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Da sinistra a destra: fotografia dell'allevamento di bovini podolici scattata durante la prova sperimentale; albero di ulivo con sansa pellettata, sottoprodotto derivato dalla lavorazione delle olive;  
in basso, un'immagine simbolica dell'economia circolare.

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# **Innovative strategies in feeding ruminants to improve product quality and reduce the environmental impact of farming**

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*Ai miei familiari*

## Table of contents

Abstract.....	1
Chapter 1. General introduction.....	6
1.1. Podolian young bulls and the use of by-products in animal feed.....	6
1.2. Olive cake production process and advantages and disadvantages of different separation methods.....	9
1.3. Use of Olive cake in Animal Production: Preservation through Ensiling and Drying.....	13
1.4. Olive cake and chemical composition: Benefits and Applications of Olive cake in Animal Feed.....	15
1.5. Linseeds and chemical composition.....	18
References.....	20
Chapter 2. Organization .....	25
Chapter 3: Effects of olive cake and linseed supplementation in the diet on performance, carcass traits, and oxidative stability of beef from young Podolian bulls .....	26
1. Introduction.....	28
2. Materials and methods.....	31
2.1. Ethical Statement.....	31
2.2. Animal management, dietary treatments and sample collection.....	31
2.3. Slaughter procedures, carcass characteristics and meat sampling.....	35
2.4. Feed and analysis meat proximate analyses.....	36
2.5. Total phenolic content (TPC) and quantification of polyphenols by HPLC analysis.....	37
2.6. Extraction and quantification of tocopherols by HPLC analysis.....	39
2.7. Lipid oxidation of meat samples.....	39
3. Statistical Analysis.....	40
4. Results .....	40
5. Discussion .....	47
6. Conclusions .....	49
References.....	51
Chapter 4. Effect of high levels of almond hulls supplementation on performance and meat oxidative stability in lambs .....	57
Chapter 5. Conclusions and Future Perspectives.....	62

ADDITIONAL ARTICLE .....	65
Appendix 1. Effect of Dietary Ensiled Olive Cake Supplementation on Performance and Meat Quality of Apulo-Calabrese Pigs .....	65
Appendix 2. Effects of feeding bergamot pulp and olive leaves on performance and meat quality in Apulo-Calabrese pigs .....	79
Appendix 3. Enhancement of quails' ( <i>Coturnix japonica domestica</i> ) meat quality: Effects of dietary supplementation of dried bergamot pulp.....	86
1. Introduction.....	87
2. Materials and methods.....	89
2.1. Animals and diet.....	89
2.2. Feedstuff analysis.....	91
2.3. Meat fatty acid, $\alpha$ -tocopherol and proximate composition determination.....	92
2.4. Lipid oxidation and colour measurements.....	93
2.5. Statistical Analysis.....	93
3. Results .....	94
4. Discussion .....	98
5. Conclusion .....	101
References.....	103
List of Figures .....	110
List of tables.....	112
Acknowledgements .....	114

## Abstract

The aim of this PhD thesis was to find "green" solutions by valorizing agro-industrial by-products or alternative feeds, particularly those derived from olive oil production (olive cake), combined with linseeds, in the feeding of young podolian bulls, evaluating their effects on growth performance, oxidative stability (shelf life), and meat quality. Due to their significant properties, olive cake and linseeds were used in the following experimental trial conducted on thirty-six young podolian bulls, divided into 4 groups: Control Group (CTRL), Olive cake Group (OC), Linseeds Group (EL), and olive cake + linseeds Group (OCEL), to evaluate animal performance and meat quality.

Podolian bulls, a rustic breed typical of Italy's mountainous regions, are traditionally fed a diet based primarily on mountain forage, such as grasses and hay, which reflects their adaptive nature to poor and challenging environments. However, the introduction of agricultural by-products such as olive cake and linseeds into the diet of these animals represents an innovative practice that, in addition to improving nutrition and the quality of animal products, also contributes to the sustainability of agricultural practices.

Olive cake is the solid residue obtained from the crushing of olives during the olive oil extraction process and contains polyphenols, antioxidant compounds that may have positive effects on the health of cattle. Polyphenols are known for their anti-inflammatory and immunomodulatory properties, which could enhance the animals' immune response, increasing their resistance to diseases and improving their overall health.

However, the high oil content in olive cake can pose a challenge. If not properly processed, olive cake can be difficult for cattle to digest, potentially leading to digestive disorders. Therefore, to optimize its use, olive cake must be treated, either by drying or oil extraction, to reduce its lipid content and improve digestibility. Additionally, the amount of olive cake fed to the animals must be carefully monitored to prevent excessive caloric intake.

Linseeds are another valuable source of nutrition for podolian bulls. These seeds are particularly rich in polyunsaturated fatty acids, specifically alpha-linolenic acid (ALA), a type of Omega-3 fatty acid that can improve the quality of fats in meat, providing animal products with a higher amount of healthy fats.

Therefore, the use of olive cake and linseeds represents an opportunity to supplement the diets of podolian bulls with healthy, nutrient-dense, and sustainable feeds. With careful and balanced management, these resources can significantly contribute to improving the quality of animal products and promoting more ecological and efficient agricultural practices.

## Sommario

Lo scopo di questa tesi di dottorato è stato quello di trovare soluzioni "green", valorizzando i sottoprodotti del settore agroindustriale o alimenti alternativi, in particolare quelli derivanti dalla produzione dell'olio di oliva (sansa di olive), combinata ai semi di lino, nell'alimentazione dei bovini podolici, valutandone gli effetti sulla crescita delle prestazioni, sulla stabilità ossidativa (shelf life) e sulla qualità della carne. Grazie alle loro importanti proprietà, la sansa di oliva e i semi di lino sono stati utilizzati nella seguente prova sperimentale condotta su trentasei bovini podolici, suddivisi in 4 gruppi, Gruppo Controllo (CTRL), Gruppo OC (Gruppo con sansa di olive), Gruppo EL (Gruppo con semi di lino) e Gruppo OCEL (Gruppo con sansa di olive + semi di lino) per valutare la performance degli animali e la qualità della carne.

L'alimentazione dei bovini podolici, una razza rustica tipica delle aree montane italiane, è caratterizzata da una dieta principalmente basata su foraggi di montagna, come erbe e fieno, che rispecchiano la loro natura adattativa a contesti poveri e difficili. Tuttavia, l'introduzione di sottoprodotti agricoli come la sansa di olive e i semi di lino nell'alimentazione di questi animali rappresenta una pratica innovativa che, oltre a migliorare la nutrizione e la qualità dei prodotti animali, contribuisce anche alla sostenibilità delle pratiche agricole.

La sansa di olive è il residuo solido che si ottiene dalla frangitura delle olive durante il processo di estrazione dell'olio d'oliva e contiene polifenoli, composti antiossidanti che possono avere effetti positivi sulla salute dei bovini. I polifenoli sono noti per le loro proprietà anti- infiammatorie e immunomodulanti, che potrebbero migliorare la risposta immunitaria degli animali, aumentando la loro resistenza alle malattie e migliorando la loro salute generale.

Tuttavia, l'alto contenuto di olio nella sansa può rappresentare una sfida. Se non trattata correttamente, la sansa può risultare difficile da digerire per i bovini, con il rischio di causare disordini digestivi. Pertanto, per ottimizzare l'alimentazione, la sansa deve essere trattata, mediante essiccazione o estrazione dell'olio, al fine di ridurre il contenuto lipidico e migliorare la digeribilità. Inoltre, la quantità di

sansa somministrata deve essere monitorata con attenzione per evitare un eccessivo apporto calorico.

I semi di lino sono un'altra fonte preziosa di nutrimento per i bovini podolici. Questi semi sono particolarmente ricchi di acidi grassi polinsaturi, in particolare di acido alfa- linolenico (ALA), un tipo di acido grasso Omega-3 che può migliorare la qualità dei grassi nella carne, conferendo ai prodotti animali una maggiore quantità di grassi sani.

Quindi l'utilizzo della sansa di olive e dei semi di lino rappresenta un'opportunità per integrare le diete dei bovini podolici con alimenti sani, nutrienti e sostenibili. Con una gestione attenta e bilanciata, queste risorse possono contribuire significativamente al miglioramento della qualità dei prodotti animali e alla promozione di un'agricoltura più ecologica ed efficiente.

## **Keywords**

By-products; olive cake; linseeds; podolian cattle; oxidative stability; circular economy.

## **Chapter 1. General introduction**

### **1.1. Podolian young bulls and the use of by-products in animal feed**

A special breed of cattle, Podolian cattle are adapted to harsh environmental conditions. These cattle, which originate from the mountainous regions of Central and Eastern Europe, are unique in that they possess several physiological and behavioral traits that allow them to survive in harsh environments, such as extreme cold during the winter and excessive heat during the summer.

They are ideal for less intensive farming systems as their innate resilience reduces the need for regular veterinary interventions.

Longevity and fertility are two other traits associated with Podolian cattle. They can maintain good reproductive health even in regions poor in resources, which helps traditional livestock production remain viable.

This breed is characterized by a strong adaptability to harsh environmental conditions and a natural resistance to diseases and parasites. Their importance in marginal areas, such as mountains and hills, is crucial for the conservation of landscapes, the maintenance of agro-pastoral traditions and the production of high-quality foods, such as caciocavallo cheese.

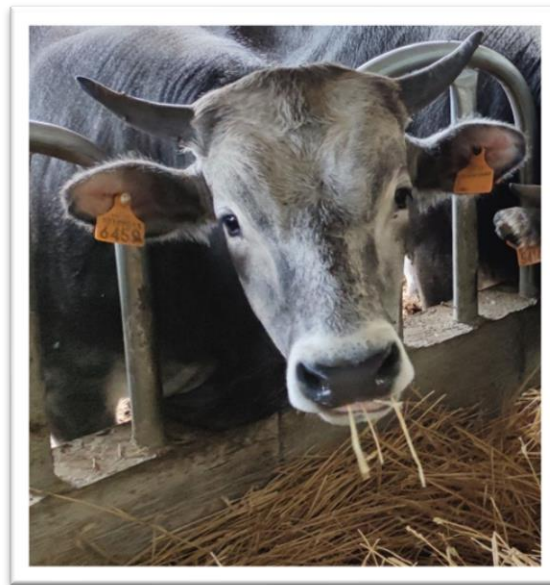
Its origin dates back to ancient times, reflecting a long evolution in difficult environments that were not favorable to intensive agriculture. Some historians believe that the breed was influenced by the prehistoric taurine breed that populated the Mediterranean, while others suggest that Podolian cattle are the result of crosses between local cattle and oriental breeds introduced into southern Italy during the historical conquests.

Over the centuries, the Podolian breed has adapted perfectly to life in mountainous territories, becoming a central element of transhumant breeding practices. Podolian cattle have been a fundamental resource for rural populations, providing meat and milk, but also a workforce for transport and cultivation of the land. This symbiotic relationship between man and animal has been the basis of the breed's long survival in the most difficult regions of the country.

Their feeding habits are strongly influenced by the resources and natural environment of the areas in which they are raised. For the most part, they are fed with natural fodder including grasses, bushes and native plants. Their freedom to exercise and exploit natural resources is enhanced by extensive grazing, which also improves the quality of their meat. It is common to supplement the diet with locally grown forages, such as hay, straw or cereals, which may vary in quality and seasonal availability, during certain seasons or periods when grazing is scarce.

This helps to provide nutrition during key times of the year. As they originate from mountainous regions, Podolian cattle need constant access to fresh, clean water sources to remain healthy and productive. However, to maintain growth, reproduction and general health of the animals, it is necessary to provide a sufficient intake of protein and micronutrients. Supplementation with concentrated feed or vitamin supplements may be necessary, particularly during periods of development or during pregnancy and lactation, to promote the best possible animal welfare.

Below is an image of a Podolian young bull used for the experimental test.



**Figure 1.** Photo of Podolian young bull experimental test

Ruminant production systems contribute significantly to enteric CH<sub>4</sub> emissions into the atmosphere (Beauchemin et al., 2020) and for this reason considerable research efforts are being invested with the goal to improve the product quality and reduce the environmental impact of livestock farming. In this context, the promotion of sustainable and safe production systems that provide high quality products is encouraged. Low-input strategies in animal farms, especially ruminants, can be implemented using alternative food resources, such as agro-industrial by-products, which often contain significant quantities of bioactive molecules capable of positively influencing meat quality.

Using by-products is a strategy that allows for the efficient utilization of available opportunities to obtain essential nutrients, ensuring a balanced diet and improving animal performance.

Utilizing agricultural byproducts in animal feed is a crucial strategy to boost the food system's effectiveness, cut waste, and lessen the livestock industry's negative environmental effects (Blanch & Aguiló, 2017; Kumar et al., 2010). Agricultural by-products may provide cattle with a substantial amount of vital nutrients, thus it's critical to thoroughly assess the nutritional elements. While the nutritional makeup of by-products varies substantially based on the kind and method of production (Blanch & Aguiló, 2017), they typically consist of the following: proteins that come from processing residues like cereal bran, oil cakes, or protein flours; fibers that come from plant residues like peels, straws, and vegetable waste; and fats that come from processing waste or vegetable oils.

Utilizing the range of nutrients found in agricultural byproducts can help animal diets be balanced and supplemented, increase feed efficiency, and lessen their reliance on concentrated feeds (Kumar et al., 2010). Additionally, using agricultural by-products in animal feed reduces waste of materials that could otherwise be sent to landfills or for treatment (Blanch & Aguiló, 2017); it lessens the need to cultivate new land for the production of livestock feed, which helps to mitigate environmental impacts and conserve natural resources (Palić & Perišić, 2018); it provides an efficient method of managing organic waste from

agricultural and food operations, encouraging more sustainable agricultural practices (Kumar et al., 2010).

Moreover, their utilization can lower feed expenses, particularly in areas where they are reasonably priced in comparison to traditional livestock feed (Blanch & Aguiló, 2017). By closing the nutrient cycle and converting waste into useful resources for animal feed, this method not only lowers production costs but also improves resource efficiency. By encouraging resource efficiency and lowering agriculture's total environmental effect, the valuation of these byproducts is consistent with the goals of the circular economy (Sadeghi et al., 2009).

In recent years, there has been a growing interest in incorporating agricultural by-products into ruminant diets. Many of these by-products contribute to the functional and nutraceutical properties of animal feed due to their high concentration of bioactive compounds (Vasta & Luciano, 2011). As a result, they serve as functional supplements that exert specific beneficial effects on health and metabolism, improving the organoleptic properties of animal-derived products. Additionally, these compounds provide antioxidant, anti-tumor, and immune-stimulating properties while also acting as inhibitors of gastrointestinal parasites, ultimately enhancing the microbiological quality of animal feed (Patra & Saxena, 2011; Durmic & Blache, 2012).

### **1.2. Olive cake production process and advantages and disadvantages of different separation methods**

The production of olive cake for animal feed follows a specific process that involves several stages, from harvesting the olives to processing the by-product into a usable ingredient in animal feed. The olive cake intended for this use must be treated with care to ensure that it is safe, nutritionally balanced, and free from contaminants. Here is a detailed description of all the stages that lead to the production of olive cake for animal feed.

Harvesting the Olives: Harvesting the olives is the first step in the olive oil production process. The olives can be harvested by hand or with specialized machines. It is ideal to harvest the olives when they are ripe but not too ripe, in order to guarantee the quality of the oil, which is essential for the production of the olive cake.

Cleaning and Selection of Olives: Once harvested, the olives are taken to the mill, where they are cleaned to remove impurities such as leaves, twigs, dust and soil. This is a crucial step to prevent foreign materials from contaminating the paste and olive cake, and to ensure the quality of the final product.

Crushing (Milling): Crushing is the process in which olives are crushed into a thick paste, composed of pulp, stones and small amounts of oil. Crushing can be done with different tools: traditionally with stone mills or hammer mills, but today more modern technologies are also used, such as hammer or disk crushers.

Kneading: Kneading is the phase in which the crushed olive paste is slowly mixed to encourage the aggregation of the oil droplets. This phase helps to improve the efficiency of oil extraction and reduce losses.

Oil Extraction (Pressing or Centrifugation): Olive oil extraction is the process of separating the oil from the olive paste. This process can be done by pressing (traditional method) or centrifugation (modern method).

Olive cake separation: After oil extraction, the solid residue that remains in the crushing or centrifugation machine is the olive cake. Olive cake is mainly composed of pulp, stones and a small amount of residual oil.

The separation process that leads to the formation of olive cake can occur through different plant configurations, each of which involves specific methods of treatment and management of by-products. The most widespread separation technology in modern oil mill lines is the 2-phase one, but there are also 2.5-phase and 3-phase configurations, each of which has distinct characteristics and advantages.

The 2-phase system is a more recent approach that has gained popularity due to its efficiency and ability to reduce water use in the process (Borja et al.,

2006). In this system, the olive paste is separated into two phases: olive oil and olive cake, through further separation of the aqueous part. The oil is extracted directly from the paste by means of a centrifugation process, and the olive cake is separated as a single solid fraction that may contain some residual water (Kapellakis et al., 2008).

The advantages of the 2-phase system are a reduction in water consumption, compared to traditional systems, which use large quantities of water to obtain oil, the 2-phase system significantly reduces the amount of water needed for extraction; the lower presence of water in the paste helps to obtain an oil with a lower quantity of free fatty acids, which is associated with a higher quality and simplified management of by-products: the olive cake produced in this system is less humid than that of 3-phase systems, which makes it easier to manage and possible reuse.

The disadvantages include an olive cake that is generally richer in water, which makes it less suitable for certain valorisation or direct combustion processes and although the amount of water in the process can be reduced, the separation of the liquid from the solid part requires more advanced and expensive technologies, increasing the initial investment costs.

The 2.5-phase system represents a middle ground between the 2-phase and 3-phase systems. In this system, the oil and the olive cake are separated, but part of the aqueous fraction is separated before the formation of the olive cake, thus reducing the amount of water present in the olive cake but without eliminating it completely.

The advantages of the 2.5-phase system are that the olive cake produced with this system is less humid than that produced in 3-phase systems, but retains enough water to be used in subsequent processes (for example, for biogas production), with an improvement in its quality and a balance between efficiency and costs, as the 2.5-phase system offers a compromise between the 2-phase system (more efficient) and the 3-phase system (more traditional), making it suitable for mills that want to balance quality and costs.

The disadvantages, on the other hand, are that compared to the 2-phase system, this system involves slightly higher costs for water management, since although it reduces the humidity of the olive cake, it does not completely eliminate the use of water and that this system does not reach the levels of efficiency in terms of oil recovery compared to more advanced systems.

As for the 3-phase system, it is the traditional method, widely used in olive mills, which separates the olive paste into three fractions: oil, vegetation water and olive cake. This system involves the use of water to facilitate the separation of the oil from the paste and obtain a separated aqueous fraction, which contains the water-soluble waste.

The advantages of the 3-phase system are a high efficiency in separating the oil from the olive paste, resulting in a high yield and providing a relatively drier olive cake compared to the 2-phase system (Preedy et al., 2020). Olives and olive oil in health and disease prevention. Academic press., which can be advantageous for some industrial applications, such as biofuel production or valorisation through distillation.

The disadvantages are certainly represented by a high use of water, which contributes to increasing operating costs and generating a greater volume of vegetation water to be treated, the presence of a greater quantity of water during the extraction process can lead to a lower quality of the oil, with possible increases in acidity and other lower organoleptic characteristics and a high volume of vegetation water that requires additional treatments for purification, increasing environmental and operating costs (Preedy et al., 2020).

In light of the above, the choice between a 2-phase, 2.5 or 3-phase system depends on multiple factors, including product quality objectives, operating costs and environmental sustainability. 2-phase systems offer higher water efficiency and oil quality, but require more complex olive cake management. The 3-phase system, while ensuring high oil yield, involves high water use and presents challenges in by-product management (Kalogeropoulos et al., 2014). The 2.5-

phase system represents a compromise solution, which seeks to balance efficiency and quality, while reducing environmental impact.

### **1.3. Use of Olive cake in Animal Production: Preservation through Ensiling and Drying**

As previously mentioned, the use of olive cake in animal nutrition represents a significant opportunity to enhance the value of by-products from the olive oil industry, contributing to the sustainability of livestock production and the circular economy. Olive cake mainly consists of residual pulp, skins, and olive pits, with its composition varying depending on the oil extraction method. The high moisture content of fresh olive cake from the decanter can limit its storability, promoting microbial growth and lipid oxidation, thus necessitating stabilization strategies such as ensiling and drying to ensure safe and prolonged preservation.

Ensiling is an effective solution for preserving olive cake, utilizing anaerobic fermentation to stabilize the product and improve its nutritional quality. This process is facilitated by the activity of lactic acid bacteria (LAB), such as *Lactobacillus plantarum* and *Pediococcus pentosaceus*, which ferment the soluble sugars present in the olive cake and produce lactic acid, lowering the pH of the material to below 4.5 (Muck et al., 2018). Effective ensiling of olive cake involves several crucial phases: chopping and compaction, which reduce oxygen presence in the silo and promote anaerobic fermentation (Weinberg & Muck, 1996); the use of microbial inoculants and additives, such as organic acids, to prevent mold and yeast growth; and finally, airtight sealing with low-oxygen-permeability plastic films, allowing optimal fermentation and product stabilization within 30–60 days. This method helps preserve the digestible energy of the olive cake and improves its palatability, making it a valuable feed option for cattle, sheep, and pigs (Tzamaloukas et al., 2021). However, the presence of phenolic compounds in olive cake, while having beneficial antioxidant properties, may be anti-nutritional at high concentrations, making it essential to balance feed rations appropriately.

Drying is an alternative to ensiling, as it reduces the moisture content of the olive cake, facilitating storage and integration into feed. Natural drying relies on solar energy and involves spreading the olive cake over large, well-ventilated surfaces for 10–15 days. However, this method is limited by climatic variability and the risk of microbial contamination. To overcome these challenges, our study utilized fluidized bed drying, an advanced and efficient technology. This method employs a stream of hot air passing through the suspended olive cake, accelerating the dehydration process while maintaining its nutritional properties. Compared to natural drying, the fluidized bed technique enables a faster reduction in moisture content (within a few hours), preventing the degradation of thermolabile compounds and minimizing lipid oxidation (Molina-Alcaide & Yáñez-Ruiz, 2008). Additionally, this technology is more energy-efficient and results in a more homogeneous and stable final product.

The choice between ensiling and drying depends on various factors, including infrastructure availability, energy costs, and the nutritional needs of the animals. Ensiling is a beneficial solution for farms with access to silos and medium-term preservation needs, whereas drying is preferable for the production of commercial feeds and greater product flexibility. In any case, both techniques contribute to enhancing the value of olive cake, reducing agro-industrial waste, and improving the sustainability of livestock production.

Below is an image of the olive cake production process.



**Figure 2.** Olive cake production process

#### **1.4. Olive cake and chemical composition: Benefits and Applications of Olive cake in Animal Feed**

Olive cake is the solid residue that remains after the olive oil extraction process. This by-product, which mainly consists of pulp, peels and stone fragments, is widely produced in olive growing areas, particularly in the Mediterranean regions (Omar, 2008).

The use of destoned olive cake in animal nutrition is of great interest due to its improved digestibility and reduced risk of gastrointestinal complications. Olive cake, a by-product of olive oil extraction, is composed of residual pulp, skin, and varying amounts of crushed olive pits, depending on the processing method. When not destoned, the high lignocellulosic content of the olive stones can pose significant challenges in animal digestion. The high fiber and lignin content of intact olive pits may lead to reduced nutrient availability and digestibility, potentially causing mechanical injuries or obstructions in the gastrointestinal

tract. Furthermore, excessive consumption of unprocessed olive cake has been associated with lower feed efficiency and slower weight gain, particularly in livestock species that lack the enzymatic capability to break down hard lignified structures. In contrast, destoned olive cake offers a more digestible feed alternative, with improved nutritional composition, including higher levels of digestible fiber and beneficial bioactive compounds, such as polyphenols and unsaturated fatty acids (Tzamaloukas et al., 2021). Studies have demonstrated that de-stoning significantly increases the energy value of olive cake, making it a more efficient and sustainable feed ingredient for ruminants (Molina-Alcaide & Yáñez-Ruiz, 2008).

Additionally, removing the olive pits facilitates the incorporation of olive cake into formulated diets, enhancing pellet durability and feed uniformity. Therefore, the selection of destoned olive cake over raw olive cake is crucial to ensuring optimal animal health, feed efficiency, and sustainability in livestock nutrition.

Traditionally considered a waste, olive cake has recently gained increasing interest in the animal feed sector, due to its nutritional characteristics, which include a significant content of healthy lipids, polyphenols, proteins and minerals. This chapter explores the chemical characteristics of olive cake and its use in animal feed, with a particular focus on ruminants.

The chemical composition of olive cake varies depending on the olive variety, extraction techniques and production region, but the main nutritional components include:

Lipids: Another significant component of olive cake is represented by lipids. Although the oil content in olive cake can vary (5-15%), it is still significant compared to other agricultural by-products. Olive cake is particularly rich in monounsaturated fatty acids, especially oleic acid (C18:1) (Uribe et al., 2013), which represents about 70-75% of total fatty acids. Monounsaturated fatty acids are known to have beneficial effects on cardiovascular health, reducing blood LDL cholesterol levels in both humans and animals. The lipid content in olive cake,

although nutritious, must be carefully managed in animal feed. If not treated correctly, the high oil content can compromise digestibility, especially in ruminants, and could lead to energy overload. Some treatments, such as drying or oil extraction, can be used to reduce the lipid content and improve digestibility.

Proteins: Olive cake contains a moderate amount of protein, usually ranging between 5% and 7% of the dry weight (Alhamad et al., 2017). Although the protein quality of olive cake is not comparable to that of traditional protein sources such as hay or soy, it can still provide a useful contribution to ruminant nutrition. Olive cake protein is mainly composed of soluble proteins and insoluble proteins. Soluble proteins are easily fermentable in the rumen, while insoluble proteins are digestible more slowly. In addition, the presence of essential amino acids such as lysine and methionine, even if in reduced amounts, can be beneficial for supporting animal growth and production, improving the quality of meat and milk in ruminants.

Polyphenols: Another important component of olive cake is polyphenols, which are powerful antioxidant compounds. Olive cake is particularly rich in hydroxytyrosol and oleuropein (Suárez et al., 2009), two polyphenols with demonstrated antioxidant and anti-inflammatory effects. These compounds may be beneficial for animal health, helping to improve the immune response and reduce oxidative stress. The antioxidant action of polyphenols may also have positive effects on the quality of meat and milk (Waqas et al 2023), helping to reduce lipid oxidation in animal products.

Minerals: Olive cake also contains minerals, including calcium, magnesium, potassium, and phosphorus (Al-Rabadi, 2017). Although the levels of minerals are relatively modest compared to other traditional mineral sources, the presence of these elements is useful to supplement the diet of animals, supporting bone health and muscle function.

The use of olive cake in the feeding of animals in zootechnical production is of recent experimentation, but the studies carried out (Pallara et al., 2014; Mele et al., 2014; Estaún et al., 2014; Dal Bosco et al., 2012) have shown the very

interesting potential of use in relation to the improvement of the nutritional quality of meat. The scenarios that see a possible use of olive cake to increase the shelf life of the meat are also very interesting. This last aspect is of particular importance in the context of the quality of the meat product; the peroxidation of fatty acids can in fact produce various substances that have a negative impact on the safety and on the nutritional and organoleptic characteristics of the meat. In this sense, olive cake can represent a more than promising option; in fact, a vast range of substances with antioxidant capacities such as carotenes, anthocyanins, tocopherols and polyphenols remain in the olive cake (Dejong and Lanari, 2009; Karantonis et al., 2008). The addition of olive cake to the diet of pigs may promote better growth, decrease the thickness of the carcass fat, or give ruminants more energy (Ferrer et al., 2020). Therefore, the use of stoned olive cake in substitution to a quote of the cereal could be a strategy to reduce the feeding cost and, at the same time, to introduce a source of oleic acid and of substances with antioxidant properties, which could contribute to improve the quality of meat (Molina-Alcaide E and Yáñez-Ruiz 2008).

### **1.5. Linseeds and chemical composition**

Another notable by-product that lends itself well to this purpose are linseeds, recognized for their high level of omega-3 fatty acids, especially alpha-linolenic acid (ALA). These fats have several health advantages for both people and animals, and they are crucial for cardiovascular health.

It has been shown that diets based on grazing nutrition and the use of linseeds (*Linum usitatissimum* L.) bring significant benefits in terms of polyunsaturated fatty acid content (PUFA) and CLA. A study by Barahona et al. (2016) demonstrated how the integration of flaxseed in beef cattle is able to significantly increase the content of  $\omega_3$  polyunsaturated fatty acids at the intramuscular level; from this it follows not only an improvement of the acidic profile of the meat, but also of the organoleptic characteristics of the same.

Linseeds varieties fed to animals contain a high level of oil (40%) with 55% 18:3 n-3 (Glasser et al., 2008). Adding linseeds in ruminant diets increase the concentration of polyunsaturated fatty acids in beef, especially omega-3 PUFAs. Ruminant products contain a variety of FAs. Some of them could provide potential benefits to human health, including polyunsaturated fatty acids of the omega-3 series. In beef, omega-3 fatty acids are composed of both 18:3 n-3 and 20- and 22-carbon omega-3 fatty acids. Omega-3 fatty acids, and more particularly 20- and 22-carbon fatty acids, may reduce the risk of cardiovascular disease (Mills et al., 2011).

According to Jenkins et al. (1993) and Saliba et al. (2014), adding linseeds to cow diets can raise the amount of omega-3 fatty acids in milk and meat, enhancing their nutritional value and perhaps having a good impact on lipid profiles. Depending on the seed kind and growth environment, their makeup can change.

They are a potential source of protein in the diet since the proteins they contain are high in important amino acids. Additionally, they are a significant source of both soluble and insoluble fiber, which supports intestinal regularity and digestive health.

According to Kristensen et al. (2012), linseeds have been linked to several advantages for human health, including improved lipid profiles, anti-inflammatory properties, and a possible lower risk of chronic illnesses including diabetes and heart disease.

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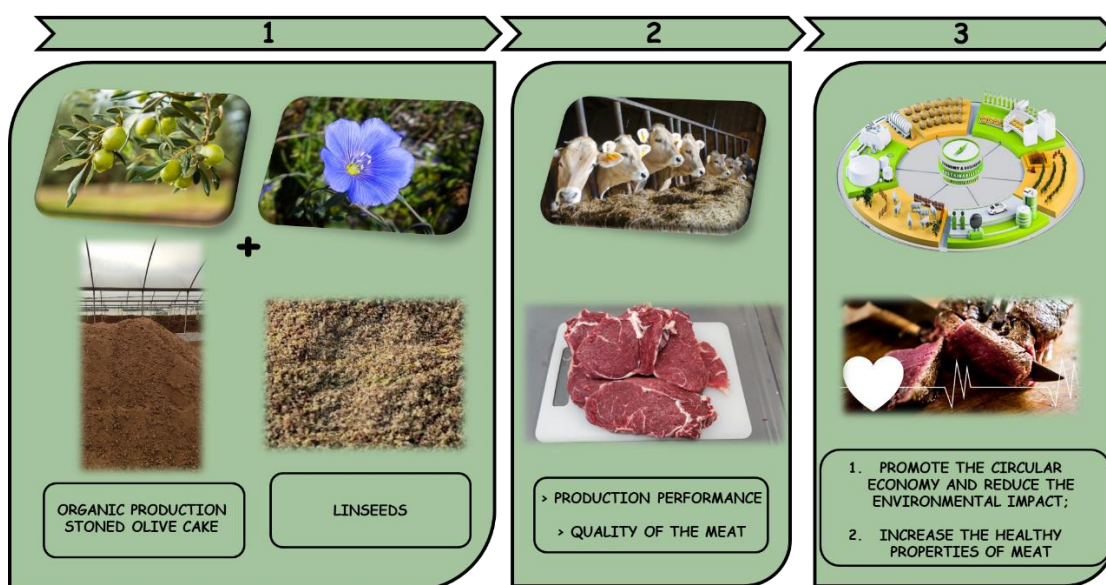
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## Chapter 2. Organization

As specified previously, the experimental test was carried out on podolian young bulls to evaluate that if on the one hand the content of polyunsaturated fatty acids increases through the use of linseed (with greater susceptibility of meat to oxidation phenomena), on the other hand, the integration of organically produced stoned olive cake into the diet of young bulls, thanks to the presence of phenolic compounds, could improve the shelf life of the product itself.

Figure 1 shows a schematic summary of the Ph.D. project.



**Figure 1.** Purpose of the PhD research

The chapters listed below in this PhD thesis take into consideration the use of by-products in animal feed. They are divided as follows:

- Chapter 3: “Effects of olive cake and linseed supplementation in the diet on performance, carcass traits, and oxidative stability of beef from young Podolian bulls”
- Chapter 4: “Effect of high levels of almond hulls supplementation on performance and meat oxidative stability in lambs”

**Chapter 3: Effects of olive cake and linseed  
supplementation in the diet on performance, carcass  
traits, and oxidative stability of beef from young Podolian  
bulls**

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## **Effects of olive cake and linseed supplementation in the diet on performance, carcass traits, and oxidative stability of beef from young Podolian bulls**

### **Abstract**

Stoned olive cake and linseeds were used in the following experimental test conducted on thirty-six young Podolian bulls, divided into 4 groups, Control Group (CON), OC Group (Group with olive cake containing 30% as-fed basis of stoned olive cake), Group EL (Group with linseeds containing 15% as-fed basis of extruded linseed) and Group OCEL (Group with olive cake + linseeds containing 20% of stoned olive cake and 10% extruded linseed) to evaluate the performance of the animals and the quality of the meat. Results show that the olive cake supplementation didn't influence performance in vita and post slaughter measurements of the animals (final body weight, DMI, FCR, ADG, carcass weight, dressing percentage and pH) ( $P > 0.05$ ), unlike TBARS and color measurements where meat samples showed excellent values ( $P < 0.001$ ), especially in diets supplemented with olive cake. In conclusion, incorporating olive cake and linseed into the diet of fattening cattle may be a way to enhance a by-product of the olive industry and naturally increase the nutritional value of meat and meat-based products in Mediterranean regions. This would reduce environmental impact and promote the valorization of this local food source in alignment with the principles of the circular economy.

**Keywords:** Shelf life; Oxidative stability; Bull meat; Olive cake; Linseed; Podolian cattle breed; local breeds; production efficiency; olive polyphenol.

## 1. Introduction

The necessity to discover alternate solutions for the disposal of by-products derived from the agri-food sector is of utmost significance given the expanding global population and the resulting requirement for wise use of food and water resources.

With the ambitious Circular Economy plan being implemented in recent years, international politics has been shifting toward a more sustainable economy with the goal of minimizing waste and reusing byproducts. Reintroducing trash into the production cycle is the main goal in order to get fresh resources that can be utilized to create new goods and provide economic value (Scarlet, 2013).

Additionally, waste management is a crucial component of plans to lower greenhouse gas emissions, air and water pollution, and health issues.

Global warming is regarded as having a number of primary causes, including the cattle industry.

The main global sources of GHG emissions in the production of feed are land use, transport, and cultivation and processing.

In this case, incorporating agro-industrial waste products into animal feed might be a key element of a global effort to lessen the environmental effect of both livestock production and agro-industrial activities.

Some of these byproducts are abundant in bioactive chemicals and may serve as an excellent supply of fresh goods with applications to the circular economy and sustainability (Sherwood, 2020). The objective of a Zero Garbage Economy, in which waste is reincorporated into the production cycle and used as a new resource, is actually the focus (Sharma et al., 2021).

By-products from the agri-food business may be reused by turning them into zootechnical feed, which has economic benefits and a positive socio-environmental effect (Vasta et al., 2008).

Since some by-products, like vitamins and unsaturated fatty acids (FA), contain bioactive compounds, using agro-industrial by-products in livestock can improve product quality and shelf stability in addition to lowering the carbon

footprint of animal products and the environmental problems caused by by-product accumulation (Salami et al., 2019; Gerber et al., 2014; Vasta et al., 2008).

There are key areas where technological innovation used for the reuse of by-products and agri-food residues in the feed sector is advantageous, such as the use the enhance of the nutritional and functional qualities of food. Due to the high concentration of bioactive chemicals in certain byproducts, which serve as functional supplements with specific positive effects on metabolism or health in addition to providing nutritional value, these byproducts are employed and incorporated in animal diets. In this instance, by-products are used similarly to food additives to enhance the health status of the animal and the quality of the products. They can also enhance the organoleptic qualities of the diet, provide antioxidants or other anticancer agents, immune system stimulants and digestive parasite inhibitors, enhance the quality of the products from a microbiological point of view, improve the stability of the lipids or conservation and shelf life, and promote organoleptic qualities of the ration.

The livestock sector may benefit economically and ecologically from the use of agro-industrial byproducts in animal feed, which will increase its sustainability and profitability. One of the most significant agro-industrial byproducts in the Mediterranean region and a significant pollutant from the manufacturing of olive oil is olive cake (OC). The environmental issues with OC management and the commercial value of components like phenols have increased interest in this byproduct's research (García-González et al., 2010).

One of its potential final applications is animal feeding, which would support the circular economy in Mediterranean nations.

The type of olive, the quantity of its components (skin, pulp, and stone), and the method used to extract the oil all have an impact on the chemical characteristics of olive cake (Alcaide et al., 1996). Olive cake is appropriate for use in animal feed due to the substantial amounts of residual oil and oleic acid present. Pigs (Joven et al., 2014; Liotta, et al., 2019), cattle (Estaún et al., 2014), small ruminants (Tzamaloukas, et al., 2021; Caparra et al., 2003), broilers (Herrero-

Encinas et al., 2020), and rabbits (Dal Bosco et al., 2012) have all shown positive results. The addition of olive cake to the diet of pigs may promote better growth, decrease the thickness of the carcass fat, or give ruminants more energy (Ferrer et al., 2020; Molina-Alcaide et al., 2008).

In order to lower the cost of feeding while also introducing a source of oleic acid and other compounds with antioxidant qualities that may help to improve the quality of meat, stoned olive cake could be used in place of some cereal.

Linseeds (*Linum usitatissimum*) were used in this work in addition to olive cake. Animal-feeding linseeds have a high oil content (40%) with a 55% 18:3 n-3 ratio (Glasser et al., 2008). When linseeds are added to ruminant diets, the amount of polyunsaturated fatty acids, particularly omega-3 PUFAs, in beef increases (Marino et al., 2018). A range of FAs can be found in ruminant products. Some of them, such as omega-3 polyunsaturated fatty acids, may have positive effects on human health. Both 18:3 n-3 and omega-3 fatty acids with 20 and 22 carbons make up the omega-3 fatty acids found in beef. According to Mills et al. (2011), omega-3 fatty acids, especially those with 20 and 22 carbons, may lower the risk of cardiovascular disease.

Consumers nowadays are more interested in natural preservatives than ever before because they want items that are good for their health. Hence, supplying meals with a high nutritional value entails not only substituting natural antioxidants for synthetic preservatives but also enhancing the goods' health benefits and shelf life.

This study will specifically focus on agro-industrial by-products rich in polyphenols used as feed additives in animal nutrition to mitigate oxidative stress-induced damage. Particular attention will be given to their impact on the physical properties, chemical composition, and sensory characteristics of the final product.

The purpose of the current study was to determine whether, on the one hand, using linseeds increases the amount of polyunsaturated fatty acids (making the meat more susceptible to oxidation phenomena), and, on the other hand,

adding organically produced stoned olive cake to the diet of young Podolian bulls could extend the shelf life of the product itself as it contains phenolic compounds.

## **2. Materials and methods**

### **2.1. Ethical Statement**

The experimental procedures in this experiment were evaluated and approved by the Animal Welfare Committee of the Mediterranean University of Reggio Calabria (Protocol Number 3015/2023) and carried out according to international guidelines on the protection of animals used for scientific research (Directive 2010/63/EU).

### **2.2. Animal management, dietary treatments and sample collection**

The experiment was conducted from February to July 2023 on a family farm (project leader) located in Melissa (province of Crotona, Calabria region, Southern Italy) at 80 m above sea level (latitude: 39° 17' 46.712" N, longitude: 17° 1' 34.651" W), which breeds Podolian cattle for meat production.

Podolian is an autochthonous cattle breed from Southern Italy, we find it especially in Calabria and Basilicata Regions, and it is particularly rustic, long-lived and well adapted to live in difficult environments with poor quality vegetation (native pastures and shrubs). It is farmed with extensive or semi-extensive systems by still using the traditional practice of transhumance which consists of the seasonal migration of livestock who move, along the ancient pathways, from the mountainous areas to those of the plains, during the winter season, or conversely during the summer season in order to find more suitable pastures.

Podolian cattle breeding is characterized by cow-calf line where calves are raised on natural pasture with their mothers and naturally weaned when they are about 7 to 8 months old. After weaning, they continue to be kept on pasture until 10-11 months of age and then are moved in housing systems made up of open

barns with pens to be finished with concentrates and straw until slaughter at 15-16 or 17-18 months of age.

The experimental involved 36 Podolian young bulls that were randomly allotted to four equal groups of 9 animals each, in accordance with dietary treatments and balanced to their age (average initial age of 315,73±19,40 days; mean ± SD) and body weight (average initial body weight of 304,31±13,50 kg; mean ± SD).

Podolian calves of each treatment were housed within the same newly constructed free-stall barn in separate contiguous pens with a feeding/resting area and an uncovered exercise area with a total space allowance of 14,5 m<sup>2</sup> per head. Each pen was equipped with individual cribs and mangers with feeding space per animal of approximately 0,80 m.

The animals of each group were fed the same basal forage consisting of wheat straw offered ad libitum once daily at 7.30 AM, and one of the following pelleted experimental concentrates: i) a concentrate without any neither olive cake nor extruded linseed supplementation (concentrate CTRL); ii) a concentrate containing 30% as-fed basis of stoned olive cake without extruded linseed supplementation (concentrate OC); iii) a concentrate containing 15% as-fed basis of extruded linseed without stoned olive cake supplementation (concentrate EL); iv) a concentrate containing 20% of stoned olive cake and 10% extruded linseed (as-fed basis) supplementation (concentrate OCEL) (Table 1).

**Table 1**

Ingredients and formulation of the experimental concentrates administered to the Podolian young bulls

	Concentrates			
	CTRL	OC	EL	OCEL
<i>Ingredient, % as fed basis</i>				
Corn meal	23,50	15,60	20,40	15,00
Barley meal	21,52	12,30	19,92	10,32
Wheat middlings	18,00	6,10	12,00	13,00
Broad bean ( <i>Vicia faba minor</i> )	15,00	15,00	15,00	13,00
Soybean extraction meal, 46% <sup>3</sup>	13,80	13,12	9,00	10,00
Extruded whole linseed	0,00	0,00	15,00	10,00

Stoned olive cake	0,00	30,00	0,00	20,00
Sugar beet pulp	4,50	4,20	5,00	5,00
Calcium carbonate	1,30	1,30	1,30	1,30
Sugar cane molasses	1,00	1,00	1,00	1,00
Lignosulfite	0,50	0,50	0,50	0,50
Sodium bicarbonate	0,40	0,40	0,40	0,40
Sodium chloride	0,10	0,10	0,10	0,10
Magnesium oxide	0,30	0,30	0,30	0,30
Mineral premix <sup>1</sup>	0,05	0,05	0,05	0,05
Vitamin premix <sup>2</sup>	0,03	0,03	0,03	0,03

<sup>1</sup> Mineral premix, contained per kilogram: Cu (copper sulfate pentahydrate), 12 mg; I (calcium iodate), 0,75 mg; Mn (manganese oxide), 45 mg; Se (sodium selenite), 0,20 mg; Zn (zinc oxide), 65 mg; Zn (zinc sulfate monohydrate), 10 mg.

<sup>2</sup> Vitamin premix, contained per kilogram: vitamin A (retinyl acetate), 6000 IU; vitamin D<sub>3</sub> (cholecalciferol), 900 IU; niacin (nicotinic acid), 77,40 mg; vitamin B<sub>1</sub> (thiamine monohydrate), 0,87 mg; vitamin B<sub>2</sub> (riboflavin), 1,92 mg; vitamin E (DL- $\alpha$ -tocopherol acetate), 15 mg.

<sup>3</sup> 46% crude protein in the dry matter.

The experimental concentrates were given to each animal twice a day at 8.00 AM and 4:00 PM, at the rate of 1,7 kg (as fed basis) per 100 kg of live weight and were completely consumed by all the animals. Concentrate offer was adjusted every two weeks according to the last body weight after weighing. Amounts of wheat straw offered and refused by each bull were weighed and recorded weekly. Water was given ad libitum for all treatments. The concentrates used in the experiment were formulated to be isonitrogenous and isoenergetics. The fattening period lasted for 170 days including 20 days as the adaptation period and 150 days as experimental period. During the entire experimental period, the general health conditions of the animals remained optimal and there was no onset of alterations of any kind, mainly with regard to the digestive system. The olive cake used in the experiment was provided by an olive farm with organic certification (project partner) located in Belcastro (province of Catanzaro, Calabria region, Southern Italy), which grows and processes olives into organic extra virgin oil of higher quality, and consequently also produces organic olive cake. In our study we preferred to use organic olive cake, free of synthetic chemical residues, so as not to compromise the extremely positive image of the

extensive Podolian farming system, environmentally and animals friendly farming method that supplies high-quality products, which is exceptionally appreciated by consumers.

The olive cake was obtained from a continuous two-and-a-half-phase olive oil mill plant (Pieralisi Group S.p.A., Jesi, Italy) by "cold extraction" (during the oil-making process the temperature never exceeds 27 °C) according to the following operative conditions: olive crushing was carried out with a hammer crusher equipped with an inverter for the adjustment of its rotating speed which due to its particular construction characteristics it is able to reduce thermal and oxidative stress on the product; olive paste malaxation (kneading) lasted round about 45 minutes at 24-26 °C; oil extraction was performed using a centrifugal extractor (two-and-a-half-phase decanter) Scorpion 5.9 model, which allows to significantly reduce water consumption compared to conventional processes, while ensuring a higher concentration of polyphenols in both the oil and the olive cake. With this technology, the moisture content of the olive cake obtained is roughly 50%.

In order to avoid high losses of phytochemicals, antioxidants and other bio-active compounds, in this experiment we have chosen to dry the of wet olive cake at room temperature by a green, simple, low-cost method developed by a small local industry (project partner), located in Belcastro (province of Catanzaro, Calabria region, Southern Italy), with our support. In detail, after olive oil extraction, the fresh virgin olive cake was transported within a short time from the oil mill to the naturally ventilated greenhouses, covered with UV stabilised polyethylene, to be dried up. In these polyhouses, the olive cake was spread on the concrete floor in layers of about 10-12 cm and periodically mixed mechanically to speed up the drying process. The drying took place through natural air convection without using any additional systems. The drying process took 8-10 days, depending on the initial humidity of the olive cake. Once drying was completed, the olive cake was stoned, using a vibration sieve with a mesh size of 2 mm, and immediately pelleted and packaged to ensure the highest quality, by

the local industry partner and subsequently sent to a feed mill for the production of the pelleted experimental concentrates (Table 1).

After drying and pitting, several representative samples of the olive cake were collected and brought to the laboratory to detect the presence of mycotoxins and other potential contaminants.

The total amounts of feed consumed (concentrate and straw) by each animal were recorded and analysed for dry matter (DM) content (in a forced air heater at 105 °C) to assess dry matter intake (DMI). During the experimental period, the bulls were individually weighed every two weeks with a cattle scale to adjust the concentrate offer and to determine the average daily gain (ADG) and feed conversion ratio values (FCR).

Representative samples of the concentrates and wheat straw were collected during the course of the experiment, and also a representative sample of dried stoned olive cake used in the experiment was taken, to be submitted to the laboratory analyses.

### **2.3. Slaughter procedures, carcass characteristics and meat sampling**

All bulls were humanely slaughtered, on the same day, in a commercial slaughterhouse after a 60 km truck journey, and after one night of fast but with access to water. All animals were slaughtered by throat cut after captive bolt stunning. The slaughter procedures comply with the welfare regulations and the EU Council Regulation the Council Regulation (EC) No 1099/2009 (EC, 2009).

After slaughtering, the carcasses were weighed to obtain hot carcass weight and hot dressing (calculated as the relation between hot carcass weight and slaughter body weight). The carcasses were then split in half and using the European Union beef carcass classification system (Council Regulation 1183/2006/EC), they were subjectively rated for conformation and fatness scores. Each score was then further classified as high (+), medium (=), and low (-). Both the conformation and fatness scales ranged from 18 (S+, very good) to 1 (P-, very poor) and 15 (5+, very high) to 1 (1-, extremely low), respectively. Once the surveys

were completed, the carcasses were stored in a refrigeration room at  $2 \pm 2$  °C following the routine refrigeration procedure used in the slaughterhouse. After refrigeration for 24 h, the carcasses were weighed again and cold dressing percentage and cooling loss were calculated. Subsequently, in the region of the 9th rib of right carcass, subcutaneous fat color was measured in triplicate as the CIELAB coordinates  $L^*$ ,  $a^*$  and  $b^*$  (CIE, 1986) using a portable colorimeter Minolta CR300 (D65 illuminant) calibrated to a standard white tile.

Carcass pH was measured at 1 and 24 h postmortem using a portable pH-meter (Hanna, HI 99163) equipped with a penetrating electrode and inserted approximately three cm into longissimus thoracis muscle between the 7th and 8th thoracic vertebrae. The electrode was calibrated to 14 °C (abattoir temperature) with two buffers (pH 7.0 and pH 4.0).

After pH testing after 48 hours of refrigeration, the part of the LD (Longissimus Dorsi), in a cranial-caudal direction, between the 6th and 10th thoracic vertebra, was taken from each right carcass for the analysis of lipid oxidation (TBARS) and color.

#### **2.4. Feed and analysis meat proximate analyses**

Crude protein, crude fat, and crude ash were measured in feed samples using respectively AOAC, 1995 procedures 984.13, 920.39, and 942.05. The analysis of the fiber fractions (NDF, ADF, and ADL) was conducted using the Van Soest, Robertson, and Lewis (1991) approach. The method developed by Gray et al. (1967) was used to determine lipids for the examination of fatty acids.

The total content of phenolic compounds was determined using the Folin–Ciocalteu reagent according to the colorimetric method described by Verma et al. (2008) and Naczki and Shahidi (2006). The phenolic compounds present in the feed were extracted using the method reported by De Bruno et al., (2020), while the extraction and quantification of tocopherols was evaluated according to Rachieru et al. (2009).

The color in the meat samples was measured in triplicate as the CIELAB coordinates L\*, a\* and b\* (CIE, 1986) using a portable colorimeter Minolta CR300 (D65 illuminant) calibrated to a standard white tile.

Lastly, for the characterization of the lipid oxidation in the meat samples the method described by (Siu & Draeper, 1978) was used.

## **2.5. Total phenolic content (TPC) and quantification of polyphenols by HPLC analysis**

Phenolic compounds were recovered as described by Verma et al. (2008) with some modification.

Powder of feeds (moisture 9%) was mixed with hydroalcoholic solvent (EtOH 80%) (5:1 solvent/row material ratio). The extractions were performed on a heating plate (60 °C) with constant stirring for 60 minutes. Later, the produced extracts were centrifuged (10000 rpm, 10 min, 4 °C) and the supernatant was recovered, filtered through a Buchner funnel, and stored at -21 °C until further analysis.

The total content of phenolic compounds was determined using the Folin–Ciocalteu reagent according to the colorimetric method described by Verma et al. (2008) and Naczka and Shahidi with appropriate modifications. Briefly, 0.5 mL of a diluted extract (1:10) was mixed with 2.5 mL of the Folin-Ciocalteu reagent (10% v/v) and 2 mL of a Na<sub>2</sub>CO<sub>3</sub> solution (7.5% w/v). The mixture was incubated for 15 minutes at 45 °C and left to cool to room temperature for 30 minutes. The absorbance was readings at 765 nm, against a reagent blank (reaction mixture without the sample), using a double-beam ultraviolet- visible spectrophotometer (Perkin-Elmer UV- Vis λ2, Waltham, Massachusetts, USA).

Total phenolics content was calculated from a calibration curve gallic acid (2-10 mg/L; r = 0.999) and expressed as µg of gallic acid equivalents (GAE) g<sup>-1</sup> dry matter.

Individual phenolic compounds were characterized by Ultra-High Performance Liquid Chromatography (UHPLC) analysis.

Phenolic compounds in the feed were extracted using the method reported by De Bruno et al., (2020). 1 g of powder of feed was extracted five times with 5 mL of methanol. The extracts were combined, the methanol was evaporated under vacuum at room temperature, and the residue was dissolved in 5 mL of methanol.

Regarding olive cake, phenolic compounds were recovered with the methods reported by Lafka, et al. (2018) and De Bruno et al., (2020), with some modification. Briefly, 2 g of olive cake were mixed with n-hexane (5:1 v/w) and placed for 1 h in an orbital shaker at room temperature. Since non-polar substances such fat-soluble vitamins and pigments, as well as fatty acids and triacylglycerols, might obstruct phenolic quantitation, this step is frequently carried out prior to phenolic compound recovery. The samples were filtered using Buchner funnel, and the filtrate was removed. Then, the residues were extracted at room temperature five times with MeOH, centrifuged (8000 rpm, 10 min, 4 °C) and the supernatant was recovered, filtered through Buchner funnel. The extracts were combined and used for the quantification of phenolic compounds.

A PLATINblue UHPLC equipped with a binary pump system using a Knauer C18 column (1.8  $\mu$ m, 100  $\times$  2 mm) coupled with a PDA-1 (Photo Diode Array Detector) and Clarity 6.2 acquisition software was used for the separation of phenolic substances contained in feed and olive cake. The eluent phases used were (A): water acidified with acetic acid (pH 3.10); (B): acetonitrile. For analysis, 5  $\mu$ L was injected into the system and an elution gradient consisting of 95% A and 5% B from 0 to 3 min; 95-60% A and 5- 40% B from 3 to 15 min; 60-0% A and 40-100% B from 15 to 15.5 min was used.

Identification and quantification analysis of the individual components was conducted by comparing the retention times with reference to external standards used to construct the calibration straight lines, and the results were expressed as  $\mu$ g g<sup>-1</sup> of dry matter.

## 2.6. Extraction and quantification of tocopherols by HPLC analysis

Tocopherol content was evaluated according to Rachieru et al. (2009), with appropriate modifications. 2 g of dried and ground feed was saponified with an ethanolic solution (55% v/v) of potassium hydroxide (11% w/v) at 80°C for 15 min. The samples were subsequently extracted with hexane. The hexane phase was recovered and concentrated using a Rotavapor. The residue obtained was dissolved in 200 µL of mobile phase.

Injection (20 µl), after filtration through cellulose syringe filters (0.45 µm), was performed in Knauer HPLC Smartline Pump 1000 equipped with a Smartline 2600 UV detector (Knauer, Germany) equipped with a C18 reversed-phase column (250 x 4.6 mm x 5 µm). The mobile phase was 100% methanol with flow rate of 1.5 ml/min and temperature 40°C. Tocopherols were monitored at 292 nm and external standards as  $\alpha$ -tocopherol and DL- $\alpha$ -Tocopherol acetate (Sigma-Aldrich, USA) were used for quantification of tocopherols. Results were expressed as µg g<sup>-1</sup> of dry matter.

## 2.7. Lipid oxidation of meat samples

The LD slices were covered with an oxygen-permeable film and stored in a refrigerator at 3°C. Analyses were conducted on the fresh product at various storage intervals: at time zero (T<sub>0</sub>), after three days (T<sub>3</sub>), six days (T<sub>6</sub>), and ten days (T<sub>10</sub>).

The analysis of thiobarbituric acid reactive substances (TBARS), used as an index of lipid oxidation, was conducted on Longissimus Dorsi muscle samples, according to the method described by (Siu & Draeper, 1978). Five grams of sample were homogenized with ultraturrax (2 minutes at 13,000 rpm) in 15 ml of 5% (w/v) trichloroacetic acid (TCA). After filtering the homogenate through filter paper (Whatman 42), a 4 ml aliquot was taken to which 1 ml of thiobarbituric acid (0.02 M) was added. Subsequently, after leaving the solution to incubate at 80°C for 90 minutes, waiting for the development of the colorimetric reaction, the

absorbance at 532 nm was measured using a double beam spectrophotometer (Shimadzu Corporation, Milan, Italy; model UV-1800). The data was expressed in mg of MDA/kg of meat.

### **3. Statistical Analysis**

The performance parameters and fatty acid composition data were examined using an ANOVA model (SAS 2009), with dietary treatments as a fixed factor. The means were compared using a Tukey test, with significance determined at  $P < 0.05$ .

Data regarding meat quality were assessed through a mixed model analysis (GLM procedure), which included the effects of the diets and storage time as fixed factors and their interactions. The least-square means (LSM) and the standard error of the least square means (SEM) were calculated. Comparisons of the least-square means were performed using the Tukey test and the differences were considered significant at  $P < 0.05$ .

### **4. Results**

The results from the tests to detect the presence of mycotoxins in samples of the olive cake collected at the end of the drying process, indicate negligible levels of mycotoxin contamination (well below the maximum levels set by the European Commission: Commission Regulation (EU) 2023/915) in all olive cake samples analyzed.

As regards tocopherols, as can be seen in Table 2, the greatest quantity of alpha tocopherol, as expected, is found in feed in which the olive cake is present (OC and OCEL).

**Table 2**

Chemical-nutritional composition and fatty acids profile of the experimental concentrates and of the straw administered to the Podolian young bulls

	Straw	Concentrates <sup>1</sup>			
		CON	OC	EL	OCEL
<i>Chemical composition</i>					
Dry matter (DM), g/kg as feed	903,2	902,9	907,8	908,4	908,7
Crude protein, g/kg DM	40,3	198,5	198,5	198,5	199,0
Ether extract, g/kg DM	14,7	23,6	62,5	67,3	82,6
Ash, g/kg DM	67,4	52,7	58,2	51,9	56,8
NDF <sup>2</sup> , g/kg DM	781,9	254,5	384,5	294,4	350,9
ADF <sup>2</sup> , g/kg DM	501,5	75,5	207,2	94,5	158,8
ADL <sup>2</sup> , g/kg DM	77,1	15,3	73,9	27,1	51,7
Net energy <sup>3</sup> , MFU <sup>2</sup> /kg DM	0,30	1,04	0,97	1,06	1,00
Total phenolic compounds, g Tae <sup>4</sup> /kg DM	1,18	6,14	15,05	7,67	14,44
<i>Tocopherols</i>					
Total tocopherols, µg/g DM		32,05	30,60	29,05	28,00
α-Tocopherol (%)		79,56	86,93	61,62	82,50
γ-Tocopherol (%)		18,41	11,44	37,18	16,07
δ-Tocopherol (%)		2,03	1,63	1,20	1,43
<i>Fatty acids, % of total fatty acids</i>					
C12:0	0,01	0,02	0,03	0,22	0,05
C14:0	2,69	1,08	1,05	1,06	1,01
C16:0	52,44	21,44	12,79	9,28	12,45
C16:1 c	0,14	0,14	0,21	0,11	0,1
C18:0	9,47	1,87	1,92	1,77	2,68
C18:1 n-9	8,44	25,96	59,87	13,82	39,57
C18:2 n-6	16,76	45,08	19,23	24,98	21,62
C18:3 n-3	4,85	1,76	1,99	45,96	19,91
C20:0	0,43	0,19	0,16	0,32	0,12
Others fatty acids	4,77	2,46	2,75	2,48	2,49
<i>Fatty acid classes</i>					
SFA <sup>2</sup>	65,04	24,6	15,95	12,65	16,31
MUFA <sup>2</sup>	8,58	26,1	60,08	13,93	39,67
PUFA <sup>2</sup>	21,61	46,84	21,22	70,94	41,53
n-3 PUFA <sup>2</sup>	4,85	1,76	1,99	45,96	19,91
n-6 PUFA <sup>2</sup>	16,76	45,08	19,23	24,98	21,62
<i>Fatty acid ratio</i>					
UFA/SFA <sup>2</sup>	0,46	2,97	5,10	6,71	4,98

<sup>1</sup> CON = containing neither linseed nor olive cake; OC = containing 30% of stoned olive cake; EL = containing 15% of extruded whole linseed; OCEL = containing 10% of extruded whole linseed and 20% of stoned olive cake. <sup>2</sup> Abbreviations: NDF, neutral detergent fiber; ADF, acid detergent fiber;

ADL, acid detergent lignin; MFU, meat forage units; SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; USFA/SFA, unsaturated fatty acids/saturated fatty acids ratio.

<sup>3</sup> The calculations of energy values, expressed in UFC, were performed using the approach and the equations proposed by INRA. INRA (Alimentation des Ruminants; Editions Quae: Versailles, France, 2018).

<sup>4</sup> Tannic acid equivalent.

As expected, oleic acid (C18:1 n-9 acid) and  $\alpha$ -linolenic acid (C18:3 n-3) are present in higher quantities in diets containing olive cake and linseeds. As shown in Table 3, the dietary treatments had no influence on the carcass weight, dressing percentage, pH, average daily gain (ADG), dry matter intake (DMI), feed conversion ratio (FCR), and final body weight.

These data suggest that the partial replacement of conventional feed with olive cake does not significantly compromise animal growth, thus proving to be a sustainable option.

**Table 3**

Performance in vita and post slaughter measurements of Podolian young bulls receiving four different supplementations during the fattening period

Items	Treatments <sup>1</sup>				SEM <sup>2</sup>	Significance
	CON	OC	EL	OCEL		
No. of bulls	9	9	9	9		
Start age (days)	314,50	315,63	316,75	316,03	7,66	NS
Initial weight (kg)	303,50	304,00	305,25	304,50	5,33	NS
Final weight (kg)	498,30	493,10	492,00	495,50	12,10	NS
ADG (kg/day)	1,30	1,26	1,25	1,27	0,04	NS
Hot carcass weight (kg)	264,38	260,06	260,56	263,25	10,58	NS
Cold carcass weight (kg)	259,50	255,25	255,56	258,31	10,43	NS
Hot dressing percentage (%)	52,95	52,70	52,87	53,09	0,55	NS
Cold dressing percentage (%)	51,96	51,73	51,85	52,10	0,54	NS
Cooling loss (%)	1,86	1,85	1,93	1,88	0,04	NS
Carcass conformation score <sup>3</sup>	8,00 (R)	7,88 (R)	8,00 (R)	8,00 (R)	0,62	NS
Carcass fatness score <sup>4</sup>	6,00 (2+)	6,22 (2+)	6,22 (2+)	6,00 (2+)	0,54	NS

pH after 1 hour <i>post mortem</i>	6,71	6,6 6	6,67	6,62	0,08	NS
pH after 24 hours <i>post mortem</i>	5,40	5,52	5,44	5,50	0,06	NS
Dry matter intake (kg/day)	8,70	8,5 4	8,3 6	8,43	0,23	NS
Feed conversion ratio (kg DM/kg gain)	6,85	7,01	7,03	6,74	0,3 2	NS
Feed conversion ratio (% Final weight)	1,75	1,74	1,71	1,71	0,10	NS

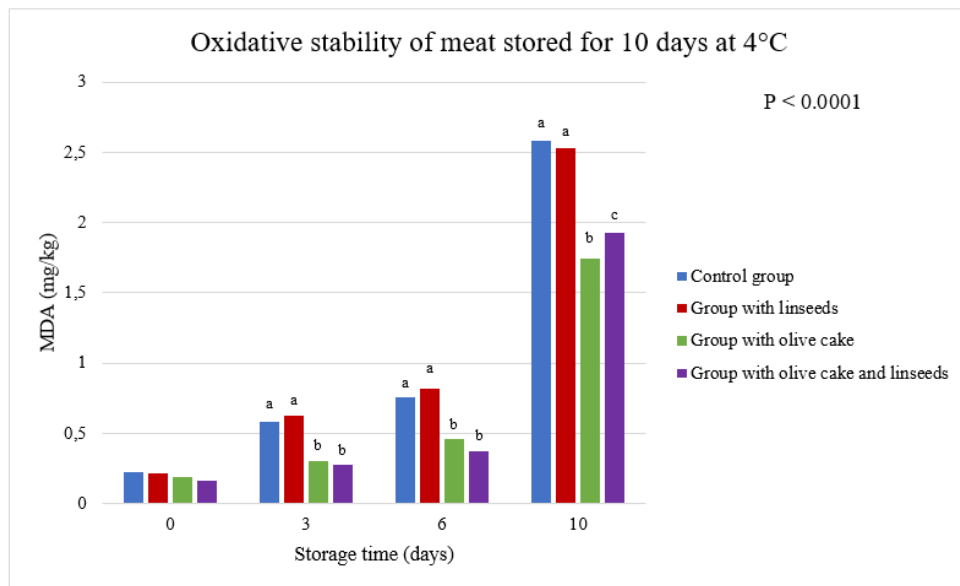
<sup>1</sup> CON=containing neither linseed nor olive cake; OC = containing 30% of stoned olive cake; EL = containing 15% of extruded whole linseed; OCEL = containing 10% of extruded whole linseed and 20% of stoned olive cake.

<sup>2</sup> Standard error of the means (SEM).

<sup>3</sup> According to EUROP classification, where 15 = E+ very excellent, 1 = P- very poor.

<sup>4</sup> According to fatness scale, where 15 = 5+ very high, 1 = 1- very low.

Regarding the oxidative stability, the Fig. 1 shows the TBARS values measured during monitoring days. The TBARS values showed highly significant differences ( $P < 0.001$ ) in the feeding-time (FxT) interaction. It is evident that the CON and EL diets exhibit a higher tendency for oxidation over time, whereas the OC and OCEL diets, influenced by the presence of olive cake, demonstrate a significant antioxidative effect, maintaining lower oxidation levels for up to 10 days.



**Fig.1** Effect of the dietary treatment and time of storage on the oxidative stability of longissimus dorsi. Treatments were: CON = containing neither linseed nor olive cake; OC = containing 30% of stoned olive cake; EL = containing 15% of extruded whole linseed; OCEL = containing 10% of extruded whole linseed and 20% of stoned olive cake.

Interactive effect of dietary treatment (CON, EL, OC and OCEL) and storage time on TBARS values measured on meat samples during aerobic storage at 4 °C. The values presented show highly significant differences ( $P < 0.001$ ).

In comparison, the linseeds group showed a reduction in oxidative stress compared to the control, but still greater than those observed in the OC and OCEL groups, suggesting that polyunsaturated fatty acids from flaxseeds may increase the susceptibility of meat to lipid oxidation.

These results are consistent with previous research demonstrating the antioxidant potential of olive derivatives in animal diets (Vasta & Luciano, 2011).

Regarding the brightness at time 0 there is a difference in values between the groups, with the OC and OCEL groups presenting the highest values. The color of the meat changes little after 3 days of storage, maintaining a higher brightness values in the EL, OC and OCEL groups even after 10 days (Table 4).

**Table 4**Variation of lightness **L\*** in meat during storage in PVC at 4° C for 10 days

	Storage time (days)				SEM	P		
	0	3	6	10		F <sup>2</sup>	T <sup>3</sup>	FxT
	CON <sup>1</sup>	39,733 <sup>Ac</sup>	39,775 <sup>Ab</sup>	39,255 <sup>Ac</sup>		38,588 <sup>Bc</sup>	0.264	0.0001
EL <sup>1</sup>	42,755 <sup>a</sup>	42,819 <sup>a</sup>	42,585 <sup>a</sup>	43,096 <sup>a</sup>	0.264	0.0001	NS	0.0001
OC <sup>1</sup>	41,683 <sup>b</sup>	42,429 <sup>a</sup>	41,765 <sup>b</sup>	42,109 <sup>b</sup>	0.264	0.0001	NS	0.0001
OCEL <sup>1</sup>	41,819 <sup>Bb</sup>	42,537 <sup>Ba</sup>	42,678 <sup>Ab</sup>	43,194 <sup>Aa</sup>	0.264	0.0001	NS	0.0001

Different capital letters in the same row indicate significant differences  $P < 0.0001$ ; Different lowercase letters in the same column indicate significant differences  $P < 0.0001$ .

<sup>1</sup> CON = containing neither linseed nor olive cake; OC = containing 30% of stoned olive cake; EL = containing 15% of extruded whole linseed; OCEL = containing 10% of extruded whole linseed and 20% of stoned olive cake.

<sup>2</sup> F = Feeding

<sup>3</sup> T = Time

The redness remains, as hypothesized thanks to the presence of the olive cake, maintaining higher values in the OC and OCEL groups unlike the C and EL groups (Table 5).

**Table 5**Variation of redness **a\*** in meat during storage in PVC at 4° C for 10 days

	Storage time (days)				SEM	P		
	0	3	6	10		F <sup>2</sup>	T <sup>3</sup>	FxT
	CON <sup>1</sup>	17,491 <sup>A</sup>	13,547 <sup>Bd</sup>	10,999 <sup>Cd</sup>		6,458 <sup>Db</sup>	0.311	0.0001
EL <sup>1</sup>	17,894 <sup>A</sup>	14,467 <sup>Bc</sup>	12,333 <sup>Cc</sup>	6,127 <sup>Db</sup>	0.311	0.0001	0.0001	0.0001
OC <sup>1</sup>	18,193 <sup>A</sup>	16,229 <sup>Bb</sup>	14,552 <sup>Ca</sup>	8,235 <sup>Da</sup>	0.311	0.0001	0.0001	0.0001
OCEL <sup>1</sup>	18,189 <sup>A</sup>	17,289 <sup>Ba</sup>	14,967 <sup>Ca</sup>	8,695 <sup>Da</sup>	0.311	0.0001	0.0001	0.0001

Different capital letters in the same row indicate significant differences  $P < 0.0001$ ; Different lowercase letters in the same column indicate significant differences  $P < 0.0001$ .

<sup>1</sup> CON = containing neither linseed nor olive cake; OC = containing 30% of stoned olive cake; EL = containing 15% of extruded whole linseed; OCEL = containing 10% of extruded whole linseed and 20% of stoned olive cake.

<sup>2</sup> F = Feeding

<sup>3</sup> T = Time

The yellowness presents lower values at time 0 in the Control group (CON) compared to the other groups, but after 10 days the lowest value is recorded in the EL group (Table 6).

**Table 6**Variation of yellowness **b\*** in meat during storage in PVC at 4° C for 10 days

	Storage time (days)				SEM	P		
	0	3	6	10		F <sup>2</sup>	T <sup>3</sup>	FxT
CON <sup>1</sup>	11,069 <sup>Ab</sup>	9,210 <sup>Bc</sup>	8,425 <sup>C</sup>	8,208 <sup>Cb</sup>	0.232	0.0001	0.0001	0.0001
EL <sup>1</sup>	12,92 <sup>Aa</sup>	10,003 <sup>Bb</sup>	8,164 <sup>C</sup>	7,740 <sup>Cb</sup>	0.232	0.0001	0.0001	0.0001
OC <sup>1</sup>	13,405 <sup>A</sup> <sub>a</sub>	12,958 <sup>Aa</sup>	11,699 <sup>B</sup>	9,520 <sup>Ca</sup>	0.232	0.0001	0.0001	0.0001
OCEL <sup>1</sup>	13,49 <sup>Aa</sup> <sub>a</sub>	13,040 <sup>Aa</sup>	12,202 <sup>B</sup>	9,741 <sup>Ba</sup>	0.232	0.0001	0.0001	0.0001

Different capital letters in the same row indicate significant differences  $P < 0.0001$ ; Different lowercase letters in the same column indicate significant differences  $P < 0.0001$ .

<sup>1</sup> CON = containing neither linseed nor olive cake; OC = containing 30% of stoned olive cake; EL = containing 15% of extruded whole linseed; OCEL = containing 10% of extruded whole linseed and 20% of stoned olive cake.

<sup>2</sup> F = Feeding

<sup>3</sup> T = Time

At time 0 the CON group presents the lowest chroma value; starting from the third day of shelf-life, in the C and EL groups the chroma value decreased to a value below 18, unlike the OC and OCEL groups in which the values remain above 18 even after the sixth day of storage (Table 7), remembering that values below 18 are not considered acceptable by consumers (Ripoll et al., 2011).

**Table 7**Variation of Chrome **C\*** in meat preserved in PVC for 10 days at 4° C.

	Storage time (days)				SEM	P		
	0	3	6	10		F <sup>2</sup>	T <sup>3</sup>	FxT
CON <sup>1</sup>	20,701 <sup>Ab</sup>	16,384 <sup>Bc</sup>	13,855 <sup>Cb</sup>	10,452 <sup>Db</sup>	0.365	0.0001	0.0001	0.0001
EL <sup>1</sup>	22,085 <sup>Aa</sup>	17,595 <sup>Bb</sup>	14,794 <sup>Cb</sup>	9,883 <sup>Db</sup>	0.365	0.0001	0.0001	0.0001
OC <sup>1</sup>	22,601 <sup>Aa</sup>	20,773 <sup>Ba</sup>	18,763 <sup>Ca</sup>	12,590 <sup>Da</sup>	0.365	0.0001	0.0001	0.0001
OCEL <sup>1</sup>	22,649 <sup>A</sup> <sub>a</sub>	21,656 <sup>Ba</sup>	19,313 <sup>Ca</sup>	13,060 <sup>Da</sup>	0.365	0.0001	0.0001	0.0001

Different capital letters in the same row indicate significant differences  $P < 0.0001$ ; Different lowercase letters in the same column indicate significant differences  $P < 0.0001$ .

<sup>1</sup> CON = containing neither linseed nor olive cake; OC = containing 30% of stoned olive cake; EL = containing 15% of extruded whole linseed; OCEL = containing 10% of extruded whole linseed and 20% of stoned olive cake.

<sup>2</sup> F = Feeding

<sup>3</sup> T = Time

Hue values increased over the storage time, whereas the chroma values had an opposite trend (Table 8). The CON and EL groups have a lower initial hue value at time 0 than the OC and OCEL groups; after 10 days the CON and EL groups present higher values than the OC and OCEL groups.

**Table 8**

Variation of Hue  $H^*$  in meat stored in PVC for 10 days at 4° C.

	Storage time (days)				SEM	P		
	0	3	6	10		F <sup>2</sup>	T <sub>3</sub>	FxT
CON <sup>1</sup>	32,305 <sup>Db</sup>	34,185 <sup>Cc</sup>	37,450 <sup>Bb</sup>	51,954 <sup>Aa</sup>	0.495	0.0001	0.0001	0.0001
EL <sup>1</sup>	35,963 <sup>Ba</sup>	34,557 <sup>Cc</sup>	33,384 <sup>Cc</sup>	51,703 <sup>Aa</sup>	0.495	0.0001	0.0001	0.0001
OC <sup>1</sup>	36,425 <sup>Da</sup>	36,629 <sup>Cb</sup>	38,777 <sup>Bab</sup>	49,165 <sup>Ab</sup>	0.495	0.0001	0.0001	0.0001
OCEL <sup>1</sup>	36,594 <sup>Ca</sup>	37,058 <sup>Ca</sup>	39,167 <sup>Ba</sup>	48,235 <sup>Ab</sup>	0.495	0.0001	0.0001	0.0001

*Different capital letters in the same row indicate significant differences  $P < 0.0001$ ; Different lowercase letters in the same column indicate significant differences  $P < 0.0001$ .*

<sup>1</sup> CON = containing neither linseed nor olive cake; OC = containing 30% of stoned olive cake; EL = containing 15% of extruded whole linseed; OCEL = containing 10% of extruded whole linseed and 20% of stoned olive cake.

<sup>2</sup> F = Feeding

<sup>3</sup> T = Time

Furthermore, as can be seen, for all the diets to which the cattle were subjected, feeding during time (FxT) showed highly significant differences ( $P < 0.001$ ).

## 5. Discussion

The color of meat is primarily influenced by the oxidation state of myoglobin, which can exist in three forms: deoxymyoglobin (purple), oxymyoglobin (red), and metmyoglobin (brown). In the fresh state, oxymyoglobin is the dominant form and gives meat its desirable bright red color. However, lipid oxidation in meat leads to the formation of free radicals and ROS that accelerate the oxidation

of myoglobin to metmyoglobin, causing the meat to turn brown (Kerry et al., 2017).

The color of the meat in the diets containing olive cake (OC and OCEL) was preserved thanks to the antioxidant's ability to limit lipid oxidation, preventing the subsequent formation of reactive aldehydes and limiting iron-catalyzed lipid oxidation (Allen and Cornforth, 2010; Dal Bosco et al., 2012).

Antioxidants from olive cake can protect against the formation of metmyoglobin by neutralizing ROS and preventing myoglobin oxidation (Ponnampalam et al., 2022). Specifically, the phenolic compounds in olive cake, such as hydroxytyrosol, have been shown to scavenge free radicals and reduce the formation of lipid peroxides in meat (Martinez et al., 2018). This antioxidant activity helps maintain the red color of meat and extends its shelf-life by slowing down the oxidative processes that cause discoloration.

This study investigated the impact of incorporating an olive waste extract as a potential natural antioxidant rich in polyphenols (Muíño et al., 2013).

Lipid oxidation is one of the primary causes of deterioration in meat products. Oxidative rancidity leads to undesirable flavors, odors, and a decrease in nutritional quality due to the formation of harmful oxidation products such as aldehydes, ketones, and peroxides (Shahidi & Zhong, 2015). The incorporation of antioxidants into livestock diets represents a promising strategy for mitigating this challenge.

Antioxidants, such as those found in olive cake, can inhibit the oxidation of lipids in meat, thereby preserving its sensory and nutritional qualities (Luciano et al., 2013).

Olive cake is rich in phenolic compounds, particularly hydroxytyrosol, oleuropein, and other polyphenols, which have been shown to exert significant antioxidant effects. These compounds scavenge free radicals and reactive oxygen species (ROS), thus protecting lipids from oxidation (Vázquez-Araújo et al., 2013). When included in the diet of ruminants, these antioxidants are transferred into

the meat, where they act to stabilize fat content, particularly polyunsaturated fatty acids (PUFAs), which are highly prone to oxidative degradation.

The phenolic compounds present in the olive cake caused that the TBARS values remained lower in the OC and OCEL groups during all the days of storage (OC and OCEL), as also reported in the study by Branciari et al. (2015). The inclusion of olive cake in livestock diets has been associated with a reduction in lipid oxidation in meat products. Studies have demonstrated that meat from animals fed olive cake has lower levels of malondialdehyde (MDA), a primary marker of lipid oxidation, compared to meat from animals on conventional diets (Barriuso et al., 2012). This reduction in lipid oxidation not only improves the flavor and texture of the meat but also enhances its nutritional profile by preserving the integrity of PUFAs, which are important for human health.

Animals fed with linseeds in the EL group exhibited a greater susceptibility to lipid oxidation, as evidenced by lower redness and chroma values and higher TBARS levels, as also reported by the study of Conte et al. (2019), already on the third day of storage.

The experiment's findings showed that the antioxidants in olive cake, which is fed to cattle, could extend the shelf life of meat by preventing lipid oxidation and maintaining the meat's color stability.

## **6. Conclusions**

This study supports our thesis according to which the integration of stoned olive cake in the cattle diet, thanks to the presence of phenolic compounds, improves the shelf life of the meat.

The integration of olive cake in the diet of Podolian young bulls did not compromise production performance and resulted in an improvement in meat quality, particularly with regard to oxidative stability. This suggests that the use of by-products from the olive oil industry may represent an effective and sustainable strategy for the production of high-quality meat.

Olive cake is a good source of antioxidants, with positive effects on meat quality, but its inclusion in diets must be carefully balanced to avoid possible negative effects due to polyphenols. Linseed, on the other hand, has been shown to be an excellent source of essential fatty acids and fiber, with positive effects on intestinal health of animals.

The use of these two ingredients could, therefore, contribute not only to the improvement of livestock performance but also to the valorization of agricultural by-products, reducing waste and promoting more sustainable practices in animal feeding. However, to optimize the benefits, precise management of doses and careful evaluation of potential interactions between ingredients are essential.

Future research should focus on long-term studies to evaluate the combined effect of these ingredients on cattle health and productivity, as well as on their overall environmental impact, considering their potential reduction of greenhouse gas emissions through the replacement of traditional feed with more sustainable by-products.

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## **Chapter 4. Effect of high levels of almond hulls supplementation on performance and meat oxidative stability in lambs**

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## Effect of high levels of almond hulls supplementation on performance and meat oxidative stability in lambs

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### ABSTRACT

The main objective of this study was to evaluate if high dietary levels of almond hulls could influence performance and meat oxidative stability in lambs. Twenty lambs, at an average body weight of  $28.8 \pm 0.30$  (SD) kg, were divided into two experimental groups and fed *ad libitum* for 40 days either with a control diet (cereal-based concentrate, control group) or with a similar diet in which part of the cereals was replaced with 40% of almond hulls on a dry matter basis (AH40 group). Dietary AH did not affect dry matter intake but reduced final body weight, average daily gain, feed conversion ratio or carcass weight. In meat, TBARS values were lower ( $P < 0.001$ ) in the AH40 group than in control group. The partial replacement of cereals with 40% almond hulls in the diet negatively affecting the growth parameters of the animals but can improve meat shelf-life by reducing lipid oxidation.

### 1. Introduction

To date, the economic emergency and the increase of the cost of raw materials is putting many livestock farms in difficulty. In Italy, for the livestock sector, in the first quarter of 2022, farmer disbursements increased by 16.6% on an annual basis, registering a further boost after +6.4% in 2021, reflecting the price increases of farm animals (+ 9.8%) and feed (+ 21%) as well as energy products (+ 61.5%) (ISMEA, 2022). Therefore, the use of alternative feeds available at lower costs than conventional feeds has become essential for the sustenance of the sector (Ponnampalam & Holman, 2022).

Almond hulls (AH) are by-products from almond fruit available in large quantities in the market. In 2019, the almond industry produced 2 million tons of AH, and this amount is expected to increase in the coming years (Almond Board of California, 2019). Recent study (Scerra et al., 2022) has shown that the replacement of cereals with AH up to 30% in lamb diets improved meat oxidative stability without compromising the animals' growth performance, proving to be an interesting strategy to reduce use of cereals. AH are cheap by-products, low in protein but with a fair amount of nonfiber carbohydrates (DePeters, Swanson, Bill, Asmus, & Heguy, 2020). Furthermore, in almond hulls a high level of phenols such as phenolic acids and tannins has been observed (An et al.,

2020), compounds that have shown health-related properties, especially to their antioxidant activity (Li, Li, & Lin, 2018). Considering the interesting results obtained by Scerra et al. (2022), the main objective of the present trial was to investigate the effect of AH inclusion at 40% in lamb diets on growth performance and meat oxidative stability.

### 2. Materials and methods

#### 2.1. Animals and diets

The experimental trial was carried out from February to November 2022 and the Animal Welfare Committee (O.P.B.A) of the University of Reggio Calabria approved all procedures (prot. No. 8937).

Twenty entire male lambs (*Sarda* breed) were weaned at 60 days of age and then received a conventional concentrate-based diet composed of maize and barley (both at 30% on a DM basis), fava bean (20%), wheat bran (17%) and vitamin mineral premix (3%) for about three months. At an average body weight of  $28.8 \pm 0.30$  (SD) kg, lambs were penned individually and randomly divided into two groups ( $n = 10$ , control and AH40 groups) and, after an adaptation period (7 days) to the treatments, fed with their respective diet *ad libitum* for 40 days. Control group were fed with the same conventional concentrate-based given

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after weaning; whereas the AH40 group received the concentrate diet in which most of the barley and maize was replaced with 40% AH (AH40 group; 9% maize, 9% barley, 32% fava bean, 7% wheat bran, 40% AH, 3% vitamin mineral premix on a dry matter basis). Hay was provided *ad libitum* in a separate feeder. The diet of the AH40 group had a higher fava bean content to maintain a similar crude protein concentration between treatments (considering the low protein content of the AH). The chemical composition of the two experimental diets was (g/kg DM): DM 896 (g/Kg wet weight), crude protein 146, ether extract 28.2, ash 32.2, NDF 221, total phenolic, in g of tannic acid equivalent (TAe)/Kg DM, 4.63, total tannins (g TAe/Kg DM) 1.54,  $\alpha$ -Tocopherol ( $\mu\text{g/g DM}$ ) 54.6,  $\gamma$ -Tocopherol ( $\mu\text{g/g DM}$ ) 60.5,  $\delta$ -Tocopherol ( $\mu\text{g/g DM}$ ) 13.1, metabolizable energy (Mcal/kg DM) 2.37 for control diet; DM 891 (g/Kg wet weight), crude protein 139, ether extract 13.9, ash 59.4, NDF 309, total phenolic (g TAe/Kg DM) 28.1, total tannins (g TAe/Kg DM) 16.9,  $\alpha$ -Tocopherol ( $\mu\text{g/g DM}$ ) 34.4,  $\gamma$ -Tocopherol ( $\mu\text{g/g DM}$ ) 32.2,  $\delta$ -Tocopherol ( $\mu\text{g/g DM}$ ) 12.1, metabolizable energy (Mcal/kg DM) 2.07 for AH40 diet.

The experimental diets were provided twice daily (0730 and 1630 h) *ad libitum*, and refusals were weighted daily to measure voluntary intake. The lambs were weighed fasting every 10 days.

After 40 days of experimental trial, after fasting for 8 h, all animals were weighted, transported to a commercial abattoir (25 min from experimental farm) and immediately slaughtered (stunned by a captive bolt) according to EU welfare guidelines.

## 2.2. Analyses of feedstuffs

Subsamples of the feedstuffs were collected 4 times during the trial (at the beginning, after 10 and 20 days and at the end of experimental trial), and vacuum-stored at  $-30^\circ\text{C}$ . After grounding (1 mm), the subsamples were pooled to obtain one representative samples of each feedstuff. The representative samples of each feedstuff were analyzed for neutral detergent fibre (NDF) (Van Soest, Robertson, & Lewis, 1991), ether extract, crude protein and ash (AOAC, 1995; methods 920.39, 984.13 and 942.05, respectively). Total phenolic compounds and total tannins were analyzed as reported by Makkar, Blümmel, Borowy, and Becker (1993) while tocopherols were determined following the method reported by Rufino-Moya, Joy, Lobón, Bertolín, and Blanco (2020).

## 2.3. Meat quality analysis

Each carcass was immediately weighed and stored at  $4^\circ\text{C}$  for 24 h. From the left side of each carcass, a portion of *Longissimus thoracis et lumborum* (LTL) muscle were collected from the 13th rib, vacuum-packed and stored at  $-30^\circ\text{C}$ , pending fatty acid and vitamins analyses. Another portion of LTL muscle from each carcass was stored at  $4^\circ\text{C}$  and used for the analyses of lipid oxidation.

Intramuscular fat where lipids was extracted from 5 g of wet meat samples with chloroform/methanol 2:1 v:v (Folch, Lees, & Sloane Stanley, 1957), methylated adding 1 mL of hexane and 0.05 mL of 2 N methanolic KOH (IUPAC, 1987), containing C19:0 as an internal standard, and analyzed using a gas chromatograph ThermoQuest (Milan, Italy) equipped with a 100 m high-polar fused silica capillary column (5 mm i.d., 0.25  $\mu\text{m}$  film thickness; Supelco Inc., Bellefonte, PA). Gas-chromatography conditions and identification of FAME was performed as described by Natalello et al. (2019).

Muscle tocopherols were extracted and quantified using an UHPLC system as reported by Natalello et al. (2022).

Thiobarbituric acid reactive substances (TBARS) were determined to evaluate lipid oxidation in meat as reported by Luciano et al. (2017) on three slices of LTL (2 cm thick), one slice for each day of storage (0, 4 and 7 days), where the absorbance (532 nm) was measured using a double beam spectrophotometer (Shimadzu Corporation, Milan, Italy; model UV-1800).

**Table 1**

Growth performance and intakes of experimental lambs, vitamins and fatty acids of the meat.

	Control diet	AH40 diet	SEM <sup>10</sup>	P value
<i>Growth performance</i>				
Final BW <sup>1</sup> , kg	37.3	33.7	0.882	0.069
Carcass weight, kg	18	16	0.472	0.025
ADG <sup>2</sup> , g/d	207	127	16.70	0.050
FCR <sup>3</sup> , g	8.17	13.1	0.937	0.015
<i>Intake</i>				
total DMI <sup>4</sup> , g/d	1691	1657	35.16	0.391
Total phenols, g TAe <sup>5</sup> /d	7.83	46.5	3.63	<0.001
Total tannins, g TAe <sup>5</sup> /d	2.60	28.1	1.92	<0.001
$\alpha$ -Tocopherol, mg/kg DM	92.3	52.0	5.54	<0.01
<i>Tocopherols, <math>\mu\text{g/g}</math> muscle</i>				
$\alpha$ -Tocopherol	3.37	3.80	0.070	0.763
$\gamma$ -Tocopherol	0.81	0.70	0.011	0.210
<i>Fatty acids, mg/100 g muscle</i>				
SFA <sup>6</sup>	1177	1293	36.50	0.193
MUFA <sup>7</sup>	1064	1123	40.61	0.276
PUFA <sup>8</sup>	122	149	5.901	0.083
OBCFA <sup>9</sup>	31.1	41.7	1.192	0.091

<sup>1</sup>BW=Body weight; <sup>2</sup>ADG = average daily gain; <sup>3</sup>FCR = feed conversion ratio, DMI/ADG; <sup>4</sup>DMI = dry matter intake; <sup>5</sup>Tannic acid equivalent; <sup>6</sup>SFA = saturated fatty acids; <sup>7</sup>MUFA: monounsaturated fatty acids; <sup>8</sup>PUFA: polyunsaturated fatty acids; <sup>9</sup>OBCFA: odd and branched chain fatty acids; <sup>10</sup>SEM = standard error of means.

## 2.4. Statistical analysis

A one-way ANOVA was applied to analyzed the effect of the dietary treatments on animal performance, feed intake, tocopherols and intramuscular fatty acids, with the diet as fixed factor (the Minitab 14 software was used for all statistical analyses). A GLM procedure for repeated measures was applied to analyzed the data of oxidative stability, with diet, time of storage and their interaction as fixed factors, while individual animal was included as a random factor. Differences between means were assessed using Tukey's multiple-comparison test. Differences were considered significant at  $P \leq 0.05$ , whereas trends toward significance were considered when  $0.05 < P \leq 0.10$ .

## 3. Results and discussion

As shown in Table 1, the replacement of part of the maize and barley with AH did not affect dry matter intake (DMI) of the lambs, but negatively influenced feed conversion ratio (FCR,  $P < 0.05$ ), average daily gain (ADG,  $P < 0.05$ ), carcass weight ( $P < 0.05$ ) and consequently tententially negatively influenced final body weight ( $P = 0.069$ ). In several previous studies (Phillips, Doyle, Harl, Carpenter, & Aschenbrenner, 2015; Rad, Rouzbehan, & Rezaei, 2016; Scerra et al., 2022), the inclusion of AH in lamb diets did not affect these parameters. In the present experimental trial, the inclusion of the AH was brought at 40%, the highest inclusion than those tested in previous experimental trials. Although AH is an excellent source of highly fermentable carbohydrates (Offeman, Holtman, Covello, & Orts., W.J., 2014), at high inclusion rates the starch reduction due to lower dietary inclusion of maize and barley was probably not compensated. In fact, the partial replacement of corn and barley with 40% almond hulls in the diet led to a 13% reduction in the metabolizable energy in the AH40 diet (2.37 vs 2.07, for control and AH40 groups respectively), negatively affecting the growth performance of the animals. Furthermore, phenols intake, of which >60% are represented by tannins, from the lambs of AH40 group probably negatively influenced these parameters, considering that a

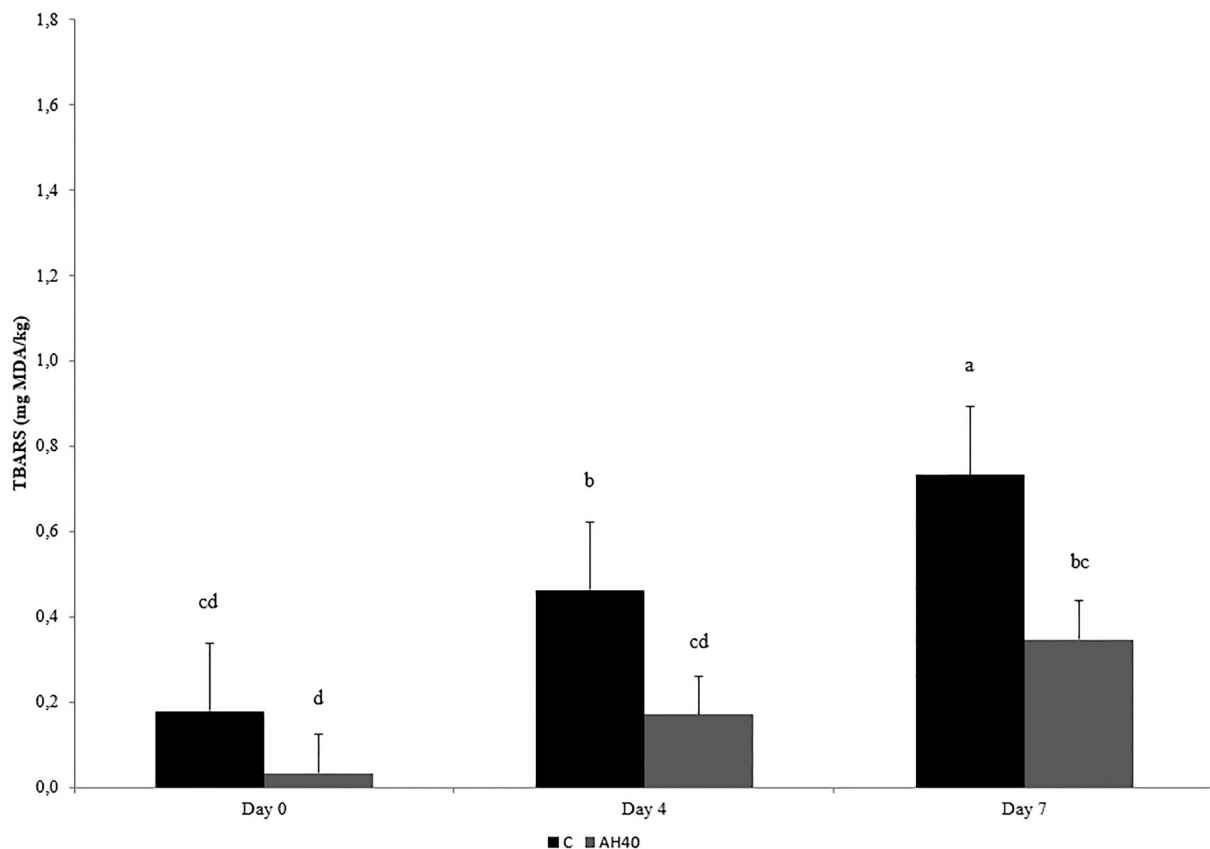


Fig. 1. Effect of the dietary treatment and time of storage on the oxidative stability of raw meat.

Interactive effect of the dietary treatment and time of storage on the TBARS values measured in raw meat slices over aerobic storage at 4 °C. Values presented are the estimated least squares means and standard error bars. <sup>a,b,c,d</sup> Values with different superscripts are significantly different ( $P \leq 0.05$ ).

high level of these compounds in the diets could lead to a slowdown in the animals' growth rates (Priolo, Waghorn, Lanza, Biondi and Pennisi, 2000). In a study of Vasta et al. (2019), it is pointed out that tannins show antimicrobial activity and protein-binding ability, make them able to affect rumen digestion and reducing biohydrogenation activity. In an *in vitro* study, Durmic et al. (2014) highlighted a reduction in methane and  $\text{NH}_3$  production when AH were incubated with sheep rumen fluid microbes. In this trial, lambs from AH40 group daily ingested an amount of phenols and tannins respectively 6 and 11 times higher ( $P < 0.001$ ) than that from control lambs. However, considering the lower cost of the diet of the AH40 group (data not shown, 198 vs 320 €/t for AH40 and control groups, respectively), the cost per 100 g of body weight gain was equivalent.

The inclusion of AH in the diet had significant effects on lipids oxidation measured in raw meat during refrigerated storage (Fig. 1). In fact, the meat from animals fed with the AH40 diet showed clearly lower ( $P < 0.001$ ) TBARS values than the meat from lambs of the control group. During the monitoring period, TBARS values increased ( $P < 0.001$ ) and a significant diet  $\times$  time interaction was observed ( $P < 0.05$ ). In particular, compared to day 0, while the TBARS values increased already after 4 days in the raw meat of the lambs from control group ( $P < 0.01$ ), the lipid oxidation was evident only after 7 days of storage in the meat of lambs whose diet was supplemented with 40% AH ( $P < 0.01$ ). Moreover, the TBARS values in raw meat after 7 days of storage were markedly lower in the AH40 group than in the control one ( $P < 0.001$ ).

Different authors reported that the increase of antioxidants compounds, such as vitamin E, is associated with improved antioxidant stability (Luciano et al., 2017), while a higher PUFA deposition makes meat more susceptible to lipid oxidation (Moloney, Kennedy, Noci,

Monahan, & Kerry, 2012).

In lamb meat, vitamin E is considered one of the compounds with the greatest antioxidant capacity (Luciano et al., 2017). In the present study, although the vitamin E level was lower in the diet received from lambs of AH40 group and although the level of PUFA in meat from AH40 group tended to be higher ( $P = 0.083$ ) than in meat from the control group, we observed a greater oxidative stability in meat from this group. These results are in agreement with those observed in our previous study (Scerra et al., 2022), where the lambs were fed a diet containing 30% AH. However, in that experimental trial the differences in TBARS values between the groups were observed only after 7 days of storage. Despite the higher vitamin E level in the control diet, no differences were observed in meat for the vitamin E levels between treatments. The latter data could be influenced by phenolic compounds present in the AH, which, due to their antioxidant activity, could have indirectly preserved vitamin E during digestion, allowing the animal to absorb a greater amount of it (López-Andrés et al., 2013). Furthermore, some authors suggest that dietary phenolic compounds could directly contribute to the antioxidant capacity of meat through their metabolites absorbed in the intestine (Soldado, Bessa and Jerónimo, 2021).

#### 4. Conclusions

The results of the present study show that the inclusion of 40% almond hulls in the diet negatively affects feed conversion ratio, average daily gain, carcass weight and final body weight in lambs. However, the inclusion of high percentage of this by-product can improve meat shelf-life by reducing lipid oxidation. This is probably influenced by antioxidant molecules contained in AH, primarily phenolic compounds.

## Author contribution statement

M. Scerra: Conceptualization, Writing-Original draft, Final approval of the version to be published; M. Bognanno: Methodology, Writing-Review & Editing, Final approval of the version to be published; F. Foti: Writing-Review & Editing, Final approval of the version to be published; P. Caparra: Writing-Review & Editing, Final approval of the version to be published; C. Cilione: Formal analysis, Writing-Review & Editing, Final approval of the version to be published; P. De Caria: Formal analysis, Writing-Review & Editing, Final approval of the version to be published; P. Fortugno: Formal analysis, Writing-Review & Editing, Final approval of the version to be published; G. Luciano: Formal analysis, Writing-Review & Editing, Final approval of the version to be published; A. Natalello: Formal analysis, Writing-Original draft, Final approval of the version to be published; L. Chies: Writing-Review & Editing, Final approval of the version to be published.

## Declaration of Competing Interest

There are no conflicts of interest.

## Data availability

Data will be made available on request.

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## Chapter 5. Conclusions and Future Perspectives

The present study rigorously explored the use of agricultural and industrial by-products in the diet of Podolian young bulls, with the aim of evaluating the benefits and potential of these alternative feeds in the animal diet. The results obtained confirm that the use of by-products, if properly managed, can represent a valid strategy to improve the sustainability of livestock production, reducing feed costs and contributing to the valorization of otherwise unused resources. In particular, it emerged that some types of by-products, such as food chain residues and agro-industrial by-products, are able to provide a significant nutritional contribution without compromising animal health and the quality of the final product. However, the effectiveness of these by-products depends on a number of factors, including their nutritional composition, treatment method and integration with other feed sources.

Although ruminant farms are essential for food production and for the maintenance of agricultural traditions, they are also a major source of environmental pollution.

Greenhouse gas emissions, water and soil pollution, and biodiversity loss are some of the most serious issues related to these production systems.

However, there are technical and management solutions that can help significantly reduce the environmental impact of livestock farming, promoting a transition towards more sustainable livestock farming systems.

Public policies and technological innovation are essential to ensure that ruminant production takes place in a way that respects the environment and natural resources, with the aim of ensuring a healthier and more sustainable future for the planet.

The use of by-products in animal feed is confirmed as a promising practice, with positive impacts on both livestock productivity and the environment. Scientific evidence supports the possibility of using these by-products as a strategic resource to improve the sustainability of livestock supply chains.

Ruminant feeding is one of the crucial aspects in the management of modern farms, as it directly affects animal health, productivity and, consequently, the economic and environmental sustainability of agricultural activities, supporting the principles of circular economy aimed at improving the resilience of agricultural and livestock practices.

In recent decades, the agri-food industry has generated a large amount of by-products, many of which have become an important resource in animal feed. The growing need to reduce production costs, combined with the growing attention to environmental sustainability, has led to considering these by-products as valid and promising alternatives for feeding ruminants.

By-products are resources that, if not valorized, can contribute to significant waste and increase the environmental impact of agri-food chains.

Specifically, the use of olive cake and linseeds in the feed of Podolian cattle presents itself as a promising solution to improve the quality of derived products, increase production efficiency and promote sustainable agricultural practices.

Scientific evidence supports the feasibility of this practice, but further studies are needed to optimize the use of these resources and to explore their potential in different farming conditions.

In view of the positive results obtained, the use of olive cake and linseed should be further explored and standardized to optimize doses and methods of administration, in order to maximize economic and productive benefits. In particular, it is desirable that more in-depth studies are conducted on the interaction between these ingredients and other feed additives, as well as on their long-term impact on animal health and the quality of final products.

It would also be useful to monitor the compatibility of these ingredients in different environmental and production conditions, especially in different types of farming, to ensure that the positive results are applicable on a larger scale.

The use of olive cake and linseed as ingredients in the diet of Podolian cattle also presents significant advantages in terms of environmental sustainability, as it reduces the ecological footprint of breeding, contributing to the reduction of

methane emissions and other pollutants, thanks to the improved digestibility of diets and the improvement of feeding practices.

Together with this, the importance of Podolian cattle for the sustainable management of pastures and mountain and hilly territories could be reassessed, as they not only contribute to the maintenance of the landscape and biodiversity, but also represent an important economic resource thanks to the quality of the meat and dairy products they produce. Their protection and valorization are essential to support rural agriculture, promote ecological practices and preserve a cultural tradition that has its roots in the centuries. The protection of Podolian cattle is not only a question of conserving the breed, but also of promoting a more sustainable agricultural model in harmony with the environment.

In conclusion, the study of olive cake combined with linseeds highlights the importance of a multidisciplinary and integrated approach for their use in the feeding of Podolian cattle, which considers the economic, ecological and nutritional needs of farms.

Only through a conscious and innovative management of these by-products will it be possible to achieve the objectives of sustainability and improvement of livestock performance in a context increasingly oriented towards circularity and waste reduction.

## ADDITIONAL ARTICLE

### Appendix 1. Effect of Dietary Ensiled Olive Cake Supplementation on Performance and Meat Quality of Apulo-Calabrese Pigs

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## Article

# Effect of Dietary Ensiled Olive Cake Supplementation on Performance and Meat Quality of Apulo-Calabrese Pigs

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**Simple Summary:** A valid strategy for increasing the sustainability of food production systems is the use of industrial by-products in livestock diets, which allows food processing industries to profitably mitigate the costs generated by waste disposal. The agro-industrial olive sector produces large quantities of olive by-products, considered extremely toxic, with a highly adverse environmental impact. Thus, to valorise a by-product of the oil industry, the aim of this study was to evaluate the inclusion of different levels of ensiled olive cake in the fattening diets of Apulo-Calabrese pigs as a strategy to partially replace the conventional cereal-based diet and improve animal performance and meat fatty acid profile. The results showed that the inclusion of ensiled olive cake in the diet improves the fatty acid composition of meat, improving health by increasing the oleic acid level (C8:1n-9,  $p < 0.001$ ), decreasing the linoleic acid level (C18:2n-6,  $p < 0.001$ ) and altering the polyunsaturated/saturated ratio (P/S,  $p < 0.001$ ). In conclusion, ensiled olive cake is suitable for dietary supplementation at up to 40% for fattening pigs and could represent a valid strategy for naturally improving the nutritional value of meat and valorising a by-product of the olive industry.

**Abstract:** The aim of this study was to evaluate the inclusion of different amounts of ensiled olive cake, a major pollutant from olive oil production, in the fattening diets of 30 Apulo-Calabrese pigs as a strategy to partially substitute the traditional cereal-based diet and improve animal performance and meat fatty acid composition. The animals, during a fattening period of 120 days, were fed with three dietary treatments containing increasing levels of ensiled olive cake: 0% (C), 20% (OC20) and 40% (OC40) on dry matter. No effect of the dietary treatment was found on the animal performance and proximate meat analysis results. The inclusion of ensiled olive cake in the diet led to differences in the fatty acid (FA) profile of intramuscular fat, with a higher proportion of monounsaturated fatty acid (MUFA;  $p < 0.001$ ) and oleic acid (C8:1n-9,  $p < 0.001$ ) and a lower concentration of polyunsaturated (PUFA,  $p < 0.001$ ) and linoleic acid (C18:2n-6,  $p < 0.001$ ). In conclusion, the supplementation of ensiled olive cake at up to 40% in the diets of fattening pigs could represent a useful strategy in Mediterranean areas to naturally improve the nutritional value of meat and valorise a by-product of the olive industry, reducing its environmental impact and promoting the exploitation of this local feed resource according to the principles of the circular economy.

**Keywords:** circular economy; olive by-product; fatty acid composition; silage olive cake; fattening pig



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## 1. Introduction

Recently, due to the global intensification of food production, which continues to generate considerable quantities of co-products and food waste, there has been consid-

erable social and environmental pressure for the effective re-use of agricultural industry residues. A valid strategy to increase the sustainability of food production systems is the use of industrial by-products in livestock diets, which allows food processing industries to profitably mitigate the costs generated by waste disposal [1].

Furthermore, in accordance with a circular economy, one of the key challenges to preserving natural resources and ensuring environmental sustainability is the use of agro-industrial by-products as replacements for traditional feedstuffs or to improve animal products [2].

The inclusion of agricultural by-products in animal nutrition could have significant economic benefits by reducing the costs of animal feeding and improving the chemical, physical and sensory characteristics of animal products [3,4] due to their high content of bioactive secondary compounds. In Italy, 1,156,344 hectares of land are used for the cultivation of olive trees, with an average annual production of 338 thousand tons of oil, of which about 70% is produced in Southern Italy [5]. The agro-industrial olive sector produces, in addition to olives and olive oil, large quantities of olive by-products. These by-products are considered extremely toxic, with a high adverse environmental impact.

The chemical properties of olive cake are affected by the type of olive, the amounts of its constituents (skin, pulp and stone) and the oil extraction process [6]. The high levels of residual oil and oleic acid in olive cake make it suitable for use in animal feed. Positive results have been obtained in pigs [7,8], cattle [9], small ruminants [10,11], broilers [12] and rabbits [13]. Incorporating olive cake into the pig diet may improve their growth performance, reduce carcass fat thickness [14] or provide more energy to ruminants [15]. The high lignin content associated with the presence of stone, on the other hand, may limit its use in piglets unless it is completely removed [16].

In finishing pigs, many authors [8,17,18] found that increasing the level of dietary olive cake supplementation results in a decrease in saturated fatty acid (SFA) and an increase in monosaturated fatty acid (MUFA), with an improved meat fatty acid composition in relation to human health. Furthermore, supplementing the diet of finishing pigs with partially defatted olive cake (120 g/kg) results in an increase in MUFA concentration in meat without affecting the growth performance, carcass quality or microbial counts [14].

Furthermore, olive by-products are available seasonally, and their use as animal feed throughout the year necessitates preservation and storage. The main issue with preserving olive cake is that it contains a high percentage of water and oil, and long-term storage may result in mould formation and nutrient losses [19]. The silage preservation method is a simple and effective way to preserve olive cake while increasing its nutritional value [20]. Thus, to valorise a by-product of the oil industry, the aim of this study was to evaluate the inclusion of different levels of ensiled olive cake in the fattening diets of Apulo-Calabrese pigs as a strategy to partially replace the conventional cereal-based diet and improve animal performance and meat fatty acid profile.

## 2. Materials and Methods

### 2.1. Ethical Statement

In this trial, the handling and experimental procedures were carried out according to international guidelines [21] and approved by the Animal Welfare Committee of the Mediterranean University of Reggio Calabria (Prot. No. 3015).

### 2.2. Diets, Animals and Experimental Design

The present trial was conducted on a private farm located in the Calabria region (Southern Italy), mainly focused on the production of heavy Apulo-Calabrese pigs. The Apulo-Calabrese pig, also known as the Nero Calabrese, is an Italian autochthonous breed from the Calabria region, reared traditionally in a free-range system that is based on natural woodland resources (acorns, chestnuts, berries, tubers, roots and pasture).

However, due to the seasonality of natural woodland products (which are only abundant in autumn and winter), the traditional pig-rearing system has some limitations. Thus,

both the maintenance of traditional outdoor systems and the development of semi-intensive farming have been suggested to improve the breed's profitability [22].

The experimental trials were carried out on a private farm whose traditional free-range rearing system for Apulo-Calabrian pigs includes a fattening period based on concentrate feeds complemented with free-range rearing.

The study was performed, during the fattening period (120 days), on 30 castrated male pigs of the Apulo-Calabrese breed that were randomly allotted to three groups in accordance with dietary treatments and balanced to their age and body weight (average initial body weight of  $84.83 \pm 3.52$  kg).

In the daytime, all pigs were kept outdoors for about six to seven hours in a fenced wooded area of approximately two hectares and fed on natural woodland products (acorns, chestnuts, berries, tubers, roots and pasture). The fenced wooded area of grazing was divided into two paddocks (one hectare each) so that animals grazed rotationally. In the evening, when the animals returned from the pasture, they were placed in individual 4 m<sup>2</sup> cages and fed with the experimental dietary treatments for 120 days of fattening.

The pigs were fed ad libitum (with an average consumption of 3.0 kg per head per day on fresh weight) with three dietary treatments (Table 1) formulated to contain: only concentrate (C; 10 pigs), concentrate with the inclusion of olive cake silage at 20% of the dry matter weight (OC20; 10 pigs) and concentrate with the inclusion of olive cake silage at 40% of the dry matter weight (OC40; 10 pigs). The control diet was formulated using conventional ingredients (mainly barley and maize grains and wheat bran) locally produced and traditionally employed in the Calabria region for pig feeding. Furthermore, to preserve the natural quality attributes of this environmentally sustainable pig-farming system, which is highly appreciated by consumers, for our research, we choose to source organic olive cake from olive farms with organic certifications. The diets containing ensiled olive cake (OC20 and OC40) were fed in the form of total mixed rations. All diets were designed to be isoproteic and isoenergetic (Table 1). Every day, before the addition of fresh feed, the amounts of feed offered and refused were recorded and the dry matter content was determined to calculate individual voluntary feed intakes (DMI). From the start to the end of the experiment, pigs were weighed every 15 days to determine the average daily gain (ADG) and feed conversion ratio (FCR).

**Table 1.** Ingredients (g/100 g of DM) and chemical composition of diets offered.

	Diet <sup>1</sup>		
	C	OC20	OC40
Barley grain	29.5	26.5	16.5
Maize grain	29.5	26.5	16.5
Wheat bran	20.0	-	-
Faba bean	20.0	26.0	26.0
Olive cake silage	-	20.0	40.0
Vitamin-mineral premix <sup>2</sup>	1.0	1.0	1.0
Chemical composition			
Dry matter (DM), g/kg wet weight	901	810	713
Crude protein, g/kg DM	162	154	155
Ether extract, g/kg DM	27	40	54
Ash, g/kg DM	32	34	37
NDF, g/kg DM	255	270	341
ADF, g/kg DM	94	151	218
ADL, g/kg DM	15	33	56
Gross energy, MJ/kg DM	18.76	18.91	18.89

Table 1. Cont.

	Diet <sup>1</sup>		
	C	OC20	OC40
Fatty acids (g/100 g of fatty acids)			
C16:0	15.92	14.49	13.69
C18:0	2.02	2.09	2.23
C18:1 n-9	20.72	31.75	42.25
C18:2 n-6	56.37	47.45	37.48
C18:3 n-3	3.75	3.19	2.52
Others	1.22	1.03	1.83

<sup>1</sup> C = Control diet; OC20 = diet with silage olive cake at 20% of dry matter; OC40 = diet with silage olive cake at 40% of dry matter. <sup>2</sup> Provided the following quantities per kilogram of diet: vitamin A, 6750 IU; vitamin D3, 1000 IU; vitamin E, 2.0 mg; vitamin B12, 0.01 mg; vitamin B1, 1.0 mg; folic acid 0.2 mg; D-pantothenic acid, 5.0 mg; cobalt, 0.05 mg; manganese, 12.5 mg; zinc, 15.0 mg; molybdenum 0.5 mg.

The fresh olive cake was obtained from a two-phase continuous centrifugation system for olive oil extraction (Pieralisi MAIP Group S.p.A., Jesi, Italy). After the oil extraction, the virgin olive cake was stoned, immediately accumulated and well compacted in the silo, which was covered with a black polyethylene film and firmly closed. The olive cake was kept in the silo for about three months before use and stored during the experimental trials. No additives were added to olive cake silages.

### 2.3. Chemical Analyses of Experimental Diets

The diet samples, relative to the dietary treatments, were collected daily and pooled weekly, followed by storage at  $-20\text{ }^{\circ}\text{C}$  until further analysis. Table 1 shows the chemical composition of experimental diets. The dry matter (DM), ether extract (EE) and ash contents of diets were performed according to AOAC [23] procedures. Fibre fractions, including NDF, ADF and ADL, were measured according to the method described by Van Soest et al. [24]. The method of Gray et al. [25] was used to analyse the fatty acid composition of dietary treatments.

### 2.4. Slaughter, Carcass Characteristics and Meat Sampling

All pigs were slaughtered on the same day at a commercial slaughterhouse with an average weight of  $145.24 \pm 7.75$  kg. All animals were treated and slaughtered according to the welfare regulations and the EU Council Regulation [26]. Following slaughter, the carcasses were moved to a  $4\text{ }^{\circ}\text{C}$  cooling chamber for 24 h.

Successively, the carcass weight was recorded, and the dressing percentage expressed was calculated. A section of 300 g ( $\pm 30$  g) of loin (*Longissimus thoracis* muscle, LT) was excised at the level of 10th/14th thoracic vertebrae from each carcass and was vacuum sealed immediately and refrigerated during transport to the laboratory. Then, 200 g of each muscle was vacuum sealed and used after 24 h of storage at  $\pm 4\text{ }^{\circ}\text{C}$  for the measurement of the physical and chemical characteristics. For fatty acid analysis, 100 g of each sample was vacuum packed and stored at  $-20\text{ }^{\circ}\text{C}$  until subsequent analysis.

### 2.5. Colour, Physical and Proximate Analyses for Meat Samples

The colour parameters were measured after blooming (30 min), using a MINOLTA CR300 spectrophotometer with D65 illuminance, with an 11 mm aperture and  $10^{\circ}$  observer angle. The parameters  $L^*$ ,  $a^*$  and  $b^*$ , representing lightness, redness and yellowness, were measured four times on the cut surface and averaged.

The pH was measured at 48 h post-mortem on LT with a portable pH meter (Hanna HI982) equipped with automatic temperature compensation. The pH was measured using calibrated pH standard buffers at pH 4.0 and pH 7.0.

The dry matter and proximate composition of meat samples were determined in duplicate using AOAC standard procedures [23].

The protein determination was performed using the Kjeldahl method, multiplying the nitrogen content by 6.25. The fat content was extracted by Soxhlet apparatus on meat samples hydrolysed by HCl 3N. The moisture was calculated on 10–15 g pieces of the meat samples, which were dried in the oven at C 105 °C overnight. The muffle furnace at 550 ± 1 °C was used for the determination of the ash content.

### 2.6. Fatty Acids Analysis

The lipids were extracted, in duplicate, from 5 g of meat samples according to the procedure of Folch et al. [27]. Aliquots of 100 mg of lipid, with nonanoic acid methyl ester (20 mg/mL; standard no. 245895, Sigma-Aldrich, St. Louis, MO, USA) as the internal standard, were methylated by adding 1 mL of hexane and 0.05 mL of 2 N methanolic potassium hydroxide [28].

Gas chromatography analysis was performed using a GC 6890N (Agilent, Inc., Santa Clara, CA, USA) instrument connected to a 60 m-length fused silica capillary column coated with 100% cyanopropyl polysiloxane (DB 23, Aligent J&W, Santa Clara, CA, USA) and with an internal diameter of 0.25 mm and film thickness of 0.25 µm. The operating conditions were as follows: a helium flow rate of 1.2 mL/min, a flame ionisation detector at 250 °C, a split–splitless injector at 230 °C with a split ratio of 1:100, and an injection volume of 1 µL. The temperature program of the column was: 5 min at 60 °C increased at a rate of 14 °C/min to 165 °C. A subsequent increase of 2 °C/min led to a final temperature of 225 °C for 20 min [29]. To identify the individual fatty acids, mixtures of standard fatty acids (Supelco FAME mix37 47885U, Sigma-Aldrich, St Louis, MO, USA) were used. Fatty acids were expressed as mg/100 gr of muscle. The atherogenic index (AI) and thrombogenic index (TI) were determined in accordance with Ulbricht and Southgate [30], while the hypocholesterolemic/hypercholesterolemic ratio was calculated as reported by Bialek et al. [31].

### 2.7. Statistical Analysis

The Sas procedure [32] was performed for data analysis, using the dietary treatments as factors in ANOVA analysis. The mean values were compared by Fisher's LSD test, and the value of  $p < 0.05$  was considered as the significant difference level. To determine the relationship between the different variables and detect the most important factors of variability, standard PCA was applied to the fatty acid composition of meat samples.

In the PCA model, only the fatty acids showing significant differences ( $p < 0.05$ ) were considered to be variables.

## 3. Results

### 3.1. Animal Performance and Meat Quality Measurements

As shown in Table 2, no effect of the dietary treatment was found on the final body weight, dry matter intake (DMI), feed conversion ratio (FCR), average daily gain (ADG) and carcass weight and dressing percentage. As for proximate meat analyses, there were no significant differences between treatments for crude protein, moisture, ether extract, ash, pH and colour parameters such as the luminosity index ( $L^*$ ), red index ( $a^*$ ) and yellow index ( $b^*$ ) (Table 3).

**Table 2.** Pig performances in vivo and data at slaughter.

	Diets <sup>1</sup>			S.E.M. <sup>5</sup>	p-Value
	C	OC20	OC40		
Number of pigs	10	10	10		
Final body weight, kg	145.10	146.33	144.08	5.781	0.945
ADG <sup>2</sup> (g/d)	412.50	421.50	402.83	9.171	0.758
Total DMI <sup>3</sup> , kg/d	3.35	3.07	2.98	0.203	0.546
FCR <sup>4</sup> , g DMI/g ADG	8.12	7.52	7.40	0.567	0.439
Carcass weight, kg	120.08	121.83	119.16	8.330	0.751
Dressing percentage, %	82.68	83.24	82.71	1.021	0.683

<sup>1</sup> C = Control diet, OC20 = diet with silage olive cake at 20% of dry matter, OC40 = diet with silage olive cake at 40% of dry matter; <sup>2</sup> ADG = average daily gain; <sup>3</sup> DMI = dry matter intake; <sup>4</sup> FCR = feed conversion ratio; <sup>5</sup> S.E.M. = standard error of the means.

**Table 3.** Effect of the dietary treatment on chemical composition (g/100 g of muscle), pH and colour of *Longissimus thoracis* muscle.

	Diets <sup>1</sup>			S.E.M. <sup>2</sup>	p-Value
	C	OC20	OC40		
Moisture	74.083	74.013	73.199	0.387	0.232
Protein	20.663	21.149	20.928	0.223	0.334
Fat	1.860	1.905	2.239	0.407	0.774
pH <sub>48</sub> <sup>3</sup>	5.766	5.800	5.850	0.066	0.682
Lightness (L*)	49.316	49.203	48.277	1.864	0.967
Redness (a*)	3.905	3.965	6.081	0.868	0.164
Yellowness (b*)	5.854	6.198	7.249	0.715	0.296

<sup>1</sup> C = Control diet, OC20 = diet with silage olive cake at 20% of dry matter, OC40 = diet with silage olive cake at 40% of dry matter; <sup>2</sup> S.E.M. = standard error of the least square means; <sup>3</sup> pH<sub>48</sub> = pH at 48 h after slaughter.

### 3.2. Fatty Acid Composition

The effects of dietary treatment on the individual FA are reported in Table 4. The total of saturated fatty acids (SFA) was not different between groups. Within the SFA class, the dietary treatment with 20% and 40% olive cake increased the levels of C12:0 ( $p < 0.001$ ), C14:0 ( $p < 0.001$ ) and C21:0 ( $p < 0.05$ ) compared to the control group. Additionally, the C15:0 content ( $p < 0.05$ ) decreased in meat samples from OC40 and OC20 treatments compared to the C group.

The total monounsaturated fatty acid (MUFA) was enhanced by olive cake inclusion ( $p < 0.001$ ). In particular, the level of oleic acid (C18:1n-9) increased in the muscle of pigs fed with the silage olive cake inclusion ( $p < 0.001$ ), although no difference was observed as the level of inclusion of silage olive cake in the diet increased from 20% to 40% (dry matter).

In relation to the monounsaturated fatty acids, the level of C16:1 n-9 ( $p < 0.001$ ) fatty acid increased in OC20 and OC40 meat samples compared to those of C group; conversely, a higher proportion of C14:1 n-9 ( $p < 0.01$ ) was observed in meat samples from C group compared to meat samples from OC20 and OC40 groups.

Feeding the ensiled olive cake diet decreased the total PUFA levels. Consequently, the polyunsaturated/saturated ratio (P/S) significantly changed ( $p < 0.001$  and  $p < 0.01$ , respectively), although no differences were observed between OC20 and OC40 groups.

The levels of linoleic acid (C18:2n-6;  $p < 0.001$ ) and  $\gamma$ -linolenic acid (C18:3n-6;  $p < 0.01$ ) decreased in the OC20 and OC40 samples compared to those of the C group.

A higher proportion of C20:3n-6 ( $p < 0.001$ ), C20:3n-3 ( $p < 0.001$ ), C20:4n-6 ( $p < 0.001$ ), C20:5n-3 ( $p < 0.001$ ) and C22:6n-3 ( $p < 0.001$ ) was noticed in muscles from C treatment compared to the OC20 and OC40 ones. Conversely, the amount of C20:2n-6 increased ( $p < 0.01$ ) in the OC40 group more than in other groups.

**Table 4.** Effect of the dietary treatment on fatty acid composition (mg/100 g of meat) of *Longissimus thoracis* muscle.

	Diets <sup>1</sup>			S.E.M. <sup>2</sup>	p-Value
	C	OC20	OC40		
C10:0	1.230	1.527	1.776	0.601	0.068
C12:0	1.019 <sup>B</sup>	1.281 <sup>A</sup>	1.325 <sup>A</sup>	0.057	0.0008
C14:0	17.153 <sup>B</sup>	23.007 <sup>A</sup>	23.908 <sup>A</sup>	0.877	0.0001
C14:1n-9	0.509 <sup>d</sup>	0.315 <sup>e</sup>	0.282 <sup>e</sup>	0.050	0.012
C15:0	1.254 <sup>a</sup>	1.004 <sup>b</sup>	0.833 <sup>b</sup>	0.092	0.019
C16:0	400.47	427.16	427.35	17.52	0.562
C16:1 t	5.372	6.021	5.564	0.363	0.304
C16:1n-9	57.987 <sup>B</sup>	72.937 <sup>A</sup>	72.027 <sup>A</sup>	2.260	0.0004
C17:0	5.423	4.877	4.382	0.482	0.272
C17:1	4.136	4.493	3.702	0.536	0.579
C18:0	207.96	203.21	195.622	8.958	0.238
C18:1t11	3.288	3.167	3.863	0.497	0.466
C18:1 n-9	682.355 <sup>B</sup>	844.561 <sup>A</sup>	921.361 <sup>A</sup>	23.283	0.0001
C18:1 n-7	75.122	82.937	85.988	2.816	0.057
C18:2t9t12	2.798 <sup>b</sup>	3.363 <sup>ab</sup>	3.501 <sup>a</sup>	0.191	0.047
C18:2 n-6	349.686 <sup>A</sup>	244.785 <sup>B</sup>	209.244 <sup>B</sup>	14.587	0.0001
C20:0	1.819	2.212	2.351	0.165	0.097
C18:3n-6	2.144 <sup>d</sup>	1.454 <sup>e</sup>	1.202 <sup>e</sup>	0.167	0.003
C20:1	1.380	1.321	1.365	0.194	0.869
C18:3 n-3	12.521	12,046	12.533	0.654	0.795
C21:0	4.347 <sup>b</sup>	5.413 <sup>ab</sup>	6.627 <sup>a</sup>	0.580	0.044
C20:2 n-6	13.847 <sup>e</sup>	17.960 <sup>de</sup>	22.045 <sup>d</sup>	1.422	0.004
C22:0	1.833	1.855	1.892	0.291	0.913
C20:3 n-6	13.162 <sup>A</sup>	9.927 <sup>B</sup>	9.653 <sup>B</sup>	0.479	0.0002
C20:3 n-3	13.949 <sup>A</sup>	7.960 <sup>B</sup>	6.809 <sup>B</sup>	1.177	0.0013
C20:4 n-6	132.431 <sup>A</sup>	61.705 <sup>B</sup>	41.953 <sup>B</sup>	13.041	0.0005
C20:5 n-3	6.197 <sup>A</sup>	4.649 <sup>B</sup>	4.526 <sup>B</sup>	0.253	0.0004
C22:2 n-6	2.094	1.261	2.145	0.699	0.614
C22:4 n-6	16.075	6.528	5.342	3.538	0.063
C22:5 n-3	18.682	14.282	8.658	3.012	0.093
C22:6 n-3	12.994 <sup>A</sup>	6.624 <sup>B</sup>	4.654 <sup>B</sup>	1.314	0.0011
n-3 <sup>3</sup>	63.293 <sup>d</sup>	45.561 <sup>e</sup>	37.183 <sup>e</sup>	4.368	0.0019
n-6 <sup>4</sup>	516.592 <sup>A</sup>	325.662 <sup>B</sup>	269.539 <sup>B</sup>	27.928	0.0001
n-6/n-3	8.161	7.147	7.249	0.388	0.309
SFA <sup>5</sup>	642.508	671.547	666.066	25.662	0.842
MUFA <sup>6</sup>	830.149 <sup>B</sup>	1015.552 <sup>A</sup>	1094.152 <sup>A</sup>	25.261	0.0001
PUFA <sup>7</sup>	596.530 <sup>A</sup>	392.544 <sup>B</sup>	332.265 <sup>B</sup>	30.609	0.0001
P/S <sup>8</sup>	0.928 <sup>d</sup>	0.584 <sup>e</sup>	0.499 <sup>e</sup>	0.079	0.0031
AI <sup>9</sup>	0.333	0.375	0.374	0.019	0.263
TI <sup>10</sup>	0.746	0.826	0.826	0.047	0.409
h/H <sup>11</sup>	3.163	2.864	2.892	0.209	0.646

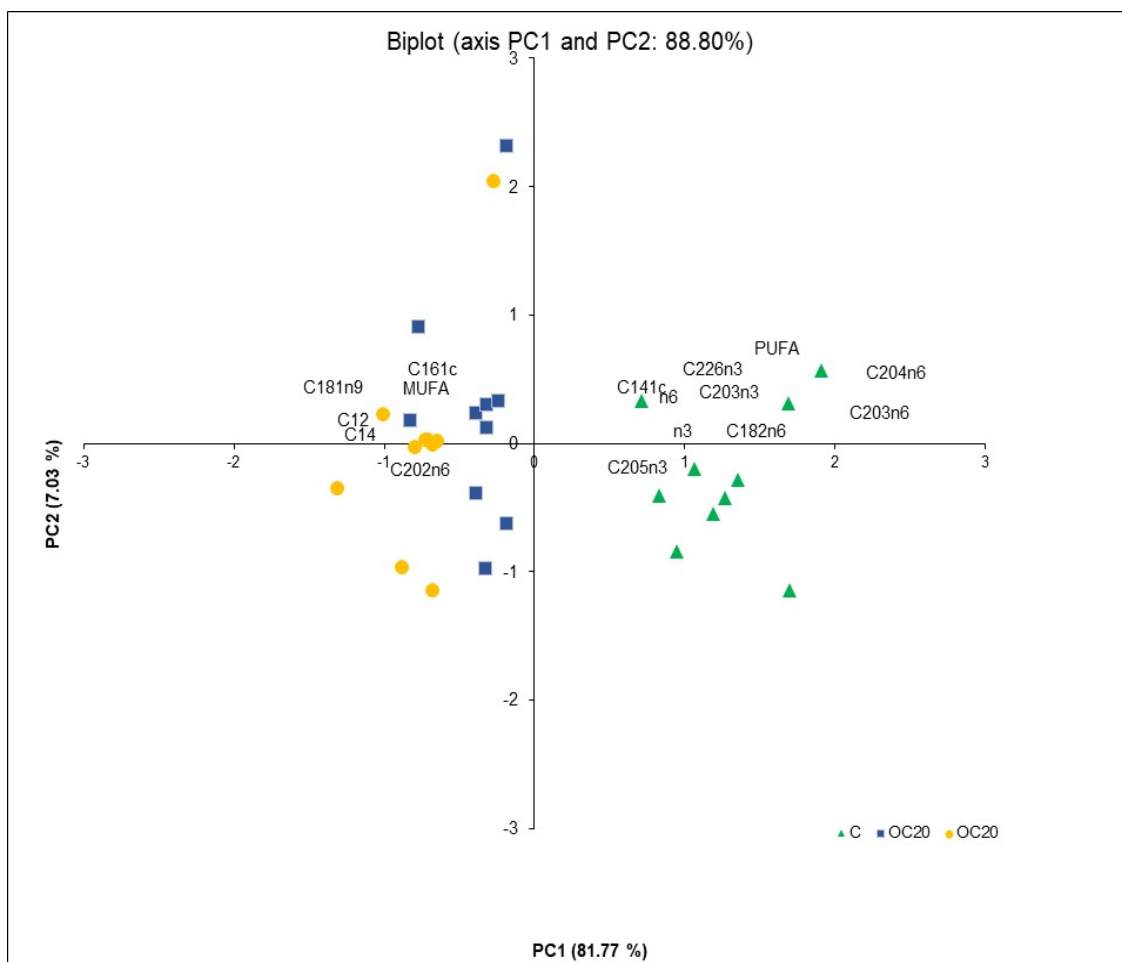
Means in the same row with different letters indicate significant differences (a, b =  $p < 0.05$ ; d, e, =  $p < 0.01$ ; A, B =  $p < 0.001$ ). <sup>1</sup> C = Control diet, OC20 = diet with silage olive cake at 20% of dry matter, OC40 = diet with silage olive cake at 40% of dry matter. <sup>2</sup> SEM, standard error of means; <sup>3</sup> n-3 = (C18:3 n-3 + C20:2 n-3 + C20:3 n-3 + C20:5 n-3 + C22:5 n-3 + C22:6 n-3); <sup>4</sup> n-6 = (C18:2 n-6 + C18:3 <sup>4</sup> n-6 + C20:2 n-6 + C20:3 n-6 + C20:4 n-6 + C22:2 n-6 + C22:4 n-6); <sup>5</sup> SFA, saturated fatty acids; <sup>6</sup> MUFA, monounsaturated fatty acids; <sup>7</sup> PUFA, polyunsaturated fatty acids; <sup>8</sup> P/S polyunsaturated fatty acids/saturated fatty acids; <sup>9</sup> AI, atherogenic index = C12:0 + 4 × C14:0 + C16:0)/(n-3 + n-6 + MUFA); <sup>10</sup> TI, thrombogenic index = (C14:0 + C16:0 + C18:0)/((0.5 × MUFA) + (0.5 × n-6) + (3 × n-3) + (n-3/n-6)); <sup>11</sup> h/H = [(C18:1n 9, C18:1n 7, C18:2n 6, C18:3n 6, C18:3n 3, C20:3n 6, C20:4n 6, C20:5n 3, C22:4n 6, C22:5n 3 and C22:6n 3)/(C14:0 and C16:0)].

The dietary treatment affected the sum of n-3 PUFA and n-6 PUFA, with a greater concentration found in meat samples from the C group ( $p < 0.01$  and  $p < 0.001$ , respectively) compared to those in other groups.

No effect of dietary treatments was observed on the atherogenic (IA) and thrombogenic indexes (TI) and the ratio between hypocholesterolemic and hypercholesterolemic fatty acids (h/H index).

### 3.3. PCA Analysis

Figure 1 shows the PCA scores plots and the corresponding loadings for the PC1 and PC2, which together account for 88.81% of the total variance, respectively. The first component (PC1) explains 81.77%, and the second component (PC2) represents 7.03% of the total information.



**Figure 1.** Principal component analysis was applied to fatty acids detected in meat samples from concentrate (C), concentrate with the inclusion of silage cake at 20% (OC20) and concentrate with the inclusion of silage cake at 40% (OC40) diets. Scores and loading plots on the first two principal components. Scores correspond to samples, and loadings correspond to variables.

The labels correspond to the following variables: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-3 = (C18:3 n-3 + C20:2 n-3 + C20:3 n-3 + C20:5 n-3 + C22:5 n-3 + C22:6 n-3); n-6 = (C18:2 n-6 + C18:3 n-6 + C20:2 n-6 + C20:3 n-6 + C20:4 n-6 + C22:2 n-6 + C22:4 n-6).

## 4. Discussion

### 4.1. Animal Performances and Meat Quality Measurements

The performance parameters were not affected by dietary treatment. No significant differences were observed in the final weight and carcass weight among the experimental groups (Table 2).

These results are in accordance with other studies in which the inclusion of olive cake in the diets was not shown to affect the growth of the animals [7,33]. Additionally, as reported by Aboagye et al. [34], the Apulo-Calabrese is a breed characterised by reduced growth and carcass performance, and this justifies the lower ADG values registered in all treatments of our trial.

As for proximate meat analyses, the finishing diet with silage olive cake did not significantly affect the pH and colour parameters of meat, according to Ferrer et al. [14].

#### 4.2. Meat Fatty Acid Composition

The inclusion of fats and oils in monogastric animal feeding is a common practice due to their high energetic input and the availability of essential fatty acids, improving production. Additionally, dietary fat alters the lipid composition as well as the nutritive and organoleptic qualities of meat. Oleic acid (C18:1 n-9; 55–83%) is particularly abundant in silage olive cake, and the consumption of this fatty acid has been strongly associated with health benefits [35,36].

As expected, the FA profile of intramuscular fat changed as a result of the inclusion of ensiled olive cake in the diet, with a higher proportion of MUFA and a lower proportion of PUFA. The high oleic acid content of olive cake dietary treatments is linked to the increase in MUFA detected in fat tissue.

These results agree with the findings of Ferrer et al. [14], who found a higher concentration of MUFA and a lower content of PUFA in pigs when olive cake (in the range of 0–15%) was included in the diet, reflecting the dietary fatty acid composition. Additionally, the modification of the FA profile with the addition of olive by-products has been described by González et al. [37] and Serra et al. [38] as being of interest due to the improved sensory quality of meat.

Considering the PUFA content, in our study, the values ranged from 28.62% in the control group to 15.35% in the OC40 group, fed with the inclusion of 40% of silage olive cake. Some interesting differences are detectable when compared to Liotta et al. [8], who found higher levels of PUFA in Pietrain pigs fed with the inclusion of olive cakes (6 to 7% more) in the diet.

The amount and type of dietary fat, the *de novo* synthesis of fatty acids, the conversion rate to other fatty acids and metabolites, the percentage of oxidation for energy consumption and the levels of dietary elements and vitamins affect the amount of PUFAs in any tissue [14,31]. In our study, the lower proportion of PUFA detected in the muscle of pigs fed with the inclusion of olive cake is likely to be associated with the lower linoleic acid contents provided by the OC20 and OC40 dietary treatments (see Table 1). Furthermore, it should be noted that the PUFA percentage in the OC 40 group has been found within the limit of 15%, which is considered the threshold above which the fat consistency and oxidative stability could be negatively affected [39].

Nutritional evaluation of the fat fractions of foods is frequently based on nutritional indexes such as the polyunsaturated/saturated (P/S) ratio and n-6/n-3 ratio [40], and the atherogenicity (AI), thrombogenicity (TI) and hypocholesterolemic/hypercholesterolemic (h/H) characteristics [40,41].

The inclusion of olive cake in the pig-fattening diet improves the P/S ratio of meat toward the required value of 0.40 in human nutrition. The P/S ratio found in meat from the OC 20 and OC 40 groups is comparable to the value reported by Oksbjerg et al. [42] in pigs raised under organic and free-range production systems and over the value of 0.16 found by Leite et al. [18] in Bisaro pigs fed with olive cake supplementation.

The n-6/n-3 PUFA ratio was not significant between treatments. However, the values were high in relation to the recommended value of 4, which is associated with benefits to human health [43].

As stated by several authors [44,45], it is difficult to decrease the n6/n3 ratio in pork due to the high concentration of C18:2n-6 found in typical feed concentrate. In our study, all dietary treatments were richer in C18:2n-6 (see Table 1); thus, it is not surprising to find

a high n-6/n-3 PUFA ratio in the meat from all experimental groups. Regarding the n6/n3 ratio, a similar value was found in the Nero Siciliano breed by Zumpo et al. [45] and in pigs fed under free-range conditions by Oksbjerg et al. [42].

The atherogenic (IA) and thrombogenic indexes (TI) are measures of lipid quality, which could serve as predictors of cardiovascular risks [41]. They were lower than 1.00. Dietary treatments did not significantly affect the IA and IT indices on the intramuscular fat of muscle, and the atherogenicity potential was within the expected range. The IA and IT values agreed with those achieved by Liotta et al. [8] in Pietrain pigs fed with 50 g/kg and 100 g/kg of olive cake incorporated into their diet.

The hypo-/hypercholesterol (h/H) ratio is an index that considers the functional activity of FAs in the metabolism of plasma cholesterol lipoproteins, whose types and quantities are associated with a higher or lower risk of cardiovascular disease. Nutritionally higher h/H values are considered more beneficial for human health. The average of h/H (2.97) is high in all experimental groups compared to the values reported by Woloszyn et al. [46] in pork (2.4); this confirms that meat from Apulo-Calabrese pigs reared in a free-range system with the supplementation of silage olive cake, is recommended for a healthy diet.

#### 4.3. PCA Analysis

The standard principal component analysis (PCA) technique was applied as a tool for reducing data dimensions to a few principal components and visualising similarities. It produces a new set of variables derived from the best linear combination of the original parameters, which accounts for more variance than any other combination. Simultaneously, this chemometric tool allows the identification of how the samples differ and which variables contribute the most to the difference. Considering the score plots for PC1 and PC2, the difference among the meat samples from different dietary treatments is apparent (Figure 1).

The meat samples from pig feed with diet C are located in the lower right of the plot (PC1 negative and PC2 positive) and can be clearly separated from all others, whereas the meat samples from pig feed with the inclusion of silage olive cake at 20% and 40% of dry matter in the diet (OC20 and OC40), clustered together on the inferior (PC1 and PC2 negative) and superior (PC1 positive and PC2 negative) left of the plot, can be partly distinguished. Moreover, as can be seen in Figure 1, the clear separation of meat samples, according to the presence or absence of the ensiled olive cake in the diets, is evident. The FAs accounting for most variation in the PCA were n-3 long-chain fatty acids, n-6 long-chain fatty acids, C18:2n-6, C20:3n-6, C20:3n-3, C20:4n-6, C20:5n-3, C22:6n-3, C14:1 ci-s9 and PUFA (positive loading), and C12:0, C14:0, C18:1n9 and MUFA (negative loading) for PC1. The second principal component (PC2) captured the variance associated with C16:1 (positive values) and C20:2n6 (negative values). From the comparison of the PCA score plot and PCA loading plot in Figure 1, it may be inferred that the contents of n-3 long-chain fatty acids, n-6 long-chain fatty acids, C18:2n-6, C20:3n-6, C20:3n-3, C20:4n-6, C20:5n-3, C22:6n-3, C14:1 ci-s9 and PUFA were higher in control samples (C), whereas meat samples from the OC20 and OC40 groups were characterised by high values of C12:0, C14:0, C18:1n-9, C16:1n-9, C20:2n-6 and total MUFA.

#### 5. Conclusions

The main finding of this study was that including ensiled olive cakes in the fattening diet of Apulo-Calabrese pigs had no effect on animal performance and carcass characteristics. Moreover, the replacement of part of the concentrate with ensiled olive cake in the diet improves the fatty acid composition of meat from a health point of view, increasing oleic acid and decreasing linoleic acid and PUFA levels. Thus, from a technological and commercial point of view, the inclusion of silaged olive cake in fattening diets results in meat with a fatty acid profile more appropriate for transformation and storage. In fact, the food industry prefers meat with a limited amount of polyunsaturated fatty acids because

they are more likely to undergo lipolytic and oxidative processes, resulting in rancidity, abnormal flavours, fat softness, and altered organoleptic properties.

This investigation may provide the basis for further studies, including the evaluation of lipid stability during the refrigerated storage of meat and the flavour of meat products, enabling broader application in the research and food industries.

Furthermore, the valorisation of local breeds, such as Apulo-Calabrese, would lead to the creation of a niche market with increasing importance and very high profits.

Finally, the incorporation of ensiled olive cake at up to 40% in diets for fattening pigs could represent a strategy in Mediterranean areas to naturally improve the nutritional value of meat and meat products as well as to valorise an olive industry by-product while reducing the environmental impact and promoting the exploitation of this local feed resource according to the principles of the circular economy.

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## **Appendix 2. Effects of feeding bergamot pulp and olive leaves on performance and meat quality in Apulo-Calabrese pigs**

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## Effects of feeding bergamot pulp and olive leaves on performance and meat quality in Apulo-Calabrese pigs

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### ABSTRACTS

To determine the effects of dietary inclusion of bergamot pulp and olive leaves on pork quality, thirty-six barrows (Apulo-Calabrese; 112.5 ± 7.40 kg initial bodyweight) were randomly assigned to four experimental treatments and fed for 100 days a control diet (control group) or a diet similar to the control diet in which part of the cereals were replaced with 20 % (DM on the diet fed) of ensiled bergamot pulp (dry matter on the diet fed, EBP group) or olive leaves (OLL group) or a 1:1 mixture of both by-products (BPOL group).

In the present study, the dietary administration of by-products did not influence the accumulation of IMF ( $P > 0.05$ ) in meat. As for individual fatty acids, no differences were observed among groups. However, the dietary supplementation of bergamot pulp tended to increase ( $P = 0.073$ ) the level of  $\alpha$ -linolenic acid in meat. In raw and cooked meat TBARS values were higher ( $P < 0.001$ ) in the Control group than in EBP, OLL and BPOL groups. The integration of 20 % of bergamot pulp and/or olive leaves by-products increased the levels of tocopherol and phenolic compounds in the diets, positively influencing the shelf life of meat. No effect of diet was observed on animal performance. The results of present study indicated mainly that partial replacement of cereals with 20 % DM on the diet fed of ensiled bergamot pulp and olive leaves, either alone or in combination, in the pig's diet led to greater oxidative stability of the meat.

### Introduction

Technological innovation applied to the reuse of agri-industrial by-products and residues in the feed sector is an advantageous situation from many aspects, including recovering the nutritional value of the by-products and improving the functionality and nutraceutical properties of diet. By-products are cheaper than grains and can reduce the ration cost. There are by-products rich in protein (corn gluten, meal extracted from vegetable oils, etc.), rich in digestible fibers (beet pulp, citrus pulp, etc.), rich in sugars and starch (meal and other by-products of the milling industry) that can be used in the formulation of complementary feeds. Moreover, some by-products are used and included in animal rations due to the particular concentration of bioactive compounds since, in addition to providing nutritional value, they play the role of functional supplement with particular beneficial effects on health or metabolism. In this case, the by-products are used similarly to feed additives and improve the health of the animal and the quality of the products; they

can also *i)* favor the organoleptic qualities of the diets, *ii)* provide antioxidants, immune system stimulants, and digestive parasite inhibitors, *iii)* improve the quality of the products from the microbiological point of view and the stability of the lipids or the conservation and duration (Santana-Méridas et al., 2012; Valenti et al., 2019; Scerra et al., 2022a).

In our recent investigations (Scerra et al., 2022a), we demonstrated that supplementing the diet of growing pigs with ensiled bergamot pulp at a 15 % DM level increased the phenolic compounds and tocopherol levels in the diet. Similar findings have been reported by other researchers (Paiva-Martins et al., 2009; Botsoglou et al., 2012) using olive leaves, a by-product available in the same seasonal period as bergamot pulp. Although the incorporation of olive leaves improved the oxidative stability of raw pork (Paiva-Martins et al., 2009; Botsoglou et al., 2012), our studies showed that integrating bergamot pulp into the diet enhanced the shelf life of salami but not raw pork (Scerra et al., 2022a, 2022b), with slight differences on meat fatty acid composition in either case.

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Considering that these by-products (i.e., bergamot pulp and olive leaves) are typically available simultaneously, it would be valuable to investigate the effects of their combined use on pork quality. Our hypothesis was that a higher inclusion of bergamot pulp — surpassing levels studied in previous research — and/or the synergetic effect with olive leaves could improve the pork shelf-life. Therefore, the aim of the present study was to assess the impacts of a 20 % DM inclusion of bergamot pulp in a pig's diet and the synergistic effects of combining bergamot pulp and olive leaves on animal performance and pork quality.

## Materials and methods

Thirty-six barrows of the Apulo-Calabrese breed obtained from a single farm were weighed ( $112.5 \pm 7.40$  kg initial body weight), randomly assigned to one of four treatments (9 animals per treatment), housed in individual pens and adapted for 7 days to the experimental treatments. Pigs received the experimental diets (Table 1) ad libitum for 100 days and had continuous access to water. The control group was fed a commercial concentrate-based diet. The other groups received a similar diet in which part of the cereals was replaced with 20 % (DM on the diet fed) of ensiled bergamot pulp (EBP group) or olive leaves (only olive leaves without olive pomace, OLL group) or a mixture (1:1) of ensiled bergamot pulp and olive leaves (BPOL group). Diet with olive leaves and/or bergamot pulp was a mixture of concentrate with the respective amount of by-product. In the diets of EBP, OLL and BPOL groups were integrated higher soybean meal content than the control diet to avoid feeding diets with different crude protein concentrations. The ingredients and chemical composition of the experimental diets are presented in Table 1.

The BP was supplied by a juice citrus industry (Bova, Bova Marina,

**Table 1**  
Ingredients (% on DM basis) and chemical composition of the experimental diets.

	Control diet	EBP diet	OLL diet	BPOL diet
Barley	30	20	20	20
Maize	30	20	20	20
Oat	15	11	11	11
Soybean meal	7	11	11	11
Faba bean	15	15	15	15
Olive leaves	-	-	20	10
Bergamot pulp	-	20	-	10
Vitamin mineral premix <sup>1</sup>	3	3	3	3
<i>Chemical composition</i>				
Dry matter (DM) g/kg wet weight	884	884	886	885
Crude protein g/kg DM	141	139	140	140
Ether extract g/kg DM	30.7	29.4	27.7	28.7
Ash g/Kg DM	38.9	39.2	36.2	38.1
NDF g/Kg DM	411	446	441	443
Total phenolic compounds (g TAe <sup>2</sup> /kg DM)	1.57	6.70	4.99	5.84
Total tannin compounds (g TAe <sup>2</sup> /kg DM)	1.19	1.71	2.11	1.91
<i>Tocopherols (µg/g DM)</i>				
α-Tocopherol	1.02	24.6	28.2	26.4
γ-Tocopherol	5.16	5.41	5.32	5.40
<i>Fatty acids (g/100 g of total fatty acid)</i>				
C10:0	0.03	0.01	0.06	0.09
C12:0	0.04	0.02	0.04	0.06
C14:0	0.82	0.72	0.88	0.80
C16:0	30.0	30.9	30.2	30.5
C18:0	4.01	3.90	4.19	4.05
C18:1 n-9	38.6	33.7	40.0	36.9
C18:2 n-6	25.6	27.0	21.7	24.3
C18:3 n-3	0.42	3.26	1.45	2.36

<sup>1</sup> The mineral vitamin premix consisted of vitamins A = 6750 UI; vitamin D3 = 1000UI; vitamin E 2 mg; vitamin B12 0.01 mg; vitamin B1 1 mg; folic acid 0.2 mg; D-pantotenic acid 5 mg; Co 0.05 mg; Mn 12.5 mg; Zn 15 mg; Mo 0.5 mg.  
<sup>2</sup> tannic acid equivalent.

RC) and then ensiled in the experimental farm, while OL were obtained at a local oil mill (Melia Trasformazioni, Monasterace, RC) during the olive oil production period (October–December), dried at 35 °C (in a ventilated oven) for three days, milled and stored at room temperature until mixed with experimental feeds.

All the pigs were fed twice daily (0800 and 1500 h). The amounts of feed offered and refused were recorded every day in order to measure dry matter intake (DMI). Pigs were weighed every 20 days from the beginning to the end of the experimental trial.

After 100 days from the beginning of the trial, the pigs were transported to a commercial abattoir (15 min from experimental farm) and immediately slaughtered (electrically stunned) according to EU welfare guidelines. The animals were slaughtered after 8 h of fasting. Twenty minutes after slaughtering, the carcasses were weighed and stored at 4 °C. After 24 h from each carcass the *longissimus thoracis et lumborum* muscle (LTL) was removed and prepared for the analyses.

## Feedstuff analysis and meat proximate analysis

Feed samples were analyzed for crude protein, crude fat, and ash (AOAC,1995, methods 984.13, 920.39, 942.05 respectively) and neutral detergent fiber (NDF, method proposed by Van Soest et al., 1991). In feed samples, total phenolic compounds (extracted in aqueous acetone) were determined using the Folin–Ciocalteu reagent (Makkar et al., 1993). The vitamins α-tocopherol and γ-tocopherol were evaluated through methanol:acetone:petroleum ether (1:1:1, v:v:v) extraction (Rufino-Moya et al., 2020) and ultra-high performance liquid chromatography (UHPLC), using a Shimadzu UHPLC (Nexera, Shimadzu Corporation, Milan, Italy) equipped with a Zorbax ODS column (25 cm × 4.6 mm, 5 µm; Agilent Technologies, CA). A Shimadzu spectrofluorometric detector (RF-20AXS) was used to determine tocopherols (295 nm excitation wavelength and 330 nm emission wavelength). A Shimadzu photodiode array detector (PDA; SPD-M40) was used to analyze retinol (absorbance at 325 nm).

The software LabSolutions controlled the UHPLC system. 10 µl of sample with methanol as mobile phase (flow rate of 1.3 ml min<sup>-1</sup>) was injected.

The analytes were identified by comparison of the retention times with those of the pure standards.

Fatty acid composition was analyzed following the procedures used by Valenti et al. (2018). Following AOAC (1995) procedures, moisture, crude fat, ash, and crude protein (methods 950.46, 991.36, 920.153 and 984.13 respectively) were determined in LTL samples.

## Meat fatty acid and antioxidant vitamins determination

Following the procedures described by Folch et al. (1957) fatty acid composition was analyzed on total lipids extracted. Briefly, fat was extracted from 10 g of homogenized samples with chloroform/methanol (1:1, v/v), then 50 mg of lipids was methylated adding 2 ml of hexane and 1 ml of 0.5 N sodium methylated in methanol (I.U.P.A.C., 1987). C19:0 was used as an internal standard and samples were analyzed using a gas chromatograph (model TRACE GC; Thermo Finnigan, Milan, Italy) equipped with a 100 m high-polar fused silica capillary column (5 mm i. d., 0.25 µm film thickness; SP. 24056; Supelco Inc., Bellefonte, PA). Gas-chromatography conditions and identification of FAME was performed as described by Scerra et al. (2022a). Fatty acids were quantified as mg/100 g of meat.

Antioxidant vitamins and cholesterol were determined in muscle following the procedures described by Natalello et al. (2023). Briefly, at 0.5 mg of meat sample were added 200 mg of L-ascorbic acid and 7.5 ml of 10 % KOH in ethanol:water (1:1) and saponified at room temperature overnight.

Them, 5 ml of 9:1 hexane:ethyl acetate with 25 mg/L of BHT was added. Subsequently, the tubes were centrifuged (at 10 °C, 5 min, 2000 × g). Supernatants were removed under nitrogen flow, dissolved in 1 ml

of methanol, vortexed and filtered into a vial for UHPLC. Cholesterol was detected by absorbance wavelength at 220 nm. Chromatographic conditions were as described before for feed samples.

#### Lipid oxidation and colour measurements

Three raw meat slices (2 cm thick) from each LTL samples were used to study the oxidative stability during 7 days of aerobic refrigerated storage. The slices were overwrapped with plastic wrap and stored in the dark at 4 °C. Colour parameters and lipid oxidation extent were determined after 2 h (for blooming), 3 and 7 days of storage. The colour descriptors  $L^*$ ,  $a^*$ ,  $b^*$ ,  $C^*$  and hue angle ( $H^*$ ) were measured using a CR300 colour-meter (Minolta Co. Ltd. Osaka, Japan; aperture 6 mm, illuminant A and 10° standard observer).

Two more 2 cm thick meat slices were used to evaluate lipid oxidation in cooked meat, where the slices were vacuum-packaged and immersed in a 75 °C water bath for 30 min. One cooked slice was immediately used for lipid oxidation determination, whereas the other one was stored at 4 °C in the dark, measuring lipid oxidation after 2 days.

To study lipid oxidation in fresh and cooked meat during storage, thiobarbituric reactive substances (TBARS) were assessed (Siu & Draper, 1978). In brief, 2.5 g of samples (in 12.5 ml of distilled water) were mixed with 12.5 ml of 10 % trichloroacetic acid and, homogenized and filtered. Then, 4 ml of clear filtrate was mixed with 1 ml of 0.06 M aqueous thiobarbituric acid and incubated in a water bath at 80 °C for 90 min. Then, absorbance of the solution was measured at 532 nm using a UV-1800 spectrophotometer (Shimadzu Corporation, Milan, Italy). The assay was calibrated as reported in a previous paper (Scerra et al., 2022a).

#### Statistical analysis

Individual pig was considered as experimental unit. All data were analyzed considering the dietary treatment as the fixed factor, using a GLM (general linear model) procedure. Data of colour descriptors and TBARS were analyzed using a GLM procedure for repeated measures, where the individual animal was included as a random factor, while the fixed factors in the model were the diet, the storage time and their interaction.

Multiple comparisons of the means were assessed using Tukey's test. The software used for statistical analyses was Minitab 14 (Minitab Inc, State College, PA).

#### Results

As shown in Table 2, the administration of the four different diets to the pigs involved in the experimental trial did not lead to different final weights of the animals of the different groups ( $P = 0.449$ ; Table 2). Also, other performance parameters, such as dry matter intake ( $P = 0.421$ ; DMI), feed conversion ratio ( $P = 0.214$ ; FCR), average daily gain ( $P = 0.586$ ; ADG) and carcass weight ( $P = 0.823$ ), were not influenced.

Regarding the chemical composition of meat (Table 2), no significant differences between groups were found for moisture, crude protein, ether extract and ash ( $P > 0.05$ ). Vitamin E was mainly represented by  $\alpha$ -tocopherol and its concentration in meat was affected by supplementing bergamot pulp and olive leaves, resulting higher ( $P < 0.019$ ) in meat from EBP, OLL and EBOL groups than in meat from Control group.

In Table 3 are reported the effects of dietary treatments on the fatty acid composition of meat. In the present study, the dietary administration of by-products did not influence the accumulation of IMF ( $P > 0.05$ ) in meat. The total of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were not different among groups ( $P > 0.05$ ). As for individual fatty acids, no differences were observed among groups.

Fig. 1 shows the TBARS values measured during monitoring days.

**Table 2**

Effect of dietary treatment on growth performance and chemical composition of *longissimus thoracis et lumborum* muscle (g/100 g wet weight).

	Dietary treatment				SEM <sup>5</sup>	P value
	Control	EBP	OLL	BPOL		
Final BW <sup>1</sup> , kg	146	144	142	145	4.740	0.449
Carcass weight, kg	122	119	120	121	4.280	0.823
Carcass yield,	83.5	82.4	84.3	83.4	3.567	0.621
total DMI <sup>2</sup> , kg/d	3.53	3.50	3.55	3.53	0.154	0.421
ADG <sup>3</sup> , g/d	340	320	300	330	24.20	0.586
FCR <sup>4</sup> , g (DMI <sup>2</sup> /g ADG <sup>3</sup> )	10.4	10.9	11.8	10.7	0.423	0.214
<i>Tocopherols and Cholesterol (µg/g muscle)</i>						
$\alpha$ -Tocopherol	2.28 <sup>b</sup>	3.14 <sup>a</sup>	3.23 <sup>a</sup>	3.13 <sup>a</sup>	0.184	0.019
$\gamma$ -Tocopherol	0.30	0.28	0.25	0.28	0.022	0.112
Cholesterol	2.57	2.59	2.64	2.55	0.012	0.063
<i>Chemical composition</i>						
Moisture	73.6	74.0	73.7	74.1	0.187	0.781
Crude protein	22	21.6	21.3	21.5	0.173	0.603
Ether extract	2.11	1.98	2.25	2.08	0.140	0.520
Ash	1.14	1.14	1.19	1.17	0.112	0.506

<sup>1</sup> BW = Body weight.

<sup>2</sup> DMI = dry matter intake; <sup>3</sup>ADG = average daily gain; <sup>4</sup>FCR = feed conversion ratio; <sup>5</sup>SEM = standard error of means.

**Table 3**

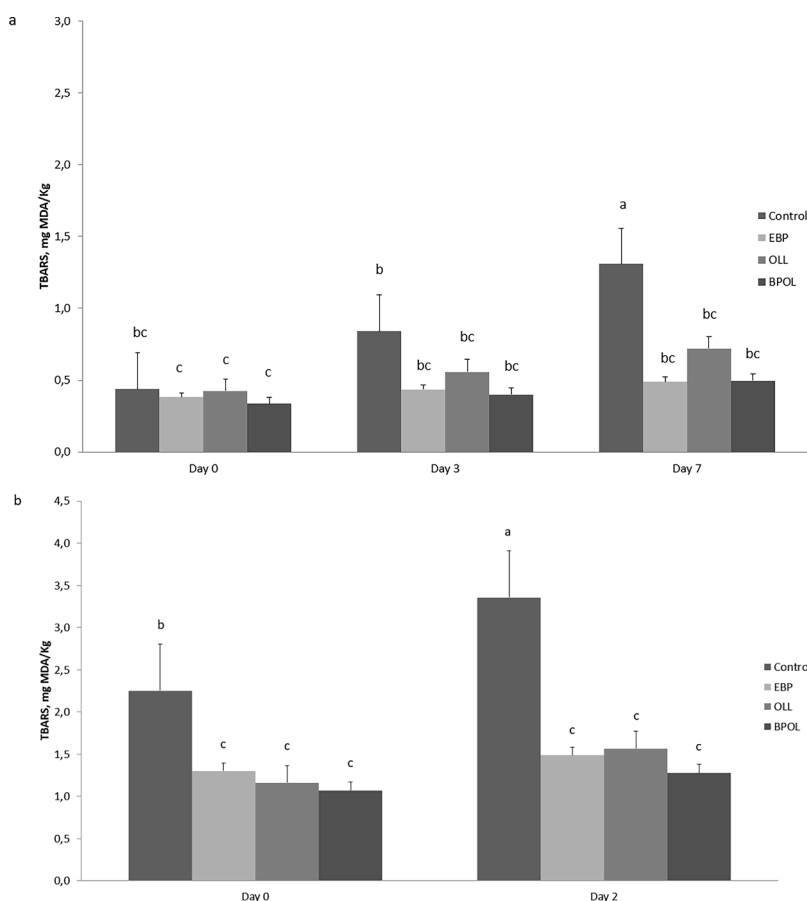
Effect of the dietary treatments on fatty acid composition of *longissimus thoracis et lumborum* muscle (mg/100 g of muscle).

Item	Dietary Treatment				SEM	P value
	Control	EBP	OLL	BPOL		
intramuscular fat, mg/100 g of muscle	1914	1949	1907	1885	195	0.092
C12:0	0.89	0.93	0.82	1.07	0.155	0.757
C14:0	18.4	20.5	17.1	20.1	2.440	0.249
C15:0	0.30	0.34	0.37	0.42	0.113	0.131
C16:0	310	350	304	335	40.8	0.195
C16:1 <i>trans</i> -9	0.23	0.37	0.41	0.62	0.085	0.459
C17:0 <i>anteiso</i>	4.34	6.39	4.76	6.32	0.928	0.162
C16:1 <i>cis</i> -9	42.2	43.1	36.3	41.7	4.670	0.335
C 17:0	3.40	5.50	4.23	5.32	0.893	0.131
C17:1 <i>cis</i> -10	2.09	3.76	2.17	3.38	0.628	0.092
C18:0	155	182	154	180	21.4	0.167
C18:1 <i>trans</i> -11 VA <sup>1</sup>	4.55	4.72	4.52	4.68	0.536	0.313
C18:1 <i>cis</i> -9	565	654	656	635	76.3	0.229
C18:1 <i>cis</i> -11	71.7	78.6	67.6	75.1	8.18	0.246
C18:2 <i>cis</i> -9, <i>cis</i> -12 LA <sup>1</sup>	146	187	153	174	21.6	0.196
C 20:1 <i>cis</i> 11	12.7	15.9	12.3	14.0	2.17	0.159
C18:3 <i>n</i> -3 ALA <sup>1</sup>	4.90	7.31	5.24	7.02	0.891	0.073
C20:2 <i>n</i> -6	4.31	5.48	4.28	5.25	0.888	0.249
C20:3 <i>n</i> -6	1.02	0.92	1.64	1.38	0.159	0.502
C20:4 <i>n</i> -6	20.2	20.5	22.3	21.4	0.613	0.736
C20:5 <i>n</i> -3 EPA <sup>1</sup>	2.43	3.22	2.73	2.95	0.211	0.848
C22:6 <i>n</i> -3 DHA <sup>1</sup>	1.29	1.98	1.73	1.47	0.196	0.327
$\sum$ SFA <sup>1</sup>	487	559	480	541	66.5	0.186
$\sum$ MUFA <sup>1</sup>	698	800	779	774	92.2	0.230
$\sum$ PUFA <sup>1</sup>	180	226	191	213	24.1	0.195
$\sum$ <i>n</i> -3	8.63	12.5	9.70	11.4	1.19	0.082
$\sum$ <i>n</i> -6	171	213	181	202	22.8	0.197
$\sum$ PUFA <sup>1</sup> / $\sum$ SFA <sup>1</sup>	0.37	0.40	0.40	0.39	0.016	0.328
<i>n</i> -6/ <i>n</i> -3	19.9	17.1	18.6	17.6	0.601	0.621

<sup>1</sup> VA: Vaccenic acid; LA: linoleic acid; ALA:  $\alpha$ -linolenic acid; EPA: Eicosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

The TBARS values increased over storage duration ( $P < 0.05$ ) only in raw and cooked meat from control group, remaining constant in meat from EBP, OLL and EBOL groups during all monitoring days, with a value of diet  $\times$  time interaction highly significant ( $P < 0.001$ ).

Regarding colour descriptors (Table 4), the redness ( $a^*$ ), lightness ( $L^*$ ) and yellowness ( $b^*$ ) were not affected by storage time and dietary treatment. Instead, hue angle ( $H^*$ ) values were affected by storage time ( $P < 0.001$ ), with progressively decreasing values.



**Fig. 1.** Effect of the dietary treatment and time of storage on the oxidative stability of longissimus thoracis et lumborum muscle, raw (a) and cooked (b). Treatments were: Control, concentrate-based diet; EBP, concentrate and ensiled bergamot pulp at the level of 20 % DM on the diet fed; OLL, concentrate and olive leaves at the level of 20 % DM on the diet fed; BPOL, concentrate and a mixture 1:1 of ensiled bergamot pulp and olive leaves at the level of 20 % DM on the diet fed. Interactive effect of the dietary treatment (Control, EBP, OLL and BPOL) and time of storage on the TBARS values measured in raw and cooked slices over aerobic storage at 4 °C. Values presented are the estimated least squares means and standard error bars. <sup>a,b,c,d</sup>Values with different superscripts are significantly different ( $P < 0.05$ ).

**Table 4**

Effect of the dietary treatments and time of refrigerated storage on meat colour stability of *longissimus thoracis et lumborum* muscle.

	Dietary treatment				Time <sup>2</sup>			SEM	P values		
	Control	EBP	OLL	BPOL	0	1	2		Diet	Time	Diet × Time
<i>L*</i> values <sup>1</sup>	46.2	48.1	47.0	47.8	47.9	46.4	47.6	0.363	0.244	0.221	0.997
<i>a*</i> values <sup>1</sup>	7.1	7.0	7.3	7.9	7.3	7.2	7.7	0.145	0.099	0.434	0.935
<i>b*</i> values <sup>1</sup>	6.1	5.8	5.8	6.4	6.5	5.9	5.6	0.138	0.110	0.097	0.939
<i>C*</i> values <sup>1</sup>	9.4	9.2	9.4	10.2	9.8	9.3	9.4	0.176	0.128	0.259	0.997
<i>H*</i> values <sup>1</sup>	41.0	39.5	38.3	39.2	42.3 <sup>x</sup>	39.5 <sup>y</sup>	36.5 <sup>z</sup>	0.576	0.538	0.001	0.226

<sup>x,y,z</sup> Within row, different superscripts indicate differences between days of storage ( $P < 0.05$ ) tested using the Tukey’s adjustment for multiple comparisons.

<sup>1</sup> *L\** = lightness; *a\** = redness; *b\** = yellowness; *C\** = Chrome; *H\** = hue angle, measured in degrees.

<sup>2</sup> Times 0, 1, 2 = days 0, 3, 7 for raw meat at 4 °C under aerobic conditions (meat slices).

**Discussion**

Scarce recent data are available in literature on the effects of the use of OL on animal performance and meat quality (Paiva-Martins et al., 2009), while interesting results have recently been reported for the use of ensiled BP (Scerra et al., 2022a) or exhausted bergamot by-product (Scerra et al., 2022b) in pig feeding from our experimental trials. However, no studies have been conducted to evaluate the effects of the simultaneous use of BP and OL - which are usually available on the same season - in pig diets on animal performance and meat quality.

The administration of the four different diets to the pigs involved in the experimental trial did not lead to a variation of performance parameters among the experimental groups. Similar results were observed

in our previous studies (Scerra et al., 2022a) in which pigs were fed a diet integrated with ensiled bergamot pulp and in a trial where pigs received a diet integrated with 10 g/kg of OL (Botsoglou et al., 2012). Conversely, dietary OL negatively affected daily gain and final body weight of pigs in a study carried out by Paiva-Martins et al. (2009), where the authors underline that the incorporation of 5 % or 10 % of olive leaves in diets for growing pigs increased the levels of dietary fibre and led to impaired growth rates and feed:gain ratio, as observed by other authors in rabbits (García et al., 1999). However, Paiva-Martins et al. (2009) also observed a decrease of DMI in the animals supplemented with OL and this probably negatively affected final body weight. In the present study, the lack of effects on animal performances could be partially explained in two different manners. (i) EBP, OLL and BPOL

diets had a higher in soybean meal content than the control diet in order to obtain isoproteic treatments. This may have led to an improved amino acid profile and, in turn, a positive influence on the growth rates of the animals in these experimental groups. (ii) This result may also have been influenced by the breed used for the experimental trial. The Apulo-Calabrese pig is a native Calabria breed that, as reported by Micari et al. (2009) and Pugliese and Sirtori (2012) has a high adaptability to different production systems compared to cosmopolitan breeds. As reported above, even the dietary supplementation of 20 % of a mixture (1:1) of BP and OL did not influence the growth performance of the pigs. Considering the results obtained using the by-products individually, this result was predictable.

The partial replacement of cereals with BP or OL or a mix of both by-products did not lead to changes in meat fatty acid composition (Table 3). Considering the higher percentage of  $\alpha$ -linolenic acid in the diets supplemented with the examined by-products, we expected also in the meat from the groups that received these diets a higher level of  $\alpha$ -linolenic acid compared to the control group, at least from the groups whose diets included 15 % BP, as observed in our previous experimental study (Scerra et al., 2022a). Actually, an increasing trend of  $\alpha$ -linolenic acid was observed in meat from EBP and BPOL groups compared to the control group, as well as in total n-3 fatty acids. No significant differences in fatty acid composition of meat were observed by Paiva-Martins et al. (2009) integrating 10 % of olive leaves in the diet of pigs.

Dietary integration of by-products tested in this trial led to greater oxidative stability of the meat during the days of storage in both fresh and cooked meat (Fig. 1). In EBP, OLL and BPOL groups the TBARS values not increased during storage in fresh and cooked meat, while increased in meat from the control group. Specifically, in raw meat from animals fed the control diet lipid oxidation increased significantly after 7 days of refrigerated storage ( $P < 0.001$ ), while TBARS values were remained stable throughout the monitoring period for the other three experimental groups. As in raw meat, in cooked meat TBARS values were higher ( $P < 0.01$ ) in the control group than in the EBP, OLL and BPOL groups after 2 days of storage. No significant differences were observed between the groups that received BP or OL or a mixture of both by-products. Similar results were observed by Paiva-Martins et al. (2009) in pigs fed a diet supplemented with dried olive leaf powder at 5 % and 10 %, where after 8 days of storage at 4 °C the peroxide and conjugated dienes values of muscles were higher in pigs fed a conventional diet. In contrast, no significant differences were observed in our previous study (Scerra et al., 2022a) in fresh and cooked meat obtained from pigs fed diets that included BP at the level of 15 % than meat from pigs fed a conventional diet. However, in this study (Scerra et al., 2022a), in the salami (a product that has been subjected to pro-oxidation conditions) obtained from animals supplemented with BP, the TBARS values were lower already after 2 days of storage than in salami from animals fed a concentrate-based diet.

Data reported by our recent study and by other authors highlight that the integration of BP (Scerra et al., 2022a) or OL (Paiva-Martins et al., 2009, 2014), into the diet increased the level of antioxidant compounds. The improvement of oxidative stability in meat products following the integration of BP into the diet was attributed to the higher amount of phenolic compounds (Scerra et al., 2022a), considering that no vitamins differences were observed in salami from the experimental groups, whereas Paiva-Martin et al. (2009) attributed the improvement in the oxidative stability of meat to the greater amount of vitamin E provided by the diets that included OL. In the present trial, meat from pigs that received EBP, OLL and BPOL diets showed a higher ( $P < 0.01$ ) amount of  $\alpha$ -tocopherol than meat from pigs of the control group. The level of  $\alpha$ -tocopherol in BP and OL was similar (data not shown, 89 and 100  $\mu\text{g/g}$  DM, respectively) and their inclusion as cereals replacement increased the ingestion of tocopherol in EBP, OLL and BPOL diets, thus leading to higher levels of this important antioxidant molecule in the meat of the animals from the groups fed the tested by-products compared to the meat from the control group.

The latter data probably influenced the different trends of lipid oxidation during the 7 days of monitoring between the EBP, OLL and BPOL groups and the control one. Furthermore, this data could also have been influenced by the level of phenolic compounds in the diets, which resulted 3–4 times higher in diets that included BP or OL or both compared to the control diet. The presence of phenolic compounds in the BP and OL could have indirectly preserved vitamin E during digestion thanks to their antioxidant activity, leading to greater absorption of tocopherols by the animals. The TBARS value at day 0 already exceeds the threshold value of 2 mg MDA/kg of meat (maximum level for positive sensory perception; Campo et al., 2006), in cooked meat from animals of the control group, while it remains below the threshold value for the entire monitoring period for the other 3 experimental groups.

Regarding meat colour, all colour descriptors were not affected by storage time or dietary treatment except the hue angle ( $H^*$ ), which was affected by storage time. These results were not expected considering that, following the typical meat discoloration pattern (Luciano et al., 2019), the descriptor  $L^*$ ,  $b^*$ , and hue angle should increase while  $a^*$  should decrease during storage. Some studies (Alderton et al., 2003; Faustman et al., 2010) reported that the oxidation of myoglobin, the main pigment of meat, is promoted by fatty acid oxidation. The TBARS values found in raw meat from the control group after 7 days of monitoring, although higher than those observed in the EBP, OLL and BPOL groups, remain well below the value considered a maximum level for positive sensory perception (Campo et al., 2006), indicating that in general the meat from all experimental groups showed good resistance against oxidative phenomena.

## Conclusion

In this study, the partial replacement of cereals with 20 % DM on the diet fed of ensiled bergamot pulp and olive leaves, either alone or in combination, in the pig's diet, led to greater oxidative stability of the meat during the days of storage in both fresh and cooked meat. These data were likely influenced by the higher levels of  $\alpha$ -tocopherol in meat from pigs that received these by products, either alone or in combination, than in meat from pigs fed a commercial concentrate. Furthermore, the level of phenolic compounds in the diets, higher in diets that included ensiled bergamot pulp and/or olive leaves, may also have influenced the improvement of oxidative stability. Conversely, no effect of diet was observed on animal performance and on fatty acid composition of meat.

Further studies are needed to evaluate bergamot pulp and olive leaves at different proportions of inclusion in the pigs' ration and with difference genetic types (e.g., cosmopolitan breeds) in order to make these two new feeds commonly employed in all pig farms present in the same production areas as the by-products.

## Availability of data

None of the data were deposited in an official repository. The data that support the study findings are available from the authors upon request.

## Declaration of generative AI and AI in scientific writing

The authors did not use any artificial intelligence-assisted technologies in the writing process.

## Ethics statement

This experiment was approved by the Animal Welfare Committee of the University of Reggio Calabria (No. 8937).

## CRedit authorship contribution statement

**Manuel Scerra:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Francesco Foti:** Writing – review & editing, Formal analysis. **Pasquale Caparra:** Writing – review & editing. **Caterina Cilione:** Writing – review & editing, Formal analysis. **Matteo Bognanno:** Writing – review & editing. **Fortugno Paolo:** Writing – review & editing, Formal analysis. **De Caria Paolo:** Writing – review & editing, Formal analysis. **Antonio Natalello:** Writing – review & editing, Investigation, Formal analysis. **Martino Musati:** Writing – review & editing, Formal analysis. **Luigi Chies:** Writing – review & editing.

## 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 3. Enhancement of quails' (*Coturnix japonica domestica*) meat quality: Effects of dietary supplementation of dried bergamot pulp**

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# Enhancement of quails' (*Coturnix japonica domestica*) meat quality: Effects of dietary supplementation of dried bergamot pulp

## Abstract

This work investigated the effects of feeding dried bergamot pulp to quails on performance and meat quality. 112 Japanese quails (*Coturnix japonica domestica*) aging 15 days were divided into two groups of 56 quail each (4 replicates of 14 quails for group) and fed, for three weeks, a basal diet (control group) or the basal diet in which part of the maize was replaced with 10 % (DM on the diet fed) of dried bergamot pulp (BP10). The BP10 treatment tended to increase eicosapentaenoic acid ( $P = 0.08$ ) and  $\omega$ -3 PUFA ( $P = 0.08$ ), positively influencing the  $\omega$ -6/ $\omega$ -3 ratio which was reduced by approximately 35%. The inclusion of bergamot pulp in quail diet, despite tending to increase the total of  $\omega$ -3 fatty acids, did not alter the TBARS value in meat. Color analysis showed that the integration of bergamot into the quail diet led to higher lightness ( $L^*$ ) and yellow index ( $b^*$ ) values compared to the control group, a result which could depend on the release of pigments in the muscle, such as carotenoids. However, this replacement in the diet led to a reduction in DMI and consequently in ADG, probably due to the lower palatability of the diet due to the presence of bergamot pulp. Finally, dietary supplementation with dried bergamot pulp to quails improved the nutritional value of meat.

Keywords: fatty acids, oxidative stability, by-product, phenols, tocopherols.

## 1. Introduction

Nowadays, meat consumption has changed. The indiscriminate use of it has raised several discussions about its possible negative effects on human health, changing consumer perceptions of it over time. Some studies reported that

excessive meat consumption (in particular, red and processed meat) is positively associated with cancerous diseases (Huang et al., 2021), leading consumers to pay more attention to what they eat, preferring healthy and safe foods (Wolfswinkel et al., 2024).

Since the COVID-19 pandemic, the relationship between nature and humans has been further questioned, with calls to reduce wild meat consumption to protect health and biodiversity (Booth et al., 2021). However, this would pose a problem for the global food system, considering the importance of wild meat for human nutrition, especially for certain populations, and its relationships with other food production systems.

Meat quail (*Coturnix coturnix*) consumption in Europe has been predominant especially in the last century, due to a different lifestyle, as the quail consumed was derived almost exclusively from hunting activity; it has been estimated that with hunting activity about 6.4 million hunters are involved in Europe with killing more than 52 million birds in total, in addition to quail (Hirschfeld et al., 2019). Today's quail meat market is based exclusively on Japanese quail (*Coturnix japonica*), later domesticated over the years for eggs and meat production (*Coturnix japonica domestica*) (Lukanov and Pavlova, 2020).

Considering the high prolificacy rate of quail within a season, its use for meat consumption, associated with other Galliformes could satisfy the growing demand for poultry production and consequently diversify the consumption of this type of meat. To date, farmers make extensive use of maize (*Zea mays* L.) as the main source of energy in the diet of quail (Morne, 2017), which, in addition to being an important food ingredient for many other farmed animals, is used in the production of various beverages, biofuels, and human foods. Particular attention is also paid to the use of alternative feeds, considering the continuous increase in the prices of raw materials and traditional feedstuffs (Wolfswinkel et al., 2024).

Recently, several trials have been carried out to test by-products from agro-industrial processing in diets for Japanese quail (Reda et al., 2022; Rejeki et al., 2022). Among the agro-industrial by-products from the industrial processing of

fresh fruit there is bergamot pulp, which would constitute waste to be disposed of. Recently, interesting results have been observed from the use of bergamot pulp (*Citrus bergamia* Risso) in diets for small ruminants and pigs, leading to positive effects on the shelf-life of the meat and in general in meat quality (Scerra et al., 2018; Scerra et al., 2022; Scerra et al., 2024). Bergamot is a citrus fruit typical of southern Italy, with the area of greatest production located in the province of Reggio Calabria (RC). Various beneficial actions for health are attributed to bergamot, especially linked to its important antioxidant propriety (Siano et al., 2023). According to our knowledge, to date there are no data on the use of bergamot pulp in diets for small animals such as quails and rabbits, therefore our objective was to evaluate the effects of the use of this by-product in the quail diet on animal performance and meat quality.

## **2. Materials and methods**

### **2.1. Animals and diet**

The Animal Welfare Committee of the University of Reggio Calabria approved this study (protocol number 1214).

In the study, 112 Japanese quails (*Coturnix japonica domestica*) aging 15 days ( $146,52 \pm 9.15$  g live-weight) were randomly assigned to 2 treatment groups (control and BP10 groups) of 56 quail each, and each group was divided into 4 replicates of 14 quails each, in an enclosed area (temperature 20–24°C). The quails were subjected to one week of adaptation to the experimental diet. The control group received only basal diet, while the BP10 group fed the basal diet in which part of the maize was replaced with 10 % (DM on the diet fed) of dried bergamot pulp (BP). A slightly greater quantity of faba beans was integrated into the BP10 diet compared to the control diet to avoid providing diets with different concentrations of crude protein (table 1).

**Table 1**

Ingredients (% on DM basis) and chemical composition of the experimental diets.

	Dried Bergamot pulp	Control diet	BP <sub>10</sub> diet
Maize		55	41
Soybean meal		10	10
Faba bean		32	36
Dried bergamot pulp		-	10
Vitamin mineral premix <sup>1</sup>		3	3
<i>Chemical composition</i>			
Dry matter (DM) g/kg wet weight	884	887	883
Crude protein g/kg DM	61.5	178	178
Ether extract g/kg DM	13.2	19.4	21.6
Ash g/Kg DM	47.9	57.3	53.9
NDF g/Kg DM	367	169	229
Total phenolic compounds (g TAc <sup>2</sup> /kg DM)	16.5	1.59	6
Total tannin compounds (g TAc <sup>2</sup> /kg DM)	2.31	1.59	1.71
α-Tocopherol (μg/g DM)	50.2	36.6	39.9
<i>fatty acids (g/100g of total fatty acid)</i>			
C10:0	0.06	0,03	0,01
C12:0	0.13	0.04	0,02
C14:0	0.27	0,13	0,20
C16:0	17.1	15,9	16,2
C18:0	3.64	2,71	3,89
C18:1 n-9	25.9	32,20	30,7
C18:2 n-6	29.8	41,60	37,2
C18:3 n-3	8.98	1.31	4.52

<sup>1</sup>The mineral vitamin premix consisted of vitamins A=6750 UI; vitamin D<sub>3</sub>=1000UI; vitamin E 2 mg; vitamin B<sub>12</sub> 0,01 mg; vitamin B<sub>1</sub> 1mg; folic acid 0,2 mg; D-pantotenic acid 5 mg; Co 0,05 mg; Mn 12,5 mg; Zn 15 mg; Mo 0,5mg; <sup>2</sup>tannic acid equivalent

The diets were fed ad libitum and the animals had continuous access to water. Each quail was weighed at the beginning, in the middle and at the end of the experimental trial which lasted, without considering the adaptation period, 3 weeks. The animals' feed consumption was calculated every day removing the amount of feed remaining in the feeder from the previous day's feeding, in order to evaluate DMI (dry matter intake).

At the end of the experiment trial, all the animals were slaughtered and the carcasses were subsequently weighed after the removal of feathers, legs and internal organs, and maintained at +4°C. After 24h, the breast from each animal was removed for the evaluation of meat quality. In detail, a total of 56 breasts (n=7 breasts/replicant, n=28 breasts/treatment) were vacuum-packaged and stored at -30 °C for analyses of proximate composition. The left side of the other 56 breasts (n=7 breasts/replicant, n=28 breasts/treatment) was vacuum-packed and delivered to the laboratory, stored at 4 °C, for the determination of lipid oxidation and color stability, while the right side was vacuum- packed and stored at -30 °C and subsequently used to evaluate the fatty acid and  $\alpha$ - tocopherol composition of the meat.

## **2.2. Feedstuff analysis**

The chemical composition (dry matter, crude ash, crude protein and ethereal extract) of the experimental diets was determined according to the methods indicated in AOAC (1995), while the crude fiber content was determined following the method of Van Soest et al. (1991). Using the Folin-Ciocalteu reagent (Makkar et al., 1993), total phenolic compounds in feed (extracted in aqueous acetone) were determined.  $\alpha$ -Tocopherol was evaluated by methanol:acetone:petroleum ether (1:1:1, v:v:v) extraction (Rufino-Moya et al., 2020) and UHPLC (Nexera, Shimadzu Corporation, Milan, Italy) equipped with a Zorbax ODS column (25 cm  $\times$  4.6 mm, 5  $\mu$ m; Agilent Technologies, CA) and a Shimadzu spectrofluorometric detector (RF-20AXS) (excitation wavelength of 295 nm and emission wavelength

of 330 nm). A Shimadzu photodiode array detector (PDA; SPD- M40) was used to analyze retinol (absorbance at 325 nm). The UHPLC system was controlled by LabSolutions software. The quantity of sample with methanol injected was 10  $\mu$ l and the analytes were identified by comparing the retention times with those of pure standards. Chromatographic conditions were described in detail in Natalello et al. (2022).

The procedures used by Valenti et al. (2018) were used to determinate fatty acid composition.

### **2.3. Meat fatty acid, $\alpha$ -tocopherol and proximate composition determination**

Fatty acid composition was analyzed on the total lipids extracted following the procedures described by Folch et al. (1957). Fat was extracted from 5 g of minced meat samples homogenized with chloroform/methanol (2:1, v/v), then with 1 mL of hexane and 0.05 mL of 2 N methanolic KOH, 100 mg of lipids were methylated (I.U.P.A.C., 1987), using C9:0 as internal standard. Fatty acid methyl esters were evaluated using a Varian gas chromatograph (model CP 3900) equipped with a 100 m (CP-Sil 88, Agilent J&W) capillary column (25 mm i.d., film thickness 0.25  $\mu$ m).

Gas chromatography conditions and FAME identification were performed as described by Scerra et al. (2022). Fatty acids (FA) were quantified in g/100 g of methyl esters.

The  $\alpha$ -tocopherol in muscle samples was determined as described by Natalello et al. (2022), using the UHPLC described above, where the chromatographic conditions were the same as described for the feed samples.

AOAC (1995) methods were used to determinate moisture, crude fat, ash, and crude protein in meat samples.

#### **2.4. Lipid oxidation and colour measurements**

To evaluate raw meat oxidative stability during refrigerated storage, three slices of meat (breast) from each sample were placed in a tray (covered with PVC film) and assessed through TBARS (thiobarbituric acid reactive substances) assay at 0, 3 and 7 days of storage at 4 °C (Siu & Draper, 1978). In brief, 2.5 g of meat samples were homogenized (in a cold-water bath) with 12.5 mL of distilled water, mixed with 10% (w/v) trichloroacetic acid (12.5 mL) and filtered (Whatman No.1 filter paper). Subsequently, 4 mL of filtrate was mixed with 1 mL of 0.06 M aqueous thiobarbituric acid and incubated in a water bath at 80 °C for 90 minutes. Using a UV-1800 Shimadzu spectrophotometer (Shimadzu Corporation, Milan, Italy) the absorbance of the samples was measured at 532 nm. The assay was calibrated as described by Scerra et al. (2022). Results were expressed as mg of malonaldehyde (MDA)/kg of meat.

Color parameters were assessed at 0 d (after 2 hours of blooming), 3 d, and 7 d of refrigerated storage, using the same slice for TBARS assay (color test before TBARS assay) with a Minolta CR300 colour-meter (illuminant A and 10° standard observer; Minolta Co. Ltd. Osaka, Japan).

#### **2.5. Statistical Analysis**

Data on animal performance, chemical composition and intramuscular FA composition of meat were analyzed using a one-way ANOVA to evaluate the effect of the dietary treatment (C and BP10). Individual animal was considered as the experimental unit for body weight measurements, while pen was the experimental unit for other performance parameters. For meat quality parameters, sample quails (from which the breast was removed) were used as the experimental unit. Data of colour and TBARS in meat were analyzed using a mixed model to evaluate the effect of experimental diets and of the time of

storage (0, 3 and 7 days), as well as of their interaction as the fixed factors, while individual animal sampled was included as a random factor.

Differences between means were assessed using Tukey's multiple comparison test. Significance was declared when  $P \leq 0.05$ , whereas trends toward significance were considered when  $0.05 < P \leq 0.10$ . Minitab software (version 19, Minitab Inc., State College, PA) was used for statistical analyses.

### 3. Results

Data relating to quail performance are represented in table 2.

**Table 2**

Quail performances *in vivo* and chemical composition of muscle (g/100g wet weight).

	Dietary treatment <sup>1</sup>		SEM <sup>6</sup>	P value
	Control	BP10		
Final BW <sup>2</sup> , g	252	234	4.39	0.041
Carcass weight, g	136	128	2.002	0.151
Total DMI <sup>3</sup> , g/d	26.1	23.7	1.000	0.039
ADG <sup>4</sup> , g/d	5.38	4.60	0.252	0.040
FCR <sup>5</sup> , g DMI <sup>3</sup> /g ADG <sup>4</sup>	4.85	5.15	0.232	0.05
<i>Chemical composition</i>				
$\alpha$ -Tocopherol, $\mu\text{g/g}$ muscle	5.59	6.65	0.234	0.01
Moisture	72.3	72.4	0.193	0.659
Crude protein	21.4	21.3	0.051	0.358
Ether extract	3.01	2.96	0.152	0.847
Ash	1.53	1.53	0.112	0.524

<sup>1</sup>Treatments were: basal diet (control) or the basal diet in which part of maize was replaced with 10 % (DM on the diet fed) of dried bergamot pulp (BP10 group).

<sup>2</sup>BW=Body weight; <sup>3</sup>DMI=dry matter intake; <sup>4</sup>ADG=average daily gain; <sup>5</sup>FCR=feed conversion ratio; <sup>6</sup>SEM=standard error of means.

The integration of dried bergamot pulp in quail diet reduced final body weight (FBW, 252 g and 234 g, in control and BP10 groups, respectively,  $P < 0.05$ ),

average daily gain (ADG, 5.38 and 4.60 g/d, in control and BP10 groups, respectively,  $P < 0.05$ ) compared to control treatment. Furthermore, a reduction of daily intake (DMI,  $P < 0.05$ ) was observed in BP10 group than in control group.

Conversely, quails from BP10 group showed a higher feed conversion ratio ( $P = 0.05$ ) than control quail. No statistical differences were observed for carcass weight ( $P = 0.154$ ).

Regarding the chemical composition of meat, no statistical differences were observed between the two experimental groups for moisture ( $P = 0.659$ ), crude protein ( $P = 0.358$ ), etheral extract ( $P = 0.847$ ) and ash ( $P = 0.524$ ), while statistical difference was observed for  $\alpha$ -tocopherol level ( $P < 0.05$ ), showing higher values in meat from quails supplemented with bergamot pulp compared to meat from control quails.

Data on fatty acid composition of meat is shown in the table 3.

**Table 3**

Effect of the dietary treatments on fatty acid composition of muscle (mg/100 g of muscle)

Item	Dietary Treatment			
	Control	BP10	SEM	<i>P</i> value
Intramuscular fat, mg/100 g of muscle	2510	2389	185	0.349
C12:0	0.71	0.53	0.13	0.439
C14:0	11.4	12.9	1.43	0.565
C14:1 <i>cis</i> -9	5.24	5.75	0.703	0.727
C16:0	516	518	39.1	0.967
C17:0	8.17	8.89	0.712	0.568
C16:1 <i>cis</i> -9	216	217	22.9	0.934
C18:0	232	190	13.2	0.119
C18:1 <i>cis</i> -9	817	771	75.9	0.754
C18:1 <i>trans</i> -11	58.1	53.9	4.601	0.582
C18:1 <i>trans</i> -9	2.11	1.98	0.292	0.812
C18:2 <i>cis</i> -9. <i>cis</i> -12 LA <sup>1</sup>	351	381	26.3	0.640
C18:3 $\omega$ -3 ALA <sup>1</sup>	10.2	11.4	1.009	0.440
C18:2 <i>cis</i> -9 <i>trans</i> -11	2.67	2.90	0.221	0.517
C 20:1 <i>cis</i> -11	3.26	1.77	0.803	0.347
C20:2 $\omega$ -6	13.2	7.13	1.450	0.028

C20:3 ω-6	6.77	1.98	1.112	0.025
C20:3ω-3	5.23	7.74	0.753	0.106
C20:4 ω-6	103	68.2	9.463	0.080
C20:5 ω-3 EPA <sup>1</sup>	9.92	11.4	0.792	0.080
C22:2 ω-6	4.55	2.83	1.049	0.410
C24:1	1.29	0.52	0.441	0.389
C22:5 ω-3 DPA <sup>1</sup>	4.17	3.90	0.417	0.867
C22:6 ω-3 DHA <sup>1</sup>	1.83	1.59	0.409	0.679
∑ ω-6	478	461	31.2	0.751
∑ ω-3	31.3	36.0	2.277	0.080
ω-6/ω-3	15.3	12.8	2.189	0.097
∑ SFA <sup>1</sup>	768	730	51.87	0.712
∑ MUFA <sup>1</sup>	1100	1052	103.2	0.813
∑ PUFA <sup>1</sup>	508	500	31.7	0.840
Thrombogenic index <sup>2</sup>	0.88	0.86	0.015	0.292
Atherogenic index <sup>3</sup>	0.27	0.28	0.005	0.412

LA: linoleic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

<sup>2</sup>Thrombogenic index:  $(C_{14:0} + C_{16:0} + C_{18:0}) / (0.5 \text{ MUFA} + 0.5 \text{ PUFA } \omega-6 + 3 \text{ PUFA } \omega-3 + \text{PUFA } \omega-3 / \text{PUFA } \omega-6)$

<sup>3</sup>Atherogenic index:  $(C_{12:0} + 4 * C_{14:0} + C_{16:0}) / (\text{MUFA} + \text{PUFA } \omega-6 + \text{PUFA } \omega-3)$

Intramuscular fat (IMF) content was not affected by diet. As regards the individual fatty acids, the control group showed higher values of C20:2 ω-6 (P = 0.030) and C20:3 ω-6 (P = 0.025) compared to the BP10 group. Furthermore, meat from control group showed a tendentially higher values of arachidonic acid (C20:4 ω-6, P = 0.08) and of the ω-6/ω-3 ratio (P = 0.097) than meat from BP10 group.

Conversely, the supplementation of bergamot pulp tended to increase the C20:5 ω-3 (EPA, P = 0.08) and the total of ω-3 fatty acids (P = 0.08) compared to the control treatment.

Finally, no statistical differences were found between experimental groups (P > 0.05) regarding the total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids.

Data on oxidative stability (TBARS) of meat is shown in table 4.

**Table 4**

Effect of the dietary treatments and time of refrigerated storage on meat colour stability

	Dietary treatment <sup>1</sup>		Time (T) <sup>3</sup>			SEM	Diet	P values	
	Control	BP10	0	1	2			Time	Diet x Time
L* values <sup>2</sup>	45.6	51.1	48.7	47.9	49.1	0.431	0.001	0.139	0.293
a* values <sup>2</sup>	14.7	12.1	13.3	13.5	12.8	0.331	0.001	0.328	0.075
b* values <sup>2</sup>	14.1	15.5	11.4 <sup>x</sup>	15.5 <sup>y</sup>	16.7 <sup>z</sup>	0.292	0.001	0.001	0.267
C* values <sup>2</sup>	20.2	19.9	18.0 <sup>x</sup>	21.2 <sup>y</sup>	21.1 <sup>y</sup>	0.301	0.331	0.001	0.139
H* values <sup>2</sup>	43.7	52.4	41.6 <sup>x</sup>	48.7 <sup>y</sup>	52.9 <sup>z</sup>	0.920	0.001	0.001	0.209
TBARS meat mg MDA/kg	0.54	0.53	0.47 <sup>x</sup>	0.48 <sup>x</sup>	0.65 <sup>y</sup>	0.019	0.972	0.001	0.617

<sup>x,y,z</sup> Within row, different superscripts indicate differences between days of storage ( $P < 0.05$ ) tested using the Tukey's adjustment for multiple comparisons

<sup>1</sup> Treatments were: only basal diet (control) or the basal diet in which part of maize was replaced with 10 % (DM on the diet fed) of dried bergamot pulp (BP10 group).

<sup>2</sup> L\*=lightness; a\*=redness; b\*=yellowness; C\*=Chrom; h\*=hue angle, measured in degrees.

<sup>3</sup> Times 0, 1, 2 = days 0, 3, 7 for raw meat at 4 °C under aerobic conditions (meat slices).

Lipid oxidation was affected by the time of storage ( $P < 0.05$ ), but not by the dietary treatment.

Regarding color descriptor in meat (table 4), the lightness (L\*) and redness (a\*) descriptor were significantly affected by the diet ( $P = 0.001$ ), 45.8 vs 51.2 for lightness and 14.6 vs 12 for redness descriptor in control and BP10 groups respectively, but not by the time of storage, whereas the yellowness index (b\*) was influenced by both diet and time ( $P = 0.001$ ), with values that increase constantly over the time and higher in BP10 group than in control one. Instead, meat saturation (C\*) was affected only by the time of storage ( $P < 0.001$ ), with values increasing from 0 to 3 days and stabilizing thereafter, whereas hue angle (H\*) increased along the storage time ( $P < 0.01$ ), accompanied by the influence of dietary treatment ( $P < 0.01$ ).

## 4. Discussion

The aim of this study was to evaluate the use of an industrial by-product in the diet of Japanese quail, dried bergamot pulp, to study the effect on animal performance and meat quality. The integration of dried bergamot pulp in the diet has led to significant differences between experimental groups for animals' performance. Dietary supplementation of 10% dried bergamot pulp, provided for approximately 3 weeks, has led to an ADG reduction in quails. This data could be due to the lower DMI of the quails in the BP10 group, probably due to the lower palatability of their diet. It has been observed that the presence of a high content of flavonoids in bergamot pulp (Russo et al., 2016), can lead to a bitter taste (Sevim et al., 2021), and taking into account that birds are highly sensitive to bitter substances, this could have had a negative effect on DMI (Roura et al., 2013). Similar results were observed in the study of Goliomytis et al. (2018), where the integration of orange pulp in laying hens' diet led to higher FCR and a lower DMI compared to the control group, as well as Ojabo et al. (2014) in pullet chicks fed sun-dried sweet orange peel, while no differences were observed by Florou-Paneri et al. (2001) on the body weight of quails in egg production by providing a diet supplemented with 6% of citrus pulp. Furthermore, the presence of other substances, such as oxalate, saponins, tannins, and phytates (also present in other citrus fruits such as mandarin, lemons, orange), could have negatively influenced the palatability and consequently DMI of quails (Ebrahimi et al., 2013). Contrary to the use of fresh or dried citrus pulp, supplementation of citrus essential oil in the diet of Japanese quail led to increase body weight, DMI and FCR (Dalkilic et al., 2015). Other studies carried out to investigate citrus pulp as an ingredient in chicks' diet (Mourao et al., 2008) have observed that it significantly increases the fiber content in the diet, especially nonstarch polysaccharides (NSP), promoting anatomical changes to the animal, such as a greater length of the small intestine. Non-starch polysaccharides are poorly degraded in the small

intestine, which may have affected total feed intake in quail, considering the correlation between feed retention time and DMI (Almirall and Esteve-Garcia, 1994). The expansion of the gastrointestinal tract with an increased length as well is due to a condition of adaptation of the bird to greater fibrousness of the diet, resulting in a variation in the absorption of nutrients (Jorgensen et al., 1996). Furthermore, bergamot peel is a by-product that contains high amount of pectins, compounds that could reduce the growth performance, as observed in several experimental trials with chicks (Patel et al., 1981; Bishawi and McGinnis, 1984; Langhout and Schutte, 1996). Patel et al. (1981) and Bishawi and McGinnis (1984) suggested that the reduction in growth performance could be due to the viscous properties of pectins leading to antinutritional effects.

Partial replacement of maize with dried bergamot pulp did not lead to any particular changes in the fatty acid composition of the meat (Table 3). Despite the higher levels of  $\alpha$ -linolenic acid in the diet of the BP10 group, no statistical differences were observed for this fatty acid between the two experimental groups. However, the total  $\omega$ -3 FA tended to increase in the meat of quails that received dried bergamot pulp supplementation. This last result led to a tendentially lower values of the  $\omega$ -6/ $\omega$ -3 ratio in the meat of the BP10 group. Similar results were observed integrating fresh bergamot pulp in lambs' diet (Scerra et al., 2018), although with much lower values of the ratio  $\omega$ -6/ $\omega$ -3 than those obtained in this experimental trial and close to those recommended (the ratio should not exceed a threshold value of 4) by the department of Health (1994). However, the integration of bergamot pulp into the quail diet led to a 35% reduction in  $\omega$ -6/ $\omega$ -3 ratio. Among the  $\omega$ -3 PUFAs, C20:5  $\omega$ -3, one of the two most important  $\omega$ -3 PUFAs well known to have beneficial effects on human health (Song et al., 2018; Kumar et al., 2022), was tendentially higher in meat from BP10 group than in meat from control group. Similar results were observed by Çiftci et al. (2016) in quails supplemented with orange peel extract, in which an increase of  $\omega$ -3 fatty acids accumulation was observed in breast muscle lipids, a reduction  $\omega$ -6/ $\omega$ -3 ratio and an increase of the amounts of desaturation and

elongation products such as C<sub>20:5</sub>  $\omega$ -3. Çiftci et al. (2018) also observed, integrating a mixture of essential oil (thyme, orange peel, bay leaf and eucalyptus) in quail diet, positive effects on  $\omega$ -3 and  $\omega$ -6/ $\omega$ -3 ratio levels. Other study on layer hens (Bölükbaşı et al., 2010) showed that the integration of bergamot oil in diet significantly increase C<sub>20:5</sub>  $\omega$ <sub>3</sub>, C<sub>22:6</sub>  $\omega$ <sub>3</sub>, and total  $\omega$ -3 concentrations and decreased the  $\omega$ -6/ $\omega$ -3 ratio in egg yolk.

The lipid oxidation of meat during the days of storage depends on several factors and, among these, dietary treatment can have a significant influence. Many studies suggest that meat with high levels of PUFAs, and in particular  $\omega$ -3 fatty acids, is more susceptible to lipid oxidation (Dunne et al., 2011; Moloney et al., 2012), while the increase in antioxidant compounds such as vitamin E and phenols compounds (Luciano et al., 2017) protect it from this phenomenon. In the present study was observed that despite the tendency to increase in  $\omega$ -3 fatty acids in the meat of animals that received the integration of bergamot pulp, the meat from the two experimental groups did not show significant differences in lipid oxidation (TBARS values). Probably, higher levels of antioxidant compounds, such as vitamins and phenols, in meat from BP<sub>10</sub> group than in meat from control group protected it from the oxidation of the highly peroxidizable PUFA (such as  $\omega$ -3 FA), making the meat from the two experimental treatments comparable. The higher level of  $\alpha$ -tocopherol in BP<sub>10</sub> meat than in control one could be influenced by the highest concentration of phenolic compounds in BP<sub>10</sub> diet that could have indirectly preserved vitamin E during digestion, considering their antioxidant activity (Menci et al., 2023). Indeed, some authors (Sales and Koukolov, 2011) reported that even a small amount of  $\alpha$ -tocopherol in meat can provide protection from lipid oxidation, a quantity of  $\alpha$ -tocopherol lower than that of the basal diet of this experimental trial. However, in both experimental groups there was a significant increase in MDA levels after 7 days of storage, although it remained below the suggested tolerance thresholds (2 mg MDA/Kg of meat, Campo et al., 2006). Some authors reported that the effects of dietary treatment on meat oxidative stability could be more evident by subjecting meat

samples to greater oxidative stress (Luciano et al., 2019). Probably the TBARS test carried out on samples of cooked meat could have revealed some differences. Unfortunately, it was not possible to carry out these analyses in our conditions. Similar results were observed by Scerra et al. (2022) in pork meat from animals fed ensiled bergamot pulp.

The data obtained from the color analysis showed that the integration of bergamot into the quail diet led to higher lightness ( $L^*$ ) and yellow index ( $b^*$ ) values compared to the control group, a result which could depend by the release of pigments in the muscle, such as carotenoids. In particular, the yellow color could derive from the accumulation of xanthophylls (Castaneda et al., 2005; Perez-Vendrell et al., 2001). Some authors have underlined that the increase of these colorimetric parameters, and in particular in lightness, could be correlated to the greater ingestion of phenolic compounds, considering the results of experimental trials carried out using by-products rich in polyphenolic compounds in lambs (Natalello et al., 2020; Menci et al., 2023). As regard the red index ( $a^*$ ), similarly to other studies (Mourao et al., 2008), this value appears to be significantly lower in the BP10 group than in control group.

## **5. Conclusion**

The partial replacement of maize with dried bergamot pulp in the quail diet led to a tendency to increase  $\omega$ -3 FA in the meat, positively influencing the  $\omega$ -6/ $\omega$ -3 ratio which was reduced by approximately 35%. Color analysis showed that the integration of bergamot into the quail diet led to higher lightness ( $L^*$ ) and yellow index ( $b^*$ ) values compared to the control group, a result which could depend on the release of pigments in the muscle, such as carotenoids. In this study it was observed that, despite the tendency to increase in  $\omega$ -3 fatty acids in the meat of animals that received the integration of bergamot pulp, the meat from the two experimental groups did not show significant differences in TBARS values, probably due to the higher levels of antioxidant compounds in BP10 diet than in

control diet. However, dietary supplementation of 10% dried bergamot pulp led to a reduction in DMI and consequently in ADG, probably due to the lower palatability of bergamot pulp.

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## List of Figures

### Chapter 1

**Fig. 1** Photo of Podolian young bull experimental test

**Fig. 2** Olive cake production process

### Chapter 2

**Fig. 1** Purpose of the PhD research

### Chapter 3

**Fig.1** Effect of the dietary treatment and time of storage on the oxidative stability of longissimus dorsi.

### Chapter 4

**Fig. 1.** Effect of the dietary treatment and time of storage on the oxidative stability of raw meat. Interactive effect of the dietary treatment and time of storage on the TBARS values measured in raw meat slices over aerobic storage at 4° C. Values presented are the estimated least squares means and standard error bars a,b,c,d. Values with different superscripts are significantly different ( $P \leq 0.05$ ).

### Appendix 1

**Fig. 1** Principal component analysis was applied to fatty acids detected in meat samples from concentrate (C), concentrate with the inclusion of silage cake at 20% (OC20) and concentrate with the inclusion of silage cake at 40% (OC40)

diets. Scores and loading plots on the first two principal components. Scores correspond to samples, and loadings correspond to variables.

## **Appendix 2**

**Fig. 1.** Effect of the dietary treatment and time of storage on the oxidative stability of longissimus thoracis et lumborum muscle, raw (a) and cooked (b). Treatments were: Control, concentrate-based diet; EBP, concentrate and ensiled bergamot pulp at the level of 20 % DM on the diet fed; OLL, concentrate and olive leaves at the level of 20 % DM on the diet fed; BPOL, concentrate and a mixture 1:1 of ensiled bergamot pulp and olive leaves at the level of 20 % DM on the diet fed. Interactive effect of the dietary treatment (Control, EBP, OLL and BPOL) and time of storage on the TBARS values measured in raw and cooked slices over aerobic storage at 4° C. Values presented are the estimated least squares means and standard error bars a,b,c,d. Values with different superscripts are significantly different (P <0.05).

## List of tables

### Chapter 3

**Table 1** Ingredients and formulation of the experimental concentrates administered to the Podolian young bulls

**Table 2** Chemical-nutritional composition and fatty acids profile of the experimental concentrates and of the straw administered to the Podolian young bulls

**Table 3** Performance in vita and post slaughter measurements of Podolian young bulls receiving four different supplementations during the fattening period

**Table 4** Variation of lightness L\* in meat during storage in PVC at 4° C for 10 days

**Table 5** Variation of redness a\* in meat during storage in PVC at 4° C for 10 days

**Table 6** Variation of yellowness b\* in meat during storage in PVC at 4° C for 10 days

**Table 7** Variation of Chrome C\* in meat preserved in PVC for 10 days at 4° C.

**Table 8** Variation of Hue H\* in meat stored in PVC for 10 days at 4° C.

### Chapter 4

**Table 1.** Growth performance and intakes of experimental lambs, vitamins and fatty acids of the meat.

### Appendix 1

**Table 1** Ingredients (g/100 g of DM) and chemical composition of diets offered.

**Table 2** Pig performances in vivo and data at slaughter.

**Table 3** Effect of the dietary treatment on chemical composition (g/100 g of muscle), pH and colour of Longissimus thoracis muscle.

**Table 4** Effect of the dietary treatment on fatty acid composition (mg/100 g of meat) of Longissimus thoracis muscle.

## **Appendix 2**

**Table 1.** Ingredients (% on DM basis) and chemical composition of the experimental diets.

**Table 2.** Effect of dietary treatment on growth performance and chemical composition of longissimus thoracis et lumborum muscle (g/100 g wet weight).

**Table 3.** Effect of the dietary treatments on fatty acid composition of longissimus thoracis et lumborum muscle (mg/100 g of muscle).

**Table 4.** Effect of the dietary treatments and time of refrigerated storage on meat colour stability of longissimus thoracis et lumborum muscle.

## **Appendix 3**

**Table 1.** Ingredients (% on DM basis) and chemical composition of the experimental diets.

**Table 2.** Quail performances in vivo and chemical composition of muscle (g/100g wet weight).

**Table 3.** Effect of the dietary treatments on fatty acid composition of muscle (mg/100 g of muscle)

**Table 4.** Effect of the dietary treatments and time of refrigerated storage on meat colour stability

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## DM 1061 del 10 agosto 2021

Dottorati PON – Bando 2021- Ciclo 37 (XXXVII)

Azione IV.4 – Dottorati e contratti di ricerca su tematiche dell'innovazione

Azione IV.5 – Dottorati su tematiche Green

<b>Dottorando</b>	Paolo De Caria
<b>Tutor</b>	Prof.ssa Amalia Rosa Maria Piscopo
<b>Co-Tutor</b>	Prof. Pasquale Caparra
<b>Coordinatore</b>	Prof. Leonardo Schena
<b>Corso di Dottorato in</b>	Scienze delle Produzioni Agrarie
<b>Ciclo</b>	XXXVII PON
<b>Codice borsa e n.</b>	DOT1647787-6
<b>CUP</b>	C35F21001320002
<b>Tipologia Green/Innovazione</b>	Green
<b>Titolo Progetto</b>	Innovative strategies in feeding ruminants to improve product quality and reduce the environmental impact of farming



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