

## SAMPLE PREPARATION AND STORAGE EFFECTS ON FATTY ACID PROFILE OF RABBIT *LONGISSIMUS THORACIS ET LUMBORUM* MUSCLE

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**Abstract:** Twenty-five Pannon White male rabbits reared and fed in similar conditions were slaughtered at 11 weeks of age. The *longissimus thoracis et lumborum* muscles (LTL; right and left) were removed at 24 h *post-mortem* and allocated to four sampling/storage treatments: the left side of LTL muscle was divided in half perpendicularly, with the posterior portion being analysed within one day (fresh), and the anterior portion *vacuum* packaged and stored for 1 mo at  $-20^{\circ}\text{C}$  (whole-frozen); the right LTL side was ground with half of the product *vacuum* packaged and frozen for 1 mo at  $-20^{\circ}\text{C}$  (ground-frozen), whereas the other half was freeze-dried, *vacuum* packaged, and stored for 1 mo at  $4^{\circ}\text{C}$  (freeze-dried refrigerated). Treatments impacted percentages of total saturated ( $P<0.01$ ), monounsaturated ( $P<0.05$ ), and polyunsaturated fatty acids ( $P<0.001$ ), whole-frozen treatment affecting the most the fatty acids profile of the meat. Method of preparation and storage of meat samples before performing fatty acid analysis had an impact on the percentage of specific fatty acids, which could render the precision of study-to-study comparisons less reliable.

**Key Words:** rabbit, meat, storage temperature, packaging, fatty acids profile.

## INTRODUCTION

The role of dietary fat intake, including specific fatty acid composition of foods, has been the centre of the diet/health controversy for many decades now. For livestock and meat, excellent reviews by Wood *et al.* (2004, 2008) have been published in relation to fat content, fatty acid profile and meat quality. This topic continues to be one of great interest and significance to the meat industry because of both the alleged positive and negative roles that individual fatty acids and ratios of saturated (SFA), monounsaturated (MUFA), and/or polyunsaturated fatty (PUFA) acids play in human health.

Rabbit meat is an important protein source in some parts of the world, and reviews on rabbit meat quality (Dalle Zotte, 2002) and its role as a functional food (Dalle Zotte and Szendrő, 2011) shed light on the unique features of this product. Cullere *et al.* (2013) have presented comprehensive work on raw and cooked proximate composition, cholesterol content, fatty acid profile and true retention of different prime cuts of rabbit, which shows the very favourable proximate composition and fatty acid profile of this product. These natural qualities and traits represent a solid foothold from which to investigate how to eventually enhance its nutritional value.

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In the last decade, fatty acid profile has been one of the meat components most studied. Dal Bosco *et al.* (2014) modified fatty acid profiles of rabbit meat by including different amounts of fresh alfalfa to a commercial diet, resulting in a significant increase of PUFA, in particular alpha-linolenic acid (C18:3 n-3 or C18:3 omega-3), eicosapentaenoic acid (C22:5 n-3) and docosahexaenoic acid (C22:6 n-3), obtaining an improvement of the omega-6/omega-3 ratio as well. However, increasing unsaturated fatty acids content, especially PUFA, may also lead to a worsening of the oxidative status of the meat if it is not balanced by a proportional inclusion of antioxidant agents. Oxidation is a chemical reaction that takes place after slaughtering and leads to tissue deterioration resulting in discoloration, oxidized toxic compounds, rancid odour and ultimately shelf-life reduction (Contini *et al.*, 2014). Unsaturated fatty acids, together with haem pigments and metal catalysts, are the main compounds that make tissues more susceptible to oxidation (Lorenzo and Pateiro, 2013). It has been already demonstrated that when the amount of PUFA outweighs the antioxidant capacity of the tissue, it leads to a significant decrease in the shelf-life of the product due to a high lipid oxidation (Dal Bosco *et al.*, 2015). Therefore, balancing the dietary increase of unsaturated fatty acids by including antioxidant agents, such as selenium (Papadomichelakis *et al.*, 2017), vitamin E (Dalle Zotte *et al.*, 2020) or liquorice (Dal Bosco *et al.*, 2019) in the diet plays a key role in preserving the high nutritional value of rabbit meat.

Besides, dietary inclusion of unsaturated fatty acid sources is not the only factor determining the fatty acid profile of rabbit meat. Loponte *et al.* (2018) compared the fatty acid profile of free-range reared fryer rabbit meat to that from rabbits born and reared in cages, all fed the same diet. They found that free-range rabbit meat had a higher PUFA level and an improved antioxidant capacity in comparison to rabbits reared in cages. Ultimately, fatty acid content may also vary among different muscles due to their different energy metabolism. As a matter of fact, loin, which is a prevalently glycolytic muscle, generally has a lower lipid content than hind leg or fore leg muscles (Rasinska *et al.*, 2018). Therefore, lots of factors consisting of both native qualities, alimentary and environmental factors are involved and may affect rabbit meat quality, either favouring or creating the conditions to enhance oxidation if meat products are not properly processed and preserved.

Packaging also has a role in preserving the nutritional value of rabbit meat, slowing down oxidation reactions. Indeed, similarly to other meats, rabbit meat stored under *vacuum* or modified atmosphere conditions also has a longer shelf-life in comparison to meat that was stored under atmospheric oxygen level (Dal Bosco *et al.*, 2018). Thus, storage methods combined with storage duration and temperature are all key factors that might affect the outcome of the fatty acids profile analysis both at the commercial level and for laboratory research.

For scientific research purposes, meat samples should preferably be freeze-dried and stored until analysis. Freeze-drying is an expensive and time-consuming analysis/preparation, so some analyses, including fatty acid profile, can be conducted on fresh samples. However, as fresh samples cannot be analysed in a short time, samples are stored in various ways, potentially modifying the analytical results and making the comparison with scientific research hardly feasible. Thus, since fatty acid composition is so important for rabbit meat research, understanding the parameters that may affect it are vital to obtaining the most accurate results possible.

To the best of our knowledge, research has yet to investigate how meat sampling (processing) may affect the fatty acid profile of rabbit meat. Thus, the aim of this study was to investigate four different sample preparation/storage treatments, to determine whether and how fatty acid profile may differ among these treatments, and to recommend the adoption of the best rabbit meat sampling technique (possibly replacing freeze-drying) before performing fatty acid profile research.

## MATERIALS AND METHODS

### **Animals**

Twenty-five Pannon White male rabbits were reared in an experimental rabbitry with the same feed and slaughtered at 11 wk of age. The *longissimus thoracis et lumborum* muscle (LTL; right and left) was removed at 24-h *post-mortem*. These muscles were considered in the study due to their larger size (about 160 g in 11-wk-old rabbit) compared to other muscles.

Muscle samples, deprived of dissectible fat, were then allocated to four pre-fatty acid analysis storage treatments: the left side of LTL muscle was divided in half perpendicularly, with the posterior portion being analysed within 1 day (T1, fresh) and the anterior portion *vacuum* packaged as a whole and stored for 1 mo at  $-20^{\circ}\text{C}$  (T2, whole-frozen); the right side of LTL muscle was ground with a Retsch Grindomix GM 200 grinder (Retsch GmbH, Hann, Germany) at 7000 r/min for 10 s, with half of the product *vacuum* packaged and frozen for 1 mo at  $-20^{\circ}\text{C}$  (T3, ground-frozen), and the other half of the ground product freeze-dried, ground again with the same grinder at 7000 r/min for 10 s, before being *vacuum* packaged and stored for 1 mo at  $4^{\circ}\text{C}$  (T4, freeze-dried-refrigerated). T1 and T2 were ground before fatty acid analysis was performed.

### **Lipid extraction, fatty acid analysis**

The meat samples were homogenised in 20-fold volume of chloroform:methanol (2:1 vol:vol), containing 100 mg/L butylated hydroxytoluene as an antioxidant. Total lipids were extracted from the homogenised muscle samples with the method of Folch *et al.* (1957).

Fatty acid transmethylation was carried out with 1%  $\text{H}_2\text{SO}_4$  in methanol, according to Christie (2003). Biphasic separation was obtained through addition of 0.5 ml distilled water and 1.5 mL of n-heptane to each sample. The fatty acid methyl esters (FAME) were quantified by gas chromatography (Shimadzu GC17A, equipped with a Omegawax 250 column (30 m $\times$ 0.25  $\mu\text{m}$  $\times$ 0.25  $\mu\text{m}$ ) and FI detector. Helium was used as carrier gas at a constant flow of 0.8 mL/min; injector and detector temperatures were  $260^{\circ}\text{C}$ . Peaks were identified based on commercially bought FAME mixture (37-Component FAME Mix, Supelco Inc., Bellefonte, PA, USA), and the data obtained were expressed as weight % of total detected FAME.

### **Statistical analyses**

Data were analysed using the SAS 9.1.3 statistical software package for Windows (SAS, 2008). A one-way analysis of variance (ANOVA) tested the storage condition as fixed effect, and the significance level was calculated at the 5% confidence level. Data normality was analysed with a Shapiro-Wilk confidence level of 85%. Group means were compared by Tukey test.

## **RESULTS AND DISCUSSION**

Table 1 shows the results on fatty acid profile of the rabbit LTL samples according to the four different preparation and storage methods considered in this study. Overall, T2 is the treatment that registered the highest variation in terms of percentage of fatty acid profile compared to the sample analysed 24 hours after slaughter (T1). However, T2 also differed significantly from T3 and T4 for many fatty acids.

In detail, total SFA were significantly higher in T2 in comparison to T3 (33.7 vs. 32.7% total FAME;  $P<0.01$ ), due to higher percentages of pentadecanoic acid (C15:0) and palmitic acid (C16:0), despite the reduction in stearic acid (C18:0). Moving to MUFA, in T2 they were significantly higher compared to T1 and T4 (26.6 vs. 25.0 and 25.1% total FAME, respectively;  $P<0.05$ ), as a consequence of the increased percentage of palmitoleic acid (C16:1 *n*-7) and oleic acid (C18:1 *n*-9). Total PUFA were significantly lower in T2 compared to T1 and T4 (39.8 vs. 42.0 and 41.8% total FAME, respectively;  $P<0.001$ ), but also compared to T3 (38.8 vs. 41.7% total FAME;  $P<0.05$ ).

The main reason for the decrease in T2 lies in the significant ( $P<0.01$ ) decrease in fatty acids of the omega-6 (*n*-6) series, and within them the dihomo- $\gamma$ -linolenic acid (C20:3 *n*-6) and arachidonic acids (C20:4 *n*-6) ( $P<0.0001$ ). Nevertheless, neither the mean of the total fatty acids of the omega-3 (*n*-3) series nor the *n*-6/*n*-3 ratio was affected by the treatments. It is worth noting that T4 exhibited the highest percentage of docosahexaenoic acid (DHA, C22:6 *n*-3), which did not differ from T3, although it resulted significantly higher compared to T1 and T2 (0.179 vs 0.131 and 0.143% total FAME, for T4 vs. T1 and T2, respectively;  $P<0.0001$ ).

Rabbit meat lipids are rich in unsaturated fatty acids in association with a rather low *n*-6/*n*-3 ratio, compared to other meat sources (Dalle Zotte, 2002). These features, together with its low cholesterol and sodium level and high potassium, phosphorus, magnesium, selenium and B vitamins content, make this meat suitable to be steadily

Table 1: Effect of sampling on fatty acid profile (% total FAME) of *longissimus thoracis et lumborum* muscle in the rabbit ( $n = 25$ ).

N.	Meat sampling				Significance	RSD
	T1	T2	T3	T4		
	Fresh	Whole-frozen	Ground-frozen	Freeze-dried refrigerated		
	25	25	25	25		
C10:0	0.107	0.096	0.100	0.105	ns	0.033
C12:0	0.107	0.115	0.111	0.117	ns	0.028
C14:0	1.52	1.66	1.50	1.47	ns	0.25
C15:0	0.476 <sup>AB</sup>	0.489 <sup>Bb</sup>	0.463 <sup>ABa</sup>	0.457 <sup>A</sup>	<0.01	0.031
C16:0	22.7 <sup>A</sup>	23.9 <sup>B</sup>	22.6 <sup>A</sup>	22.8 <sup>A</sup>	<0.0001	0.9
C17:0	0.614	0.626	0.608	0.609	ns	0.051
C18:0	7.42 <sup>B</sup>	6.64 <sup>A</sup>	7.23 <sup>B</sup>	7.52 <sup>B</sup>	<0.0001	0.48
C20:0	0.105 <sup>ABa</sup>	0.112 <sup>Bb</sup>	0.104 <sup>A</sup>	0.106 <sup>ABa</sup>	<0.01	0.008
C22:0	0.0000	0.0060	0.0004	0.0028	ns	0.010
C23:0	0.0005	0.0068	0.0050	0.0041	ns	0.010
Total SFA	33.0 <sup>AB</sup>	33.7 <sup>B</sup>	32.7 <sup>A</sup>	33.1 <sup>AB</sup>	<0.01	0.9
C14:1	0.082	0.091	0.085	0.088	ns	0.043
C15:1	0.153	0.155	0.145	0.142	ns	0.040
C16:1	1.17 <sup>a</sup>	1.85 <sup>b</sup>	1.70 <sup>ab</sup>	1.65 <sup>ab</sup>	<0.05	0.79
C17:1	0.190	0.204	0.181	0.188	ns	0.034
C18:1 <i>n</i> -9	21.4 <sup>ab</sup>	22.2 <sup>b</sup>	21.4 <sup>ab</sup>	21.0 <sup>a</sup>	<0.05	1.45
C18:1 <i>n</i> -11	1.80	1.80	1.87	1.84	ns	0.175
C20:1 <i>n</i> -9	0.187	0.195	0.186	0.180	ns	0.024
C22:1 <i>n</i> -9	0.019	0.023	0.017	0.011	ns	0.023
Total MUFA	25.0 <sup>a</sup>	26.6 <sup>b</sup>	25.5 <sup>ab</sup>	25.1 <sup>a</sup>	<0.05	1.86
C18:2 <i>n</i> -6	29.7	29.2	29.5	29.1	ns	1.36
C18:2 <i>n</i> -c9t11	0.027	0.012	0.014	0.019	ns	0.021
C18:3 <i>n</i> -6	0.242	0.231	0.245	0.248	ns	0.027
C18:3 <i>n</i> -3	2.62	2.59	2.63	2.51	ns	0.311
C20:2	0.353	0.368	0.401	0.379	ns	0.119
C20:3 <i>n</i> -6	0.792 <sup>B</sup>	0.629 <sup>A</sup>	0.768 <sup>B</sup>	0.801 <sup>B</sup>	<0.0001	0.127
C20:3 <i>n</i> -3	0.103	0.100	0.107	0.109	ns	0.014
C20:4 <i>n</i> -6	7.16 <sup>B</sup>	5.69 <sup>A</sup>	6.99 <sup>B</sup>	7.46 <sup>B</sup>	<0.0001	1.39
C20:5 <i>n</i> -3	0.076 <sup>A</sup>	0.109 <sup>B</sup>	0.077 <sup>A</sup>	0.089 <sup>AB</sup>	<0.01	0.033
C22:2	0.085 <sup>B</sup>	0.068 <sup>A</sup>	0.079 <sup>AB</sup>	0.084 <sup>B</sup>	<0.01	0.016
C22:5 <i>n</i> -3	0.758 <sup>ABb</sup>	0.639 <sup>Aa</sup>	0.749 <sup>AB</sup>	0.798 <sup>B</sup>	<0.01	0.148
C22:6 <i>n</i> -3	0.131 <sup>A</sup>	0.143 <sup>A</sup>	0.156 <sup>AB</sup>	0.179 <sup>B</sup>	<0.0001	0.035
Total PUFA	42.0 <sup>B</sup>	39.8 <sup>Aa</sup>	41.7 <sup>ABb</sup>	41.8 <sup>B</sup>	<0.001	2.14
Total <i>n</i> -3	2.93	2.94	2.97	2.88	ns	0.29
Total <i>n</i> -6	37.8 <sup>B</sup>	35.7 <sup>Aa</sup>	37.5 <sup>ABb</sup>	37.6 <sup>ABb</sup>	<0.01	2.06
<i>n</i> -6/ <i>n</i> -3	13.1	12.2	12.7	13.3	ns	1.68

Means with different letters (a, b) within a row are significantly different at  $P < 0.05$ .

Means with different letters (A, B) within a row are significantly different at  $P < 0.01$ .

included in human diet to help prevent cardiovascular diseases and other illnesses, especially in pregnant women, children and people suffering from high blood pressure (Dalle Zotte and Szendrő, 2011; Redondo-Solano *et al.*, 2022). Therefore, given these valuable nutritional properties and peculiar characteristics of rabbit meat, it is necessary to pay particular attention to the processing/storage methods, with a view to keeping the oxidation of proteins and rancid deterioration of lipids as low as possible. Oxidation is a chemical process that negatively affects nutritional value and sensory characteristics of the meat and may also be responsible for cancer and cardiovascular disease (Cullere *et al.*, 2018). Due to its high content in unsaturated fatty acid, especially PUFA, rabbit meat, as well as other PUFA-rich meats, is particularly prone to oxidation. For this reason, information on the best combination of processing and storage methods for laboratory use is essential to preserve the nutrients of rabbit meat.

To date, several studies have been conducted investigating the best storage and temperature combination to properly preserve meat, whether originating from rabbit or other livestock species (Muela *et al.*, 2010; Leygonie *et al.* 2012; Lan *et al.*, 2016; Dal Bosco *et al.*, 2018; Wang *et al.*, 2018). However, these research works mostly focused on choosing the best time, temperature, or their best combination, as well as the best packaging, to effectively extend meat shelf-life, and none of these studies have considered the effect of the storage processing of meat matrix/structure (i.e. grinding before or after freezing) in order to verify differences in the fatty acid profile.

*Vacuum* packaging to store meat has been widely demonstrated to limit oxidation processes compared to atmospheric oxygen storage (Dal Bosco *et al.*, 2018; Chmiel *et al.*, 2019; Redondo-Solano *et al.*, 2022); *vacuum* packaging and freezing should be one of the sampling solutions also for the rabbit meat. Nevertheless, in the current study the meat samples that were frozen as a whole and *vacuum* packaged for one month at  $-20^{\circ}\text{C}$  (T2) were the most affected in terms of fatty acid profile in comparison to the fresh samples (T1), showing the highest variations within the PUFA, reasonably due to oxidation reactions. A possible explanation could lie in the freezing-thawing-grinding process. The freezing treatment turns unbound water into ice crystals, which stick among muscle cell fragments, causing *ex vivo* tissue damage and resulting in an increase in free water and spreading of pro-oxidant agents in the muscle (Wang *et al.*, 2018). As the muscle is frozen, oxidation is slowed down, but when thawing starts, oxidation reactions take over, decreasing the PUFA level. Indeed, it has already been demonstrated that freezing, despite its high potential for prolonging the shelf-life of even highly perishable meat products, also causes the most severe tissue damage (Lan *et al.*, 2016). Moreover, meat which is first frozen and then thawed is heavily affected by oxidation processes, resulting in a significant decrease in long chain PUFA (Dominguez *et al.*, 2019). The combination of these two factors may have taken place in T2 during sample preparation for the fatty acid profile analysis, when it was first thawed and then ground: once the temperature was increasing, the combination of free water intrinsically present in the muscle along with tissue damage caused by the freezing process and the following spreading of pro-oxidant agents, the latter accelerated by meat grinding, found the best condition for oxidation, resulting in the highest loss of PUFA in comparison to T1, but also to T3, which was first ground and then frozen to  $-20^{\circ}\text{C}$ .

Another possible reason behind the differences among T1 and the further treatments to be considered is the fact that LTL is a relatively large anatomical formation, and its energy metabolism differ slightly along the muscle (Vigneron *et al.*, 1986; Ouhayoun and Dalle Zotte, 1993; Hulot and Ouhayoun, 1999), possibly resulting in a different FA profile (Martinez-Álvarez *et al.*, 2018).

As regards storage temperature, it is widely demonstrated that  $-40^{\circ}\text{C}$  is the minimum required to effectively start turning oxidation down, whereas at  $-20^{\circ}\text{C}$  oxidation reactions are slowed down but still ongoing (Pérez-Palacios *et al.*, 2008; Rahman *et al.*, 2015). In the present study, both frozen samples (T2 and T3) were stored at  $-20^{\circ}\text{C}$  so as to be in practical laboratory conditions even in less technologically advanced countries. This could have had an impact on the fatty acid profile of T2 and T3 samples, although storage temperature is assumed to have exerted the same effect on both treatments.

As for grinding, it is a process that completely breaks down the texture of the tissue and destroys muscular fibres. For this reason, ground meat is expected to be more prone to microbial spoilage and oxidation due to the increase in its surface area and the amount of free water available in the tissue (Redondo-Solano *et al.*, 2022).

However, the results of this study showed a different scenario, as ground-frozen samples were expected to show higher PUFA losses than those found in whole-frozen samples. A possible further explanation may depend on the grinding process: if performed before freezing, it might have led to a reduction in the unbound water available in

the tissue, but also to have freed the natural antioxidants still present in the meat, thus making the sample a less favourable environment for lipid peroxidation (Warner, 2014). Conversely, the lipids of the T2 samples, ground after thawing and shortly before analysis, would have been less protected from oxidation, also due to the depletion of natural antioxidants, such as vitamin E, naturally present in rabbit meat and of feed origin (Dalle Zotte *et al.*, 2020), thus grinding after thawing could have exacerbated the process compared to grinding before freezing.

Considering that freeze-drying dramatically decreases of the amount of free water available in the tissue, the combination with *vacuum* packaging and storage at +4°C created the perfect conditions to prevent oxidation. Therefore, the fatty acid profile of freeze-dried-refrigerated samples mirrored that of the fresh samples.

Summarising, this study proved that pre-fatty acid analysis sampling treatments affect the fatty acid profile of rabbit meat. T4 was found to be the preparation that least affected the fatty acid profile of the meat due to the allegedly lowest amount of free water available in the tissue, which was supposed to be the key element determining the different PUFA oxidation levels between the different treatments. However, when the rabbit meat was ground and then frozen before fatty acid profile analysis (T3), it provided results comparable to those obtained in freshly analysed meat (T1). Conversely, when rabbit meat samples are frozen whole and subsequently ground prior to fatty acid profile analysis (T2), an alteration of the fatty acid profile occurs, thus recommending avoiding the use of this sampling method.

Attention is drawn to the fact that this study is limited to providing sampling indications for fatty acid analysis. Furthermore, due to the lack of related studies, further research is needed to optimise both sample preparation and storage conditions of rabbit meat samples intended not only for fatty acid profile analysis, but also for other physico-chemical sensory analyses.

## CONCLUSIONS

The method of preparation and storage of rabbit meat samples before performing fatty acid profile analysis has an impact on the percentage of specific fatty acids. Although these differences are quite subtle, they can greatly limit comparison between studies, but especially fail to highlight the correct information when studies involve feeding aimed at improving the fatty acid profile of meat.

**Ethics approval:** The research was not an activity requiring permission. All animals were handled according to the principles stated in European directive 86 609/EEC on the protection of animals used for experimental and other scientific purposes (European Union, Directive EC, 2010).

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