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1 # Metabolic fingerprinting of in-bag dry- and wet-aged lamb with Rapid
2 Evaporative Ionisation Mass Spectroscopy

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Abstract

The effect of in-bag dry- and wet-ageing on metabolite profiles of lamb legs was determined using Rapid Evaporative Ionisation Mass Spectrometry (REIMS). Using orthogonal projection to latent structures-discriminant analysis (OPLS-DA) with REIMS, 1705 metabolite ions were identified ($Q^2 = 0.86$) in four muscles: *m. semimembranosus, m. biceps femoris*, *m. vastus lateralis* and *m. rectus femoris*. A total of 663 metabolites differed between ageing methods ($P < 0.05$) which mainly resulted from proteolysis and lipid metabolism. Dry-aged lamb had higher pH ($P = 0.016$) and lower moisture content ($P = 0.034$) than the wet-aged. Dry-ageing produced more ($P < 0.05$) smaller sized metabolites including dipeptides and free amino acids and lipid oxidation metabolites compared to wet-aged equivalents. Different muscles had distinct REIMS metabolic profiles. Outcomes of this study demonstrated that REIMS can be used for authentication between in-bag dry- and wet-aged lamb based on their metabolic fingerprints.

Keywords: Meat ageing; in-bag dry-aged lamb; REIMS; metabolic fingerprinting; dipeptides.

1 Introduction

Demand for meat products with premium quality is increasing to meet the expectations of an exceptional eating experience. Post-mortem ageing is a widely used processing technique in the meat industry to improve meat quality, in terms of tenderness and characteristic aged flavour (Koutsidis, Elmore, Oruna-Concha, Campo, Wood, & Mottram, 2008; Nishimura, 1998). Post-mortem ageing commonly refers to wet-ageing, an anaerobic maturation process accomplished in the water-impermeable vacuum packaging. Wet-ageing of fresh meat at -1.5 °C is the common practice which is widely utilised in meat industry to produce premium quality meat with extended shelf life. Dry-ageing is another form of post-mortem ageing which allows for moisture evaporation during ageing process under aerobic conditions for developing distinctive dry-aged flavour, such as intense nutty, buttery, and meaty flavour notes with umami taste (Li, Babol, Bredie, Nielsen, Tománková, & Lundström, 2014; O'Quinn et al., 2016).

Traditional dry-ageing is generally carried out without packaging at 0-3 °C to allow moisture evaporation during ageing process and at the same time retard the proliferation of spoilage microorganisms (Kim et al., 2018). A novel dry-ageing technique called “in-bag dry-ageing” has emerged over the last decade. With the use of a water-permeable ageing bag, issues arising from the traditional practice of dry ageing, such as microbial contamination and excessive trimming leading to loss in saleable yield, were resolved without compromising the eating quality of dry-aged meat (Kim et al., 2018; Li et al., 2014; Zhang, Yoo, & Farouk, 2019). The in-bag dry-ageing processing technique is likely to have major economic implications for the meat industry in producing consistent premium quality dry-aged products cheaper.

57 Extensive studies have been carried out using beef samples to investigate the
58 differences in meat quality, microbial safety, sensory and flavour profiles arising from the use
59 of different ageing treatments, but rarely on lamb (Kim et al., 2018; Li et al., 2014; O'Quinn
60 et al., 2016; Zhang et al., 2019). Lamb is favoured by the consumers across the world due to
61 its characteristic flavour profile. However, the impact of different ageing regimes on the
62 biochemical and metabolic changes of lamb and the underlying mechanisms for such changes
63 have not been explored.

64 Metabolic fingerprinting is an emerging approach for understanding of the changes in
65 biological samples arising from inherent properties and/or external factors (Kosmides,
66 Kamisoglu, Calvano, Corbett, & Androulakis, 2013). For example, the dehydration process
67 during dry-ageing could be associated with metabolic changes to the meat that contributed
68 towards the development of distinct flavour profile. Determining such changes could be
69 useful for understanding the dry-ageing process, and identifying biomarkers or fingerprints as
70 the signature for a distinct product or associated with a unique response to a processing
71 treatment. In the case of dry-ageing, the potential signature could be related to the level of
72 dehydration (Lee et al., 2019), formation of unique volatile compounds (O'Quinn et al., 2016)
73 and/or metabolites such as peptides and free amino acids (Kim, Kemp, & Samuelsson, 2016).
74 Dry-ageing of beef has been suggested to produce more abundant free amino acids including
75 tryptophan, phenylalanine, valine, tyrosine, glutamic acid, isoleucine, leucine and histidine,
76 compared to those wet-aged for 3-4 weeks (Kim et al., 2016; Lee et al., 2019).

77 Rapid evaporative ionisation mass spectrometry (REIMS) is a relatively novel mass
78 spectrometry-based metabolic fingerprinting technique which has demonstrated accurate and
79 reliable differentiation of meat according to their species and breeds (Balog et al., 2016).
80 Through the use of REIMS, rapid determination of whole tissue samples is possible to enable
81 detection of adulteration in minced beef (Black et al., 2019), which would impose a

82 significant merit in addressing food safety issues. Compared to other metabolomics
83 techniques, such as liquid or gas chromatography – mass spectrometry (LC or GC-MS) and
84 nuclear magnetic resonance, no sample preparation is required, which removes this as a
85 source of error, and allows a much higher throughput of samples. This reduces cost per
86 sample and can allow many more samples to be analysed in the same timeframe as
87 conventional metabolomics methods, potentially improving statistical power for detecting
88 small changes. The trade-off is less certainty around compound identification and ion
89 suppression, as well as the production of chemical artefacts due to the thermal process
90 required for volatilising samples for REIMS (Ross et al., 2020).

91 The main purpose of this study was to use REIMS fingerprinting (1) to test the
92 hypothesis that in-bag dry-ageing of lamb would produce different metabolic profiles
93 compared to wet-ageing; (2) to understand the underlying biochemical mechanisms which
94 differed between in-bag dry-ageing and wet-ageing of lamb, as observed by lipid oxidation,
95 protein and free amino acids profiles.

96 2 Materials and methods

97 2.1 Ageing treatments

98 Lamb legs for ageing were collected from a local abattoir (Ram, approx. 46 weeks and
99 26 kg carcass weight) following 24 h post-mortem. Paired legs (bone-in, shank and chump
100 off, three pairs, n = 6) from the same animal were randomly assigned to two ageing methods:
101 in-bag dry-ageing and wet-ageing. In-bag dry-ageing was carried out in a water permeable
102 ageing bag (TUBLIN® 10, 50 μm thick, polyamide mix with water vapor transmission rate
103 920 g/50 $\mu/m^2/24$ h at 7 °C, 50% RH, and oxygen transmission rate 660 $g/m^2/24$ h at 7 °C, 50%
104 RH, TUB-EX ApS, Denmark) at 2 ± 0.5 °C, 0.5 $m.s^{-1}$ air velocity and relative humidity of 75
105 ± 5%. Wet ageing of lamb legs using water impermeable barrier bags (Cryovac® A600

106 barrier bag, oxygen transmission rate 20-50 g/m^2/24 h at 23 °C, Sealed Air®, New Zealand)
107 was performed at -1.5 ± 0.5 °C. After 21 days of ageing, the lamb samples from both ageing
108 methods were removed from ageing packages and directly fabricated into chops (1.5 cm thick)
109 without trimming the surface. Lamb chops used for metabolic profiling by REIMS were
110 frozen at -80 °C immediately after ageing. Lamb chops used for biochemical analyses were
111 then deboned and finely minced for further analysis.

112 2.2 pH and proximate analysis, and lipid oxidation

113 The pH of fresh lamb chops before and after the ageing treatments was measured by
114 inserting a calibrated pH probe (Hanna 99,163 pH meter with a FC232D combined
115 temperature and pH insertion probe, Rhode Island, USA) directly into the meat. A 2-point
116 standard buffer solution of 7.01 and 4.01 was used for calibration of the pH meter at ambient
117 temperature. Four measurements were taken from four random positions of each sample.

118 Subsamples (approx. 5 g) were taken from the minced chop (section 2.1) for
119 measurement of moisture content using oven drying (AOAC 950.46) and crude fat content by
120 Soxhlet extraction (AOAC 960.39) according to the AOAC standard analytical methods
121 AOAC (2010). The extent of lipid oxidation arising from different ageing methods was
122 determined by measuring the formation of thiobarbituric acid reactive substances (TBARS)
123 in the minced lamb samples following the method described by Buege and Aust (1978). The
124 TBARS results were expressed as μg of malondialdehyde (MDA)/g fat. All measurements
125 were carried out in triplicate.

126 2.3 REIMS

127 REIMS analysis was carried out on four major muscles of lamb chops: *m.*
128 *semimembranosus* (SM), *m. biceps femoris* (BF), *m. vastus lateralis* (VL), and *m. rectus*
129 *femoris* (RF). Metabolic profiling of lamb samples was performed using an electronic

130 monopolar surgical knife (Electrosurgical pencil, Erbe Medical UK Ltd, UK) coupled to a
131 Waters Xevo® G2 qToF mass spectrometer (Waters Corp., UK). The surgical knife was used
132 to directly cut the thawed lamb chops (each cut or 'burn' was approx. 2 mm deep and 15 mm
133 long) with 3 seconds per cut at 15 W power in cutting mode (Erbe VIO 50C generator, Erbe
134 Medical UK Ltd, UK). Five technical replicates were carried out per muscle with 10 seconds
135 delay between each cut. To avoid loss of conductivity and carry-over effects, the knife was
136 cleaned by scraping off the residues and wiping with a clean tissue (Kimwipes, Kimtech
137 Science™) which had been dampened with distilled water. Aerosol produced from the
138 samples was infused with isopropanol (LC-MS grade, Fisher scientific) at a constant flow
139 rate of 100 $\mu L.min^{-1}$ into the spectrometer. The mass spectra were acquired between the mass
140 range of m/z 50-1500 using a negative ionisation mode with a scan rate of 0.5 seconds per
141 scan. In-house prepared minced lamb sample was analysed for every 10-cuts cycle for quality
142 control and accuracy of the collected data.

143 REIMS data was processed first using ProGenesis Bridge (Waters UK) which converts
144 each individual REIMS measurement ('burn') into a separate file and at the same time
145 subtracts the background signal from ambient air and performs mass alignment, in this case
146 to m/z 281.25 for fatty acid C18:1 $[M-H]^-$ which was one of the main ions observed in all
147 samples. Data were then further processed using ProGenesis QI (Waters, UK) to group
148 adducts belonging to the same compound and perform tentative identification based on
149 matching the measured masses with the Human Metabolome Database (http://www.hmdb.ca)
150 and Lipid Maps database (www.lipidmaps.org), with a mass error of 5 ppm. Here, we have
151 focused on the m/z values with significant variations between treatments and identified those
152 masses which were associated with mammalian metabolism. There were several potential
153 identifications assigned to one high-resolution m/z value, especially for the lipid compounds

154 which have large number of potential isobaric and isomeric forms. For this reason, we have
155 reported the lipid class rather than individual lipids.

156 The identification of metabolites based on database search met level 2 criteria for
157 identification in metabolomics (Sumner et al., 2007). While it is useful for understanding the
158 underlying biochemical mechanisms arising from different ageing treatments, and/or other
159 potential factors involved. Measurements of lipid oxidation (TBARS), protein and free amino
160 acids profile were also performed to support the present findings from REIMS.

161 ## 2.4 SDS-PAGE gel electrophoresis

162 Whole muscle protein of minced lamb chops was extracted with a buffer made of
163 50mM Tris-HCl (pH = 5.8, Tris base, ACS reagent grade, Millipore®), 10% glycerol (AR
164 grade, Fisher Scientific), 2% Sodium dodecyl sulfate (ACS grade, Sigma-Aldrich®), and 2%
165 β-mercaptoethanol (AR grade, 99.0% purity, Sigma Aldrich®) according to the procedures
166 described by Zhang et al. (2019). The protein content in the extract was determined by a
167 commercial protein assay kit (RC-DC™ protein assay, Bio-Rad® Laboratries, Hercules,
168 USA). The protein extract was diluted to a constant concentration of 5 $\mu g.\mu L^{-1}$ with the
169 extraction buffer then loaded to a Novex™ NuPAGE™ 10% Bis-Tris Midi Protein Gels
170 (Invitrogen, UK), by 8 µL aliquot protein extract (equivalent to 40 µg protein) per well.
171 Electrophoresis separation was carried out at ambient temperature in a Bio-Rad Criterion cell
172 system at 150 V equipped with a PowerPac™ HC High-Current Power Supply (Bio-Rad®
173 Laboratories, Hercules, CA, USA). An 8 µl aliquot of Novex™ Sharp Pre-stained protein
174 standard (Invitrogen, UK) was used to assist with identification of the protein molecular
175 weight ranging from 3.5 kDa to 260 kDa. Staining was carried out in a SimplyBlue SafeStain
176 (Invitrogen™) for 4 hours. The stained gels were washed twice with distilled water for 2
177 hours before the images were captured with a GS900 calibrated densitometer scanner (Bio-

178 Rad® Laboratories). Identification and semi-quantitative analysis of protein bands were
179 carried out in Image Lab™ software (Version 6.0.1, Bio-Rad® Laboratories, Inc.). Seven
180 molecular weight regions were grouped according to the protein standards, including < 20,
181 20-30, 30-40, 40-50, 50-60, 60-110 and > 110 kDa. Relative optical density of protein groups
182 was calculated by % relative optical density = (optical density of the protein bands in the
183 group/total density of all the bands) × 100%. The analysis was carried out in duplicate.

184 2.5 Free amino acids analysis

185 Free amino acids were extracted from lamb chops with 80% methanol (HPLC grade,
186 Sigma-Aldrich®). Sub-samples (5 g) were taken from the minced chop as described in section
187 2.2 and homogenised with 80% methanol (15 mL) using an ultra-turrax (Ultra-turrax, IKA) at
188 14,000 rpm for 20 s in an ice bath. The clear supernatant containing free amino acids was
189 obtained after centrifuging at 15,000 g for 10 min at 4 °C. Chromatographic separation and
190 quantification of free amino acids were performed by LC-MS/MS according to Salazar,
191 Armenta, and Shulaev (2012) with some adaptions. In brief, aliquots of free amino acids
192 extract were spiked with 10 $mg.L^{-1}$ d_4-alanine (DL-Alanine-2,3,3,3-d4, Sigma-Aldrich®) as
193 an internal standard (1:1, v/v), and derivatised (1:1, v/v) with 2.8 $mg.mL^{-1}$ 6-aminoquinolyl-
194 N-hydroxysuccinimidyl carbamate (in dry acetonitrile, Apollo Scientific, UK) in a 70 μL of
195 200 mM sodium tetraborate decahydrate buffer (pH = 8.8, Pure Science Ltd, New Zealand) at
196 55 °C for 15 min. The amino acid derivatives were then neutralised with of 10% formic acid
197 (1:9, v/v) prior to the chromatographic analysis by the Agilent 1260 Infinity HPLC system
198 intergrated with Agilent 6420 Triple Quadrupole LC/MS system (Agilent Technologies New
199 Zealand Limited, New Zealand). A C18 core shell Kinetex LC column (100 × 2.1 mm, 1.7
200 μm particle size, 00D-4726-AN, Kinetex®) was used for the separation of amino acid
201 derivatives using separation gradient as following: 0-8.0 minutes (13.0% A and 87.0% B),
202 8.0-16.0 minutes (17.0% A and 83.0% B), 16.0-17.0 minutes (80.0% A and 20.0% B), and

203 18.0-18.5 minutes (1.5% A and 98.5% B). The working buffer A was prepared with 0.1%
204 formic acid in acetonitrile, and B with 0.6% formic acid in MilliQ water. Constant column
205 flow rate (0.2 $mL.min^{-1}$) and set temperature (22.6 °C) were used. Identification of free
206 amino acids was carried out using MRM-MS with positive electrospray ionization (ESI+)
207 mode to select the most sensitive parent-daughter ion transition of m/z $[M-H]^+ > 171$.
208 Ionisation source settings in mass spectrometry included the temperature at 325 °C, capillary
209 voltage of 2.0 kV and gas flow rate of 6.0 $L.min^{-1}$.

210 Quantitative analysis of free amino acids was performed using external standard
211 calibration. The standard solution was prepared with 40 amines including 37 amines mix,
212 asparagine, glutamine (A9906, A0884 and G3126, Sigma-Aldrich®) and d_4-alanine as an
213 internal standard. The amino acid standard (100 µM) was then serially diluted to 0.78 µM to
214 generate a standard curve for the identification and quantitation of free amino acids using
215 MassHunter software (Agilent Technologies). The final concentration of free amino acids
216 was expressed as two forms on wet basis and dry basis. Measurements of each lamb samples
217 were performed in duplicate.

218 ## 2.6 Statistical analysis

219 The statistical analyses of pH, proximate content, TBARS, free amino acids and SDS-
220 PAGE were carried out using linear mixed effect regression analyses in R software (version
221 3.4.1) using "lme4" and "predictmeans" packages. The ageing treatments were considered as
222 a fixed effect, and carcass IDs and sides were random effects fitted into the model. Analysis
223 of variance (ANOVA, one-way) was used to investigate the changes in lamb chops due to
224 different ageing treatments with a post-hoc comparison of means performed using Fisher's
225 least significant differences (LSD) and Tukey's (HSD) test at 5% significance level.

226 The normalised abundance of the identified ions generated from REIMs were collected
227 for determining the effect of ageing treatments, carcass sides and types of muscle on the
228 metabolic profiles of lamb chops. Five technical replicates were combined into an average
229 abundance for each sample/muscle. Differences on metabolic profiles were determined using
230 Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA) (SIMCA,
231 Umetrics, Sweden) on the abundance of identified ions in each sample/muscle. R^2
232 (cumulative) and Q^2 (cumulative) scores were used to describe the robustness and accuracy
233 of the statistical model used in this study. An OPLS-DA model with the Q^2 score > 0.2 is
234 suggested to be of interest due to a predictability of separation. The normalised abundance
235 between treatments were compared using one-way ANOVA with t-test to separate the means
236 at $P < 0.05$. As a high number of identifications with significant changes in abundance were
237 observed in the present study, we have focused on two groups of ions: (1) dominant
238 metabolites with the highest average abundance (minimum threshold = 100); (2) high fold
239 change metabolites with the largest fold changes between two treatments (fold change > 1.1,
240 or < 0.9).

241 # 3 Results

242 ## 3.1 Proximate analysis, pH and lipid oxidation (TBARS)

243 As shown in Table 1, similar pH was observed for lamb samples before ageing as
244 expected ($P = 0.510$). Increase ($P = 0.008$) of pH following 21 days of ageing was detected
245 regardless of ageing methods. A lower ($P = 0.034$) moisture content was observed in dry-
246 aged lamb compared to the wet-aged equivalents. No difference ($P = 0.856$) in fat content
247 was found between the ageing methods. Higher ultimate pH ($P = 0.016$) and TBARS ($P =$
248 0.007) levels were observed in in-bag dry-aged lamb compared to the wet-aged equivalents.

249 3.2 Metabolic profile

250 In this study, the REIMS metabolic fingerprinting of in-bag dry- and wet-aged lamb
251 resulted in the detection of approximately 4000 features and the identification of 1705 ions.
252 Due to the large number of identified compounds, the current study focused on the ions
253 which showed the greatest (P < 0.05) fold change and abundance in the sample matrix
254 between the two ageing treatments (Table 2 and Table 3). The standard operation of REIMS
255 does not include chromatographic separation and MS/MS fragmentation. Thus, several lipid
256 molecules with high ion mass (> m/z 600) have been assigned to one or more lipid classes
257 instead of a specific lipid, owing to a large number of isobaric and isomeric forms which
258 cannot be distinguished by REIMS. The lipid classes putatively identified in this study
259 include ceramides (Cer), diacylglycerols (DG), phosphatidic acids (PA),
260 phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylglycerols (PG),
261 glycerophosphoinositols (PI), phosphatidylserines (PS), sphingomyelins (SM) and
262 triacylglycerols (TG).

263 3.2.1 In-bag dry-ageing *vs.* wet-ageing

264 A clear separation was observed between the metabolite profile of in-bag dry- and wet-
265 aged lamb using OPLS-DA model, with the Q^2 value of 0.86 (Figure 1a). Out of 1705
266 identified metabolites, 663 metabolites (38.89%) differed significantly between the two
267 ageing treatments. There were 318 metabolites (18.65%), which were significantly more
268 abundant in dry-aged lamb over the wet-aged equivalents. These mainly consisted of low ion
269 mass metabolites (178 ions, < m/z 500), including amino acids, dipeptides and amines (Table
270 2). Wet ageing, on the other hand, produced significantly more abundant high molecular
271 weight metabolites (284 of total 345 ions, > m/z 500), mainly associated with phospholipids
272 metabolism.

273 3.2.2 Carcass sides and different muscles

274 A negative Q^2 value (Q^2 = -0.06) was observed using OPLS-DA modelling between the
275 carcass sides suggesting there was no significant variation in metabolic profiles arising from
276 using the different sides of carcasses, regardless of the ageing methods (data not shown).
277 Only 1.94% of the detected metabolites differed ($P < 0.05$) between the carcass sides, well
278 within what would be expected by random chance.

279 A weak OPLS-DA model was seen between the four major muscles (SM, BF, VL and
280 RF) of the lamb chops with Q^2 value of 0.12. As shown in Figure 1b, muscle SM and BF and
281 muscles RF and VL were separated into two groups in the OPLS-DA model with a Q^2 value
282 of 0.62, suggesting that a good prediction of different metabolic profiles between the two
283 muscle groups was observed. Through further quantitative analysis, significant ($P < 0.05$)
284 difference in 418 metabolites (24.52%) was observed. There were 313 metabolites (18.36%)
285 being significantly more abundant in SM + BF muscles, where around 44.41% (139 ions) of
286 the metabolites had low-medium ion mass (< m/z 500). Another 105 metabolites (6.16%)
287 were significantly more abundant in RF + VL muscles and most of them (88.57%) had high
288 ion masses (> m/z 500).

289 3.3 SDS-PAGE gel electrophoresis

290 As shown in Figure 2a, there was no clear difference observed in SDS-PAGE protein
291 profile between the two ageing methods. This finding was further confirmed by semi-
292 quantitative analysis on the relative optical quantity of seven molecular weight groups (< 20,
293 20-30, 30-40, 40-50, 50-60, 60-110 and > 110 kDa) to determine the changes in protein
294 degradation arising from different ageing methods. As shown in Figure 2b, no significant
295 difference ($P > 0.05$) was observed for all seven groups. The large size (> 110 kDa) protein
296 fragments were the most predominant group in both ageing methods, followed by the

297 intermediate size (30-50 kDa), together accounting for about 70% of the total proteins.
298 Around 10% of the total proteins were small sized protein fragments, which may be
299 associated with protein degradation, and these were also observed in both aged lamb samples.

300 3.4 Free amino acids analysis

301 Total of 21 free amino acids have been detected in both ageing methods including 9
302 essential amino acids, as shown in Table 4. In-bag dry-ageing resulted in a higher ($P = 0.049$)
303 level of total free amino acids compared to the wet-aged. The most predominant amino acid
304 released from the aged lamb samples was glutamine, followed by alanine, leucine, threonine,
305 arginine and serine, accounting for over 50% of total free amino acids. There were no
306 significant differences ($P > 0.05$) observed in most of the dominant free amino acids (> 5% of
307 total free amino acids) except for arginine ($P = 0.001$, fold change = 1.43), leucine ($P = 0.005$,
308 fold change = 1.43) and valine ($P = 0.017$, fold change = 1.41), which were more abundant in
309 dry-aged lamb. However, there was no significant difference in all the amino acids when the
310 concentrations were determined based on dry weight (Table 4). Dry ageing process tended to
311 result in higher levels (fold change > 1.0) of free amino acids than the wet-aged even after
312 accounting for the differences in moisture content of the samples from two methods.

313 4 Discussion

314 A series of biochemical changes occur post-mortem. These mainly include energy
315 depletion, degradation and oxidation of lipids and proteins, which play a key role in
316 determining the meat quality (Matarneh, England, Scheffler, & Gerrard, 2017). It is vital to
317 understand how these biochemical changes interact with the different processing techniques
318 in order to assure the meat quality. In this study, our hypothesis that in-bag dry-aged lamb
319 would produce different metabolic profiles compared to wet-aged lamb was confirmed by
320 REIMS fingerprinting. The following discussion was divided into four sections focusing on

321 the biochemical (proteolysis, energy metabolism and oxidation) and physiological (type of
322 muscles) mechanisms which resulted in the observed changes of metabolites in this study.
323 Results of lipid oxidation and protein and free amino acid profiles were used to aid the
324 understanding of the underlying biochemical changes behind the different ageing methods.

325 4.1 Proteolysis

326 Enzymatic proteolysis by calpains system during post-mortem ageing has been widely
327 accepted as the primary pathway to produce free amino acids and peptides (Nishimura, 1998).
328 In-bag dry-ageing resulted in significantly increased levels of metabolites contributed to
329 developing the flavour profile of dry-aged products, including peptides, amino acids and
330 amines. As shown in Table 2, there were 20 metabolites produced from proteolytic
331 degradation and 18 of them were found significantly more abundant in dry-aged lamb over
332 the wet-aged equivalents. These suggest that different proteolytic processes may have
333 occurred in those two ageing methods. These metabolites were identified as a tetrapeptide, 15
334 dipeptides and 4 free amino acids. Wet-ageing of lamb resulted in significantly higher levels
335 of 2 dipeptides – arginine-aspartic acid and alanine-lysine – compared to dry-aged
336 equivalents.

337 Small peptides and free amino acids are the primary taste-active compounds and/or
338 flavour precursors which considerably contribute to the flavour and other sensory properties
339 of meat (Mottram, 1998). In-bag dry-ageing of lamb produced significantly increased level of
340 free amino acids with ion masses of m/z 116.1 ($[M-H]^-$), 128.0 ($[M-H_2O-H]^-$), 130.1 ($[M-H]^-$)
341 and 154.1 ($[M-H]^-$). These were identified as valine, glutamic acid, leucine/isoleucine and
342 histidine, respectively (Table 2). The current findings have been further supported by
343 chromatographic profiling of free amino acids, as shown in Table 4 (wet basis). Although
344 there was no statistically significant difference observed when determined on dry basis,
345 numerically dry-ageing tended to produce higher levels of free amino acids compared to the

346 wet-aged. The increase of small peptides and free amino acids in dry-aged samples could be
347 partially associated with dehydration during dry-ageing process, where the components were
348 concentrated due to the significantly reduced moisture level in dry-aged lamb. The impact of
349 moisture evaporation may account for about 4% of the differences observed in REIMS
350 metabolites. As shown in Table 2 and Table 3, over 10% of variations in metabolites were
351 observed suggesting other factors may contribute towards the changes between two ageing
352 treatments. Previous studies on beef have suggested that dry ageing may have a higher rate of
353 protein hydrolysis than wet-ageing (Kim et al., 2016), that could also support the present
354 findings. Significantly increased level of the free amino acids glutamic acid, valine, leucine,
355 isoleucine and histidine in dry-aged beef has been reported in comparison to wet-aged
356 equivalent (Kim et al., 2016; Lee et al., 2019). These amino acids contribute directly towards
357 the meat flavour or participate later in the Maillard reactions or via the Strecker degradation
358 to produce flavour compounds (Koutsidis et al., 2008).

359 Glutamic acid is one of the most important taste active compounds produced from
360 ageing process and contributed towards the improvement of umami taste, which are
361 associated with distinctive flavour of dry-aged products (Kim et al., 2018; Li et al., 2014).
362 Post-mortem ageing has been reported to release a significant amount of bitter taste amino
363 acids, including valine, leucine and isoleucine. These amino acids can also act as important
364 flavour precursors participating in the production of meat aroma compounds, including 2-
365 methylbutanal, 3-methylbutanal and pyrazines, through the Strecker degradation with
366 dicarbonyl compounds generated in the Maillard reaction (Koutsidis et al., 2008). The
367 increased level of 2- and 3-methylbutanal have been associated with pronounced
368 browned/grilled, buttery and nutty flavour of dry-aged beef (O'Quinn et al., 2016). Histidine
369 is another amino acid with bitter taste found in more abundance in dry-aged lamb. Free
370 histidine in meat products may come from the enzymatic proteolysis during ageing process as

371 described above. Another pathway could be associated with the histidine biosynthesis
372 through enzymatic oxidation of histidinol by histidinol dehydrogenase from yeast to produce
373 histidine (Kulis-Horn, Persicke, & Kalinowski, 2014). This hypothesis could be supported by
374 the significantly reduced level of histidinal (m/z 160.0, $[M+Na-2H]^-$), which was observed in
375 dry-aged lamb (P = 0.004, Table 2). Histidinal is an important intermediate product acting as
376 a substrate for the biosynthesis of histidine (Kulis-Horn et al., 2014).

377 The changes in protein degradation due to ageing methods are shown in Figure 2. There
378 was no difference in protein profile observed for dry- and wet-aged lamb. Appearance of
379 fragments with molecular weight of 32 and 31 kDa following post-mortem ageing has been
380 associated with enzymatic degradation of troponin T (Negishi, Yamamoto, & Kuwata, 1996)
381 and actin (Longo, Lana, Bottero, & Zolla, 2015), and consequently resulted in improvement
382 of tenderness. No difference in these two protein fragments has been detected suggesting
383 similar proteolytic pattern is present between two ageing regimes. Similar protein profile
384 (SDS-PAGE) have also been observed in beef muscles (Kim et al., 2018; Choe, Park, Lee, &
385 Jo, 2020). Therefore, the primary protein degradation due to endogenous enzymes (mainly
386 calpains and cathepsins) are very likely to be similar between the two ageing methods
387 throughout 21 days of ageing time. However, the secondary proteolysis pathway due to the
388 proliferation of microorganisms may differ between ageing methods and this may have
389 consequently led to the variations in the metabolites observed in REIMS. As shown in Table
390 1, significantly reduced moisture content was found in lamb samples following 21 days of in-
391 bag dry-ageing. The decrease in water activity due to the dehydration along with the exposure
392 to oxygen during dry-ageing process could enhance the proliferation of microorganism, such
393 as yeast and moulds. The growth of yeast has been reported in dry-aged beef using both
394 traditional out-of-bag dry-ageing and in-bag dry-ageing regimes (Li et al., 2014; Zhang et al.,
395 2019). The proteolytic activities of yeast on meat during dry-ageing process could account for

396 the increased levels of small peptides and free amino acids in dry-aged lamb observed in this
397 study.

398 Several amines have also been detected in the dry- and wet-aged lamb, including
399 biogenic amine, N-acyl amines and glycerophosphoethanolamines, as shown in Table 2.
400 Histamine (110.1, $[M+Na-2H]^-$) is a biogenic amine found in meat and meat products (Ruiz-
401 Capillas & Jimenez-Colmenero, 2005). A significantly higher level ($P < 0.001$, fold change =
402 1.42) of histamine was detected in dry-aged lamb over the wet-aged equivalents. The
403 production of histamine during post-mortem processing of meat has been associated with the
404 decarboxylation of histidine by microorganisms including yeast (Gardini, Özogul, Suzzi,
405 Tabanelli, & Özogul, 2016). As described above, the significantly more abundant histidine
406 observed in dry-aged lamb could also act as substrate to impart the formation of histamine.

407 Another ion of interest was m/z 241.1 ($[M-H_2O-H]^-$) which was identified as
408 cyclo(Leu-Phe). This is a cyclodipeptide commonly found in biological tissues and it is
409 mainly produced from protein metabolism by microorganisms, including bacteria and fungi
410 (yeast) (Prasad, 1995). Several cyclodipeptides have been reported previously in cooked dry-
411 aged beef (Chen et al., 2009). As shown in Table 2, significantly more abundant cyclo(Leu-
412 Phe) found in dry-aged lamb could be attributed to the action of yeast during the ageing
413 process, as described above.

414 4.2 Energy metabolism

415 Some metabolites associated with energy metabolism have also been identified; these
416 include the metabolism of glycogen, adenosine triphosphate (ATP) and creatine (Table 2).

417 Sugar-related metabolites in meat, including hexoses and trioses, are mainly produced
418 from the post-mortem glycolysis within the first 24-48 hrs of slaughter (Matarneh et al.,
419 2017). Hexose has been identified with ion mass of 201.0 ($[M+Na-2H]^-$) as shown in Table 2.

420 Many monosaccharides (e.g. glucose, mannose, fructose and galactose) have the same
421 molecular weight which cannot be differentiated based on high resolution mass alone.
422 Glucose and mannose have been reported in aged beef while no significant difference was
423 found between dry- and wet-ageing (Kim et al., 2016). In this study, a significantly (P =
424 0.021, fold change = 1.40) increased level of hexose has been observed in dry-aged lamb
425 compared to the wet-aged equivalents. Hexose (glucose, fructose and mannose) content in
426 beef has also been reported to increase significantly during the extended post-mortem ageing
427 up to 21 days (Koutsidis et al., 2008). Hexose are the taste-active compounds contributing
428 towards the sweet taste of meat and are also important water-soluble precursors in the
429 Maillard reaction formation of flavour volatiles (Koutsidis et al., 2008). Hypoxanthine is
430 another water-soluble flavour precursor in meat produced from the metabolism of ATP
431 within 24 h post-mortem. Upon the exhaustion of ATP, hypoxanthine has been suggested to
432 accumulate during the extended ageing period over 3-4 weeks, due to the degradation of 5'-
433 monophosphate (IMP) (Koutsidis et al., 2008). Hypoxanthine was identified with ion mass of
434 m/z 135.0 ($[M-H]^-$) as shown in Table 2. A significantly (P = 0.002, fold change = 1.18)
435 increased level of hypoxanthine was observed in the dry-aged lamb. The enhanced levels of
436 hexose and hypoxanthine observed in dry-aged lamb could have resulted from the slightly
437 different biochemical reactions caused by the activities of microorganisms during dry-ageing
438 compared to the wet-ageing, as described above.

439 Another metabolite associated with energy metabolism was identified to be creatinine
440 with ion mass of m/z 148.0 ($[M+Cl]^-$). A significantly more abundant (P = 0.008, fold change
441 = 2.28) creatinine was observed in dry-aged lamb compared to the wet-aged equivalents
442 (Table 2). The formation of creatinine in meat could be due to the non-enzymatic cyclisation
443 of creatine (Dvořák, 1981). Creatine is the key component participating in the post-mortem
444 energy metabolism of skeletal muscles upon slaughter (Toldrá, 2006). Post-mortem ageing

445 has been suggested to significantly increase the level of creatinine in meat products
446 (Koutsidis et al., 2008). The increased levels of creatinine in dry-aged lamb in this study
447 could be explained by the significant increase of pH value following in-bag dry-ageing (5.98
448 *vs.* 5.87), which may have played a role in the conversion of creatine to creatinine during
449 post-mortem processing (Mora, Hernández-Cázares, Sentandreu, & Toldrá, 2010). Another
450 possible biosynthesis pathway of creatinine from creatine could be associated with the
451 activity of yeast under limited oxygen level inside ageing bags (Soda, Yoshida, & Oikawa,
452 1953).

453 ## 4.3 Oxidation

454 As shown in Table 2, in-bag dry-ageing of lamb resulted in significantly more abundant
455 metabolites arising from lipid oxidation than the wet-aged equivalents. These metabolites
456 included aldehydes, acylcarnitine, aryl alkyl ketones, fatty alcohols and oxidised
457 glycerophosphocholine. Hexanal (m/z 135.1, $[M+Cl]^-$) is one of the main aldehydes formed
458 from the oxidation of linoleic and arachidonic acids (Tamura, Kitta, & Shibamoto, 1991).
459 Hexanal has a low odour threshold which could be suggested as an indicator for oxidative
460 stability and changes in meat flavour during the post-mortem handling (Shahidi & Pegg,
461 1994). Tetracosatetraenoyl carnitine (m/z 524.4, $[M+Na-2H]^-$) is an acylcarnitine which
462 could result from the β-oxidation of fatty acid followed by the esterification with carnitine in
463 the mitochondria (Reuter & Evans, 2012). The production of ketones (m/z 175.0 and 217.1,
464 $[M+Na-2H]^-$) and fatty alcohol (1-Nonanol, m/z 179.1, $[M+Cl]^-$) observed in this study could
465 have been associated with β-oxidation of fatty acid (Toldra, 1998).

466 Another oxidation-derived metabolite has been observed with significantly higher level
467 in wet-aged lamb over the dry-aged equivalents ($P < 0.0001$, fold change = 1.62), which is
468 tetradecanedioic acid (m/z 773.5, $[3M-H]^-$). Tetradecanedioic acid is a saturated dicarboxylic
469 fatty acid, which has been associated with the oxidation of long chain polyunsaturated fatty

470 acids (Passi, Picardo, De Luca, Nazzaro-Porro, Rossi, & Rotilio, 1993). However, increased
471 levels (P < 0.05) of unsaturated fatty acids, including but-2-enoic acid (m/z 123.0, $[M+K-2H]^-$)
472 and tetracosahexaenoic acid (m/z 711.5, $[2M-H]^-$), have also been detected using wet-
473 aging regime due to the lipolysis. On the other hand, the higher level of free fatty acids
474 released from wet-ageing could contribute towards the lower ultimate pH observed in wet-
475 aged lamb compared to the dry-aged equivalents (Table 1).

476 Therefore, lipid oxidation occurred in both ageing methods, with higher oxidative stress
477 using in-bag dry-ageing regime. This was in line with the significantly higher level of
478 TBARS observed in dry-aged lamb over the wet-aged equivalents, as shown in Table 1. Lipid
479 is susceptible to oxidative damage during post-mortem storage, especially under aerobic
480 conditions (Ladikos & Lougovois, 1990). In this study, dry-ageing of lamb was carried out in
481 a water permeable bag with limited level of oxygen present to simulate the aerobic
482 maturation of traditional out-of-bag dry-ageing regime. Therefore, a moderate oxidation level
483 is expected in the dry-aged products which may be one of the key contributors to the
484 signature dry-aged flavour. The severe oxidative damage of lipid has been reported to result
485 in deterioration in sensory quality and generation of toxic products (Min & Ahn, 2005).
486 However, a moderate level of lipid oxidation has also been suggested to produce flavour
487 precursors that may contribute to the unique aroma during ripening stage of dry-cured meat
488 production (Domínguez, Pateiro, Gagaoua, Barba, Zhang, & Lorenzo, 2019). Similar process
489 could be speculated to occur during dry-ageing of meat which may account for the distinctive
490 flavour of dry-aged meat.

491 ## 4.4 Type of muscles

492 The hindleg of lamb consist of multiple muscles. The compositions and properties of
493 different muscles may differ due to their unique roles in supporting the physiological
494 activities of the animals. In this study, the formation of two muscle groups (SM + BF and VL

495 + RF, Figure 1b) suggested a certain level of similarity of metabolic profile within the group.
496 As shown in Table 3, SM + BF muscles had significantly increased level of metabolites
497 associated with metabolic reactions of proteins and lipids, mainly including proteolysis (m/z
498 620.4), decarboxylation (m/z 410.2) and lipid degradation and oxidation (m/z 124.0, 338.2,
499 354.2, 396.2 and 467.3). Different muscles have variable metabolic responses to the post-
500 mortem ageing treatments owing to muscle fibre types (Ithurralde et al., 2018; Ouali &
501 Talmant, 1990). All four muscles analysed in this study consisted of multiple fibre types,
502 mainly including slow oxidative fibres (type I) and fast glycolytic fibres (type IIA and IIB).
503 SM and BF in lamb generally contain higher levels of slow oxidative fibres than VL and RF
504 which predominantly consist of fast glycolytic fibres (Hou et al., 2013; Ithurralde et al., 2018).
505 In general, muscles made of predominantly fast glycolytic fibres are more susceptible to post-
506 mortem biochemical changes than those dominated by slow oxidative fibres. This could be
507 attributed to the increase in activity of calpastatin in slow oxidative fibres which has
508 inhibitory effects on calpains, resulting in a slower response to the ageing treatments (Ouali
509 et al., 1990). Therefore, we hypothesise the primary enzymatic protein metabolism due to the
510 endogenous enzymes (mainly calpains) may be slow in SM and BF, while these muscles
511 could be susceptible to oxidation and secondary biochemical reactions by microorganisms.

512 # 5 Conclusion

513 The effect of in-bag ageing methods on the metabolic profiles of lamb legs was
514 successfully determined using REIMS based on 1705 identified metabolite ions. These
515 metabolites did not differ between carcass sides as expected but did differ between muscles
516 which may be associated with different fibre types in different muscles. The primary
517 enzymatic degradation of proteins in the current lamb samples was similar between the two
518 ageing methods as observed using gel electrophoresis. Different ageing methods resulted in
519 significant changes of 663 metabolites, including more abundant dipeptides and some free

520 amino acids observed for in-bag dry-ageing compared to the wet-aged equivalents. Thus,
521 such changes in peptides and amino acids could be used as the biomolecular signatures for in-
522 bag dry-aged lamb. A moderate level of lipid oxidation caused by the in-bag dry-ageing
523 process might contribute to the distinctive flavour of in-bag dry-aged lamb.

524 6 Implications

525 The outcomes from the present study have the following implications:

526 - The use of REIMS to monitor processing in meat science is novel, with real
527 time profiling of metabolites with no sample preparation. REIMS has potential
528 as a rapid food fraud screening and quality control tool in the food industry.
529 - The thousands of metabolic features generated using REIMS allows for the in-
530 depth differentiation between samples and the understanding of the underlying
531 biological differences due to inherent properties and the processing treatments,
532 which could be used for meat processing control and product developments.
533 Thus, future study can be performed to use REIMS to discriminate other factors,
534 such as different ageing times, shelf stability and changes over frozen storage.
535 - Due to the large number of small peptides observed in the present study, which
536 could be associated with the proliferation of yeast; further studies focusing on
537 determining the role of yeast on formation of small peptides should be carried
538 out to validate the current findings.

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8 Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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670

Table(s)

1 Table 1 Effect of in-bag dry- and wet-ageing on pH, moisture, crude fat and lipid oxidation
2 (TBARS) of lamb legs.

	In-bag dry-ageing	**Wet-ageing**	**SED**	***P*-values**
pH $_{ageing\ 0d}$	5.84	5.80	0.05	0.510
pH $_{ageing\ 21d}$	5.98	5.87	0.03	0.016
Moisture (%)	68.46	72.52	1.28	0.034
Crude fat (%)	6.49	6.04	2.34	0.856
TBARS (µg MDA/g fat)	14.51	5.81	1.68	0.007

3 SED is the standard error of a difference between means.

4

5 Table 2 Effect of in-bag dry-ageing and wet-ageing on metabolic profile of lamb legs.

		Dominant metabolites					**High fold change metabolites**		
m/z	**Ion/Adduct**	**Putative compounds/Lipid class**	***P*-values**	**#Fold change**	**m/z**	**Ion/Adduct**	**Putative compounds/Lipid class**	***P*-values**	**#Fold change**
More abundant in in-bag dry-aged lamb									
98.0	$[M-H_2O-H]^-$	L-2-amino-3-oxobutanoic acid	**	1.22	110.1	$[M+Na-2H]^-$	Histamine	*	1.42
127.1	$[M-H_2O-H]^-$	Alanine-glycine dipeptide	0.008	1.14	116.1	$[M-H]^-$	Valine	**	1.55
128.0	$[M-H_2O-H]^-$	Glutamic acid	*	1.55	130.1	$[M-H]^-$	Leucine/isoleucine	**	1.32
135.0	$[M-H]^-$	Hypoxanthine	0.002	1.18	148.0	$[M+Cl]^-$	Creatinine	0.008	2.28
135.1	$[M+Cl]^-$	Hexanal	0.011	1.20	175.0	$[M+Na-2H]^-$	1-(2-Thienyl)-1-butanone	*	1.69
141.1	$[M-H_2O-H]^-$	Alanyl-alanine	0.024	1.12	201.0	$[M+Na-2H]^-$	Hexoses (glucose/fructose/mannose/galactose)	0.021	1.40
154.1	$[M-H]^-$	Histidine	0.015	1.17	205.1	$[M-H]^-$	Glycine-methionine dipeptide	**	2.73
179.1	$[M+Cl]^-$	1-Nonanol	0.005	1.17	217.0	$[M-H_2O-H]^-$	Aspartate-cysteine dipeptide	*	1.49
194.1	$[M+Cl]^-$	2-Aminooctanoic acid	0.010	1.19	217.1	$[M+Na-2H]^-$	1-(2-Thienyl)-1-heptanone	**	1.59
369.2	$[M-H_2O-H]^-$	Hydroperoxy fatty acid	0.006	1.21	219.1	$[M-H]^-$	Alanine-methionine dipeptide Cysteine-valine dipeptide	0.005	1.33
452.3	$[M+Cl]^-$	N-acyl amine	0.012	1.17	222.1	$[M+Cl]^-$	7-oxo-8-amino-nonanoic acid	*	1.81
482.3	$[M-H]^-$	PS(O-16:0/0:0)	0.005	1.21	231.0	$[M-H_2O-H]^-$	Cysteine-glutamate dipeptide	*	1.78
524.4	$[M+Na-2H]^-$	Tetracosatetraenoyl carnitine	**	1.99	231.1	$[M-H_2O-H]^-$	Methionine-threonine dipeptide	0.020	1.35
552.3	$[M-H]^-$	PS(20:0/0:0)	0.012	1.23	237.1	$[M-H_2O-H]^-$	Histidine-threonine dipeptide	*	1.34
614.3	$[M+Na-2H]^-$	PC(16:0/5:0(CHO))	0.019	1.22	245.1	$[M-H]^-$	Methionine-proline dipeptide	0.027	1.13
757.6	$[M-H]^-$	Cholesteryl-6-O-myristoyl-alpha-D-glucoside	0.003	1.27	260.1	$[M-H_2O-H]^-$ $[M-H]^-$	Asparagine-phenylalanine dipeptide Glycine-tryptophan dipeptide	0.005	1.25
796.5	$[M+K-2H]^-$ $[M+Na-2H]^-$	Phosphatidylcholine Phosphatidylethanolamine	0.003	1.36	275.1	$[M+Na-2H]^-$	Histidine-valine dipeptide	0.007	1.26
798.6	$[M-H_2O-H]^-$	Phosphatidylcholine	0.008	1.27	332.1	$[M-H_2O-H]^-$	Phenylalanine-tryptophan dipeptide	0.021	1.30
828.5	$[M-H]^-$	Oxidized glycerophosphocholine	*	1.31	570.3	$[M-H]^-$	Arginyl-histidyl-prolyl-tyrosine	*	1.29
854.6	$[M+Cl]^-$	Phosphatidylcholine	0.002	1.16	609.3	$[2M-H]^-$	Arginine-methionine dipeptide	0.003	1.43
More abundant in wet-aged lamb									
655.5	$[M-H]^-$	Ceramide phosphoethanolamine	*	1.34	123.0	$[M+K-2H]^-$	But-2-enoic acid	**	1.34
657.5	$[M-H]^-$	Ceramide phosphoethanolamine	**	1.31	160.0	$[M+Na-2H]^-$	Histidinal	0.004	1.44
671.5	$[M-H_2O-H]^-$	DG(20:3(5Z,8Z,11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	**	1.42	196.1	$[M-H]^-$	N-Acetylhistidine	0.008	1.52
673.5	$[M+K-2H]^-$	1-(8-[3]-ladderane-octanyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerol	**	1.30	270.1	$[M-H_2O-H]^-$	Arginine-aspartic acid dipeptide	0.015	1.53
679.5	$[M+Na-2H]^-$	Ceramide phosphoethanolamine	**	1.58	433.3	$[2M-H]^-$	Alanine-lysine dipeptide	**	1.31
680.5	$[M+Cl]^-$	CerP(d18:1/18:0)	**	1.57	445.3	$[M-H_2O-H]^-$	7-Dehydrocholesterol-3-sulfate ester	**	1.71
681.5	$[M-H_2O-H]^-$	PE-Cer(d14:2(4E,6E)/22:1(13Z)(2OH)) PE-Cer(d16:2(4E,6E)/20:1(11Z)(2OH))	**	1.54	463.3	$[2M-H]^-$	3-Mercaptohexyl hexanoate	0.006	1.20
683.5	$[3M-H]^-$	11-Hydroxy-9-tridecenoic acid	**	1.47	489.3	$[M-H]^-$	Saccharolipid	**	1.35
685.5	$[M-H_2O-H]^-$	Phosphatidic acid	*	1.42	643.5	$[2M-H]^-$	15(S)-Hydroxyeicosatrienoic acid	**	1.49
695.5	$[2M+Hac-H]^-$	Xylene	**	1.49	677.5	$[M+Na-2H]^-$	PE-Cer(d14:2(4E,6E)/20:1(11Z))	**	1.45
699.5	$[M+K-2H]^-$	Ceramide	**	1.41	773.5	$[3M-H]^-$	Tetradecanedioic acid	**	1.62
701.5	$[M-H]^-$	Ceramide phosphoethanolamines	0.007	1.16	816.6	$[M-H]^-$	Phosphatidylcholine	**	1.45
705.5	$[M+Na-2H]^-$	PE-Cer(d14:2(4E,6E)/22:1(13Z)) PE-Cer(d16:2(4E,6E)/20:1(11Z))	**	1.75	830.6	$[M-H]^-$	Phosphatidylserine	**	1.64
711.5	$[2M-H]^-$	Tetracosahexaenoic acid	*	1.58	842.6	$[M-H_2O-H]^-$	Glycosphingolipid	**	1.45
713.5	$[M+K-2H]^-$	Ceramide	0.001	1.35	844.6	$[M-H]^-$	Phosphatidylserine	**	1.64
721.5	$[M+Na-2H]^-$	Phosphatidylglycerol Diacylglycerol	0.004	1.28	845.6	$[M-H_2O-H]^-$	Glycerophosphoinositol	**	1.46
733.5	$[M+Na-2H]^-$	PE-Cer(d14:2(4E,6E)/24:1(15Z)) PE-Cer(d16:2(4E,6E)/22:1(13Z))	**	1.73	847.6	$[M+Cl]^-$	Phosphatidic acid	**	1.51
818.6	$[M+Na-2H]^-$	Phosphatidylcholine	*	1.34	850.6	$[M-H_2O-H]^-$	Phosphatidylserine	**	1.43
863.6	$[M-H]^-$	PG(21:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	**	1.40	874.6	$[M+Cl]^-$	Phosphatidylcholine	**	1.47

886.6	$[M+K-2H]^-$	Phosphatidylcholine Phosphatidylethanolamine	**	1.26	876.6	$[M-H_2O-H]^-$	Phosphatidylethanolamine Phosphatidylserine	**	1.48

6 "*" denotes $P < 0.001$, "**" denotes $P < 0.0001$

7 #Fold change is calculated as the ratio of metabolite intensity between in-bag dry- and wet-aged lamb legs.

8

9

10 Table 3 Comparison of the metabolite profiles of the two muscle grouping *m. semimembranosus* + *m.*
11 *biceps femoris* and *m. vastus lateralis* + *m. rectus femoris*.

m/z	Ion/Adduct	Putative compounds/Lipid class	*P*-values	#Fold change
More abundant in muscle group SM + BF				
124.0	$[M-H_2O-H]^-$	Vinylacetylglycine	0.004	1.20
144.1	$[M-H]^-$	4-Guanidinobutanoic acid	0.009	1.14
169.1	$[M+Na-2H]^-$	1-Methyl-4-(1-methylpropyl)-benzene	0.005	1.18
210.1	$[M+K-2H]^-$	9-amino-nonanoic acid	0.006	1.22
250.2	$[M+Cl]^-$	12-amino-dodecanoic acid	0.006	1.12
338.2	$[M+Na-2H]^-$	3-hydroxynonanoyl carnitine	0.002	1.31
354.2	$[M+K-2H]^-$	N-methyl arachidonoyl amine	0.001	1.22
370.3	$[M+K-2H]^-$	2,4,12-Octadecatrienoic acid isobutylamide	*	1.28
396.2	$[M+Cl]^-$	N-Arachidonoyl glycine	*	1.27
410.2	$[3M-H]^-$	Tyramine	0.009	1.20
411.2	$[M+Cl]^-$	10'-Apo-beta-carotenal	0.005	1.20
467.3	$[M-H_2O-H]^-$	N-(2'-(4-benzenesulfonamide)-ethyl) arachidonoyl amine	*	1.25
614.3	$[M+Na-2H]^-$	PC(16:0/5:0(CHO))	0.002	1.28
620.4	$[3M-H]^-$	Phenyl-leucine dipeptide	0.003	1.33
651.4	$[M+H]^-$	Phosphatidylglycerol	0.001	1.33
665.4	$[M+K-2H]^-$	PE-Cer(d14:2(4E,6E)/18:1(9Z))	*	1.32
768.5	$[M-H_2O-H]^-$ $[M+Na-2H]^-$	Glycerophosphoethanolamine Diacylglycerophosphocholine	0.010	3.39
More abundant in muscle group VL + RF				
332.3	$[M-H]^-$	Docosahexaenoic acid	*	0.65
388.2	$[M+Cl]^-$	Hexadecaphinganine-1-phosphate	0.010	0.76
388.3	$[M+K-2H]^-$	Anandamide	0.005	0.66
657.5	$[M-H]^-$	Ceramide phosphoethanolamine	0.017	0.83
698.5	$[M-H_2O-H]^-$ $[M-H]^-$	Phosphatidylcholine Phosphatidylethanolamine	*	0.78
701.5	$[M-H]^-$	Ceramide phosphoethanolamine	0.008	0.86
703.5	$[M-H]^-$	Ceramide phosphoethanolamine	0.006	0.83
712.5	$[M-H_2O-H]^-$	N-tryptophanyl-35-aminobacteriohopane-32,33,34-triol	0.006	0.78
714.5	$[M-H_2O-H]^-$ $[M-H]^-$	Phosphatidylcholines Phosphatidylethanolamine	0.004	0.78
724.5	$[M-H]^-$	Phalluside-1	0.003	0.79
726.6	$[M-H]^-$	Cerebroside B	0.002	0.79
727.6	$[M-H]^-$	Diacylglycerol	*	0.76
808.6	$[M+Na-2H]^-$	Phosphatidylcholine Phosphatidylethanolamine	0.007	0.84
979.8	$[M+Cl]^-$ $[M+K-2H]^-$	Triacylglycerol	0.001	0.72
981.8	$[M+Cl]^-$ $[M+K-2H]^-$	Triacylglycerol	0.010	0.72
989.8	$[M+K-2H]^-$	Triacylglycerol	0.011	0.76
991.8	$[M+K-2H]^-$ $[M+Na-2H]^-$	Triacylglycerol	*	0.71

12 "*" denotes $P < 0.001$, "**" denotes $P < 0.0001$

13 #Fold change is calculated as the ratio of metabolite abundance between *m. semimembranosus* (SM) +
14 *m. biceps femoris* (BF) and *m. vastus lateralis* (VL) + *m. rectus femoris* (RF).

15

16

17 Table 4 Effect of ageing methods on the release of free amino acids in lamb legs.

	Wet basis (mg/100g meat)					Dry basis	
	In-bag dry-ageing	**Wet-ageing**	**SED**	***P*-values**	**#Fold change**	***P*-values**	**#Fold change**
Essential amino acids							
Histidine	13.58	8.19	0.97	0.005	1.66	0.206	1.23
Isoleucine	34.02	23.57	5.29	0.120	1.44	0.451	1.24
Leucine	47.21	33.03	2.52	0.005	1.43	0.085	1.20
Lysine	24.71	19.64	1.18	0.013	1.26	0.363	1.11
Methionine	17.70	13.93	1.17	0.032	1.27	0.252	1.12
Phenylalanine	26.91	19.46	3.39	0.093	1.38	0.188	1.21
Threonine	42.13	29.00	5.24	0.066	1.45	0.131	1.27
Tryptophan	7.26	5.08	0.77	0.046	1.43	0.128	1.26
Valine	30.36	21.56	2.24	0.017	1.41	0.073	1.24
Non-essential amino acids							
Alanine	52.06	41.49	10.66	0.378	1.25	0.744	1.08
Arginine	39.62	27.80	1.45	0.001	1.43	0.138	1.18
Asparagine	14.14	9.26	1.41	0.026	1.53	0.058	1.30
Aspartic acid	13.93	6.93	0.82	0.001	2.01	0.170	1.37
Cysteine	0.84	0.85	0.09	0.948	0.99	0.137	0.87
Glutamine	84.43	69.62	17.19	0.438	1.21	0.831	1.05
Glutamic acid	27.80	16.84	3.74	0.043	1.65	0.542	1.18
Glycine	29.68	24.94	3.91	0.292	1.19	0.746	1.04
Hydroxyproline	2.39	2.13	0.16	0.180	1.12	0.843	0.98
Proline	16.53	11.00	1.84	0.039	1.50	0.281	1.24
Serine	35.04	24.96	4.35	0.081	1.40	0.284	1.18
Tyrosine	29.10	20.22	3.61	0.070	1.44	0.265	1.20
% Essential amino acids	41.65	40.71	2.77	0.752	1.02	0.698	1.03
Total free amino acids	589.45	429.50	57.24	0.049	1.37	0.282	1.16

18 SED is the standard error of a difference between means.

19 #Fold change is calculated as the ratio of amino acid levels between in-bag dry- and wet-aged lamb
20 legs.

21

Figure(s)
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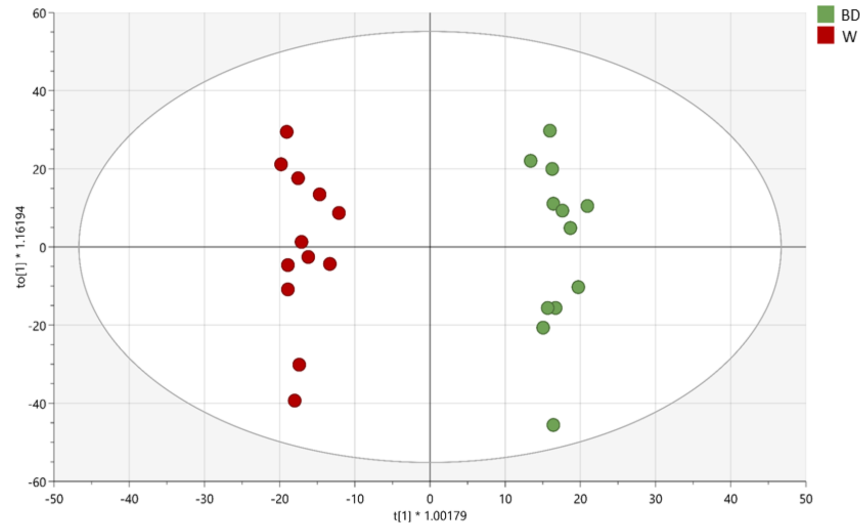

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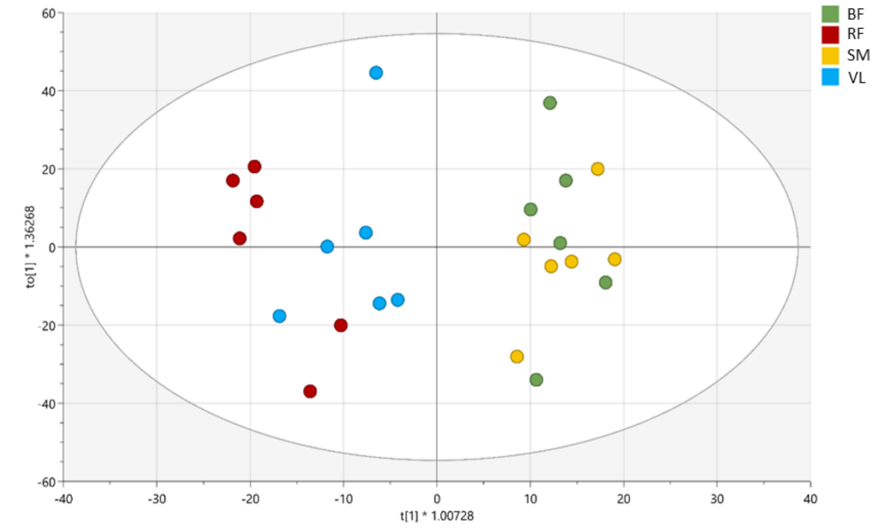
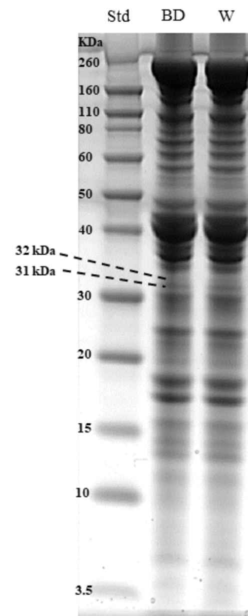


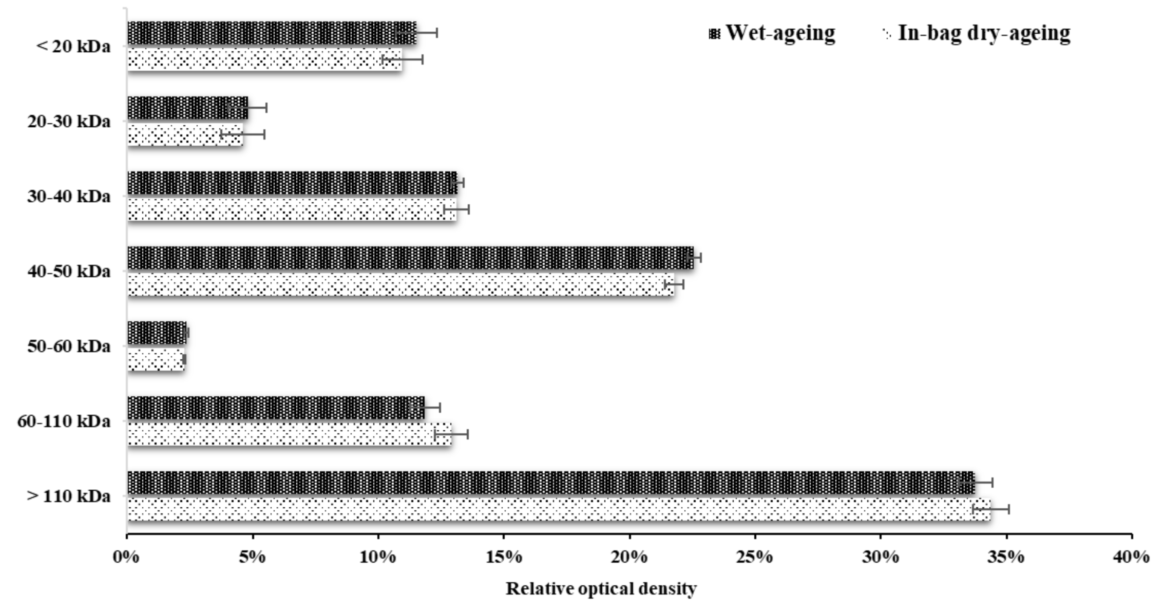
b

1

2 Figure 1 OPLS-DA score plot: (a) in-bag dry-ageing (BD) *vs.* wet-ageing (W) ($R^2X = 0.64$, $R^2Y = 0.98$, $Q^2 = 0.86$); (b) comparison between
3 four muscles: *m. semimembranosus* (SM), *m. biceps femoris* (BF), *m. vastus lateralis* (VL), and *m. rectus femoris* (RF): $R^2X = 0.52$, $R^2Y = 0.33$,
4 $Q^2 = 0.12$, and for muscle groupings SM + BF muscles and VL + RF muscles: $R^2X = 0.52$, $R^2Y = 0.92$, $Q^2 = 0.62$.

5





a

b

6

7 Figure 2 (a) Representative SDS-PAGE protein profile of in-bag dry-aged (BD) and wet-aged (W) lamb chops; (b) Relative optical density
8 (mean ± standard error) of seven major molecular weight protein groups from SDS-PAGE gel electrophoresis of BD and W lamb samples.

9