISO 1841-2 (1996) using a potentiometric  
122 titrator 785 DMP Titrino (Metrohm, Herisau, Switzerland) and results were expressed  
123 as percentage of NaCl.

### 124 2.4. Proteolysis index

125 Total nitrogen content (NT) was determined with Kjeldahl method (ISO R-937,  
126 1978) using the Vapodest 50S analyser (Gerhardt, Königswinter, Germany). It concerns  
127 a semi-micro rapid routine method using block-digestion, copper catalyst and steam  
128 distillation into boric acid. A known quantity of the sample ( $1 \pm 0.1$  g) was taken in the  
129 Kjeldatherm digestion tube of the Vapodest. Added 20 mL of H<sub>2</sub>SO<sub>4</sub> solution to the  
130 tube. Then, the tube was placed onto Vapodest and steam digestion was started for 4  
131 minutes. The steam vapor was collected and titrated in a 250 mL volumetric flask.

132 For non-protein nitrogen, preparation of sample was performed as described by  
133 Lorenzo, García Fontán, Franco, & Carballo (2008). 2.5 g of sample was homogenised  
134 in 25 mL of deionized water and centrifuged. Afterwards, 10 mL of 20% trichloroacetic  
135 acid (99.5% purity, Merck, Darmstadt, Germany) was added, stirred well and let to  
136 stabilize for 60 min at room temperature. After centrifugation, the supernatant was  
137 filtered and 15 mL of filtrate was used for determination of nitrogen as described above  
138 for total nitrogen (NT, ISO R-937, 1978). The **proteolytic index** was calculated as the  
139 ratio (**non-protein nitrogen /nitrogen total**)  $\times$  100 according to Ruiz-Ramírez *et al.*  
140 (2006).

#### 141 **2.5. Protein extraction for proteomic analysis**

142 Total protein from *biceps femoris* muscle was extracted from 50 mg of lyophilized  
143 dry-cured ham. Samples were homogenized with 1 mL of lysis buffer (7 M urea; 2 M  
144 thiourea; 4% CHAPS; 10 mM DTT, and 2% Pharmalyte™ pH 3-10, GE Healthcare,  
145 Uppsala, Sweden) and sonicating (Sonifier 250, Branson, Danbury, CC, USA) in short  
146 pulses at 0 °C. Excess salts and other interfering substances were removed twice using  
147 the 2-D Clean-Up Kit (GE Healthcare) following manufacturer's indications. This  
148 method for selectively protein precipitating was carried out using 200 µL of sonicated  
149 sample and the resulting pellet was dissolved in 210 µL of lysis buffer. The protein  
150 concentration was assessed using a commercial CB-X protein assay kit (G-Biosciences,  
151 St. Louis, MO, USA) according to the manufacturer instructions in a Chromate®  
152 microplate reader (Awareness Technology, Palm City, FL, USA).

#### 153 **2.6. Two-dimensional electrophoresis (2-DE)**



154 The 2-DE was performed according to Franco *et al.* (2015a). Briefly, 250 µg of  
155 protein in lysis buffer was mixed with rehydration buffer (7 M urea, 2 M thiourea, 4%  
156 CHAPS, 0.002% bromophenol blue), reaching 450 µL of total volume. Finally, 0.6%  
157 DTT and 1% IPG buffer (Bio-Rad Laboratories) were added. This protein extract was  
158 loaded into immobilized pH gradient (IPG) strips (24 cm, pH 4-7 linear, Bio-Rad  
159 Laboratories, Hercules, CA, USA). The isoelectric focusing (IEF) was carried out on a  
160 PROTEAN IEF cell system (Bio-Rad Laboratories). Low voltage (50 V) was applied to  
161 rehydrate the strips and then an increasing voltage ramp until to reach 70 kVh. After  
162 IEF, strips were soaked in equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 2% SDS,  
163 30% glycerol) successively supplemented with 1% DTT and 2.5% iodoacetamide for  
164 15 min each. Second dimension was performed using an Ettan DALTsix vertical gel  
165 system (GE Healthcare) with 12% SDS-PAGE gels at 18 mA/gel until the bromophenol  
166 blue dye front reached the end of the gels. The 2-DE gels were stained with SYPRO  
167 Ruby fluorescent stain (Lonza, Rockland, ME, USA).

### 168 **2.7. Image analysis of 2-DE gels**

169 Gels were visualized and digitalized using the Gel Doc XR+ system (Bio-Rad  
170 Laboratories). The detection and quantification of spot volumes were performed with  
171 PDQuest Advanced software v. 8.0.1 (Bio-Rad Laboratories) after background  
172 subtraction. Relative volumes of spots were obtained considering the total intensity  
173 value of image pixels. Observed values of molecular mass ( $M_r$ ) were determined across  
174 protein spots from standard molecular mass markers ranging from 15 to 200 kDa  
175 (Fermentas, Ontario, Canada), whereas those of isoelectric point (pI) were established  
176 according their position on the IEF-strips.

## 2.8. Protein identification by mass spectrometry (MS)

For MALDI TOF/TOF MS analysis, selected spots were excised from the gel and they were dehydrated with acetonitrile using a vacuum centrifuge. The gel piece was washed with Ambic buffer (50 mM ammonium bicarbonate in 50% methanol). The proteins were reduced with 10 mM DTT in 50 mM ammonium bicarbonate and alkylated with 55 mM acetoamide in 50 mM ammonium bicarbonate. Extracts were repeatedly rinsed with Ambic buffer, dehydrated by addition of acetonitrile and dried in a SpeedVac. Then the proteins were hydrolysed with 20 µg/µL of trypsin in 20 mM ammonium bicarbonate for a total volume of 30 µL overnight at 37 °C. The total digest was incubated three times in 40 µL of 60% acetonitrile with 5% formic acid, concentrated in a SpeedVac and stored at -20 °C until analysis. Dried samples were dissolved in 4 µL of 0.5 % acetic acid. Equal volumes (0.5 µL) of peptide and matrix solution, consisting of 3 mg of  $\alpha$ -cyano-4-hydroxycinnamic acid dissolved in 1 mL of 50 % acetonitrile and 0.1 % trifluoroacetic acid, were deposited onto a 384 Opti-TOF MALDI plate (Applied Biosystems, Foster City, CA, USA) using the thin layer method. Mass spectrometric data were obtained in an automated analysis loop using 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA, USA). MS spectra were acquired in positive-ion reflector mode with a Nd:YAG, 355 nm wavelength laser, averaging 1000 laser shots, and at least three trypsin autolysis peaks were used as internal calibration. All MS/MS spectra were performed by selecting the precursors with a relative resolution of 300 (FWHM) and metastable suppression. Automated analysis of mass data were achieved using the 4000 Series Explorer Software v. 3.5 (Applied Biosystems, Foster City, CA, USA). Peptide mass fingerprint and peptide

200 fragmentation spectra data of each sample were combined using the GPS Explorer  
201 Software v. 3.6 and Mascot software v. 2.1 (Matrix Science, Boston, MA, USA) to  
202 search against UniProt/SwissProt database. A 50 ppm precursor tolerance, 0.6 Da  
203 MS/MS fragment tolerance, carbamidomethyl cysteine were used as fixed modification,  
204 oxidized methionine as variable modification and permitting one missed cleavage.  
205 Proteins with at least two matched peptides and statistically significant ( $P$ -value <0.05)  
206 MASCOT scores were selected as positively identified.

## 207 2.9. Statistical analysis

208 Statistical analysis of the results for physico-chemical parameters was performed  
209 by an analysis of variance (ANOVA) using IBM SPSS Statistics V21.0 (SPSS, Chicago,  
210 USA) software package.

211 Quantitative changes of 2d gel spot volumes in sample groups were assessed  
212 using the measures “fold change” ( $FC$ ) and “relative change” ( $RC$ ) (Franco et al.,  
213 2015a, b). The measure fold change is given by  $FC = V_{\text{high}}/V_{\text{low}}$ , where  $V_{\text{high}}$  and  $V_{\text{low}}$   
214 are the mean volumes in samples with high and low proteolysis level, respectively. Fold  
215 change values less than one were represented as their negative reciprocal. The relative  
216 change is provided by the relationship  $RC = DV / |DV_{\text{max}}|$ , where  $DV = V_{\text{high}} - V_{\text{low}}$  and  
217  $DV_{\text{max}}$  is the maximum observed value of  $DV$  over spots.

218 Bootstrapping was used to obtain 95% confidence intervals for the means of spot  
219 volume across replicates as previously described (Franco *et al.*, 2015a, b). For each set  
220 of  $N$  (= 4) volume estimates, 20,000 bootstrap samples of size  $N$  were obtained  
221 following a Monte Carlo algorithm. The 95% bootstrap confidence intervals were  
222 obtained by the bias-corrected percentile method from distribution of bootstrap mean

223 replications (Efron, 1982). Confidence intervals were adjusted for multiple hypothesis  
224 testing with the Bonferroni procedure.

### 225 3. Results and discussion

#### 226 3.1. Proteolysis index and instrumental adhesiveness of dry-cured hams

227 A total of two hundred dry-cured hams were analyzed for the following physico-  
228 chemical parameters: proteolysis index, instrumental adhesiveness, moisture, salt  
229 content, non-protein nitrogen and total nitrogen. Four biological replicates exhibiting  
230 extreme and statistically significant ( $P$ -value  $<0.05$ ) differences in proteolytic index  
231 were eventually selected for proteomic analysis. Mean ( $\pm$  SE, standard error) proteolytic  
232 index values in the selected sample groups with low and high samples proteolytic index  
233 were  $30.3 \pm 0.68$  and  $38.0 \pm 0.88$ , respectively. Differences in proteolytic index can be  
234 attributed to a large number of factors such as variable raw materials, salting  
235 procedures, ripening process, duration of the different steps involved in the elaboration,  
236 as well as variations of temperature and relative humidity in dry-cured ham processing  
237 (García-Garrido *et al.*, 1999; Pugliese *et al.*, 2015; Škrlep *et al.*, 2011; Zhao, Tian, Liu,  
238 Zhou, Xu, & Li, 2008). In the present study, however, hams were elaborated under  
239 uniform conditions. It suggests that proteolysis can undergo large variations even under  
240 similar processing systems.

241 Table 1 shows mean ( $\pm$  SE) values of instrumental adhesiveness, moisture, salt  
242 content, total nitrogen and non-protein nitrogen in samples with different proteolytic  
243 index (low and high samples) selected for proteomic analysis. It must be highlighted  
244 that adhesiveness of sliced dry-cured ham was assessed, for the first time, by  
245 mechanical procedures as alternative to sensory analysis panel. We found that the

246 instrumental adhesiveness was significantly ( $P<0.001$ ) higher in **high proteolysis** batch  
247 (100.43 g) than in **low proteolysis** batch (66.75 g). Hams with a defective texture can  
248 exhibit high moisture/protein ratios as result of both increased moisture and decreased  
249 protein contents related to ham with a normal texture (García-Garrido *et al.*, 1999). In  
250 addition, several authors (Bermúdez *et al.*, 2014a; Ruiz-Ramírez *et al.*, 2006; Virgili,  
251 Parolari, Schivazappa, Bordini, & Borri, 1995) noticed that proteolytic activity in ham  
252 is governed by salt. However, García-Garrido *et al.* (1999) showed hams with normal  
253 and defective texture containing salt contents from 6.2% to 8.1% by wet weight. In this  
254 study, there were **no** significant differences between sample groups for moisture and salt  
255 content. In contrast, non-protein nitrogen showed significant ( $P<0.01$ ) differences  
256 between treatments, since the lowest values were observed in **low proteolysis** batch  
257 (1.50 vs. 1.84%, for **low and high proteolysis** groups, respectively). This finding is in  
258 agreement with data reported by García-Garrido *et al.* (1999) who observed that non-  
259 protein nitrogen levels were 30% higher in hams of defective texture than in normal  
260 pieces.

### 261 3.2. Comparison of proteomic profiles by 2-DE

262 High-quality 2-DE gels were obtained despite dry-cured ham salt content.  
263 Representative 2-DE gel images of **low and high proteolysis** proteomes **were** shown in  
264 Fig. 1. The identification, matching and volume evaluation of 2-DE spots were obtained  
265 by PDQuest software. The total number of selected spots for proteomic analysis was 92  
266 and 123 spots in **low and high proteolysis** groups, respectively; after the elimination of  
267 saturated or faint spots, as well as non-reproducible spots over replicates. We found that  
268 proteomic profiles of **low and high proteolysis** samples were remarkably differentiated

269 (Table 2). In total, 58 protein spots showed statistically significant differential  
270 abundance by the bootstrap re-sampling statistical method. Note that Bonferroni-  
271 corrected 95% bootstrap **confidence intervals** for means of spot volumes did not overlap  
272 in matched spots of different intensity or did not overlap zero in unique spots. It is  
273 important to highlight that only eight unique spots were observed in **low proteolysis**  
274 samples, whereas in **high proteolysis** were 37 spots ( $P < 0.001$ , Fisher's exact test). This  
275 difference probably reflects an increased protein fragmentation in **high proteolysis**  
276 samples.

### 277 3.3. Evaluation of protein fragmentation

278 Protein fragmentation in **low and high proteolysis** hams was evaluated by the  
279 following procedure. First, protein identification of differentially abundant spots was  
280 performed by MALDI-TOF/TOF MS. Second, spots containing protein fragments were  
281 assessed by comparing the theoretical molecular mass of each protein with the  
282 molecular mass observed on 2-DE gel. Protein fragments were eventually validated  
283 when the ratio between theoretical and empirical masses was above 1.5 kDa. We found  
284 that most differentially abundant protein spots in **low and high proteolysis** ham samples  
285 (40 out of 58 spots) were successfully identified ( $P < 0.05$ ) by MALDI-TOF/TOF MS  
286 (Table 3). The comparison of theoretical and observed molecular masses revealed that  
287 an important number (55%) of identified spots contained protein fragments (Table 3). It  
288 is noteworthy, however, that most (86%) of these spots were actually unique spots  
289 present only in **high proteolysis** samples (Table 2). Accordingly, the proteomic profile  
290 in dry-cured ham samples of higher **proteolysis index** showed increased levels of  
291 protein fragmentation. It also shows that **proteolysis index** scores can be good indicators

292 of differential proteolysis over proteomes. The remaining spots, with theoretical and  
293 empirical mass ratios below 1.5 kDa, were excluded for further analysis. It is not  
294 possible to assess whether they actually contain either entire or slightly degraded  
295 proteins at the level of resolution of 2-DE.

296 All fragments detected in our study corresponded to seven non-redundant  
297 myofibrillar or sarcoplasmic muscle proteins: myosin-1 (MYH1), myosin-4 (MYH4),  $\alpha$ -  
298 4 glucan phosphorylase (F1RQQ8),  $\alpha$ -actin (ACTS or ACTA1), heat shock 70 kDa  
299 protein 1-like (HS71L), myosin-7 (MYH7) and vinculin (VINC). However, most  
300 fragments (86%) resulted from hydrolysis of myosin heavy chain and  $\alpha$ -actin  
301 myofibrillar proteins: nine MYH1 spots, four MYH4 spots, one MYH7 spots and five  
302 ACTS spots (Table 3). It is noteworthy, however, that the amount of protein fragments  
303 does not provide determinant information by itself to reliably evaluate the extent of  
304 differential proteolysis over proteins and sample groups. A complete characterization of  
305 differential proteolysis not only requires determining the number of protein fragments,  
306 but also the quantification of their volumes.

#### 307 *3.4. Candidate biomarkers for differential proteolysis and adhesiveness*

308 Quantitative differences in proteolysis intensity between low and high proteolysis  
309 ham batches were assessed by fold and relative change statistics from protein fragment  
310 volumes. Table 4 shows fold and relative change values for each protein found to be  
311 differentially affected by proteolysis. There can be seen that fold and relative change  
312 provide very discrepant information about the extent of proteolysis across proteins. It is  
313 worth noting that fold change is a measure traditionally used to quantify differential  
314 protein abundance between treatments. But it has the disadvantage that its range varies

315 from  $-\infty$  to  $+\infty$  and range boundaries are achieved with the presence of unique spots  
316 independently of the existing differences in volume. In contrast, **relative change** always  
317 ranges from -1.0 to +1.0. It provides; therefore, a more intuitive measure of the strength  
318 of change and maximum values of its range are not necessarily achieved with the mere  
319 occurrence of unique spots (see Table 4). Accordingly, **relative change** is particularly  
320 appropriate measure for the analysis of degraded proteome profiles exhibiting large  
321 number of unique spots. In the present study, we found that **relative change** values over  
322 proteins ranged between -0.04 and +1.0 (Table 4). Only five proteins (i.e. MYH1,  
323 ACTS, MYH4, HS71L and F1RQQ8) showed positive **relative change** values,  
324 indicating that their fragments were over-represented in **high proteolysis** hams. In  
325 contrast, MYH7 and VINC proteins underwent decreased proteolysis in **high proteolysis**  
326 samples given that their **relative change** values were of negative sign. This result  
327 suggests that MYH7 and VINC proteins are not useful biomarkers of proteolysis  
328 intensity.

329 MYH1, ACTS and MYH4 proteins showed the highest level of degradation in  
330 **high proteolysis** samples (**relative change** values  $> 0.40$ ). Previous proteomic studies  
331 based on one-dimensional electrophoresis and 2-DE have systematically demonstrated  
332 that myosin heavy chain and  $\alpha$ -actin are main targets of proteolysis in the *biceps femoris*  
333 muscle, particularly at the end of ripening (Larrea *et al.*, 2006; Tabilo, Flores, Fiszman,  
334 & Toldrá, 1999; Théron *et al.*, 2011; Toldrá, Rico, & Flores, 1993). In 12-month old  
335 Parma and S. Daniele dry-cured ham, most isoforms of myosin and actin were found to  
336 be completely hydrolysed (Di Luccia *et al.*, 2005). We found that MYH1 (**relative**  
337 **change** = +1) was a more sensitive biomarker for proteolysis than ACTS (**relative**



338 **change** = +0.60). This difference can be attributed to the fact that the myosin is more  
339 sensitive to denaturation by salt content (Graiver, Pinotti, Califano, & Zaritzky, 2006).  
340 However, we found that two specific isoforms of the myosin heavy chain (MYH1 and  
341 MYH4) were intensively degraded in response to proteolysis. It suggests that these two  
342 myosin heavy chain isoforms might exhibit differential susceptibility to degradation by  
343 proteolytic enzymes during dry-cured ham processing. In this regard, Théron *et al.*  
344 (2011) reported differential MYH1 or MYH4 fragmentation in *biceps femoris* and  
345 *semimembranosus* muscles with different proteolytic activity due to differences in salt  
346 and moisture content in the course of dry-cured ham processing. Specifically, fragments  
347 of these two myosin heavy chains isoforms were overrepresented in *biceps femoris*  
348 muscle that is an internal muscle with lower NaCl concentration, higher water content  
349 and increased proteolytic activity. Taken together, the available evidence suggests that  
350 MYH1 and MYH4 can be suitable biomarkers for proteolysis under different scenarios.

351 Of the five differentially fragmented proteins in the present study, two were  
352 sarcoplasmic proteins: HS71L and F1RQQ8. They are proteins with a considerably  
353 lower relative representation in the proteome of *biceps femoris* muscle, which explains  
354 their low **relative change** values (<0.10). The HS71L protein is a molecular chaperone  
355 that appears to play a critical role in multiple cellular functions, including protection of  
356 the proteome in response to stress, activation of proteolysis of misfolded proteins and  
357 controlling the targeting of proteins for subsequent degradation (Archivald *et al.*, 2010;  
358 Radons, 2016; The UniProt Consortium, 2017). On the other hand, the F1RQQ8 protein  
359 is a phosphorylase that catalyzes and regulates the breakdown of glycogen to glucose-1-  
360 phosphate for the generation of ATP during glycogenolysis (Archivald *et al.*, 2010;

361 Gautron, Daegelen, Menecier, Dubocq, Kahn, & Dreyfus, 1987; The UniProt  
362 Consortium, 2017). Fragments of F1RQQ8 result from proteolytic activity were also  
363 detected in post-mortem *longissimus dorsi* porcine muscle (Lametsch, Roepstorff, &  
364 Bendixen, 2002), as well as in dry-cured *biceps femoris* and *semimembranosus* muscles  
365 (Théron *et al.*, 2011). Specifically, the *biceps femoris* muscle showed more F1RQQ8  
366 fragments than the *semimembranosus* muscle during the ripening of dry-cured ham due  
367 to its higher proteolytic activity (Théron *et al.*, 2011). It follows F1RQQ8 is a good  
368 biomarker of proteolysis in agreement with our observations.

369 In the present study, we found that the proteolytic activity correlated positively  
370 with the extent of sliced dry-cured ham instrumental adhesiveness. Therefore, the  
371 identified biomarkers also apply for the meat quality trait of adhesiveness. These  
372 biomarkers provide non-invasive tools alternative to sensory analysis panel or  
373 mechanical measures in order to assess variations in adhesiveness. The identified  
374 proteins can also be potential biomarkers for other proteolysis-related porcine quality  
375 traits. It is particularly true in the case of pastiness considering that pastiness variations  
376 are closely related with the extent of proteolysis and adhesiveness (Morales *et al.*, 2008;  
377 Škrlep *et al.*, 2011).

#### 378 **4. Conclusions**

379 Comparison of dry-cured ham proteomic profiles with extreme proteolysis index  
380 scores, based on two-dimensional electrophoresis coupled to tandem mass spectrometry,  
381 allowed us to identify novel candidate biomarkers for differential proteolytic activity  
382 underlying meat quality traits. First of all, we found that the proteolytic index is a  
383 reliable indicator of the extent of protein hydrolysis at proteomic scale and instrumental

384 adhesiveness of sliced dry-cured ham. A total of five myofibrillar and sarcoplasmic  
385 proteins of *biceps femoris* muscle were identified as candidate markers for proteolysis  
386 and adhesiveness. However, two distinct isoforms of the myosin heavy chain (myosin-1  
387 and myosin-4) and  $\alpha$ -actin exhibited the strongest response to variable proteolysis as  
388 well as to adhesiveness according to the measure of relative change. These proteins  
389 could also be potential candidate biomarkers for quality traits closely linked to  
390 proteolysis such as pastiness. Further research is clearly needed to precisely assess the  
391 relationship of these markers with proteolysis-related quality traits under a wide range  
392 of dry-cured ham elaboration conditions.

#### 393 **Conflict of Interest statement**

394 The authors declare no conflict of interest.

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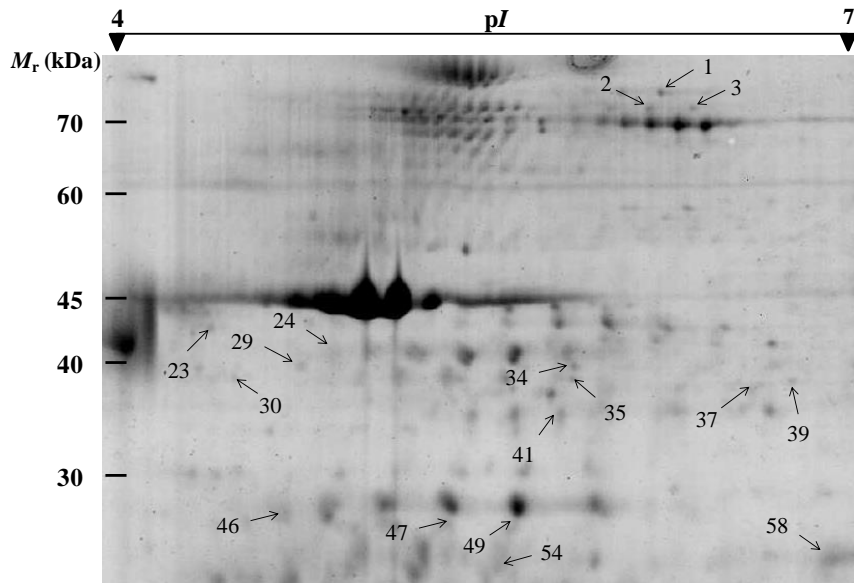


518 **Figure captions**

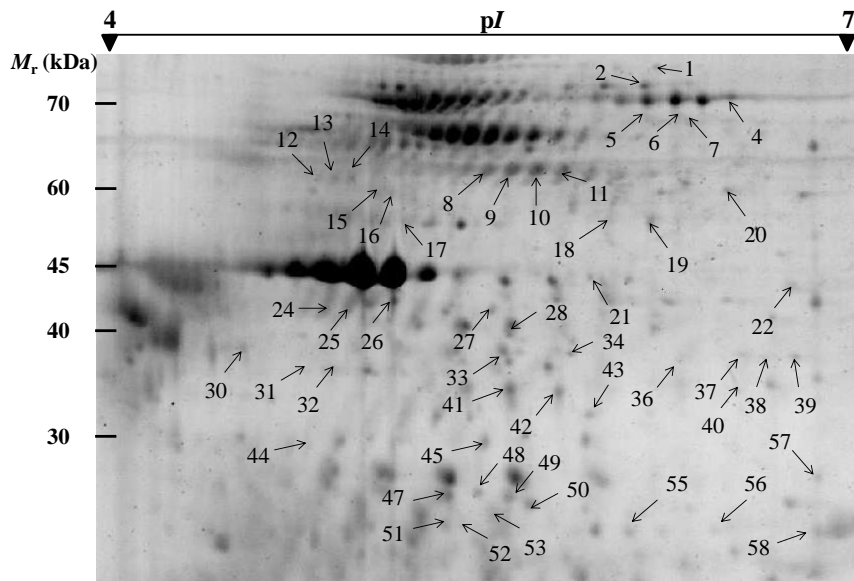
519 **Fig. 1.** 2-DE gel images showing the proteome profile of dry-cured ham with low  
520 (A) and high (B) proteolysis index (LP and HP samples, respectively). Protein spots  
521 with statistically significant qualitative (presence/absence) and quantitative (changes in  
522 intensity) differences are marked and numbered. All these spots were excised for further  
523 analysis by MALDI-TOF/TOF MS.

**Figure 1**

**A) Low proteolysis index (LP samples)**



**B) High proteolysis index (HP samples)**



## **Highlights**

- Instrumental adhesiveness was assessed for the first time in dry-cured ham
- The proteolysis index is indicator of differential adhesiveness
- Myosin-1, myosin-4 and actin underwent the strongest response to proteolysis
- Novel candidate biomarkers for proteolysis and adhesiveness

**Table 1.-** Mean ( $\pm$  SE) values of physico-chemical parameters in dry-cured hams with different proteolysis index selected for proteomic analysis.

Parameters	Batch		<i>p</i> -value
	LP	HP	
Instrumental adhesiveness (g)	66.75 $\pm$ 4.87	100.43 $\pm$ 2.86	0.001
Moisture (%)	59.10 $\pm$ 0.14	58.57 $\pm$ 0.16	0.052
Salt content (%)	4.67 $\pm$ 0.05	4.69 $\pm$ 0.10	0.884
Non-protein nitrogen (%)	1.50 $\pm$ 0.07	1.84 $\pm$ 0.04	0.010
Total nitrogen (%)	4.97 $\pm$ 0.19	4.84 $\pm$ 0.03	0.539

Batches: LP = low proteolysis ( $PI < 33\%$ ); HP = high proteolysis ( $PI > 36\%$ ).

**Table 2.-** Spot volumes with statistically significant ( $p$ -value < 0.05) differential abundance in dry-cured hams of low and high proteolysis level.

Spot No.	Low proteolysis (LP)			High proteolysis (HP)		
	Mean ( $\pm$ SE) Volume	$P(\hat{\theta}_B \leq \hat{\theta})$	95% bootstrap CI (CL, CU)	Mean ( $\pm$ SE) Volume	$P(\hat{\theta}_B \leq \hat{\theta})$	95% bootstrap CI (CL, CU)
1	684 $\pm$ 31	0.57	617, 746	280 $\pm$ 75	0.53	79, 409
2	741 $\pm$ 150	0.53	353, 962	1531 $\pm$ 128	0.52	1259, 1742
3	392 $\pm$ 81	0.55	247, 554	–	–	–
4	–	–	–	1360 $\pm$ 215	0.54	815, 1712
5	–	–	–	307 $\pm$ 18	0.75	281, 333
6	–	–	–	271 $\pm$ 25	0.73	236, 306
7	–	–	–	366 $\pm$ 113	0.58	121, 566
8	–	–	–	2010 $\pm$ 419	0.60	1241, 2904
9	–	–	–	2186 $\pm$ 473	0.56	1320, 3073
10	–	–	–	2360 $\pm$ 500	0.53	1348, 3212
11	–	–	–	1174 $\pm$ 342	0.56	647, 2156

12	–	–	–	$688 \pm 95$	0.49	520, 881
13	–	–	–	$667 \pm 219$	0.54	53, 1014
14	–	–	–	$1302 \pm 257$	0.58	976, 1830
15	–	–	–	$661 \pm 58$	0.55	509, 764
16	–	–	–	$508 \pm 43$	0.56	422, 589
17	–	–	–	$655 \pm 185$	0.64	377, 1074
18	–	–	–	$619 \pm 194$	0.60	229, 1003
19	–	–	–	$582 \pm 193$	0.56	237, 974
20	–	–	–	$163 \pm 13$	0.75	145, 182
21	–	–	–	$468 \pm 116$	0.53	259, 695
22	–	–	–	$798 \pm 176$	0.49	437, 999
23	$234 \pm 16$	0.75	211, 257	–	–	–
24	$725 \pm 183$	0.49	341, 993	$1801 \pm 212$	0.68	1419, 2259
25	–	–	–	$1459 \pm 56$	0.76	1379, 1537
26	–	–	–	$1980 \pm 327$	0.75	1518, 2443
27	–	–	–	$477 \pm 112$	0.51	248, 602

28	–	–	–	3396 ± 855	0.62	2016, 5152
29	283 ± 122	0.52	67, 510	–	–	–
30	235 ± 65	0.67	84, 310	489 ± 65	0.67	409, 639
31	–	–	–	324 ± 95	0.51	99, 541
32	–	–	–	507 ± 160	0.61	185, 826
33	–	–	–	477 ± 112	0.51	248,602
34	1079 ± 177	0.75	829, 1329	443 ± 178	0.62	318, 652
35	524 ± 99	0.77	394, 674	–	–	–
36	–	–	–	387 ± 16	0.61	359, 422
37	255 ± 6	0.76	246, 263	333 ± 40	0.64	284, 426
38	–	–	–	142 ± 66	0.67	37, 289
39	252 ± 29	0.54	172, 302	455 ± 98	0.58	338, 658
40	–	–	–	266 ± 47	0.53	158, 358
41	1756 ± 408	0.56	957, 2485	3274 ± 249	0.56	2990, 3783
42	965 ± 267	0.55	649, 1511	2041 ± 254	0.56	1577, 2555
43	–	–	–	544 ± 82	0.52	372, 667

44	–	–	–	1103 ± 113	0.74	943, 1264
45	1145 ± 197	0.56	814, 1556	–	–	–
46	465 ± 43	0.76	405, 525	–	–	–
47	475 ± 86	0.73	354, 597	1469 ± 302	0.56	722, 1963
48	–	–	–	608 ± 31	0.63	567, 679
49	779 ± 34	0.62	706, 843	1517 ± 312	0.58	1112, 2441
50	–	–	–	1370 ± 46	0.59	1277, 1462
51	–	–	–	622 ± 33	0.71	0.569, 0.697
52	1089 ± 344	0.66	543, 1862	–	–	–
53	–	–	–	2544 ± 665	0.62	1485, 4037
54	1622 ± 462	0.55	654, 2496	–	–	–
55	–	–	–	313 ± 116	0.58	46, 537
56	–	–	–	661 ± 292	0.61	28, 1180
57	683 ± 67	0.74	589, 777	352 ± 62	0.75	264, 440
58	643 ± 90	0.63	634, 849	399 ± 121	0.56	156, 623

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Gel position of spots is shown in Fig. 1.



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Mean ( $\pm$  SE) volumes were obtained from four biological replicates.

Bootstrap confidence intervals (CIs) were obtained by the bias-corrected percentile method from 20,000 bootstrap mean replications; Bonferroni method was applied to obtain simultaneous CIs over comparisons; CL and CL are the lower and upper bounds, respectively.

The bootstrap distribution was median biased if  $P(\hat{\theta}_B \leq \hat{\theta}) \neq 0.50$ , where  $\hat{\theta}_B$  and  $\hat{\theta}$  are the bootstrap and sample mean estimates, respectively.

**Table 3.-** Protein identification by MALDI-TOF/TOF MS of differentially ( $p$ -value < 0.05) represented 2-DE spots in dry-cured hams with low and high proteolysis index.

Spot No.	Protein	Abbrev.	Accession number (Uniprot)	Mascot score	Sequence coverage (%)	Number of matched peptides	$pI$ Th/Obs	$M_r$ Th/Obs (kDa)	
1	Vinculin	VINC	P26234	60	19	17	5.6/6.2	124.4/76.1	Fragment
2	Serum albumin	ALBU	P08835	144	21	13	6.1/6.1	71.6/72.9	
3	Serum albumin	ALBU	P08835	125	21	14	6.1/6.3	71.6/73.2	
4	Serum albumin	ALBU	P08835	601	42	19	6.1/6.5	71.6/70.7	
5	Serum albumin	ALBU	P08835	56	10	7	6.1/6.1	71.6/66.3	
9	Myosin-1	MYH1	Q9TV61	503	17	36	5.6/5.6	224.4/59.6	Fragment
10	Myosin-1	MYH1	Q9TV61	373	15	31	5.6/5.6	224.4/62.6	Fragment
11	Myosin-1	MYH1	Q9TV61	493	16	35	5.6/5.7	224.4/62.8	Fragment
12	Myosin-1	MYH1	Q9TV61	582	16	30	5.6/4.7	224.4/53.3	Fragment
13	Myosin-1	MYH1	Q9TV61	331	8	15	5.6/4.8	224.4/52.9	Fragment
14	Myosin-1	MYH1	Q9TV61	467	15	28	5.6/4.9	224.4/52.8	Fragment

15	Myosin-1	F1SS62	Q9TV61	287	24	34	5.5/5.1	171.0/61.2	Fragment
16	Myosin-4	MYH4	Q9TV62	249	11	19	5.6/5.1	224.0/60.8	Fragment
17	Myosin-1	MYH1	Q9TV61	249	15	25	5.6/5.2	224.4/59.4	Fragment
20	$\alpha$ -1,4 glucan phosphorylase	F1RQQ8	F1RQQ8	102	13	10	6.7/6.5	97.7/55.4	Fragment
21	$\alpha$ -actin, skeletal muscle	ACTS	P68137	180	28	9	5.2/5.9	42.4/45.5	
22	Heat shock 70 kDa protein 1-like	HS71L	A5A8V7	66	6	4	5.6/6.7	70.7/45.1	Fragment
23	Myosin-7	MYH7	P79293	380	13	21	5.6/4.4	223.0/44.2	Fragment
24	$\alpha$ -actin, skeletal muscle	ACTS	P68137	96	14	4	5.2/4.9	42.4/40.1	
25	Myosin-4	MYH4	Q9TV62	241	12	21	5.6/4.9	224.0/43.4	Fragment
26	Myosin-4	MYH4	Q9TV62	701	15	30	5.6/5.1	224.0/43.8	Fragment
28	$\alpha$ -actin, skeletal muscle	ACTS	P68137	255	34	10	5.2/5.6	42.4/40.1	
29	$\alpha$ -actin, skeletal muscle	ACTS	P68137	69	19	5	5.2/4.7	42.4/39.1	
30	Desmin	DESM	P02540	87	10	4	5.2/4.4	53.6/38.0	
31	$\alpha$ -actin, skeletal muscle	ACTS	P68137	94	13	4	5.2/4.8	42.4/42.6	
32	Myosin-4	MYH4	Q9TV62	424	11	22	5.6/4.9	224.0/39.3	Fragment

34	F-actin-capping protein subunit alpha-2	CAZA2	Q29221	269	47	11	5.6/5.8	33.1/39.1	
36	F-actin-capping protein subunit alpha-2	CAZA2	Q29221	67	9	2	5.6/6.1	33.1/35.7	
40	$\beta$ -enolase	ENOB	Q1KYT0	92	23	7	8.1/6.5	47.4/35.1	
44	F-actin-capping protein subunit beta	CAPZB	A0PFK7	395	46	13	5.5/4.9	31.6/31.0	
45	$\alpha$ -actin, skeletal muscle	ACTS	P68137	149	17	5	5.2/5.3	42.4/32.6	
46	$\alpha$ -actin, skeletal muscle	ACTS	P68137	159	30	8	5.2/4.5	42.4/25.5	Fragment
47	$\alpha$ -actin, skeletal muscle	ACTS	P68137	117	12	4	5.2/5.3	42.4/25.4	Fragment
48	Myosin-1	MYH1	Q9TV61	415	15	32	5.6/5.5	224.4/62.5	Fragment
49	$\alpha$ -actin, skeletal muscle	ACTS	P68137	180	17	5	5.2/5.6	42.4/25.4	Fragment
50	Peroxiredoxin-6	PRDX6	Q9TSX9	665	58	15	5.7/5.7	25.0/25.5	
51	$\alpha$ -actin, skeletal muscle	ACTS	P68137	126	14	4	5.2/5.3	42.4/24.0	Fragment
53	$\alpha$ -actin, skeletal muscle	ACTS	P68137	180	14	4	5.2/5.5	42.4/24.2	Fragment
55	Multiprotein bridging factor 1	A6N8P5	A6N8P5	70	49	10	10.0/6.1	16.4/24.0	
56	Triosephosphate isomerase	TPIS	Q29371	85	33	8	7.0/6.6	26.9/24.0	

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All identified proteins were matched to *Sus scrofa*.(pig) proteins.

The Mascot baseline statistically significant ( $p$ -value  $< 0.05$ ) score was 56.

Sequence coverage (%): percentage of coverage of the entire amino acid sequence by matched peptides.

Number of matched peptides: total number of identified spectra matched for the protein.

Theoretical (Th) isoelectric point ( $pI$ ) and molecular mass ( $M_r$ ), were obtained from UniProtKB/Swiss-Prot databases.

Observed (Ob)  $pI$  and  $M_r$  were obtained from the spot position on the gel.

Protein fragments:  $M_r$  (Th)/ $M_r$  (Obs) ratio higher than 1.5.

**Table 4.-** Fold change (*FC*) and relative change (*RC*) of differentially ( $P < 0.05$ ) represented protein fragments in dry-cured ham with different proteolysis index.

Spot No.	Protein (abbrev.) fragment	Fold change ( <i>FC</i> )	Relative change ( <i>RC</i> )
9-17, 48	Myosin-1 (MYH1)	$+\infty$	+1.00
46, 47, 49, 51, 53	$\alpha$ -actin, skeletal muscle (ACTS)	13.23	+0.60
16, 25, 26, 32	Myosin-4 (MYH4)	$+\infty$	+0.43
22	Heat shock 70 kDa protein 1-like (HS71L)	$+\infty$	+0.08
20	$\alpha$ -1,4 glucan phosphorylase (F1RQQ8)	$+\infty$	+0.02
23	Myosin-7 (MYH7)	$-\infty$	-0.02
1	Vinculin (VINC)	-2.44	-0.04