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Preliminary study on the influence of the geographical origin and farming system on 'Nero dei Nebrodi' pig using chemical and isotopic fingerprinting

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ABSTRACT

In this study, the geographical origin of *longissimus dorsi* meat from 'Nero dei Nebrodi' pigs reared in two distinct regions of north-eastern Sicily was indagated by correlating chemical-nutritional parameters, stable isotope composition, fatty acid and sterol profiles, and mineral element content. Significant differences ($p \leq 0.05$) between the 'Nebrodi group' (NG) and the 'External Nebrodi group' (ENG) were found for 51 over 80 variables. The δ^2H, δ^{18}O, δ^{13}C and δ^{15}N of the defatted meat were statistically different ($p<0.01$) between NG and ENG animals, indicating a change in the composition of the diet and drinking water consumed. The results showed that the characteristic rich endemic vegetation of the Nebrodi influences not only the chemical-nutritional parameters of the meat but also its isotopic ratios, allowing for a geographical characterization required for the traceability of this peculiar product, aspiring to a protected designation of origin.

1. Introduction

The performance of the pig, the quality and the composition of both carcass and derived products depend on many factors, such as the genotype and the rearing conditions, both in intensive (indoor) and free-range systems (Tejeda et al., 2020). Particularly in the free-range system, the peculiarity of the vegetation and the seasonality can confer unique characteristics to the products. The Mediterranean production systems include the use of local breeds that are extensively grazed and slaughtered at an older age to obtain high quality matured products (Lebret, 2008). Animals certified geographical origin, breed and production system usually increase the value of the derived products. Moreover, products respecting animal welfare and environmental sustainability are given additional value. Indeed, consumers are generally willing to pay a higher price for products with a clearly defined geographical origin, as for Protected Designation of Origin (PDO) brands (Flinzberger et al., 2024). The 'Nero dei Nebrodi' pig is an indigenous breed from Sicily, distinguished among others by its small size, dark bristly coat, straight frontal-nasal profile, and small ears pointing obliquely upwards with the tips carried horizontally forward. Known for their high degree of rusticity, these pigs are primarily reared outdoors in the Nebrodi area, a hilly and mountainous region reaching the maximum altitude of 1847 m. This breed has been the subject of both genomic (Chen et al., 2021; D'Alessandro et al., 2007; Guastella et al., 2010) and growth performance (Pugliese et al., 2003) evaluations, and of meat and fat quality in indoor and outdoor rearing (Pugliese et al., 2004). Following the demographic decline of the 'Nero dei Nebrodi' pig, which occurred up to 15 years ago, conservation and development programs were implemented to avoid the extinction of this native breed. These efforts increased the population to approximately 900 sows, up from 450 in 2010 (ANAS, 2024). The animals are mainly bred in Sicily, in 127 farms out of a total of 128, where pigs are reared in the open air for traditional and social reasons rather than for strictly commercial purposes. In particular, 104 farms are located in the Nebrodi area, where 685 over 874 total sows reared in Sicily (78.38 %) are grazed (Fig. 1).

In recent years, with the development in "Nero dei Nerbrodi" pig breeding, the interest in the historical tradition of this breed and the processing and transformation of its meat has also increased. To protect

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this unique Sicilian pig biodiversity, it would be useful to link the breed to the products, their specific labelling and quality certification.

The PDO is a type of geographical indication of the European Community, which refers to a typical and high-quality product, whose distinctive characteristics, related to the area of origin and to the traditional production methods, guarantee protection from counterfeiting (Flinzberger et al., 2024). Recent studies showed that the PDO designation can also be an indicator of the sustainable management of the landscape (Flinzberger et al., 2024), from breeding to grazing. Considering PDO products as directly linked to a specific landscape may imply recognising that their production interacts with the socio-ecological trends of that geographic area, including environmental and biodiversity aspects, traditions, food culture, local identity, rural development and tourism. This means that the influence of PDO products goes beyond productivity and economic aspects.

In this context, the Department of Agriculture of the Sicilian Region established a promotion committee to request the Ministry of Agricultural, Food and Forestry Policies (MASAF) to recognize the PDO designation for 'Nero dei Nebrodi' fresh meat and its processed products. This procedure has already approve for products like Kintoa pig in France or Cinta Senese in Italy. To ensure the authenticity of products with designation, food traceability techniques have been used to correlate chemical composition with geographical origin, employing chemometric tools (Fontanesi, 2017; Bandoniene et al., 2018; Potortì et al., 2018). Although some studies on 'Nero dei Nebrodi' pig are available in the literature (Liotta et al., 2001; Chiofalo et al., 2003; Madonia et al., 2000; Chicoli, 1870) no research has yet evaluated the stable isotope and mineral element composition of 'Nero dei Nebrodi' meat as a tool for traceability. Furthermore, there is still limited information on the impact of the rearing area on meat quality.

This study examined the correlation between the geographical origin of 'Nero dei Nebrodi' *longissimus dorsi* meat, coming from pigs raised in two different north-eastern Sicily areas, and various factors, including chemical-nutritional parameters, stable isotopic composition, fatty acid and sterol profiles, and mineral elements. The aim was to establish a reliable link between "Nero dei Nebrodi" meat and its specific characteristics, supporting traceability efforts to protect consumers from fraud and resolve commercial disputes.

2. Material and methods

2.1. Animals, study area and sampling

This study was conducted on commercial meat samples purchased directly from farm stores. Consequently, it did not involve animal testing or the introduction of agricultural practices other than those normally used and, therefore, it was not necessary to request the evaluation and approval of an ethics committee. The study took place in two geographical areas of northern Sicily, in the province of Messina: Mirto Arale (38°05'04''N 14°44'33''E, 515 a.s.l.), within the Nebrodi National Park, and Valle del Mela Arale (38°09'33'' N 15°16'13''E, 144 a.s.l.), outside the Nebrodi National Park. A total of 20 12-month-old black Nebrodi females were randomly divided into two homogeneous groups: the 'Nebrodi group' (NG) and the 'External Nebrodi group' (ENG). The animals came from the same commercial farm but from different litters and were weaned 90 days after birth. The NG group remained on the farm of origin, in Mirto Areale, while the ENG group was transferred to a farm outside the Nebrodi area. As it can be seen in Fig. 1, Mirto Areale

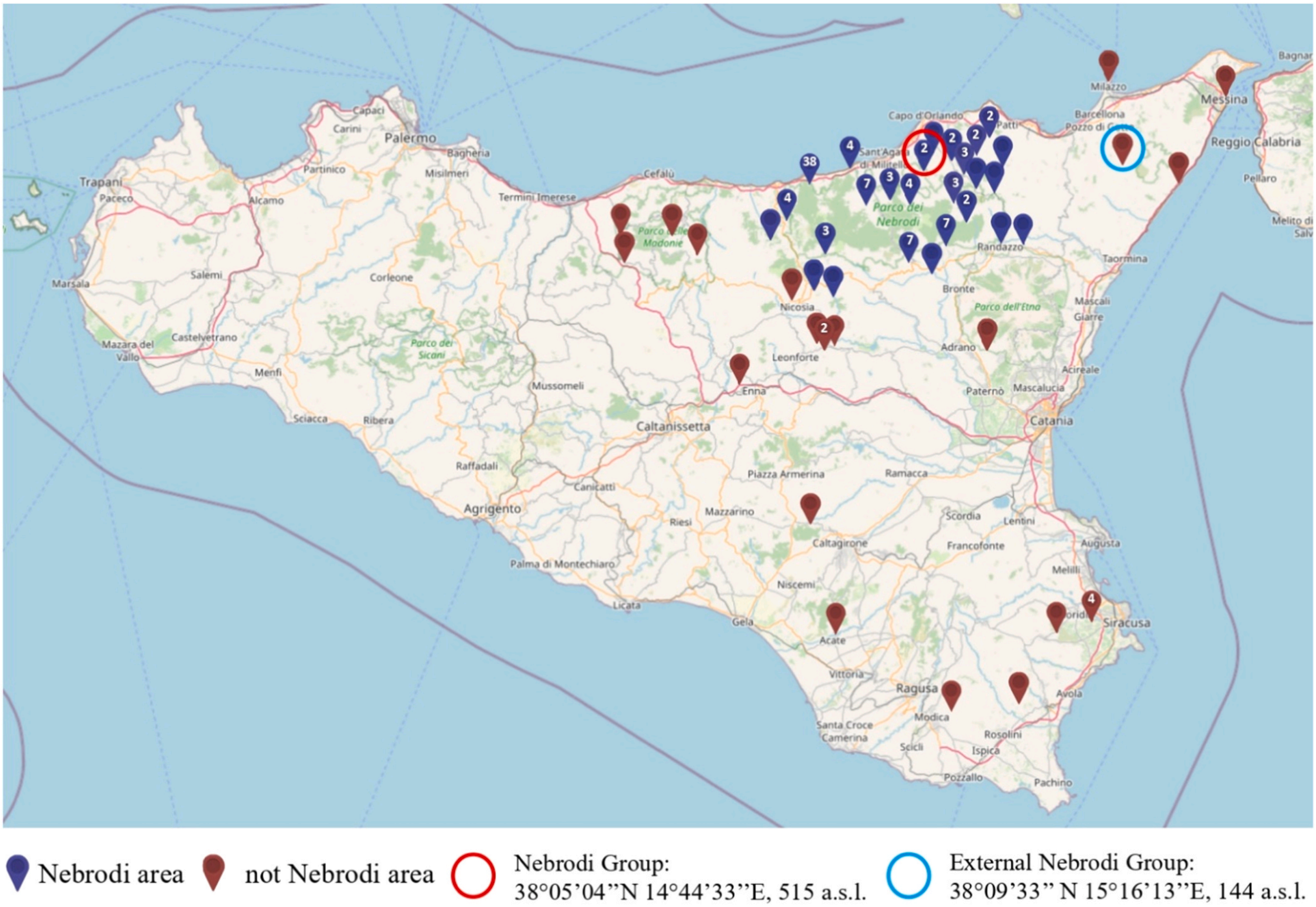

Fig. 1. Map of Sicily illustrating the geographical location of all "Nero dei Nebrodi" pig farms and two farms considered in this study.

lies in the heart of the Nebrodi National Park; the Nebrodi Mountains are part of the Sicilian Apennines, facing the Tyrrhenian Sea to the north, while their southern limit is marked by Mount Etna. The main elements that characterise the natural landscape of Nebrodi are the dissymmetry of the two faces, the rich vegetation, and the wetlands. The Valle del Mela Areale, outside the Nebrodi Natural Park, is located on the western slopes of the Peloritani Mountains and offers a rich variety of landscapes.

The study was conducted during the finishing phase, from June 2022 to January 2023. All pigs were housed under the same conditions in a traditional outdoor system in the two geographical areas mentioned above; the diet was mainly based on natural pasture with some supplementation of cereals (mainly barley, while corn has never ben given to the animals) and broad beans during periods of low pasture availability. All pigs had *ad libitum* access to water throughout the study. Approximately 24 hours after slaughter (20 ± 2 months of age; 110 ± 10 kg), a meat sample corresponding to the *longissimus dorsi* (LD) between the 10th and 14th ribs was taken from the left half-carcass of each animal. All samples were vacuum-packed and shipped to the laboratory at a controlled temperature of 4–5 °C within 2 hours of collection. Each muscle sample weighed approximately 300 g. Once at the laboratory, samples were trimmed of fat, minced, homogenised and analysed in triplicate.

2.2. Analytical grade chemical and reagent

The fatty acid methyl ester (FAME) mixture reference standard was obtained from Sigma-Aldrich (Supelco 37-Component FAME Mix, Darmstadt, Germany). Individual standards not included in the mix, such as trans-palmitoleic acid (C16:1 n-7), cis-vaccenic acid (C18:1 n-7), 15-docosenoic acid (C22:1 n-7) and acid 6, 9,12-hexadecatrienoic acid (C16:2 n-4), were purchased from Merck (Darmstadt, Germany). Merck (Darmstadt, Germany) also provided the internal standard 5α-cholesterol for the determination of sterols. Nitric acid (HNO_3, 65 % v/v) and hydrogen peroxide (H_2O_2, 30 % v/v) were provided by J. T. Baker (Mallinckrodt Baker, Milan, Italy). The standard solution Re as internal standard (IS) and the standards Ca, Na, Mg, Mn, Fe, Zn, Be, Co, Cr, Cu, Mo, Ni, Sb, Sn, Pb, Cd and As used for the curves calibration tests were obtained from Supelco (Bellefonte, PA, USA). All the standars used had a purity of 99 %.

2.3. Chemical composition

Each LD sample was homogenized individually after removal of the fat cover. Moisture and protein were determined in triplicate for each sample AOAC (Association of Official Analytical Chemists), (2002). Intramuscular fat (IMF) was instead determined by extraction with chloroform/methanol (2:1 v/v) according to the rapid solvent extraction method described by Folch et al., (1957).

2.4. Stable isotope ratio analysis

2.4.1. Preparative and analysis procedures

After cutting the meat, it was minced and subsequently freeze-dried with a 5PASCAL freeze-dryer (Trezzano sul Naviglio MI, Italy) model LIO5P DIGITAL. Subsequently, a Soxhlet apparatus was used to degrease the samples using petroleum ether, and the fat-free residue (or dry mass) was collected and stored for C, N, S, O, and H analyses (Perini et al. 2009; Camin et al. 2007).

2.4.2. Stable isotope ratio analysis

All samples were weighed into silver and tin capsules for OH− and CNS−isotope measurements, respectively. The $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios were measured using an isotope ratio mass spectrometer (IRMS) (Finnigan DELTA XP, Thermo Scientific, Bremen, Germany) coupled with a pyroliser (Finnigan DELTA TC/EA, high-temperature conversion elemental analyser, Thermo Scientific). Before analysis, the silver capsules containing the samples or standards were kept for 72 h in a vacuum desiccator with silica. The measure of $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ were performed by using an isotope ratio mass spectrometer (IsoPrime, Isoprime Limited, Langenselbold, Germany) after total combustion in an elemental analyser (VARIO CUBE, Isoprime Limited).

The measured isotope ratios are reported in the delta (δ) notation corresponding to the relative deviations of the molar ratio (R) of the heavy elements (^{h}E i.e., ^{13}C, ^{2}H, or ^{18}O) to light elements (^{l}E i.e., ^{12}C, ^{1}H, or ^{16}O) isotopes in the samples from those in international standards V−PDB (Vienna−Pee Dee Belemnite) for $\delta^{13}C$, V−SMOW (Vienna−Standard Mean Ocean Water) for $\delta^{18}O$ and $\delta^{2}H$, V−CDT (Vienna−Canyon Diablo Troilite) for $\delta^{34}S$, and Air (atmospheric N_2) for $\delta^{15}N$, as shown in the following equation according to the IUPAC protocol (Brand and Coplen, 2012):

$$\delta^{h}E_{sample} = \left[\frac{R\left({}^{h}E/{}^{l}E\right)_{sample}}{R\left({}^{h}E/{}^{l}E\right)_{standard}}\right] - 1 \tag{1}$$

The delta values are here multiplied by one thousand and expressed in the more common unit "per mil" (‰) rather than, as required by the International System of Units (SI), in milliurey units (mUr) (Brand and Coplen, 2012).

Data normalization to the VPDB-LSVEC and VSMOW-SLAP scales was performed using three or four-point calibration with international reference materials (RMs). International reference materials or internal working standards calibrated against them were used for the analyses. For details of the standards used, see Carullo et al. (2024).

By placing the samples at the beginning and end of each daily analysis group, the samples were analyzed in duplicate. To verify the effectiveness of the measure, a control sample was also included in the analyzes of each sample group. The maximum standard deviations of repeatability accepted were 0.3 ‰ for $\delta^{13}C$ and $\delta^{15}N$, of 0.4 ‰ for $\delta^{34}S$, 0.5 ‰ for $\delta^{18}O$ and of 3 ‰ for $\delta^{2}H$.

2.5. Fatty acid and sterols content

The collected lipid fraction was subjected for one hour to hot esterification with a 9:1 methanol: sulphuric acid (v/v) mixture in an oven at 110 °C. The supernatant was collected and diluted 1:2 with hexane and analysed on a gas chromatograph (GC) equipped with a split/splitless injector and a flame ionisation detector (FID) (Dani Master GC, Dani Instrument, Milan, Italy) according to the method proposed by Di Bella et al. (2022). Aliquots of the lipid extracts were saponified with a solution of ethanol and potassium hydroxide after the addition of the internal standard (5α-cholesterol). The unsaponifiable fraction was extracted with ethyl ether. After separation by chromatography on silica gel, the sterol fraction was analysed by gas chromatography after derivatisation with derivatising reagent BSTFA: TMCS (99:1) to obtain the corresponding trimethylsilyl ethers. The extraction was carried out according to the EU Regulation 1348/2013, following the method proposed by Martini et al., (2021).

2.6. Analyses of mineral elements

Mineral elements screening (Ca, Na, Mg, Mn, Fe, Zn, Be, Co, Cr, Cu, Mo, Ni, Sb, Sn, Pb, Cd and As) was performed using our optimised method for several food matrices (Potortì et al., 2024; Massous et al., 2023; Nava et al., 2022). The analysis was performed with a quadrupole ICP-MS iCAP-Q (Thermo Scientific, Waltham, MA, USA). Briefly, muscle aliquots of 0.5 g each were digested with 2 mL H_2O_2 and 7 mL HNO_3 using the Ethos 1 microwave digestion system (Milestone, Bergamo, Italy). All samples were analysed in triplicate together with analytical blanks and data acquisition was performed using Qtegra™ Intelligent Scientific Data Solution (Thermo Scientific™). Each analyte was quantified by constructing a six-point calibration curve (R^2 between 0.9984

(Na) and 0.9999 (Cd)). The samples were analyzed in triplicate, together with the analytical blanks. The ICP-MS procedure has been validated analytically for linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and both intra- and inter-assay variability, as detailed in our recent study (Potortì et al., 2024) (for more details see Table S1 in Supplementary Material). Quality control of the analysis was achieved by measurement of the blank and the certified reference material 'ERM®- BB184 Bovine Muscle' (Joint Research Centre Institute for Reference Materials and Measurements, Belgium), prepared under the same conditions as the samples. The following mineral elements were contained in the certified reference material: Ca, Mg, Na, As, Cd, Cu, Fe, Mn, Zn. The matrix was fortified with a known amount of these analytes if the element was not certified in the reference material, as in the case of Be, Co, Cr, Mo, Ni, Sb, Sn and Pb.

2.7. Statistical analysis

Statistical analyzes were conducted using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). The initial multivariate matrix included 20 cases (samples analyzed) and 80 variables (all parameters analyzed). The data were categorized into two groups based on the area of origin: NG (inside the park) and ENG (outside the park). Differences between groups were initially assessed using the nonparametric Mann-Whitney U test. Subsequently, the data set was normalized to ensure the independence of the scaling factors between the variables. Only variables that showed significant differences ($p < 0.05$ or $p < 0.01$, depending on the variable) between the two groups were included. After having verified the congruity of the initial data with the Kaiser-Meyer-Olkin (KMO) test and with the Bartlett test, a factor analysis with principal component extraction (PCA) was carried out to distinguish the samples based on their area of origin.

3. Results and discussions

3.1. Chemical composition and sterols content

Table 1 shows sterol profile and chemical composition of the samples analysed. Protein and moisture contents were significantly different between groups ($p < 0.05$). Moisture was significantly higher in the ENG samples than in the NG samples. Both values are in line with those found in other studies, e.g. Ren et al., (2008) found moisture values between 69.65 % and 72.00 % in Queshan black pigs and Yorkshire pigs, respectively, while Kim et al., (2009) found average values of 70 % in native Korean black pigs. The protein content was significantly higher in NG samples with a mean value of 23.08 %. Although not statistically different between the two groups, the lipid content was on average lower in NG samples than in ENG samples ($p > 0.05$). However, the results obtained are in line with those of other authors (Muhlisin et al., 2014; Perna et al., 2014; Ren et al., 2008).

Table 1
Chemical composition and sterol profile in muscle samples from the two geographical areas of origin: NG "Nebrodi group", ENG "External Nebrodi group".

Item	NG	ENG	SEM[a]	*p-value*[b]
Moisture %	66.94	70.46	0.01	**0.027**
Proteins %	23.08	20.88	0.01	**0.001**
Lipids %	4.30	6.08	0.09	0.269
Sterols profile (mg/100 g)				
Cholesterol	56.96	63.55	0.03	0.215
Brassicasterol	0.54	0.07	0.26	**0.000**
Campesterol	0.42	0.17	0.12	**0.000**
Campestanol	0.09	0.05	0.10	**0.009**
Δ−5,24-Stigmastanol	0.11	0.07	0.11	**0.009**
Δ−7-Stigmastanol	0.06	0.05	0.12	0.964
Δ−7-Avenasterol	0.04	0.05	0.11	0.685

[a] SEM (Standard Error of Mean)

[b] Bold values are significant at p<0.05

The sterol profile is also shown in Table 1; in addition to cholesterol, brassicasterol, campesterol, campestanol, Δ-5,24-stigmastanol, Δ-7-stigmastanol, Δ-7-avenasterol were found in all samples analysed. The cholesterol content of the two groups was not statistically different, with a mean value of 56.96 mg / 100 g in the NG samples and 63.55 mg / 100 g in the ENG samples. In contrast, brassicasterol, campesterol, campestanol and Δ-5,24-stigmastanol were significantly higher in the NG samples ($p<0.05$).

As reported by Nashed et al., (2005) among the important roles played by phytosterols are immune regulation and positive effects on disease resistance and anti-inflammatory activity and may benefit animal health and growth.

As demonstrated by Song et al. (2023) plant sterol supplementation has positive effects on pig growth performance and biochemical indicators, by improving both nutrient digestibility and lipid status in animal blood. Furthermore, another study conducted by Naji et al., (2014) evaluated the effects of dietary phytosterols on growth and gene expression in broiler muscle and concluded that the administration of phytosterols was a good dietary programme for adequate morphological development of pectoral muscle.

The mountain flora of Nebrodi is particularly rich in regional endemic vegetation (Tuttolomondo et al., 2014) such as the pug thistle (*Carlina nebrodensis*), the Nebrodi broom (*Genista aristata*), the Boccone hellebore (*Helleborus bocconei*), the Boccone oak (*Quercus gussonei*) and the wild brassica (*Brassica montana* and *Brassica rupestris*), which are a source of valuable biomolecules for the animals bred in the area. The results of our study could relate to the rich wild vegetation in the Nebrodi area, which has a positive influence on meat quality.

3.2. Stable isotope composition and geographical discrimination between areas

Table 2 shows the results of the stable isotopes ratios analysis of carbon, nitrogen, sulphur, oxygen, and hydrogen of *longissimus dorsi (*LD) muscle of pig samples raised in both NG and ENG areas. The defatted meat δ^{13}C and δ^{15}N resulted statistically different ($p<0.01$) when comparing NG and ENG animals, revealing a difference in the composition of their diet. Carbon isotopic composition is mainly correlated with plant photosynthetic cycles, according to which they can be classified into three distinct groups: C3, CAM and C4 plants (O'leary et al. 1992; O'Leary, 1981). C3 plants, which represent about eighty-five percent of the planet's plant species, use the Calvin cycle to fix carbon via the enzyme Rubisco. These plants have a δ^{13}C ranging between −33 ‰ and −23 ‰. To adapt to hot, arid environments while minimizing water loss through stomatal evapotranspiration, both C4 and CAM plants developed alternative photosynthetic pathways to the C3 one. C4 plants use an enzyme other than Rubisco to fix CO_2, forming a four-carbon (oxaloacetic) acid, after which they are named. These plants typically have δ^{13}C values ranging from −14 ‰ to −12 ‰. Finally, CAM plants have δ^{13}C values between C3 and C4 and adopt a water-saving mechanism known as Crassulaceae acid metabolism (CAM), which

Table 2
Isotopic ratios of defatted meat samples (‰) divided into two homogeneous groups: the 'Nebrodi group' (NG) and the 'External Nebrodi group' (ENG).

Item	**δ^{13}C (‰, vs V-PDB)**	**δ^{15}N (‰, vs AIR)**	**δ^{34}S (‰, vs V-CDT)**	**δ^{2}H (‰, vs V-SMOW)**	**δ^{18}O (‰, vs V-SMOW)**
NG					
Mean	−22.8	6.3	−0.1	−86	14.3
SD	0.4	0.3	0.5	2	0.4
ENG					
Mean	−23.9	7.5	−0.3	−78	15.6
SD	0.2	0.7	0.4	2	0.6

temporarily separates CO_2 fixation from sugar synthesis (Pereira et al., 2021). In the analysed samples, the reported values (average −22.8 ‰ for NG and −23.9 ‰ for ENG) are compatible with a diet mainly based on C3 plants, the representative vegetation of the sampling area (see Section 3.1). In the two breeding sites, the animals had the same composition supplements based on germinated barley (C3) with no C4 ingredient (such as corn) included. A possible explanation for the higher $\delta^{13}C$ of the NG group could be represented by the exclusive presence of some C4 plants in the Nebrodi area vegetation, as reported by Tuttolomondo et al., (2014). These include the *Amaranthus* genus (e.g. *cruentus, hybridus, hypocondriacus, retroflexus)*, ingested by grazing animals along with other plants. The presence of C4 species in the Nebrodi area exclusively can result in the discrimination between their meat and other competitor products, based on the $\delta^{13}C$.

The $\delta^{15}N$ and $\delta^{34}S$ of defatted meat are correlated with the same parameters of fodder plants consumed by animals. Also, it has long been known that the $\delta^{15}N$ of animal tissues is higher than the $\delta^{15}N$ of the diet they consume (trophic level effect) (Deniro and Epstein, 1981). Plant and soil $\delta^{15}N$ values are generally correlated, being the former more negative than the latter. The difference between the parameters ($\delta^{15}N_{plant}$-$\delta^{15}N_{soil}$) increases with decreasing mean annual temperatures (MAT) (Amundson et al., 2003). While no fractionation processes occur to sulphur between the soil and the plant roots, resulting in no differences between their $\delta^{34}S$, leaves and grains (for instance, wheat ones) are enriched up to 2 ‰ compared to soil sulphate (Tcherkez and Tea, 2013). Different factors influence soil nitrogen and sulphur isotopic composition. The effect of fertilization (chemical or organic), which is reported to strongly affect both $\delta^{15}N$ and $\delta^{34}S$ (Vitòria et al., 2008), can be excluded in the present study, since we are dealing with fertilizers free pastures. As plants absorb nitrogen from the soil through the roots, their $\delta^{15}N$ will reflect the value of the soil (average values ranging between −2 ‰ and +10 ‰) (Amundson et al. 2003). Leguminous plants opt for an alternative way to absorb nitrogen, picking it up directly from the air through the leaves. Therefore, the $\delta^{15}N$ of these plants will reflect the atmospheric N_2 one (around 0 ‰). Leguminous plants probably had different concentrations in NG and ENG diets, providing a possible explanation for the difference between the nitrogen values ($p<0.05$) of their meat (Table 2). Among Nebrodi area exclusive endemic plants are the legumes *Genista aristata C. Presl* and *Trifolium bivonae Guss.* (Fam. Leguminosae) (Mazzola, 2020).

The geology of the soil on which a plant is grown, such as the type of underlying local bedrock or the presence of sulfides or sulfates, is one of the factors that can influence the $\delta^{34}S$ values (Mizota and Sasaki, 1996), as well as the proximity to the sea (sea-spray effect), since seawater sulphates reaching the coast have $\delta^{34}S$ values of 21–22 ‰ approximately (Krouse and Mayer, 2000). Volcanic, igneous rocks have a $\delta^{34}S$ value close to 0 ‰, while values diverging from 0 ‰ indicate rocks containing sulphur of sedimentary, biological or (bio) chemical origin (Tcherkez and Tea, 2013). The $\delta^{34}S$ signal is transferred from plants to animals and it is not altered in food chains (Camin et al. 2007). The reported correlation between the $\delta^{34}S$ value of biomass and primary sulphur sources is only valid for bulk materials and not for individual compounds or tissues (Tanz and Schmidt, 2010). The $\delta^{34}S$ values measured in the defatted meat of the sample group ENG and NG are not significantly different ($p>0.05$). This probably reflects a certain homogeneity in the geological composition of the soil (Calabride Units, pre-Paleozoic and Paleozoic crystalline basement nappes sutured by a syntectonic terrigenous deposit) as already demonstrated by Lentini and Carbone (2014). The distance from the sea (about 6 Km) is also the same for the two sampling sites, excluding a different influence of the sea-spray effect.

As reported in the literature, there is a correlation between the isotopic composition of hydrogen and oxygen of the defatted animal meat and that of both feed and water ingested by the animal (Camin et al. 2007; Heaton et al. 2008). The hydrogen isotopic composition of intracellular water (resulting in the defatted meat one) depends on extracellular water hydrogen (81 %) and intracellular metabolic hydrogen (19 %) (Ehleringer et al., 2008).

The δ^2H of the defatted meat fraction is mainly influenced by the δ^2H of the plants the animals have been consuming, while only approximately 30 % of it depends on the isotopic composition of the drinking water. The $\delta^{18}O$ isotope ratio mainly depends on the water source the animals have been drinking, be it groundwater (tap water), surface water or feed water (for example, water in pasture grass). Relatively high local temperature and humidity enhance soil and plant evapotranspiration, leading to greater ^2H and ^{18}O enrichment in plant tissues (i.e., higher ^2H and ^{18}O values). This, in turn, results in greater enrichment of these isotopes in animal products compared to the primary source, namely precipitation (Sternberg, 1989). Rainfall figures as the ultimate factor of influence for plants and drinking water δ^2H and $\delta^{18}O$.

According to the literature, the main variability factors determining rainfall δ^2H and $\delta^{18}O$ are the temperature effect, the continental effect (the greater the distance from the coast, the more depleted the precipitation, resulting in "heavier" precipitation on the coast with respect to the inland (Ingraham, 1998)) and the altitude (the isotopic composition of precipitation is lower as the air temperature decreases). While the δ^2H and $\delta^{18}O$ of rain and of the water resulting from plants evapotranspiration are essentially the same, the water contained in fresh food (e.g. grass) is significantly enriched in its isotopic composition compared to the groundwater the plant absorb (White, 1989; Dawson and Ehleringer, 1993). In the absence of direct measurement of δ^2H and $\delta^{18}O$ on rainwater in the sampling sites, the Water Isotope database was used. On the site http://wateriso.utah.edu (accessed on 1 March 2024) monthly weighted average precipitation data are available for sites around the world (Bowen et al., 2018). The GPS coordinates and the altitude in meters above sea level of the two pig breeding locations were entered into the portal to obtain the expected δ^2H and $\delta^{18}O$ values of the rainwater in those sampling sites. The NG site of Mirto (ME) presents rainwater average values of −6.3 ‰ for $\delta^{18}O$ and −38 ‰ for δ^2H, while the ENG site of Valle del Mela of −5.6 ‰ for $\delta^{18}O$ and −34 ‰ for δ^2H. Therefore, rainfall $\delta^{18}O$ and δ^2H values were significantly lower ($p<0.05$) for the Nebrodi park compared to the external sampling site ENG, which seems to justify the results obtained for meat $\delta^{18}O$ and δ^2H (mean $\delta^{18}O = +14.3$ ‰ and mean $\delta^2H = -86$ ‰ for NG, and mean $\delta^{18}O = +15.6$ ‰ and mean $\delta^2H = -78$ ‰ for ENG). The correlation between the isotopic composition of the rain and the proteinic animal fraction is therefore confirmed and it allows to discriminate the samples coming from the two studied areas.

To the best of our knowledge, no studies in the literature reported isotopic values of pigs raised in Sicily, probably because this is not a typical intensively farmed region (such as Friuli, for instance). In a recent study on cattle raised on open air, values similar to the results reported in the present research are reported, with averages for the δ^2H isotopic ratio (-87 ± 3.5 ‰) (Bontempo et al. 2023).

3.3. Fatty acids profile between areas

Meat quality is closely related to the fatty acid composition of IMF. Table 3 shows the fatty acid profile of the samples analysed and as can be seen, most of the fatty acids are statistically different in the two groups. Regarding the fatty acid classes, SFA, MUFA, PUFA, n3, n6 and n6/n3 were statistically different in the two groups. Specifically, SFA and MUFA were higher in the NG samples (55.65 % vs 42.37 %) compared to the ENG samples where PUFA, n3, n6 and n6/n3 were higher (8.15 % vs. 14.10 %, 0.89 % vs. 1.02 %, 6.05 % vs. 12.96 %, 6.91 % vs. 12.85 %, respectively). Among the SFAs, the most abundant fatty acid in both groups was C16:0, followed by C18:0, but the differences between the two groups in these fatty acids were not significant (p > 0.05). The fatty acids C12:0, C15:0, C17:0, C22:0, C24:0 was significantly lower in the NG.

Lipids in the *longissimus dorsi (*LD) muscle of NG samples were distinguished by the highest content of total monounsaturated fatty acids, with C18:1 n-9 (oleic acid) constituting 41.49 % of this fraction.

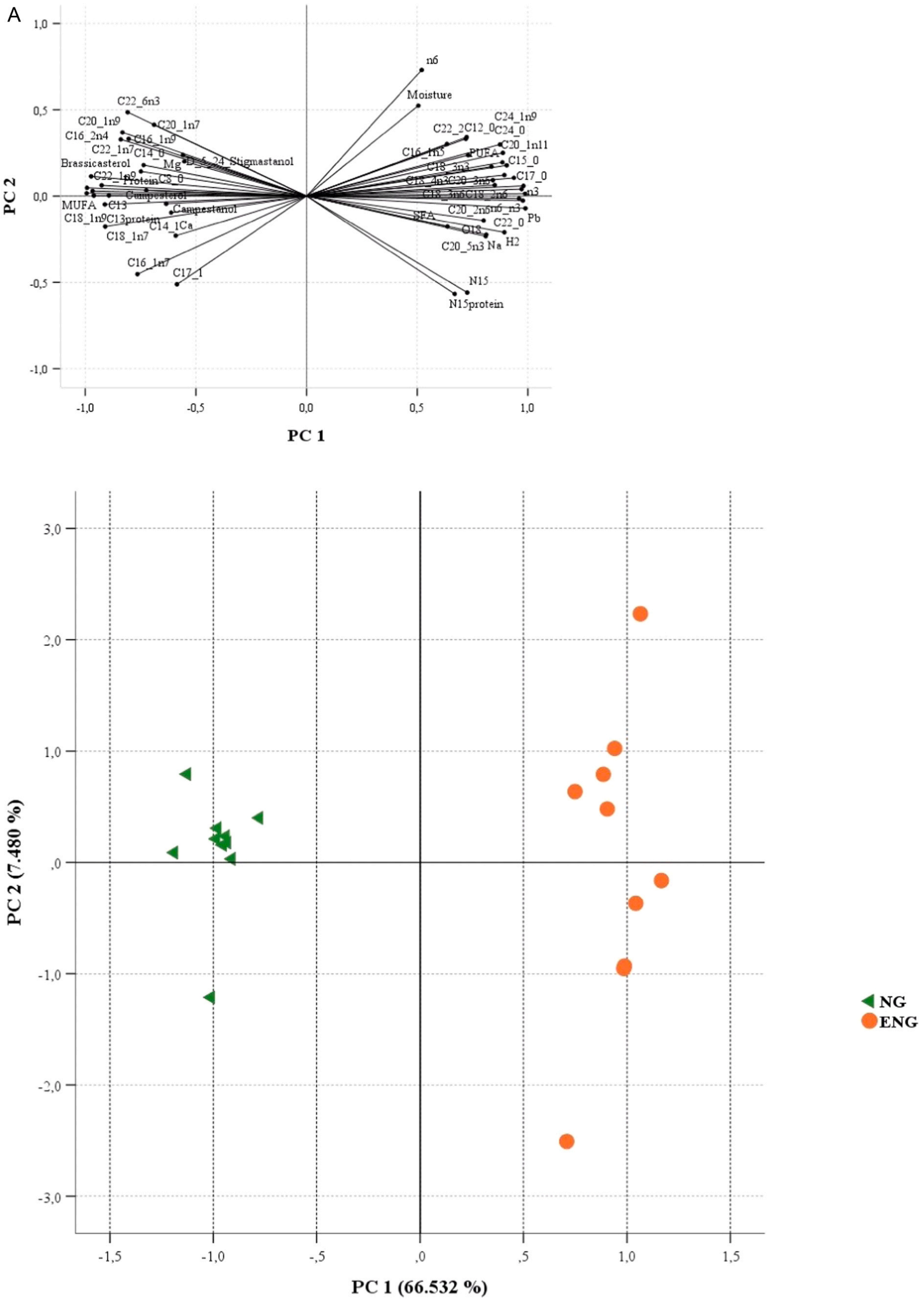

Fig. 2. A. 2D scatter plots with loading diagram for PC1-PC2 for fresh muscle samples classified by area of origin. **B.** 2D scatter plots with loading diagram for PC1-PC3 for fresh muscle samples classified by area of origin.

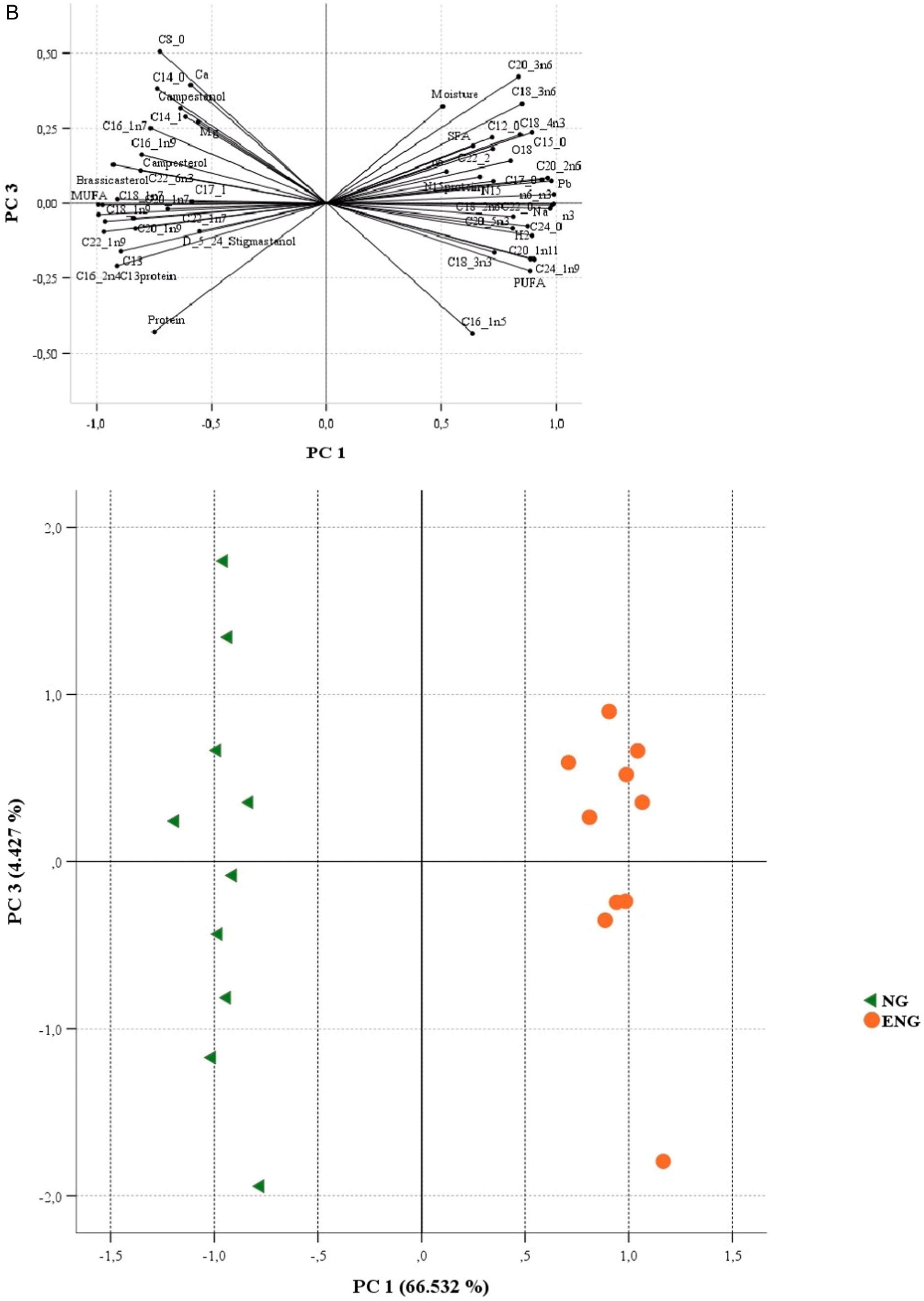

Fig. 2. (*continued*).

Table 3
Fatty acids composition expressed as % of total FA in muscle samples.

	Fatty acids	NG	ENG	SEM[a]	p-value[b]
SFA	C6:0	0.01	0.01	0.07	0.146
	C8:0	0.03	0.01	0.17	**0.002**
	C10:0	0.12	0.13	0.06	0.689
	C12:0	0.09	0.25	0.14	**0.000**
	C14:0	1.48	1.23	0.03	**0.005**
	C15:0	0.05	0.13	0.12	**0.000**
	C16:0	23.57	23.88	0.01	0.755
	C17:0	0.18	0.36	0.09	**0.000**
	C18:0	9.65	10.91	0.02	0.057
	C20:0	0.15	0.22	0.07	0.081
	C22:0	0.03	0.11	0.16	**0.000**
	C23:0	0.04	0.04	0.13	0.123
	C24:0	0.03	0.10	0.15	**0.001**
MUFA	C14:1	0.04	0.01	0.18	**0.011**
	C16:1 n−9	0.24	0.14	0.07	**0.000**
	C16:1 n−7*	3.99	2.16	0.11	**0.001**
	C17:1	0.27	0.17	0.09	**0.008**
	C18:1 n−9	41.49	34.95	0.02	**0.000**
	C18:1 n−7*	4.05	1.95	0.09	**0.000**
	C20:1 n−7*	3.35	1.61	0.12	**0.003**
	C20:1 n−9	1.83	0.29	0.32	**0.000**
	C22:1 n−7*	0.10	0.03	0.16	**0.000**
	C22:1 n−9	0.06	0.01	0.22	**0.000**
	C24:1 n−9	0.04	0.13	0.18	**0.001**
PUFA	C16:2 n−4*	1.14	0.01	0.57	**0.000**
	C18:2 n−6	4.87	10.28	0.09	**0.000**
	C18:3 n−6	0.02	0.07	0.17	**0.000**
	C18:3 n−3	0.20	0.27	0.05	**0.005**
	C18:4 n−3	0.06	0.15	0.13	**0.000**
	C20:2 n−6	0.17	0.74	0.18	**0.000**
	C20:3 n−6	0.14	0.24	0.08	**0.000**
	C20:3 n−3	0.05	0.06	0.08	0.087
	C20:5 n−3	0.03	0.06	0.09	**0.002**
	C22:2	0.11	0.06	0.16	**0.003**
	C22:5 n−3	0.52	0.31	0.10	0.652
	C22:6 n−3	0.03	0.16	0.07	**0.001**
	SFA	35.46	37.42	0.01	**0.011**
	MUFA	55.65	42.37	0.03	**0.000**
	PUFA	8.15	14.10	0.26	**0.001**
	n3	0.89	1.02	0.06	**0.000**
	n6	6.05	12.96	0.04	**0.037**
	n6/n3	6.91	12.85	0.09	**0.000**

[a] SEM (Standard Error of Mean)
[b] Bold values are significant at $p<0.05$

This high level of oleic acid may be linked both to its higher concentration in the diet and to the increased activity of Δ9-desaturase in pig adipose tissue (Kouba et al., 1999).

The increased MUFA content in the NG group was due to higher levels of C16:1, C18:1, C20:1, and C22:1 of the n-7 and n-9 series, which were significantly elevated in the NG samples ($p<0.05$). This result is particularly interesting as it indicates a correlation between these fatty acids and the rearing environment in the NG group. As highlighted by several studies, the Nebrodi area is known for its high plant biodiversity and is considered one of the most important and interesting natural regions of the Mediterranean (Tuttolomondo et al., 2014). Furthermore, Tuttolomondo et al., (2014) in their research on the use of wild plant species for medicinal purposes in the Nebrodi Regional Park, highlighted the presence of many endemic species unique to this region. It is the *Brassicaceae* that caught our attention, as another study by Barthet, (2008) identified the n-7 and n-9 fatty acids C16:1, C18:1, C20:1 and C22:1 in 12 Brassica species. In our study, as shown in Table 3, these n-7 and n-9 fatty acids were always significantly higher in the Nebrodi group. This is probably because the animals in this area could access a very rich and widespread wild vegetation. In contrast, the ENG samples had significantly higher PUFA contents (14.10 %). The most common PUFAs were C18:2 n-6, C20:2 n-6, C18:3 n-3, C20:3 n-6. Nevrkla et al., (2017) stated that a higher proportion of fatty acids in pig meat, mainly PUFAs, increased the palatability of the meat. In our study it could be therefore deduced that meat from pigs of the Nebrodi group (which have a significantly higher PUFA content, see Table 3) may be more appreciated by consumers.

3.4. Mineral elements content

The performance of the method in this study is shown in the supplementary material (Table S1 and Figure S1). Compared to other studies by the same authors, the LOD and LOQ values are significantly lower for many elements, which may also depend on the type of matrix. The LOQ values range from a minimum of 0.003 µg/kg for Cd to a maximum of 18.15 µg/kg for Ca. The results obtained show that the method used was optimally validated in terms of linearity, accuracy and sensitivity. Furthermore, the even lower LOD and LOQ values indicate that the method described in this study achieves better analytical sensitivity. As reported by several authors (Ballin, 2010; Katerinopoulou et al., 2020), the content of mineral elements in animal meat, measurable with the accurate ICP-MS technique, is influenced by the diet, reflecting the composition of the soil, pastures and drinking water and correlating the results with the geographical origin. The mineral content of the muscle samples, expressed in mg/kg fresh meat, is shown in Table 4. Seventeen mineral elements were sought. Concentrations of magnesium (Mg), sodium (Na), calcium (Ca), iron (Fe) and zinc (Zn) were found in all muscle samples. Among the macro-elements, Mg and Ca were significantly higher in the NG samples than in the ENG samples ($p<0.05$). The most important element was Mg, with an average concentration of 2940.79 mg/kg in NG samples and 2724.97 mg/kg in ENG samples. Literature indicates that Mg sources have a positive impact on various aspects of pig production (Djinovic-Stojanovic et al., 2017). High concentrations of Mg are known to improve animal behavior, reduce sensitivity to stress and improve the quality of pig (Lipiński et al., 2011). The Mg concentrations observed in this study are higher than those reported by other researchers (Batista et al., 2012; Tomović et al., 2011; Qi et al., 2021). Regarding the two geographical areas considered in this study, it is hypothesised that the higher concentration of Mg in pigs in the NG group is related to the higher concentration of this element in this area, as confirmed by the study conducted by Raab et al., (2017). Na was also significantly different between the groups, but the ENG samples had higher concentrations of this element (2680.46 mg/kg vs 2066.54 mg/kg). As shown in Table 4, Fe and Zn contents were found in all samples analysed but were not significantly different between the two groups ($p>0.05$).

Among the potentially toxic elements, Pb concentrations with an average value of 1.07 mg/kg were found in the ENG samples. In contrast, NG samples showed Pb concentrations <LOQ. The maximum level of Pb in pig as reported in Commission Regulation (EU) 2023/915 (2023) on maximum levels of certain contaminants in food must not exceed 0.1 mg/kg of fresh product. In all samples analysed from the ENG group, Pb levels were above the established limit. This is probably due to the various anthropogenic activities in the Valle del Mela area, such as the refining industry, oil-fired power plants, waste incineration and road traffic, which release fine atmospheric particles that can contaminate soils. This probability is supported by several studies carried out on the

Table 4
Mineral contents expressed as maximum, minimum, mean and standard deviation and results of Mann-Whitney test for n = 10 muscle samples.

Item	NG	ENG	SEM	p-value
mg/kg				
Mg	2940.79	2724.97	0.02	**0.019**
Na	2066.54	2680.46	0.04	**0.000**
Ca	56.66	44.90	0.04	**0.008**
Fe	20.66	22.94	0.03	0.216
Zn	20.37	23.38	0.03	0.085
Pb	-	1.07	0.48	**0.000**

area's soil (Duplay et al., 2014), water (Di Bella et al., 2018), and animal (Fazio et al., 2020).

3.5. PCA analysis

The significance of the differences in all the parameters analysed between the NG and ENG samples was estimated using the Mann-Whitney U test with a significance level below 0.05. The comparison of all parameters between the samples from different geographical areas, presented in Tables 1, 2, 3 and 4, showed that the NG samples had significantly higher values for proteins, 13 C, C16:1 n-9, C16:1 n-7, C18:1 n-9, C18:1 n-7, C20: 1 n-9, C22:1 n-7, C22:1 n-9, C16:2 n-4, MUFA, brassicasterol, campesterol, campestanol, Δ-5,24-stigmastanol, Mg and Ca; while the samples ENG had significantly higher values of moisture, δ^{15}N, δ^{2}H, δ^{18}O, C18:2 n-6, C18:3 n-3, C20:2 n-6, SFA, PUFA, n3, n6, n6/n3, Na, Fe, Zn and Pb. The suitability of the data for factor analysis was checked. The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy yielded a value of 0.735, indicating adequate sampling (greater than 0.500). Bartlett's test of sphericity yielded an approximate chi-square value of 153.869, confirming that the correlation matrix was suitable for principal component analysis (PCA). Seven principal components were extracted, accounting for 91.296 % of the total variance (66.532 %, 7.480 %, 4.427 %, 4.134 %, 3.662 %, 2.816 %, and 2.246 %, respectively). All variables showed high saturation in each factor, with commonalities consistently above 0.723, ensuring that the extracted components effectively represented all variables. Correlation matrix analysis revealed the highest positive correlations for n6/n3-C18:2 n-6 (0.995), Pb-C22:0 (0.974), C18:2 n-6-C22:0 (0.968), MUFA-C18:1 n-9 (0.964), Pb-C17:0 (0.920), C13(protein)-MUFA (0.913), and C13(protein)-C16:2 n-4 (0.902). In contrast, the highest negative correlations were found for Pb-MUFA (-0.992), MUFA-C22:0 (-0.981), Pb-C22:1 n-9 (-0.977), brassicasterol-Pb (-0.968), Pb- C18:1 n-9 (-0.958), C13(protein)-C20:2 n-6 (-0.928) and C13(protein)-C18:2 n-6 (-0.900).

4. Conclusion

To authenticate the 'Nero dei Nebrodi' pig meat coming from two distinct geographical areas of north-eastern Sicily, a complete characterization was carried out combining chemical-nutritional parameters with stable isotopes and mineral elements analyses. The rich endemic vegetation of the Nebrodi area was found to significantly influence chemical-nutritional parameters, including higher levels of brassicasterol, campesterol and n-7 and n-9 fatty acids in the NG samples. In contrast, anthropogenic activities in the "Valle del Mela" area resulted in elevated Pb levels in the ENG samples. The δ^{13}C, δ^{15}N, δ^{2}H and δ^{18}O values of the defatted *longissimus dorsi* muscle differed statistically between animals from the two sampling areas. These isotopic variations are primarily linked to differences in diet (δ^{13}C, δ^{15}N) and drinking water sources (δ^{2}H and δ^{18}O). The isotopic findings underscore the potential of these parameters as reliable tools for product traceability.

In conclusion, this study offers authorities a pioneering model for authenticating the 'Nero dei Nebrodi' breed. It lays the groundwork for using these correlations to ensure traceability and protect consumers from fraud and commercial disputes. As sampling and analysis increase, this model can be further validated, providing stronger assurances in verifying the breed's authenticity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2024.106918.

Data Availability

Data will be made available on request.

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