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(Article begins on next page)

1 **Describing backfat and *Semimembranosus* muscle fatty acid**

2 **variability in heavy pigs: analysis of non–genetic factors**

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18 **Abstract**

19 This study aimed to describe the multivariate structure of *Semimembranosus* muscle and backfat
20 fatty acid (FA) composition in 798 Italian Large White heavy pigs and to investigate the effects of
21 environmental factors and carcass characteristics on FA variations. The total FA variability in
22 muscle and backfat was characterized by a negative correlation between saturated and
23 polyunsaturated FAs, which strongly depended on the carcass adiposity. Slaughtering season was
24 also relevant, with pigs slaughtered in autumn having more *n*-6 FAs and eicosadienoic acid in
25 backfat, while pigs slaughtered in winter displayed more saturated FAs.
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28 Regarding *Semimembranosus* muscle, pigs with heavier belly cuts and slaughtered in autumn had
29 higher proportions of *cis*-vaccenic and palmitoleic acids, while those slaughtered in summer had
30 more saturated FAs. Slaughtering season emerged as a relevant factor shaping both backfat and
31 muscle FA composition, indicating that more studies and attention should be paid to environmental
32 factors, which may have effects on FA metabolism and deposition in finishing pigs.

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35 **Keywords:** fatty acid composition; subcutaneous fat quality; *Semimembranosus* muscle; swine;
36 Principal Component Analysis.

37 38 39 **1 Introduction**

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41 Global meat demand is expected to be 16% higher in 2025 over the 2013-2015 period, with poultry
42 and pork production and demand leading the trend in developing countries (OECD, 2016). The
43 demand for both fresh and processed meat products is expected to increase. Italy is a top producer
44 of processed meat products, particularly of Protected Designation of Origin (PDO) products,
45 contributing to about one-third of the European heritage meat product (Dalle Zotte, Brugiapaglia, &
46 Cullere, 2017). Parma and San Daniele PDO hams accounted for more than half of the total
47 turnover generated by the Italian PDO pork products in Italy in 2017 (ISMEA, 2019). These high-
48 quality dry-cured hams are obtained from heavy pig hind legs, just salted, and ripened for a period
49 that is generally not shorter than 13 months. Most of the Italian heavy pig production relies on
50 animals slaughtered at a minimum age of 9 months and an average live weight of 160-170 kg.
51 These pigs come from a specific selection scheme by the national herdbook, or from selection
52 schemes with comparable selection goals (Consorzio del Prosciutto di Parma, 1992; Lo Fiego,
53 Santoro, Macchioni, & De Leonibus, 2005; MIPAAF, 2007; Lo Fiego, Macchioni, Minelli, &
54 Santoro, 2010).

55 The amount and quality of covering adipose tissue and intramuscular fat (IMF) are relevant for pigs
56 used to produce seasoned meat products. The amount of subcutaneous, as well as IMF, strongly

57 affects the technological yield of green hams limiting excessive seasoning losses (Bosi & Russo,
58 2004). Indeed, adipose tissue represents a barrier to water diffusion and salt penetration. Because of
59 the inverse relationship of fat thickness with seasoning losses and salt content, leaner hams are
60 expected to have a higher salt content (Čandek-Potokar, Monin, & Zlender, 2002), which is
61 generally deemed negative for a human healthy diet. Furthermore, it has been reported that a
62 suitable IMF content has a beneficial effect on juiciness (Ventanas, Ruiz, García, & Ventanas,
63 2007) and texture of dry-cured hams (Ruiz Carrascal et al., 2000). On the contrary, because of its
64 influence on water loss and salt penetration dynamics, a high level of fat infiltration in the muscles
65 was found to be associated with excessive softness and pastiness (Parolari, Rivaldi, Leonelli,
66 Bellatti, & Bovis, 1988; Gou, Guerrero, & Arnau, 1995). Pigs with greater fat deposition tend to
67 have a higher proportion of saturated fatty acids (SFAs; Tibau et al., 2002), which has positive
68 effects on fat firmness and oxidative stability during the long maturation process of green hams
69 (Virgili & Schivazappa, 2002; Bosi & Russo, 2004). A lower fat level in hams is associated with
70 more polyunsaturated fatty acids (PUFAs; Bosi & Russo, 2004), mainly confined to phospholipids.
71 Among PUFAs, *n*-3 are preferred by consumers for their positive effects on human health.
72 However, PUFAs are also more prone to incur in lipolytic and oxidative processes causing
73 rancidity, abnormal flavors, fat softness, and altered organoleptic properties of dry-cured hams
74 (Wood et al., 2003; Juárez et al., 2011). On the other hand, meat fat content is important for the
75 technological and sensory quality of dry-cured hams, because lipolysis and subsequent fat oxidation
76 cause the development of volatile organic compounds determining the ham aroma (López et al.,
77 1992; Pinna, Simoncini, Toscani, & Virgili, 2012). Different environmental, physiological, and
78 molecular factors affect fat deposition and composition, contributing to the variability in the
79 technological and sensory features of dry-cured hams and other meat products. For that reason,
80 factors affecting fatty acid (FA) composition of different tissues have been under investigation for
81 many years. FA composition showed in general high-to-moderate heritability estimates in pigs
82 slaughtered at about 100 kg live weight, which were intended for fresh meat products (Suzuki et al.,

83 2006; Sellier, Maignel, & Bidanel, 2010), and in Duroc pigs slaughtered at about 125 kg live weight
84 (Ros-Freixedes, Reixach, Bosch, Tor, & Estany, 2014). Recent studies carried out on Italian Large
85 White (ILW) heavy pigs (slaughtered at about 155 kg live weight) found that the FA composition of
86 fat stored in muscle and backfat (BF) are the result of moderately heritable traits (Davoli et al.,
87 2019; Zappaterra et al., 2020) and associated with genetic markers (Zappaterra, Ros-Freixedes,
88 Estany & Davoli, 2018; Catillo et al., 2020). Diet has also a major role in the variability noticed in
89 pork FA composition, as proved by the considerable literature produced over the years (Morgan,
90 Noble, Cocchi, & McCartney, 1992; Leskanich, Matthews, Warkup, Noble, & Hazzledine, 1997;
91 Carrapiso, Tejada, Noguera, Ibáñez-Escriche, & González, 2020). However, except for the studies
92 concerning the effects of genetics and diet on the FA metabolism and deposition, very few
93 researchers have noted the role other factors play in determining FA composition in heavy pigs
94 (Catillo, Zappaterra, Lo Fiego, Steri, & Davoli, 2021).

95 The purpose of this research was to describe and investigate the possible effects of environmental
96 factors and carcass characteristics on the FA composition of *Semimembranosus* muscle (SM) and
97 BF tissues in a population of ILW heavy pigs selected for the production of dry-cured hams. A
98 multivariate approach was used to identify possible metabolic patterns explaining concentrations of
99 individual FAs in different tissues and relate these patterns with environmental factors and carcass
100 characteristics.

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2. Material and methods

2.1 Animals and tissue samplings

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107 A sample of 798 purebred ILW pigs was used in the present study. These samples were included in
108 a previous work (Davoli et al., 2019). Briefly, the experimental pigs came from the sib-testing
109 station of the Italian Pig Breeder National Association (Associazione Nazionale Allevatori Suini,
110 ANAS, <http://www.anas.it>). Their sib-testing program is based on the performances of triplets of
111 full sibs (two gilts and one barrow) reared in the same environmental conditions in a unique testing

112 station. The experimental population came from 323 litters by 87 boars and 371 sows. Each group
113 of siblings entered the sib-testing station located near Reggio Emilia (Italy) at the age of 30-45 days
114 and the testing period lasted a maximum of 145 days, with an average final live weight of about 155
115 kg. During the testing period, siblings were allotted in a natural-ventilated facility and fed the same
116 diets. The finishing diet (Supplementary Table S1) was fed from about 90-100 kg live weight until
117 slaughter weight was reached at *a quasi ad libitum* feeding level (i.e. 60% of the pigs were able to
118 ingest the whole ration). Pigs were slaughtered on 26 different dates between 2011 and 2012 at the
119 same commercial abattoir. Each litter was slaughtered on at least two different dates. Handling and
120 slaughtering of the animals used in this study were performed in compliance with European rules on
121 the protection of animals during transport and at slaughtering (Council Regulation (EC) No. 1/2005
122 and Council Regulation (EC) No. 1099/2009). Sampling occurred with ANAS permission.

123 BF and SM tissues were sampled on the trimming line from the carcass left sides. BF samples were
124 collected approximately between the fifth and the sixth lumbar vertebra, close to the point where the
125 hind leg is separated from the rest of the carcass, at the level of BF maximum thickness. BF and SM
126 samples were wrapped in aluminum foil, immediately put in vacuum-sealed bags, frozen in liquid
127 nitrogen, and kept at -80°C for further use.

128 129 **2.2 Phenotyping**

130 At slaughtering, hot carcass weight (kg) and optical measures (expressed in mm) of loin and BF
131 thicknesses were taken by Fat-O-Meat'er (FOM - CrometecGmbH, Lünen, Germany) between the
132 third and fourth last ribs, 8 cm off the carcass midline. These measures were used to estimate the
133 percentage of carcass lean meat, which was then used for EUROP carcass grading following EU
134 Decision 2001/468/CE of June 8th, 2001 (European Commission, 2001). BF thickness (BFT;
135 expressed in mm) was also measured at the level of the *Gluteus medius* muscle by a caliper.
136 Furthermore, on the left side, the weights (in kg) of belly and jowl cuts were also recorded.
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139 Intramuscular fat content (IMF) was determined in the SM by extraction with petroleum ether from
140 1 g fresh sample using an XT15 Ankom apparatus (Macedon, NY, USA), according to Official
141 procedure AOCS Am 5-04 (AOAC, 2005). IMF was determined in % as g of IMF per 100 g of
142 tissue.

143 BF FA composition was determined as described in Catillo, Zappaterra, Lo Fiego, Steri, & Davoli
144 (2021) and Serra et al. (2014), and was expressed as g FA per 100 g of total FA (i.e. percent FA
145 composition). SM FA determination was described in Catillo et al. (2020). Briefly, the total muscle
146 lipids destined for the gas-chromatographic analysis were extracted from SM using a mixture of
147 chloroform: methanol (2:1, v/v) (Carlo Erba Reagents, MI, Italy) according to Folch, Lees, and
148 Sloane Stanley (1957). Methylation was performed with a 2N solution of potassium hydroxide
149 (KOH) in methanol (CH₃OH) (Carlo Erba Reagents, Milan, Italy) according to Ficarra, Lo Fiego,
150 Minelli, & Antonelli (2010). Tridecanoic acid (C13:0) (Larodan Fine Chemicals AB, Solna,
151 Sweden) was used as an internal standard in SM FA determination. Intramuscular fatty acid methyl
152 esters (FAMES) were then submitted to gas-chromatographic analysis using TRACE™GC Ultra
153 (Thermo Electron Corporation, Rodano, MI, Italy) equipped with a Flame Ionization Detector, a
154 PVT injector, and a TR-FAME Column 30 m × 0.25 mm i.d., 0.2 µm film thickness (Thermo
155 Scientific, Rodano, MI, Italy). The Chrom-Card software (vers.2.3.3, Thermo Electron Corporation,
156 Rodano, MI, Italy) was used to record and integrate the peaks of FAMES. Individual FAME were
157 identified by comparing their retention times with the retention times of a standard FAME mixture
158 prepared in-house with known quantities of each methyl ester (Larodan Fine Chemicals AB, Solna,
159 Sweden). In order to present data in the same way as BF, the amount of each FA determined in SM
160 was reported as g FA per 100 g of total FA (i.e. percent FA composition).

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2.3 Statistical analysis

2.3.1 Data handling

166 The 26 slaughtering dates were grouped into a new variable with four levels corresponding to the
167 four slaughtering seasons (i.e. six dates in spring; six in summer; nine in autumn; five in winter).

168 169 **2.3.2 Multivariate analysis of the two tissues**

170 In order to identify underlying structures in the dataset and patterns linking individual FAs, a
171 Principal Component Analysis (PCA) was applied to the FA composition of BF and SM. Each
172 tissue was independently analyzed with the aim of investigating the main non-genetic factors that
173 could shape the variability of BF and SM FA composition. A PCA was run for each tissue including
174 all the individual FAs. First, the projection of the samples in the Principal Components (PC) space
175 (scores) was calculated. Samples with a high value for at least one of the distances within and
176 orthogonal to the projection plane (Hubert, Rousseeuw, & Vanden Branden, 2005) were considered
177 as outliers and not further included in the PCA analysis. A total of four and one outliers were
178 removed for BF and SM tissues, respectively. After outlier removal, a PCA was run again for each
179 of the considered tissues and PC scores were obtained. Each PC was determined by a specific
180 combination of the original variables, which, based on their weight in each PC, contribute to explain
181 total variance. The weights of individual FAs within each PC were then used to discuss possible
182 metabolic pathways capable to explain the combinations found. To test whether the distribution of
183 samples in the PCA scoreplot may have been influenced by major factors of variability, the
184 distribution of samples on the projection plane was evaluated by plotting the variables of
185 slaughtering season, sex, and EUROP carcass grading.

186 PCAs were performed using the *ropls* package (Thévenot, Roux, Xu, Ezan, & Junot, 2015) in the R
187 environment (R Core Team, 2020).

188 189 **2.3.3 Univariate models for the FA composition of the two tissues**

190 191 **2.3.3.1 Stepwise multiple regression model of the PC scores**

195 The results of the multivariate approach (PCs) were further integrated by a univariate approach
196 aimed to evaluate the effects of the categorical variables on the phenotypic variability noticed for
197 each FA or FA class. The scores of the first two PCs obtained for each PCA were then included as
198 dependent variables in backward stepwise multiple linear regression models. The initial model
199 evaluated with the backward stepwise automatic elimination was the following:

$$200 y_{ijk} = \mu Ss_i + Sex_j + b_1(Age_k) + b_2(\text{hot carcass weight}_k) + b_3(\text{Carcass lean}_k) + b_4(\text{BFT}_k) + b_5(\text{IMF}_k) \\ 201 + b_6(\text{belly weight}_k) + b_7(\text{jowl weight}_k) + e_{ijk} \\ 202 \\ 203$$

204 where: y_{ijk} was the vector of the scores of the first PCs identified with the PCAs; μ was the overall
205 mean; Ss_i is the fixed effects of the i^{th} slaughter season ($i=1$ to 4) and Sex_j is the fixed effect of the
206 sex ($j=1,2$); age at slaughtering, hot carcass weight, carcass lean %, BFT measured with a caliper,
207 IMF percentage in SM, and the weights of belly and jowl were considered as covariates; $b_1, b_2, b_3,$
208 b_4, b_5, b_6, b_7 were the regression coefficients; e_{ijk} random residual effect for the k^{th} pigs.

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210 Generalized Linear Models (GLMs) were performed with the *glm* function of the *stats* package (R
211 Core Team, 2020) in the R environment. Backward stepwise multiple linear regression models were
212 performed using the *step* function of the *stats* package (R Core Team, 2020) in the R environment.
213 *Anova* function of *car* package in R environment (R Core Team, 2020) was used to adjust the
214 results of the stepwise multiple linear regression models for the type III errors.

215 To complete the obtained results, the effect of the covariates for slaughtering season (4 levels) and
216 sex of the animals (2 levels) were also tested on BFT, carcass lean % and IMF % with the *glm*
217 function of the *stats* package, and *Anova* function of *car* package in the R environment (R Core
218 Team, 2020). The results of the GLM for slaughtering season effects are reported as Least Squares
219 Means (L.S.M.) and Standard Errors (S.E.), obtained with *lsmeans* function of *lsmeans* package in
220 the R environment.

221 *P*-values < 0.05 were considered significant and the trend towards significance was set for *P*-values
222 comprised between 0.10 and 0.05.

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2.3.3.2 Multiple regression models for the individual FAs and FA classes

In order to highlight the effects of the independent categorical variables on each FA or FA category, the FA compositions of BF and SM were analyzed with a linear model in R environment (R Core Team, 2020). The linear models used for BF and SM FA composition were based on the results of the backward stepwise multiple linear regression models performed for the relative PCs. For each tissue, variables displaying a *P*-value less than or equal to 0.05 in at least one of the stepwise models were considered as independent variables in the linear model.

3. Results

3.1 BF FA composition

The PCA for BF FA composition identified two PCs, jointly explaining 52% of the total variance. Weights of individual FAs entering each PC are reported in Table 1 and the PCA scoreplot is displayed in Supplementary Figure 1. FAs showing the highest and lowest weights contributed the most in determining the variability of the PC they belonged to. The first PC (PC1), explaining 33% of the total variance, was mainly determined by the saturated FAs (SFA) stearic, arachidic and palmitic, while arachidonic, linoleic, dihomo- γ -linolenic, docosapentaenoic (DPA), heptadecenoic unsaturated FAs (UFAs), and lauric acid had negative loadings in PC1. Most of the total variance was thus determined by the antagonism shown by the animals located in the right side of the PCA scoreplot (characterized by more stearic, arachidic, and palmitic acids in BF) against those placed on the left side of Supplementary Figure S1 (with BF having greater proportions of arachidonic, linoleic, dihomo- γ -linolenic, DPA, heptadecenoic and lauric acids). The second PC (PC2), explaining 18% of the total variance, was mainly determined by the opposition between pigs having BF with greater proportions of palmitoleic acid and of the myristic, capric, palmitic, and lauric SFAs (pigs on the upper side of the PCA scoreplot), and animals displaying more eicosadienoic, gadoleic, and erucic acids in their BF tissue (on the bottom side of Supplementary Figure S1).

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256 **Table 1.** Backfat (BF) individual fatty acids (FAs), identified by their shorthand notation and their
257 common nomenclature between brackets, and the relative Principal Component (PC) loadings. The
258 total variance explained by each PC is between brackets. Bold PC loadings indicate the lowest and
highest PC loadings.

BF FAs (%)	PC1 (33%)	PC2 (18%)
C10:0 (capric acid)	-0.059	-0.375
C12:0 (lauric acid)	-0.210	-0.285
C14:0 (myristic acid)	-0.119	-0.409
C16:0 (palmitic acid)	0.216	-0.335
C16:1 <i>cis</i> -9 (palmitoleic acid)	-0.184	-0.285
C17:0 (margaric acid)	-0.215	0.071
C17:1 <i>cis</i> -9 (heptadecenoic acid)	-0.277	0.000
C18:0 (stearic acid)	0.292	0.066
C18:1 <i>cis</i> -9 (oleic acid)	0.021	0.180
C18:1 <i>cis</i> -11 (<i>cis</i> -vaccenic acid)	-0.205	0.036
C18:2 <i>cis</i> -9, <i>cis</i> -12 (linoleic acid)	-0.317	0.019
C18:3 <i>n</i> -3 (α -linolenic acid)	-0.152	-0.027
C20:0 (arachidic acid)	0.269	0.168
C20:1 <i>cis</i> -11 (gadoleic acid)	0.134	0.259
C20:2 <i>n</i> -6 (eicosadienoic acid)	-0.071	0.404
C20:3 <i>n</i> -6 (dihomo- γ -linolenic acid)	-0.296	0.105
C22:1 (erucic acid)	-0.157	0.238
C20:4 <i>n</i> -6 (arachidonic acid)	-0.327	0.023

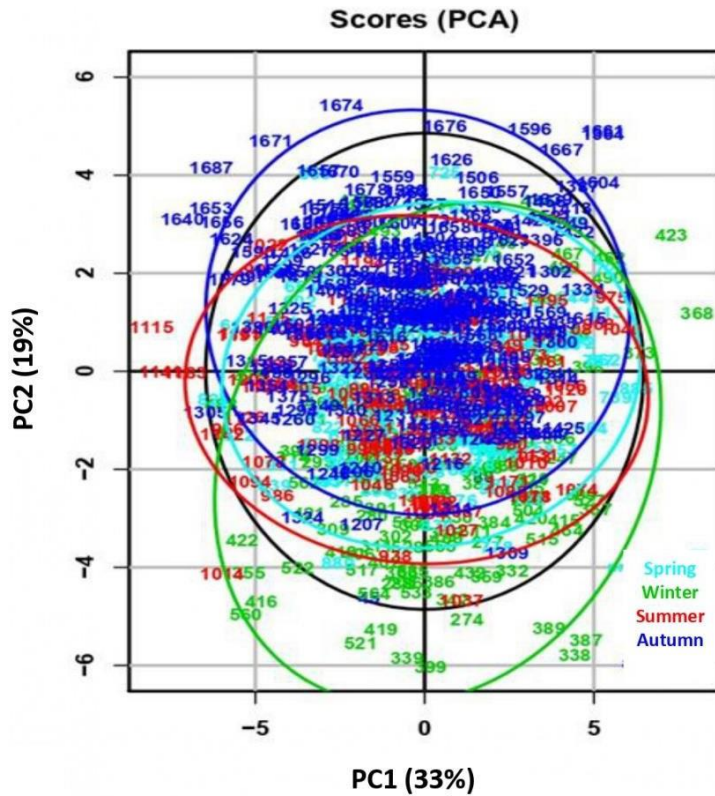
C22:4 <i>n</i> -6 (adrenic acid)	-0.235	0.173
C22:5 <i>n</i> -3 (docosapentaenoic acid-DPA)	-0.282	0.120
C22:6 <i>n</i> -3 (docosahexaenoic acid-DHA)	-0.184	0.054

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262 The samples in the scoreplot were then labeled with their levels for the independent variables of
263 slaughtering season, sex, and EUROP carcass grading, in order to test whether these factors had a
264 major role in the dataset variability. Samples in the scoreplot showed to be clustered based on
265 slaughtering seasons, as pigs slaughtered in autumn showed positive PC2 loadings and those
266 slaughtered in winter negative PC2 loadings (Figure 1). Therefore, the animals slaughtered in
267 autumn had the highest contents of eicosadienoic, gadoleic, and erucic acids, while those
268 slaughtered in winter had more myristic, capric, palmitic, palmitoleic, and lauric acids in BF.

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Figure 1. Principal Component Analysis (PCA) scoreplot for backfat (BF) fatty acids (FAs) with
272 the samples (plotted with their ID number) identified by different colors based on their slaughtering
273 season.

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277 No clear cluster in the scoreplot was observed for sex and EUROP carcass grading.

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PC scores of the samples were then submitted to backward stepwise multiple linear regression and

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the results are reported in Table 2 and Table 3. For PC1 scores, the stepwise selection process

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retained BFT, the % of carcass lean meat content, and age in the final multiple linear regression

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model. Slaughtering season showed a trend towards significance and animal sex was also retained,

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but its *P*-value was above the threshold of 0.10. As can be noticed from Table 2, animals with

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negative PC1 scores have a thinner BF, are older, and have leaner carcasses.

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Table 2. The covariates retained in the backward stepwise multiple linear regression model for PC1

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scores obtained from the Principal Component Analysis (PCA) of backfat (BF) fatty acids (FAs).

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The estimate, *F*-value, and *P*-value are reported for each covariate.

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Covariates		Estimated effect on PC1 scores		
Name	Classes	Estimate	<i>F</i> -value	<i>P</i> -value (F)

Sex	Barrows	Ref	2.260	0.133
	Gilts	0.324		
Age (days)	-	-0.026	4.514	0.034
BFT (mm)	-	0.121	23.354	<0.001
Carcass lean meat (%)	-	-0.129	8.020	0.005
Slaughtering season	Spring	Ref	2.260	0.061
	Summer	-0.099		
	Autumn	0.404		
	Winter	0.630		

Ref: reference class. The effect size of the other classes is expressed using the Ref class as a reference.

- indicates covariates with continuous values.

Table 3 shows the results of the stepwise selection process with the final multiple linear regression model obtained for PC2 scores. The strongest effect was found for slaughtering season, in agreement with the results reported in Figure 1. The estimate for slaughtering season confirmed that pigs slaughtered in autumn have positive PC2 scores and those slaughtered in winter tend to have negative PC2 scores. IMF, carcass lean meat %, jowl and belly weights were also significant, and a trend towards significance was observed for BFT. Pigs having higher contents of IMF in SM, leaner carcasses, and heavier jowl cuts are significantly associated with positive scores for PC2, while animals with heavier belly cuts are associated with negative PC2 scores.

Table 3. The covariates retained in the backward stepwise multiple linear regression model for PC2 scores obtained from the Principal Component Analysis (PCA) of backfat (BF) fatty acids (FAs). The estimate, *F*-value, and *P*-value are reported for each covariate.

Covariates		Estimated effect on PC2 scores		
Name	Classes	Estimate	<i>F</i> -value	<i>P</i> -value (F)

Slaughtering season	Spring	Ref		
	Summer	-0.339	45.827	<0.001
	Autumn	0.877		
	Winter	-1.383		
IMF (%)	-	0.162	7.306	0.007
Carcass lean meat (%)	-	0.070	5.778	0.016
BFT (mm)	-	-0.031	3.443	0.064
Jowl weight (kg)	-	0.394	6.725	0.010
Belly weight (kg)	-	-0.167	4.086	0.044

Ref: reference class. The effect size of the other classes is expressed using the Ref class as a reference.

- indicates covariates with continuous values.

The results of the multivariate approach (PCs) were further integrated by the univariate approach aimed to evaluate the effects of the categorical variables on the phenotypic variability noticed for each FA or FA class. Supplementary Table S2 displays the effects of slaughtering season, age, BFT, carcass lean meat %, belly weight, jowl weight, and IMF% on the individual FAs and FA categories in BF. The L.S.M. of individual FAs and FA categories estimated for the slaughtering seasons are reported in Supplementary Table S3. In accordance with the results identified by the multivariate approach, slaughtering season showed to affect the majority of the individual FAs and FA classes, followed by BFT, carcass lean meat %, and jowl weight. Belly weight was associated with changes in lauric, myristic, palmitoleic, margaric and *cis*-vaccenic acids, and age was significantly related to palmitic, stearic, linoleic, α -linolenic, gadoleic acids and the classes of SFAs and PUFAs.

3.2 Muscle FA composition

The PCA for the muscle FA composition identified two PCs, jointly explaining 53% of the total variance. Weights of individual FAs entering each PC are reported in Table 4 and the PCA

342 scoreplot is reported in Supplementary Figure S2. The first PC (PC1) explained 39% of the total
 343 variance noticed for SM: animals located in the right side of the PCA scoreplot were characterized
 344 by more oleic and myristic acids in SM, while those placed on the left side of Supplementary Figure
 345 2 had SM with greater proportions of erucic, DPA, adrenic, dihomo- γ -linolenic, arachidonic,
 346 docosahexaenoic (DHA), and eicosadienoic acids. The second PC (PC2), explaining 14% of the
 347 total variance, was mainly determined by the opposition between pigs with greater proportions of
 348 *cis*-vaccenic and palmitoleic acids on one hand (pigs on the upper side of the PCA scoreplot), and
 349 animals displaying more stearic, palmitic, lauric, arachidic, and myristic acids in their SM tissue (on
 350 the bottom side of Supplementary Figure S2).

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 353 **Table 4.** Muscle individual fatty acids (FAs) in *Semimembranosus*, identified by their shorthand
 354 notation and their common nomenclature between brackets, and their Principal Component (PC)
 355 loadings. In brackets, the fraction of total variance explained by each PC. Bold PC loadings indicate
 the lowest and highest PC loadings.

Muscle FAs (%)	PC1 (39%)	PC2 (14%)
C10:0 (capric acid)	0.140	0.023
C12:0 (lauric acid)	0.113	-0.256
C14:0 (myristic acid)	0.220	-0.210
C16:0 (palmitic acid)	0.181	-0.281
C16:1 <i>cis</i> -9 (palmitoleic acid)	0.171	0.387
C17:0 (margaric acid)	-0.220	-0.175
C17:1 <i>cis</i> -9 (heptadecenoic acid)	-0.169	0.082
C18:0 (stearic acid)	-0.071	-0.415
C18:1 <i>cis</i> -9 (oleic acid)	0.265	0.204
C18:1 <i>cis</i> -11 (<i>cis</i> -vaccenic acid)	0.037	0.509

C18:2 <i>cis</i> -9, <i>cis</i> -12 (linoleic acid)	-0.298	-0.113
C18:3 <i>n</i> -3 (α -linolenic acid)	-0.131	-0.139
C20:0 (arachidic acid)	0.001	-0.247
C20:1 <i>cis</i> -11 (gadoleic acid)	0.143	0.026
C20:2 <i>n</i> -6 (eicosadienoic acid)	-0.224	-0.112
C20:3 <i>n</i> -6 (dihomo- γ -linolenic acid)	-0.283	0.075
C22:1 (erucic acid)	-0.318	0.107
C20:4 <i>n</i> -6 (arachidonic acid)	-0.279	0.104
C22:4 <i>n</i> -6 (adrenic acid)	-0.312	0.076
C22:5 <i>n</i> -3 (docosapentaenoic acid-DPA)	-0.317	0.079
C22:6 <i>n</i> -3 (docosahexaenoic acid-DHA)	-0.265	0.067

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359 When plotting sample labels of IMF FA composition for the independent variables of slaughtering
360 seasons, animal sex, and EUROP carcass grading, no cluster was observed in the muscle FA PCA
361 scoreplot.

362 PC scores of the samples were then submitted to backward stepwise selection analysis. Table 5
363 reports the final multiple regression model for PC1 scores. The independent variables of
364 slaughtering season, sex, age, EUROP carcass grading, BFT, hot carcass weight, belly weight, jowl
365 weight, and IMF% were retained. In particular, IMF% was the covariate showing the strongest
366 association with PC1 scores, as pigs with higher IMF deposited in SM were associated with positive
367 PC1 scores. Animals with lower percentages of lean meat (i.e. U, R, and O carcasses vs. E
368 carcasses) were also associated with positive PC1 scores. Animals with lower hot carcass weights,
369 heavier jowl and belly weights, older, and with a thicker BF tend to have positive scores for the
370 PC1. Also, winter and autumn as slaughtering seasons showed opposed effects, with autumn being
371 associated with negative and winter with positive PC1 scores.

372 **Table 5.** The covariates retained in the backward stepwise multiple linear regression model for PC1
 373 scores obtained from the Principal Component Analysis (PCA) of *Semimembranosus* muscle (SM)
 374 fatty acids (FAs). The estimate, *F*-value, and *P*-value are reported for each covariate.

Covariates		Estimated effect on PC1 scores		
Name	Classes	Estimate	<i>F</i> -value	<i>P</i> -value (F)
Slaughtering season	Spring	Ref		
	Summer	0.550	3.34	0.018
	Autumn	-0.267		
	Winter	0.381		
Sex	Barrows	Ref	10.59	0.001
	Gilts	-0.724		
Age (days)	-	0.027	4.64	0.031
EUROP carcass grading	E	Ref		
	U	1.330	3.85	0.009
	R	1.907		
	O	2.464		
BFT (mm)	-	0.050	3.85	0.050
Hot carcass weight (kg)	-	-0.052	8.75	0.003
Belly weight (kg)	-	0.399	6.03	0.014
Jowl weight (kg)	-	0.663	6.25	0.012
IMF (%)	-	0.718	58.06	<0.001

378
 379 Ref: reference class. The effect size of the other classes is expressed using the Ref class as reference.
 380
 381 - indicates covariates with continuous values.
 382
 383

384 Table 6 shows the results of the backward stepwise selection process for the PC2 scores estimated
 385 for the samples. Two variables entered with strong significant effects in the model: slaughtering
 386

387 season, and belly weight. Pigs with heavier belly cuts and slaughtered in autumn had higher PC2
 388 scores, while summer as slaughtering season was associated with negative scores for PC2.

389
 390 **Table 6.** The covariates retained in the backward stepwise multiple linear regression model for PC2
 391 scores obtained from the Principal Component Analysis (PCA) of *Semimembranosus* muscle (SM)
 392 fatty acids (FAs). The estimate, *F*-value, and *P*-value are reported for each covariate.
 393

Covariates		Estimated effect on PC2 scores		
Name	Classes	Estimate	<i>F</i> -value	<i>P</i> -value (F)
Slaughtering season	Spring	Ref		
	Summer	-0.114	7.68	<0.001
	Autumn	0.625		
	Winter	0.213		
Belly weight (kg)	-	0.246		

397
 398
 399 The results of the multivariate approach were further integrated with the univariate approach.
 400 Supplementary Table S4 displays the effects of slaughtering season, age, sex, EUROP carcass
 401 grading, hot carcass weight, belly weight, jowl weight, and IMF % on the individual FAs and FA
 402 categories in SM. The L.S.M. of individual FAs and FA categories estimated for the slaughtering
 403 seasons are reported in Supplementary Table S5. In accordance with the results of the multivariate
 404 approach, slaughtering season, IMF content, sex, carcass weight and conformation (i.e. EUROP
 405 carcass grading, belly weight, and jowl weight) showed to affect the majority of the individual FAs
 406 and FA classes. Age was significantly related to palmitic, margaric, heptadecenoic, stearic, oleic,
 407 eicosadienoic, erucic, arachidonic, adrenic, DHA acids, and with the classes of SFAs and MUFAs.
 408 To gain a more complete view of the relationships occurring between the covariates considered, the
 409 effects of the slaughtering season and animals' sex were also tested on BFT, SM IMF%, and carcass
 410 lean %. Gilts had significantly lower contents of IMF in SM ($P = 0.003$), thinner BFT ($P = 0.002$),

411 and leaner carcasses ($P < 0.001$) when compared with barrows. Pigs slaughtered in spring had
412 thicker BFT (L.S.M. \pm S.E; 29.00 ± 0.36 mm), and lower carcass lean % (47.10 ± 0.20 %) compared
413 with those slaughtered in autumn (25.40 ± 0.29 mm and 49.60 ± 0.16 %, respectively). The animals
414 slaughtered in winter and spring had BFT and carcass lean % displaying values of L.S.M.
415 intermediate between those observed in spring and autumn (27.90 ± 0.47 mm for BFT and $48.90 \pm$
416 0.26 % for carcass lean % in winter; 27.30 ± 0.37 mm for BFT and 48.50 ± 0.21 % for carcass lean
417 % in summer). The slaughtering season did not affect IMF% in SM.
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422 **4 Discussion**

423
424 The results obtained in the present study allowed the characterization of the environmental factors
425 and carcass features associated with changes in BF and SM FA composition in ILW heavy pigs fed
426 the same diet. To the best of our knowledge, this is the first study that has used a multivariate
427 approach (PCA) to reach this objective. PCA is a dimensionality reduction technique that is used to
428 uncover hidden structures in multidimensional data (Simmons et al., 2015), and provide key
429 insights on the relationships linking the variables. For these reasons, PCA has been used in the
430 present study to characterize the patterns linking the proportions of FAs in the BF and SM tissues.
431 The characterization of the metabolic profile of a tissue produces high-dimensional data, where
432 variables are often interconnected in metabolic patterns and share portions of their variances.
433 Similarly, the FA composition of a tissue is determined by a complex of metabolic processes
434 regulating the fluxes of *de novo* FA biosynthesis, lipolysis, and FA deposition. Previous studies
435 have used PCA or other multivariate statistics to investigate changes in the multidimensional
436 structure of FA composition in porcine tissues in relation to breed (Aboagye et al., 2020), divergent
437 levels of boar taint compounds (Mörlein & Tholen, 2015; Liu et al., 2017), and different diets
438 (Bermúdez, Franco, Franco, Carballo, & Lorenzo, 2012). PCA has also been used in this work, but
439 with a different purpose. This statistical analysis has indeed been selected to highlight possible
440 metabolic patterns linking FAs in BF and SM of ILW purebred heavy pigs. The obtained new

441 variables (PCs) have been investigated to test which factors may influence the variability of the
442 linearly dependent FAs included in each PC; the identification of these factors may be useful to
443 better understand whether some environmental factors can affect the organoleptic and nutritional
444 qualities of the final pork products.

445 The PCA for BF FAs was able to capture the negative relation linking SFAs and PUFAs, which had
446 opposite loadings in the first two PCs. The variability of the first PC for BF was determined on one
447 hand by the SFAs stearic, arachidic, and palmitic, with positive PC loadings, and on the other hand
448 by arachidonic, linoleic, adrenic, and DPA, having negative loadings in PC1. These latter FAs are
449 mainly PUFAs participating in the endogenous synthesis of *n*-6 FAs. Linoleic acid is, indeed, one
450 of the essential FAs, and is used as a substrate for further elongation and desaturation steps. The
451 proportion of *n*-6 PUFAs in tissues is dependent on diet and complex enzymatic systems, consisting
452 of desaturases and elongases, responsible for the conversion of linoleic acid into longer chain *n*-6
453 PUFAs (Brenner, 1989; Raes, De Smet & Demeyer, 2004). Linoleic acid may undergo subsequent
454 desaturation and elongation steps to produce dihomo- γ -linolenic, arachidonic, and adrenic acids
455 (Brenner, 1989; Raes, De Smet & Demeyer, 2004), which in this study were all related by negative
456 PC1 loadings. These negative weights in PC1 may thus be linked to the fact that linoleic,
457 arachidonic, adrenic, and DPA share a large covariance amount, as they are all linked to the
458 endogenous synthesis of *n*-6 PUFAs. Hence, PC1 possibly captured this shared variability linking
459 the amounts of these *n*-6 PUFAs in BF. Together with those FAs, DPA showed also a negative
460 loading in PC1, indicating that also the variation of this metabolite is partly determined by the same
461 sources of variability of the *n*-6 PUFAs synthesized from linoleic acid. This result may be related to
462 the fact that DPA can be synthesized from α -linolenic acid (C18:3 *n*-3) through desaturation and
463 elongation steps controlled by the same enzymes catalyzing the elongation/desaturation steps
464 required for the transformation of linoleic acid into longer *n*-6 PUFAs (Brenner, 1989; Raes, De
465 Smet & Demeyer, 2004). In humans and several other animal species, these steps are controlled by
466 the enzymes encoded by the genes *Fatty acid desaturase 1 (FADS1)*, *FADS2*, *ELOVL elongase 2*

467 (*ELOVL2*), and *ELOVL5* (Castro, Tocher & Monroig, 2016; Gol, Pena, Rothshild, Tor & Estany,
468 2018). In particular, as reported in the literature, *FADS1* and *FADS2* display markers associated
469 with the amounts of MUFAs and PUFAs in porcine BF tissue of crossbred pigs (Crespo-Piazuelo et
470 al., 2020) and in IMF and BF of Duroc pigs (Gol, Pena, Rothshild, Tor & Estany, 2018).

471 Furthermore, some studies conducted in different pig breeds indicated that arachidonic acid
472 contents in BF and muscle were positively correlated with carcass lean mass (Gol, Pena, Rothshild,
473 Tor & Estany, 2018; Davoli et al., 2019; Zappaterra et al., 2020). In agreement with those studies,
474 the present research indicated that pigs with leaner carcasses tended to have negative PC1 scores for
475 BF, and thus were characterized by higher contents of arachidonic, linoleic, adrenic, and DPA FAs.
476 The linoleic acid percentage in BF is of great importance for ham quality and covering fat stability
477 during ham processing, as a percentage of linoleic acid above 15% of total FA is associated with a
478 content of PUFAs that can increase the oxidability of ham fat (Bosi & Russo, 2004). Hence, PDO
479 ham production rules set threshold values for the linoleic acid percentage that must not exceed 15%
480 (Consorzio del Prosciutto di Parma, 1992; MIPAAF, 2007). Leaner carcasses may therefore have an
481 amount of linoleic acid above the permitted amount, making those thighs unsuitable for PDO ham
482 production. On the other hand, individuals displaying high BFT were significantly associated with
483 positive PC1 scores. These animals had, thus, higher contents of palmitic, stearic, and arachidic
484 acids. These three FAs originate from subsequent elongation steps in the endogenous biosynthesis
485 of the SFAs: in mammals, palmitic acid may, indeed, undergo elongation steps and can be
486 transformed into stearic and arachidic acids (Miyazaki & Ntambi, 2008). The first PC for BF FAs
487 thus captured the negative correlation linking SFAs and PUFAs, and their association with carcass
488 composition; higher fat depots are mainly determined by triacylglycerols, the main neutral lipids
489 used to store energy, which mainly consists of SFAs and MUFAs (De Smet, Raes & Demeyer,
490 2004). Fatter animals are therefore characterized by increased proportions of SFAs and MUFAs
491 deposited in tissues, causing a decrease in the relative amount of PUFAs on the total FAs (De Smet,
492 Raes & Demeyer, 2004; Lo Fiego, Santoro, Macchioni, & De Leonibus, 2005; Matthews, 2011). On

493 the other hand, it is well known that lower amounts of stored fat are associated with lower
494 depositions of SFAs and total FAs, which in turn cause an increase in the relative amount of PUFAs
495 (Monziols, Bonneau, Davenel, & Kouba, 2007; Matthews, 2011). As suggested in the literature, this
496 increased proportion of PUFAs stored in tissues of leaner animals is not due to a rise in PUFA
497 synthesis, but rather in a higher percentage of PUFAs on the reduced amount of total FAs
498 (Matthews, 2011). While SFAs are, indeed, quite fluctuating in tissues as they depend on the
499 nutritional state of the animal, the amount of PUFAs deposited in tissues tends to be highly
500 dependent on dietary *n*-6 and *n*-3 PUFAs contents. Therefore, in individuals fed the same diet,
501 PUFA content tends to remain more stable than SFAs, as UFAs play essential roles in membrane
502 flexibility, inflammation control, eicosanoid production, plasma triacylglycerol synthesis, and gene
503 expression (reviewed in Fernandez & West, 2005). Because the pigs used in the present study were
504 fed the same diets, it is possible to hypothesize that the higher proportion of PUFAs characterizing
505 some of the studied pigs might be due to their lower adiposity and thus lower amount of total FAs
506 stored in their BF.

507 The variability noticed for BF PC2 scores was strongly associated with the slaughtering season,
508 with pigs slaughtered in winter being characterized by greater proportions of capric, lauric, myristic,
509 palmitic, and palmitoleic acids, and those slaughtered in autumn showing higher contents of
510 eicosadienoic, gadoleic, and erucic acids. BF is one of the first fat depots to develop in pigs, while
511 IMF develops later, particularly in the muscles of the hind leg (Kouba & Bonneau, 2009). In heavy
512 pigs, BFT and FA composition are mainly determined by the diet and environmental conditions
513 applied during the finishing period, which lasts from 110-120 kg live weight to slaughtering at
514 about 160 kg. The finishing period takes about three months in Italian heavy pigs and has the main
515 objective to improve meat quality. FA composition of IMF and subcutaneous fat depots are thought
516 to take a long time to vary, so that different fattening period lengths did not affect BF and IMF FA
517 composition in extensively reared Iberian pigs (Ayuso, González, Peña, Hernández-García &
518 Izquierdo, 2020). Given that changes in the FA composition of tissues occur slowly, it is reasonable

519 to assume that the association found in the present study between PC2 scores and slaughtering
520 season may reflect the consequence of the whole finishing period on the BF FA composition found
521 at slaughter. The studied animals were fed the same diets and were reared in the same genetic
522 station located in Po Valley (Italy), a geographical region characterized by a hot and highly humid
523 weather during the late spring and summer. Prolonged periods with high temperature humidity
524 indices cause heat stress in pigs, which lack functional sweat glands and poorly dissipate heat
525 (White et al., 2008). Increasing temperatures and humidity have been indicated as factors affecting
526 the performance of growing-finishing pigs as heat stress was proved to affect growth, feed intake,
527 and caloric and feed efficiency (Renaudeau, Gourdine, & St-Pierre, 2011; Kellner, Baumgard,
528 Prusa, Gabler, & Patience, 2016). In the present study, pigs slaughtered in autumn (and particularly
529 in early autumn) spent their finishing period in the hottest months. These environmental conditions
530 may have led pigs slaughtered in early autumn to have thinner BFT when compared to those
531 slaughtered in winter and spring. In those animals, a reduction in BFT and therefore in SFAs may
532 explain why their BF was characterized by higher proportions of eicosadienoic acid, an *n*-6 PUFA.
533 On the other hand, pigs slaughtered in winter (and in particular in late winter) may have not
534 experienced a hot and muggy environment during the finishing period, which may have caused
535 higher BFT and thus greater proportions of SFAs being stored in subcutaneous fat. Therefore,
536 taking into account these suggestions, it might not be so surprising that the two most different
537 seasons were autumn and winter. Stearic acid, instead, did not follow the same pattern evidenced in
538 PC2 for the other SFAs. This FA entered with a high weight in PC1, and its content in BF tissue
539 was higher in pigs slaughtered in summer and autumn. Several studies suggest the role of stearic
540 acid and its monounsaturated counterpart (i.e. oleic acid) in the regulation of cellular membrane
541 fluidity in animals living at different environmental temperatures (Roy, Das & Ghosh, 1997;
542 Malekar et al., 2018). Changes in oleic and stearic acid contents are particularly visible in
543 poikilothermic animals, such as fish (Roy, Das & Ghosh, 1997; Malekar et al., 2018), with
544 increased stearic acid incorporation in membranes as environmental temperatures rise (Malekar et

545 al., 2018). Accordingly, the enzyme catalyzing the unsaturation of stearic to oleic acid (i.e. Stearoyl
546 Co-A desaturase, SCD) has been suggested as an important regulator of cellular endoplasmic
547 reticulum membrane fluidity in mammals and fat globule fluidity in cow milk (Timmen & Patton,
548 1988). Stearic acid has a melting point higher than the body temperature of animal species (69.6°C),
549 and its increased incorporation permits the maintenance of cellular membrane characteristics also
550 during high-temperature seasons. The higher content of stearic acid in the BF of pigs slaughtered in
551 summer and autumn may therefore reflect the attempt of the adipocyte membranes to maintain
552 membrane integrity by incorporating higher contents of this SFA, and consequently increasing their
553 resistance to high-temperature environments.

554 Similar to what was observed for BF, the first PC for SM FAs was able to capture the negative
555 relation linking SFAs and MUFAs with PUFAs. Unlike BF, however, the results of the multiple
556 regression models for the PCs of SM indicated that the effect of slaughtering season (and thus of the
557 finishing period season) on muscle FA composition was mediated by other factors, which strongly
558 influenced the muscle FA patterns. Together with slaughtering season, SM IMF% and animals' sex
559 were highly significant for PC1 variability. Pigs displaying higher IMF % had indeed increased
560 contents of oleic and myristic acids, and lower amounts of erucic, DPA, adrenic, dihomo- γ -
561 linolenic, arachidonic, DHA, and eicosadienoic acids. This observation is in agreement with the
562 positive relation linking IMF deposition, and the amounts of SFAs and MUFAs found in muscle fat
563 depots (Bosch, Tor, Reixach, & Estany, 2012). In particular, oleic acid has been found to share a
564 consistent proportion of genetic variance with IMF deposition in different muscles of Duroc pigs
565 (Ros-Freixedes, Reixach, Bosch, Tor, & Estany, 2014), and a moderate positive genetic correlation
566 with SM IMF% in ILW pigs (Zappaterra et al., 2020). An association was also identified between
567 pigs' age and muscle PC1 scores, with older animals having higher contents of oleic and myristic
568 acids in SM. This is consistent with the fact that IMF increases with age and IMF saturation level is
569 enhanced by greater IMF deposition (Bosch, Tor, Reixach, & Estany, 2012). Pigs slaughtered at
570 later ages are therefore expected to have more IMF, SFAs and MUFAs in muscles, as SFA and

571 MUFA amounts increase with lipid deposition in porcine muscles (Bosch, Tor, Reixach, & Estany,
572 2012; Ros-Freixedes, Reixach, Bosch, Tor, & Estany, 2014).

573 The variability noticed in SM PC2 scores was mainly determined by the antagonism shown by *cis*-
574 vaccenic and palmitoleic MUFAs against major SFAs (i.e. lauric, myristic, palmitic, stearic, and
575 arachidic). Pigs slaughtered in summer showed higher proportions of SFAs in SM IMF. This
576 positive association between the summer as slaughtering season and SFAs may originate from the
577 attempt of the muscle-interspersed adipocytes to maintain membrane integrity by incorporating
578 higher contents of SFAs, which increase membrane resistance to a high-temperature environment.
579 However, unlike BF, slaughter season did not determine changes in IMF%, suggesting that different
580 environmental conditions may affect FA metabolism and deposition, but they do not change the
581 amount of fat deposited in muscle. Based on these results, further studies proving the effects of
582 different environmental temperatures on lipid and energy metabolism in heavy pigs may be of
583 interest.

584
585

586 **Conclusions**

587 The multivariate approach applied to the FA composition of porcine BF and SM allowed the
588 identification of patterns in the FA deposition shaping the variability in the FA composition of the
589 two studied tissues. An inverse relationship of the deposition of SFAs with PUFAs resulted to be
590 among the major patterns characterizing both BF and SM. The overall variability in the FAs
591 deposited in subcutaneous fat and muscle showed to be strongly related to the slaughtering season
592 and carcass features. In agreement with the literature, leaner carcasses were associated with higher
593 proportions of PUFAs, confirming that carcasses with high lean mass deposition may have FA
594 composition unsuitable for the processing into PDO dry-cured hams. Remarkably, slaughtering
595 seasons emerged as relevant factors shaping both BF and muscle FA composition. More efforts
596 should be applied to understand the effect that high environmental temperatures may have on FA
597 metabolism and deposition in finishing heavy pigs.
598

599

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828

829 **Highlights**

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- 831 • Various intrinsic and extrinsic factors affect muscle and backfat fatty acids.
- 832 • The multivariate structure of pig muscle and backfat fatty acids was investigated.
- 833 • The antagonism of saturated vs. *n*-6 fatty acids was the main relation identified.
- 834 • The dataset structure was associated with slaughtering season and carcass traits.
- 835 • Pigs had more vaccenic and palmitoleic acids in muscle when slaughtered in autumn.